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Receptor-Mediated Hypertrophic Signaling Via Protein Kinase D and Histone Deacetylase 5 in Adult Myocytes

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LOYOLA UNIVERSITY CHICAGO

RECEPTOR-MEDIATED HYPERTROPHIC SIGNALING VIA PROTEIN
KINASE D AND HISTONE DEACETYLASE 5 IN ADULT MYOCYTES

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CELL AND MOLECULAR PHYSIOLOGY

BY

CHIA-WEI JENNY CHANG

CHICAGO, IL

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LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
ACE	Angiotensin-converting enzyme
AKAP	A kinase anchoring protein
ANOVA	Analysis of variance
ARB	Adrenergic receptor blockers
ATP	Adenosine 3,4,5-triphosphate
AV	Atrioventricular
β -AR	Beta adrenergic receptor
β ARK	Beta adrenergic receptor kinase
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CaMKII	Ca^{2+} /calmodulin-dependent kinase II
CFP	Cyan fluorescence protein
CMV	Cytomegalovirus
Co-IP	Co-immunoprecipitation
CPE	Cytopathic effects
CsCl	Cesium chloride
DAG	Diacylglycerol
DKAR	D kinase activity reporter
DNA	Deoxyribonucleic acid

DMEM	D-glucose minimal essential medium
ECC	Excitation-contraction-coupling
EPAC	Effector protein activated by cAMP
ER	Endoplasmic reticulum
ET-1	Endothelin-1
ET _A R	Endothelin A receptor
ET _B R	Endothelin B receptor
FBS	Fetal bovine serum
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
GAPDH	Glyeraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescence protein
GPCR	G protein coupled receptor
GRK	G protein receptor kinase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	Heart failure
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
IP	Immunoprecipitation
IP ₃	Inisitol 1,4,5-trisphosphate
ISO	Isoproterenol
KCL	Potassium chloride
LB	Buria-bertani
MgCl ₂	Magnesium chloride
MEF2	Myocyte enhancer factor 2
MEM	Minimal essential medium

MOI	Multiplicity of infection
MW	Molecular weight
NaCl	Sodium chloride
NE	Norepinephrine
NES	Nuclear export sequence
NFAT	Nuclear factor of activated T cells
NF κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHERF-1	Na/H exchanger regulatory factor-1
NLS	Nuclear localization sequence
NRVM	Neonatal rat ventricular myocytes
Nuc	Nuclear
PBS	Phosphate buffered saline
PE	Phenylephrine
PdBu	Phorbol 12,13-dibutyrate
PDE	Phosphodiesterase
PDZ	PSD-95, Disc large and ZO-1
PH	Pleckstrin homology
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PLB	Phospholamban
PLC	Phospholipase C
PRK	Protein kinase C-related kinase
ROI	Region of interest
S259	Serine 259
S279	Serine 279
S427	Serine 427
S498	Serine 498
S916	Serine 916

SS744/748	Serine 744 and serine 748
SA	Sinoatrial node
SDS	Sodium dodecyl sulphate
SL	Sarcolemma
SR	Sarcolemma reticulum
TAC	Transverse aortic constriction
TBS	Tris buffered saline
TBST	Tris buffered saline Tween
TIRF	Total internal refraction fluorescence
WT	Wildtype

ABSTRACT

Hemodynamic stress and neurohumoral signaling are common causes of cardiac hypertrophy. These extrinsic stress stimuli typically act on GPCR and induce a cascade of signal transduction to re-program terminally differentiated myocytes to grow in length or width. The compensatory hypertrophic response can enhance cardiac output briefly due to increased work load. However, prolonged stress results in maladaptive changes in the heart and gradually deteriorates ventricular function to supply blood throughout the body. Sustained hypertrophic signaling can also progress toward heart failure.

My dissertation research focuses on the hypertrophic signaling in adult cardiac myocytes in response to neurohumoral stimuli, ET-1 and PE. In particular, I am interested in the spatiotemporal activation of PKD and its functional role in regulation of HDAC5 translocation. HDAC5 is a transcriptional repressor whose dynamic shuttling between the nucleus and the cytosol determines the outcome of transcriptional control for MEF2-dependent genes. I use a combination of biochemical approach with fluorescence imaging techniques to study the regulation of epigenetic re-programming mediated by PKD and HDAC5 in adult cardiac myocytes during hypertrophy and heart failure.

CHAPTER 1

INTRODUCTION

1. Cardiac Physiology and Overview

Anatomy of the Heart

The human heart plays a central role in the circulatory system to pump and supply oxygenated blood to the organs in our body. On average, the heart beats about 100,000 times a day, pumping approximately 2,000 gallons (equivalent of 7,571 liters) of blood. The heart is situated in the middle of the chest between two lungs, and it is surrounded by a double-layered membrane, called the pericardium. The heart has four chambers and is divided into the left and right side by the separation of the septum. The upper chambers are named the atria and the bottom chambers are called the ventricles. The ventricles are anatomically structured to have thicker muscular walls than the atria due to their contractile role in ejecting the blood from the heart. In particular, the left ventricular wall is the thickest as demanded by its role in delivering the blood to the peripheral organs.

There are four valves that separate the atria from the ventricles and the ventricles from the two major arteries connected to the heart. The right side of the heart contains the tricuspid valve, located between the right atrium and the right ventricle. The pulmonary valve separates the right ventricle from the

pulmonary artery. The mitral (bicuspid) valve keeps the left atrium apart from the left ventricle while the aortic valve separates the left ventricle from the aorta. The anatomy of the heart is illustrated in Figure 1.

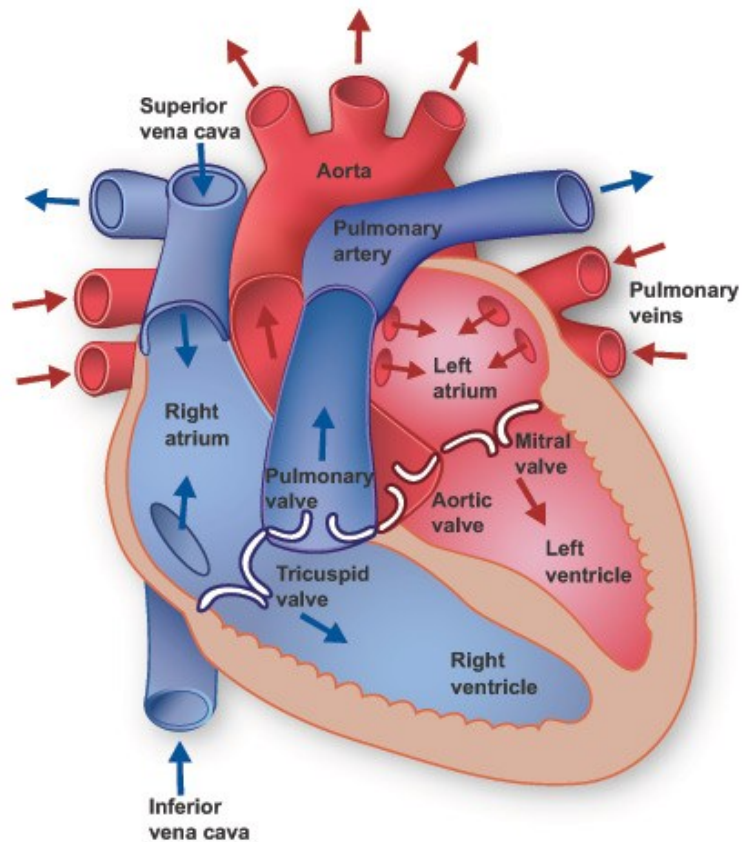


Figure 1. Anatomy of the heart. The heart is divided into 4 chambers, namely the right atrium and right ventricle along with the left atrium and right ventricle. The deoxygenated blood is shown in blue while the oxygenated blood is shown in red. The image was taken from Texas Heart Institute website: www.texasheartinstitute.org/HIC/Anatomy/anatomy2.cfm.

Function of the Heart

The function of the heart serves to pump blood throughout the body and keeps a constant flow of our circulatory system. More specifically, the heart pumps the deoxygenated blood into the pulmonary artery from the right ventricle, and the oxygenated blood into the aorta from the left ventricle respectively. The heart also receives the deoxygenated blood from the superior vena cava into the right atrium. The venous blood then flows through the right atrium into right ventricle and the pulmonary artery to be delivered to the lungs. Oxygenation of the venous blood occurs at the lungs. The oxygenated blood then returns back to left atrium via the pulmonary veins, and subsequently enters the left ventricle to be pumped throughout the body via the aorta.

Electrical System of the Heart

In order to function as the pump of the circulatory system, the heart is an organ that has intrinsic conduction properties to synchronize the beating of cardiac myocytes to pump blood effectively. The synchronization of the conduction system of the heart is of particular importance since it controls every electrical activity of the heart. Consequently, arrhythmia or irregular heart beat may occur when the conduction system is perturbed in anyway.

The electrical impulse originates at the sinoatrial (SA) node located on the wall of right atrium. The SA node is also termed the “pacemaker” of the heart because of its inherent ability to trigger electrical impulses. Each electrical

impulse is then transmitted to the atrioventricular (AV) node via special conductive tissue of the heart. In case the SA node becomes unavailable or unable to produce electrical impulses, the AV node can replace the function of the SA node to generate electrical signals. The AV node is positioned between the right atrium and the right ventricle. It transmits the electrical impulse onto the Bundle of His, which divides into right and left bundle branch to allow conduction of the right and left ventricles respectively. The two bundle branches then connect to the Purkinje fibers, which innervate the right and left ventricular walls to conduct electrical events. The conduction system of the heart is illustrated in Figure 2.

Electrical System of the Heart

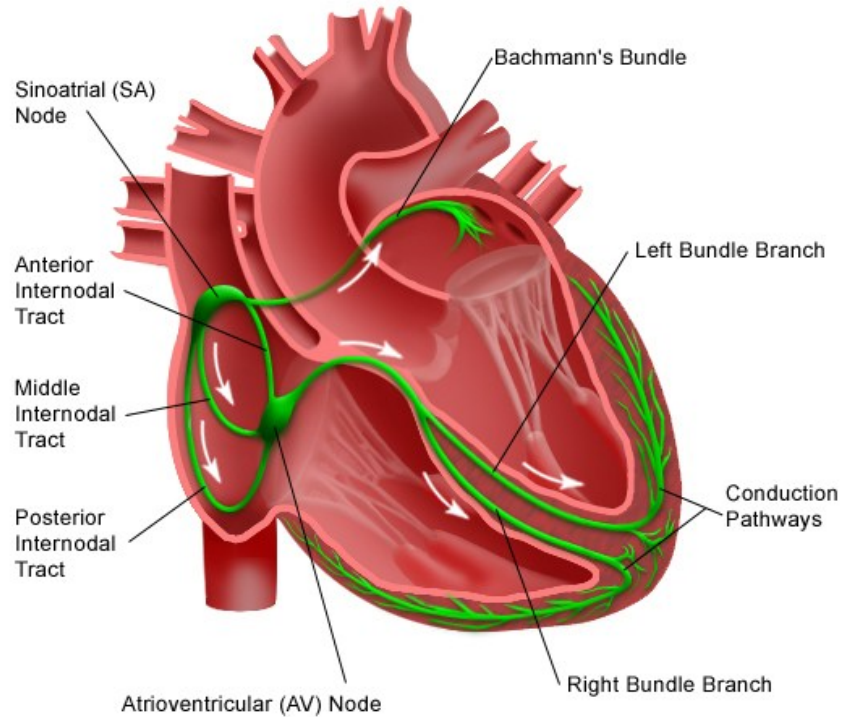


Figure 2. Electrical System of the Heart. The diagram illustrates the conduction pathways of the electrical impulse throughout the heart. Electrical impulses are transmitted in this order to synchronize the electrical activity of the myocytes of the heart: SA node → AV node → Bundle of His → Purkinje fibers. Image was taken from: <http://mdmedicine.wordpress.com/2011/04/24/heart-conduction-system/>.

2. Cardiovascular Disease

Heart disease is the leading cause of death in United States. Most recent statistics from Center for Diseases Control and Prevention indicated that there were over 616,000 people died of heart disease in 2008, and heart disease caused >50% of all death in United States. In general, heart disease is used to describe many conditions or problems triggered by small plaque builds up in the wall of the arteries creating difficulties for blood flow. This condition typically increases risks for a heart attack or stroke. There are several risk factors contributing to heart diseases, such as high cholesterol, diabetes, high blood pressure, obesity, smoking and inactivity. The American Heart Association estimates that 81 million Americans suffer from one or more forms of cardiovascular diseases. Therefore, we have a huge demand for basic cardiovascular research to understand the cellular mechanism of heart disease and design suitable therapeutics for these patients.

3. Cardiac Hypertrophy

As mentioned previously, the heart has remarkable intrinsic adaptive properties to adjust to a wide range of stress stimuli to maintain its contractile function. When the heart is subject to prolonged stress or hindrance in electrical activity, the organ undergoes a remodeling process in an attempt to compensate for its increased work load. The remodeling process results in the enlargement of

the heart, also termed “hypertrophy”. Hypertrophy can be defined as myocyte growth in length or width to increase cardiac function to pump blood throughout the body. The increase in myocyte length and width may or may not be uniform, and are categorized into eccentric hypertrophy or concentric hypertrophy.

Eccentric hypertrophy is characterized by homogenous cardiac growth response that is associated with a similar increase in ventricular wall thickness, septum and chamber dimensions. This condition is typically accompanied by more prominent increase in myocyte length than the increase in myocyte width. Conversely, concentric hypertrophy is characterized by cardiac growth resulting thickening of ventricular walls and septum without any net change in chamber volume. There is more growth in myocyte width than length in concentric hypertrophy.

Hypertrophy can be further separated into physiological hypertrophy and pathological hypertrophy. Physiological hypertrophy is usually observed in athletes and pregnant women who demand a higher amount of oxygenated blood to be pumped throughout the body due to exercise or bearing a baby. This type of hypertrophy is beneficial and can compensate for the increased work load by enhancing myocyte growth to preserve cardiac function (Heineke and Molkentin, 2006). In contrast, pathological hypertrophy is characterized by enhanced myocyte growth with reduced cardiac performance and results in heart failure over time. Pathological hypertrophy typically develops as a response to excess hemodynamic work load, and it can be induced by numerous stimuli, such as

biomechanical stress, stretch-sensitive mechanism or neurohumoral signaling via the release of cytokines, growth factors and hormones.

The objective of my dissertation work is focused on understanding the neurohumoral signaling pathway triggering pathological hypertrophy and heart failure in adult cardiac myocytes. The first evidence of hypertrophic responses inducible by neurohumoral stimulus was identified by Paul Simpson in cultured neonatal myocytes (Simpson, 1983). Others then soon follow to examine the possibility of neurohumoral stimuli to induce characteristic changes in kinase activation and cardiac gene expression (Sadoshima and Izumo, 1993; Shubeita et al., 1990). To date, neurohumoral signaling represents a common mechanism of inducing cardiac hypertrophy in both neonatal and adult myocytes. Nevertheless, due to the nature of neurohumoral signaling being G-protein coupled receptors, the corresponding signaling network is inherently complex and agonist-specific.

4. G-Protein Coupled Receptor Signaling and Hypertrophy

As mentioned previously, neurohumoral signaling is one of the mechanisms that can induce cardiac hypertrophy. Most neurohumoral molecules or agonists act on their receptors, which are typically coupled to heterotrimeric guanine-nucleotide-binding regulatory proteins (G proteins) to mediate a cascade of intracellular response. These agonist receptors consist of the largest class of drug targets and are called G-protein coupled receptors (GPCR). GPCR family

has highly conserved seven transmembrane structure linked to the G-proteins via its intracellular domains. GPCR signaling is characterized by the action of ligand binding to its receptor, causing a conformational change of the associated heterotrimeric G-proteins. Upon receptor activation, the $G\alpha$ subunit typically dissociates from the $G\beta\gamma$ subunits and targets its downstream effector to initiate intracellular signaling pathway. The $G\beta\gamma$ subunits can also target their own substrates to result in divergent signaling in parallel. This type of cascade signaling orchestrates a powerful amplification of original extracellular signal to guarantee a biological response. GPCR is coupled into 3 major G-protein signaling families, including G_s , G_i , and G_q/G_{11} . Each subfamily has a hallmark intracellular signaling pathway once the heterotrimeric G proteins are activated upon receptor stimulation. Rockman et al. has summarized a table of important GPCR and their association with intracellular G-protein signaling (Rockman et al., 2002).

For example, G_s activation stimulates activation of adenylyl cyclase (AC) and triggers the production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) to activate downstream effectors, such as protein kinase A (PKA) or exchange protein activated by cAMP (EPAC). In contrast, G_i activation can turn on phosphodiesterases (PDE), which break down cAMP molecules to terminate further cAMP signaling, forming a negative feedback loop. The action of G_s signaling is antagonized by G_i and this prevents any feed forward signaling mechanism.

Gq/G₁₁ activation is paired with activation of phospholipase C (PLC) to create two important intracellular 2nd messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerols (DAG). IP₃ and DAG can further activate several downstream targets. IP₃ production elicits calcium (Ca²⁺) release from intracellular IP₃ stores, such as the endoplasmic reticulum (ER), the sarcoplasmic reticulum (SR) or the nuclear envelope. With Ca²⁺ being an universal 2nd messenger, intracellular Ca²⁺ release in turn triggers several cellular effectors, such as calcineurin, Ca²⁺-calmodulin-dependent protein kinase II (CaMKII) and nuclear factor of activated T-cell (NFAT). The family of NFAT transcription factors are critical mediators of pathological hypertrophy. In parallel, DAG can activate its targets, for instance, the classical and novel protein kinase C (PKC) isoforms. These isoforms possess inherent DAG-binding domains which are recruited to the plasma membrane or to microdomains near the DAG pools facilitating their activation via cross-phosphorylation.

Many adaptive mechanisms have evolved from our biology to curtail the GPCR signal. The first is known as receptor “desensitization” whereby receptor responsiveness is attenuated even in the presence of continuous agonist stimulation. The receptor desensitization is categorized into homologous or heterologous desensitization depending on whether the process is initiated by the same ligand receptor or another ligand receptor. Alternatively, the receptors can also be “downregulated” to minimize the triggered physiological response. The downregulation of GPCR can be achieved by a decrease in receptor

synthesis or an increase in receptor degradation. The receptor desensitization and downregulation mechanisms are typically mediated through modification of the receptors by receptor kinases, such as G-protein receptor kinase (GRK) family. Lastly, the GPCR signal can also be terminated by uncoupling of the receptor from its associating G-proteins.

GPCR Signaling and Hypertrophy

Almost all the GPCR linked to Gq signaling are capable of triggering cardiac hypertrophy. The most common and most studied GPCR receptor types in the heart include the adrenergic receptors (α and β subtypes) for norepinephrine (NE) and phenylephrine (PE), the endothelin (ET) receptors (ET_AR and ET_BR) for endothelin-1 (ET-1) and the angiotensin-1 receptor for angiotensin II. A body of published works has supported the notion that GPCR activation associated with Gq signaling via different surface receptors can sufficiently induce cardiac hypertrophy. For instance, α -AR activation is reported to trigger hypertrophy in response to NE stimulation and pressure overload (Bishopric et al., 1987; Long et al., 1989; O'Connell et al., 2003; O'Connell et al., 2006; Simpson, 1983). Similarly, endothelin receptors are also involved in hypertrophic response induced by multitude stress stimuli (Bossuyt et al., 2011; Higazi et al., 2009; Huang et al., 2011; Mueller et al., 2011; Shubeita et al., 1990). My dissertation research primarily focuses on the intracellular hypertrophic signaling induced by Gq-agonists, ET-1 and PE in isolated adult

cardiac myocytes. More pertinently, I examine how these two Gq-signaling pathways diverge from each other to play a regulatory role in epigenetic control of cardiac remodeling process.

5. β -Adrenergic Signaling and Hypertrophy

Background

β -adrenergic activation represents one of the major pathways to increase cardiac performance during sympathetic stimulation or acute stress signaling. There are three different β -adrenergic receptors (β -AR) identified in humans (β_1 , β_2 and β_3). They share approximately 60% amino acid sequence homology in the transmembrane region where it binds ligands, the catecholamines. β_1 -AR and β_2 -AR are the dominant receptor types expressed in the heart, and their relative distribution is 75% vs. 25% respectively (Lohse et al., 2003; Rockman et al., 1997). They are also responsible for the majority cardiac adrenergic response during endogenous catecholamine signaling by norepinephrine and epinephrine. The functional role of β_1 -AR is widely accepted to be coupled to Gs signaling, which in turn triggers cAMP production via activation of AC. The classical cAMP signaling effector is PKA, and EPAC is recently identified to be another novel effector of cAMP signaling (Berthouze et al., 2011; Dodge-Kafka et al., 2005; Pereira et al., 2007; Pereira et al., 2012). β_1 -AR activation increases cardiac performance by improving cardiac contractility, increasing heart rate and lusitropy. β_2 -AR are coupled to both Gs and Gi signaling pathways, and its

functional role is more complex than β_1 -AR. It has been shown that β_2 -AR are coupled to AC with similar affinity, yet they exhibit minor functional role in regulation of contractility (Xiang, 2011; Zhao et al., 2011). Coupling of β_2 -AR to G_i has been indicated to gradually become more significant in failing animal and human cardiac myocytes (Gong et al., 2002; Xiao et al., 1999a; Xiao et al., 1999b).

β_3 -AR are initially found to be present only in adipose tissues, however, recent research shows that they are also expressed on the nuclear membranes of cardiac myocytes (Boivin et al., 2006; Vaniotis et al., 2011). Unfortunately, their functional role in the heart remains poorly defined as their expression density is dominated by the presence of β_1 -AR and β_2 -AR. They are not speculated to participate in the regulation of cardiac contractility because transgenic double knockout mice for both β_1 -AR and β_2 -AR exhibit significantly reduced contractile function despite the presence of β_3 -AR (Devic et al., 2001). It is therefore evident that these β -AR subtypes have non-redundant functional roles in cardiac myocytes.

Cardiac Distribution and Function of β_1 vs. β_2

Recent efforts have been devoted to examine the cellular distribution of β_1 -AR vs β_2 -AR in cardiac myocytes with development of new molecular tools in imaging and fluorescence resonance energy transfer (FRET) based biosensor to detect cAMP production (Nikolaev et al., 2006; Nikolaev et al., 2010). The

findings suggest that β_1 -AR are distributed to both the T-tubules and cell surface while β_2 -AR are localized mostly at the T-tubular region. The different cellular distributions of these two receptors reflect the corresponding divergent cAMP signaling patterns. β_1 -AR activation induced cAMP signaling is reported to be more global and propagates throughout the entire myocyte. Conversely, activation of β_2 -AR results in more local cAMP signaling that is confined to the T-tubules (Nikolaev et al., 2006; Nikolaev et al., 2010).

The compartmentalized cAMP signaling of β_2 -AR activation is achieved by local cAMP buffering via association of PKA activity and PDE activation (Abi-Gerges et al., 2009; Fischmeister et al., 2006; Leroy et al., 2008; Nikolaev et al., 2010). In addition, the family of scaffolding proteins called the A Kinase Anchoring Protein (AKAP) also contributes to the spatiotemporal cAMP signaling by associating PKA with PDE in various compartments within cardiac myocytes (Appert-Collin et al., 2006; Appert-Collin et al., 2007; Diviani et al., 2006; Hall and Lefkowitz, 2002). PDE are the enzymes which degrade cAMP molecules to impose a negative feedback loop for cAMP signaling. The divergent cAMP signaling pathways induced by these two receptors represent an elegant intrinsic regulation of spatiotemporal cAMP signaling in healthy myocytes.

Nuclear Signaling of β -AR

β -AR are known prototypical GPCRs, and the classical paradigm of these receptor signaling is confined only at the plasma membrane. Increasing amount

of studies is exploring the notion of GPCR signaling at the nuclear membrane. In particular, β_1 -AR and β_3 -AR (but not β_2 -AR) are also found to localize at the nuclear membrane in rat and mouse adult myocytes (Boivin et al., 2006; Boivin et al., 2008). One of the nuclear functions of β_1 -AR and β_3 -AR is to trigger *de novo* transcription, and this is achieved in part via being differentially coupled to $G_{\alpha s}$ and $G_{\alpha i}$ signaling pathways in isolated cardiac nuclei. The findings that ribosomal RNA and mRNA synthesis can be modulated by β -AR stimulation further support the notion that β -AR signaling plays a role in nuclear signaling (Vaniotis et al., 2011). However, future work on the functional role of β -AR signaling in the nucleus remains to be continued to explore further understanding of β -AR regulation on gene modification in cardiac myocytes.

β -AR Signaling in Hypertrophy and Heart Failure

Chronic catecholamine stimulation is well known to trigger hypertrophy and heart failure (Chidsey et al., 1963; Lefkowitz et al., 2000; Lohse et al., 2003), thus emphasizes the importance of β -AR activation and its ability to regulate cardiac disease. Moreover, reduced β -AR response and β -AR downregulation are leading characteristics for deteriorating cardiac function. To date, β -blockers are the most effective treatment for chronic heart failure even though its mechanism of action remains paradoxical (Braunwald and Bristow, 2000; Bristow, 2000; Bristow, 2011; Kubon et al., 2011).

Even though both β_1 -AR and β_2 -AR respond to adrenergic stimulation,

selective activation of either receptor type exhibits differential physiological responses. For instance, transgenic overexpression of β_1 -AR results in progressive cardiac hypertrophy and heart failure (Bisognano et al., 2000; Engelhardt et al., 1999), while β_2 -AR overexpression in mice produces enhanced myocardial function (Milano et al., 1994). Furthermore, β_2 -AR knockout mice develop more severe cardiac hypertrophy in response to pressure overload by transverse aortic constriction (TAC) compared to β_1 -AR deletion knockout (Zhao et al., 2011). The two β -AR subtypes also show opposing effect on apoptosis and cell survival in cardiac myocytes (Communal et al., 1999; Zhu et al., 2003; Zhu et al., 2001). Overall, the β_2 -AR signaling appears to be more cardioprotective than β_1 -AR activation. However, the implicit differential coupling of both subtypes to Gs or Gi does not sufficiently explain the opposing physiological responses produced by selective receptor stimulation.

As mentioned previously, β_1 -AR activation stimulates Gs signaling to activate PKA as its major effector to phosphorylate numerous intracellular targets. For example, active PKA is identified to phosphorylate several key proteins involved in excitation-contraction coupling (ECC), such as the L-type Ca^{2+} channel, the ryanodine receptor and phospholamban (PLB) (Bers, 2002; Hagemann and Xiao, 2002; Valdivia et al., 1995; Xiao et al., 2007; Zhou et al., 2009). However, recent studies on the time-dependence activation of β_1 -AR show that additional signaling pathways can be evoked under sustained β_1 -AR stimulation. Indeed, sustained β_1 -AR stimulation results in a shift from PKA

activation to CaMKII activation, and active CaMKII is involved in cardiac apoptosis, fetal gene activation and phosphorylating PLB at threonine 17 site (different from the serine 16 phosphorylation by PKA) (Sucharov et al., 2006; Wang et al., 2004; Zhu et al., 2001).

It is well documented that β_1 -AR are desensitized and downregulated by internalization, resulting in reduced adrenergic response, a hallmark of deteriorating cardiac function and heart failure (Bristow et al., 1982; Brodde, 1996; Choi and Rockman, 1999; Fowler et al., 1986; Lohse et al., 2003). The desensitization of β -AR during hypertrophy is caused by increased β -adrenergic receptor kinase (β ARK), which phosphorylates the β -AR to initiate their internalization (Choi et al., 1997; Ungerer et al., 1993). In parallel, the expression and activity of PDE are decreased during hypertrophy, thus prolonging the cAMP signal and activity of β ARK to desensitize β -AR (Abi-Gerges et al., 2009; Senzaki et al., 2001; Takahashi et al., 2002).

In contrast to the downregulation of β_1 -AR, there is no significant reduction of β_2 -AR during heart failure (Engelhardt et al., 1996; Kiuchi et al., 1993). Hence, the downregulation of β_1 -AR could substantially alter the relative distribution of β receptors in the heart. Moreover, research in heart failure myocytes demonstrates that β_2 -AR mediated cAMP signaling is altered to resemble the β_1 -AR induced response, where cAMP signaling propagates throughout the myocyte and is no longer confined to local signaling (Nikolaev et al., 2006; Nikolaev et al., 2010). This is attributed partially by loss of T-tubulation in heart

failure myocytes. This causes the re-distribution of β_2 -AR within the myocyte and represents a mechanism to exacerbate heart failure.

6. Protein Kinase D in Hypertrophy

Background

Protein kinase D (PKD) was initially identified as PKC mu (PKC μ), as a member of the PKC family, by two different groups (Johannes et al., 1994; Valverde et al., 1994). However, it was subsequently characterized to be part of the CaMKII superfamily based on its catalytic function and substrate specificity (Manning et al., 2002; McKinsey, 2007). PKD is a serine/threonine kinase consisting of 918 amino acids. To date, three different isoforms (PKD 1, 2 and 3) have been reported in various cell types (Avkiran et al., 2008; Rozengurt, 2011). All three isoforms are highly conserved in their sequence homology and function (Avkiran et al., 2008; Wang, 2006). PKD1 is the isoform ubiquitously expressed throughout different tissues and the dominant isoform expressed in the heart. In general, PKD is emerging as a critical signal transducer of extrinsic stress signal to intracellular processes due to its implication in multiple cellular functions ranging from cell proliferation (Bollag et al., 2004; Ernest Dodd et al., 2005; Liu et al., 2007), immune signaling (Matthews et al., 2006; Spitaler et al., 2006), apoptosis (Johannes et al., 1998; Trauzold et al., 2003), Golgi formation (Ghanekar and Lowe, 2005), cancer regulation (Eiseler et al., 2009; Jaggi et al., 2007) to cardiac hypertrophy via transcriptional regulation of HDAC5 (Bossuyt et

al., 2011; Bossuyt et al., 2008; Harrison et al., 2006; Vega et al., 2004) and cardiac contractile function via altering its Ca^{2+} sensitivity (Cuello et al., 2007; Goodall et al., 2010; Haworth et al., 2004).

PKD Function

Like many other cellular kinases, PKD functions as a kinase that phosphorylates at the serine and threonine residues of its target substrates. PKD requires a particular substrate sequence for its targets. Specifically, relative to its targeted serine/threonine residue, it prefers an aliphatic amino acid at the -5 position and a basic amino acid (especially arginine) at the -3 position (Avkiran et al., 2008). Studies have suggested that PKD is a highly mobile kinase in many cellular compartments, such as cytosol, Golgi, mitochondria and the nucleus. Some of its targets include cardiac troponin I, histone deacetylase 5 (HDAC5), c-jun and cAMP-response element binding protein (Avkiran et al., 2008; Rozengurt, 2011; Wang, 2006).

With a diverse group of target substrates, PKD is involved in numerous cellular functions. With regards to its cardiac function, PKD has been reported to regulate hypertrophic signaling via its role in phosphorylating transcriptional repressor, HDAC5 (Bossuyt et al., 2011; Bossuyt et al., 2008; Harrison et al., 2006; Vega et al., 2004), and to modulate myocyte contractile function via its role in altering Ca^{2+} sensitivity of cardiac troponin I (Cuello et al., 2007; Goodall et al., 2010; Haworth et al., 2004). The importance of PKD activity during hypertrophy

has also been demonstrated in the several ways. For example, constitutively active PKD induces cardiac hypertrophic in transgenic mice (Harrison et al., 2006), and cardiac specific deletion of PKD1 in knockout mice display a reduced hypertrophic heart in response to pressure overload (Fielitz et al., 2008). Moreover, PKD expression and activity both are found to be increased in failing human and animal myocardium (Bossuyt et al., 2008; Harrison et al., 2006), validating its regulatory role in cardiac hypertrophy.

Specifically, my work focuses on the functional role of PKD in the heart. I will examine the role of PKD activation and its functional effects on hypertrophic signaling in response to neurohumoral stimuli. Understanding the underlying cellular mechanism of how PKD is activated and its function during stress stimulation in adult cardiac myocytes will strengthen its potential as a future therapeutic for cardiac disease treatment.

PKD Structural Domains

The structure of this kinase includes the N-terminal inhibitory region with two cysteine-rich domains, an internal pleckstrin homology (PH) domain and catalytic domain near the C-terminus (Avkiran et al., 2008; Fu and Rubin, 2011; Wang, 2006). The N-terminal region of PKD exhibits autoinhibitory effect on its catalytic activity since various mutations and deletions of this region can activate PKD to different degrees (Iglesias et al., 1998a; Iglesias et al., 1998b). PKD can be activated by several extrinsic stimuli, such as growth factors, GPCR agonists,

B cell/T cell receptors and phorbol esters (Fu and Rubin, 2011; Rozengurt, 2011; Rozengurt et al., 2005). It is involved in several important cellular functions from proliferation, differentiation, cell motility to protein transport, membrane trafficking, apoptosis and cell survival. An illustration of the PKD domains is depicted in Figure 3.

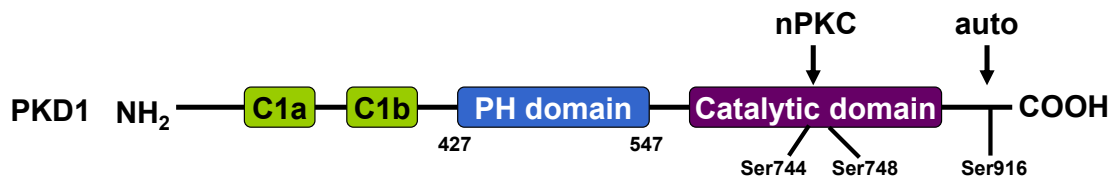


Figure 3. Schematic diagram of PKD1 domains.

The C1 domains of PKD are similar to those found in the PKC family and are designated as C1a and C1b domains. These tandem cysteine-rich domains impart reverse membrane translocation function via their ability to bind to diverse phospholipids (Colon-Gonzalez and Kazanietz, 2006; Gallegos and Newton, 2008; Valverde et al., 1994). Nevertheless, the relative lipid-binding affinity of these two C1 domains remains to be clarified. Some suggest that the C1a and C1b domains display equivalent binding properties (Irie et al., 1999) while other report disparate binding affinities (Iglesias et al., 1998a; Oancea et al., 2003; Wang et al., 2003). In addition, these two motifs contribute to the autoinhibitory effect of PKD activity, and such autoinhibition can be relieved by C1a and C1b

binding to intracellular DAG or phorbol ester. The tandem domains also display dissimilar characteristics in binding DAG and phorbol ester across all three isoforms. Jun Chen et al. characterizes individual C1a, C1b or C1a-C1b domain of PKD *in vitro*. Their findings suggest that C1a confers the majority of the DAG-binding ability. It should be noted that these two motifs have differential selectivity and ligand-binding affinities for DAG and phorbol ester on an individual basis. Accordingly, intact C1a and C1b domains together are sensitive to structural context to modulate their selectivity and ligand-binding affinities (Chen et al., 2008).

The PH domain is commonly found in proteins that mediate signal transduction or cellular trafficking (Liao and Hung, 2010; Mashanov et al., 2004). This domain is typically found to bind to phosphoinositol phospholipids. However, the role of PH domain within PKD to bind phosphoinositide is unclear since it shows low affinity for phospholipids (Wang et al., 2003). Thus far, the role of PH domain of PKD is reported to be autoinhibitory based on the evidence that tyrosine phosphorylation, protein interaction with itself or $G\beta\gamma$ or activation loop phosphorylation can trigger PKD activation (Jamora et al., 1999; Storz et al., 2003; Waldron and Rozengurt, 2003).

At its C-terminus, the last six amino acid residues of PKD1 and PKD2 makes up a motif that can bind to PSD-95, Disc large and ZO-1 (PDZ) domain, which is known to mediate protein interaction. This domain typically plays a part in protein targeting and complex assembly (Hung and Sheng, 2002). Its

functional role is achieved by interacting with the PDZ domain of other proteins or its PDZ recognition sequences. For example, the C-terminal amino acid sequence ERVSIL of PKD1 and ERISVL of PKD2 are PDZ-binding motifs (Sanchez-Ruiloba et al., 2006), which are absent in PKD3. This allows PKD1 and PKD2 to form potential protein-protein interactions with numerous cellular proteins with PDZ domains or PDZ-binding sequence although such interaction is largely unknown.

Accordingly, the S916 of PKD1 and S873 of PKD2 are at the -2 position relative to the PDZ-binding residue, thus phosphorylation at these two serines has significant impact in determining the intracellular translocation and localization of PKD1 and PKD2 via interaction with other PDZ domains. For instance, Kunkel et al. identified that PKD binds to the Na/H exchanger regulatory factor 1 (NHERF1) complex via PDZ domain interaction upon PdBu stimulation (Kunkel et al., 2009). The study indicates that PKD activation at its autophosphorylation site enhances its association with another molecular complex intracellularly. This adds another mechanism to modulate PKD activity via possibly tethering to any scaffolding complex, such as the NHERF1. Therefore, future findings of PKD interaction with other proteins via PDZ domain can provide useful information regarding its activation in the heart.

PKD Phosphorylation and Activation

In general, basal cellular PKD activity remains low due to its regulatory

autoinhibitory mechanism imposed by the N-terminal region. The canonical activation pathway of PKD is via a PKC-dependent mechanism (Rey et al., 2004; Waldron and Rozengurt, 2003; Wang, 2006) although a PKC-independent mechanism has also been described (Jacamo et al., 2008; Sinnott-Smith et al., 2009). The PKC-dependent mechanism triggers membrane recruitment of PKD along with upstream PKC isoforms attributed to DAG production and DAG-binding via C1a and C1b domains. Active PKC then cross-phosphorylates PKD near the plasma membrane at its serine 744 and serine 748 (SS744/748) sites within the catalytic domain of PKD. Consequently, SS744/748 phosphorylation of PKD relieves the autoinhibition from the regulatory N-terminal region, and the partially active PKD autophosphorylates itself at serine 916 (S916) residue. The fully active PKD subsequently undergoes reverse translocation to intracellular compartments to phosphorylate its substrate targets. The predominant PKC involved in this canonical PKD activation are the Ca^{2+} -independent novel PKC isoforms (δ , ϵ , η and θ) (Brandlin et al., 2002a; Brandlin et al., 2002b; Rey et al., 2004; Waldron and Rozengurt, 2003).

Conversely, the PKC-independent mechanism of PKD activation is also reported. This type of activation is proposed to occur by distinct autophosphorylation at the activation loop (Jacamo et al., 2008; Sinnott-Smith et al., 2009). The PKC-independent mechanism of PKD activation in cardiac myocytes remains to be explored. Lastly, there is also indirect evidence in immortal cell lines suggesting possible Ca^{2+} -dependent activation of PKD. The

study reported PKD activity upon addition of thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase pump and ionomycin, a Ca^{2+} ionophore. CaMKII inhibition by KN93 confirmed that the Ca^{2+} -induced response was specific to PKD and not CaMKII activation (Kunkel et al., 2007). The findings point to an interesting mechanism of PKD regulation by intracellular Ca^{2+} , however, further studies are required to dissect the nature and pathway of such regulation.

There is a significant decrease in PKD expression in the heart during development from neonates to adult myocytes (Haworth et al., 2000). While the studies above have uncovered certain PKD activation mechanisms, more work remains to be done in investigating the PKD activation in adult cardiac myocytes. In particular, my research will focus on dissecting the spatiotemporal PKD activation during the hypertrophic signaling of adult cardiac myocytes in response to neurohumoral stimulation.

7. Histone Deacetylase 5 in Hypertrophy

Background

Histone deacetylases (HDACs) are transcriptional repressors crucial to the regulation of gene transcription in many cell types (Haberland et al., 2009; Johnson and Turner, 1999; Martin et al., 2007; Martin et al., 2009). HDACs can preclude access to transcriptional factors via chromatin condensation and consequently suppress transcriptional activation of its target genes (Bacs and

Olson, 2006; Bush and McKinsey, 2009). As epigenetic regulators, HDACs are involved in multitude of cellular processes, such as proliferation, cardiac function, bone formation, vasculature formation and myogenesis (Haberland et al., 2009; Martin et al., 2009; Yang and Gregoire, 2005). Due to their role as essential modulators of gene transcription or repression, HDACs have many implications in physiology and disease development. Consequently, recent effort has been focused on inhibition of HDACs function and their potential use as clinical therapies for treating a wide range of disease (Haberland et al., 2009; McKinsey, 2011; McKinsey, 2012; Stimson et al., 2009).

To date, with 18 identified mammalian isoforms, the HDAC superfamily is divided into 4 distinct classes (class I, II, III and IV) with a relatively conserved catalytic domain (Bertos et al., 2001; Yang and Gregoire, 2005). Compared to other subtypes, class II HDACs (4, 5, 6, 7, 9 and 10) exhibit tissue-specificity with predominant expression in skeletal muscles, heart, brain and T-cells while class I and III are found more ubiquitously expressed in other cell types (McKinsey and Olson, 2005; Parra and Verdin, 2010; Yang and Gregoire, 2005). Additionally, class II HDACs are further grouped into class IIa (4, 5, 7 and 9) and IIb (6 and 10) and its members are characterized by a long N-terminal extension available for interactions with other transcriptional activators and co-repressors (Haberland et al., 2009). Lastly, class II HDACs are signal-dependent repressors, which can be subject to phosphorylation at particular residues in response to agonist stimulations (Parra and Verdin, 2010).

HDAC5 Function

The functional role of HDACs is to remove acetyl groups from histone tails, hence condensing the chromatin structure. Their function opposes the action of histone acetyl transferases (HAT), which results in relaxation of the chromatin structure by adding acetylation and encouraging transcriptional activity (Bacs and Olson, 2006; Bertos et al., 2001; McKinsey and Olson, 2005). Many *in vivo* studies have examined the functional role of HDAC5 in the heart and its consequent stress-induced exacerbated cardiac hypertrophy in knockout mice with a loss-of-function phenotype (Chang et al., 2004; Haberland et al., 2009; Kee and Kook, 2011), highlighting the importance of its cellular localization and function in regulating cardiac physiology and pathology. Specifically, fundamental regulation of HDAC5 is crucial to cardiac remodeling and hypertrophy at the cellular level, where activation of “fetal gene program” is initiated by relieving transcriptional inhibition to activate myocyte enhancer factor 2 (MEF2) controlled genes (Bertos et al., 2001; Calalb et al., 2009; Chang et al., 2004; Kee and Kook, 2011). Recent studies have focused on the understanding the trafficking of HDAC5 between the nucleus and the cytoplasm as its gene repression function is highly correlated with its localization within the cell (Harrison et al., 2010; Harrison et al., 2004; McKinsey and Olson, 2005; Wu et al., 2006; Zhang et al., 2002).

HDAC5 Structural Domains

HDAC5 belongs to the class IIa family of HDACs and shares highly conserved sequence homology with HDAC 4, 7 and 9 (Bertos et al., 2001; Martin et al., 2007; Martin et al., 2009). While several *in vivo* mouse knockout studies have demonstrated the importance of these class IIa members during development and disease, HDAC5 is the isoform most pertinent to cardiac pathophysiology since genetic deletion creates cardiac hypertrophy and ventricular defects in the knockout mouse (Bush and McKinsey, 2009). Like the rest of the class IIa members, HDAC5 contains an N-terminal MEF2-binding domain, followed by a nuclear localization sequence (NLS) with a catalytic deacetylase domain near the nuclear export sequence (NES) at the C-terminus. With its inherent NLS and NES signals, the cellular localization of HDAC5 in the nucleus or the cytosol can be altered depending on the current modification of either signal.

There are mixed evidence supporting the catalytic activity of HDAC5 in cardiac myocytes by itself. For example, some reports demonstrate that HDAC5 oligomerizes with HDAC4 to achieve its catalytic function to suppress gene transcription (Backs et al., 2008). Others also observe an association of HDAC3 with HDAC5 to account for the cellular deacetylase activity of HDAC5 (Greco et al., 2011). In contrast, transcriptional activation is also detected with nuclear export of HDAC5, relieving transcriptional inhibition of MEF2-dependent genes (Bossuyt et al., 2011; Bossuyt et al., 2008; Harrison et al., 2004; Lu et al., 2000a;

Lu et al., 2000b). Therefore, the functional role of HDAC5 remains to be further investigated in adult cardiac myocytes.

HDAC5 Phosphorylation and Regulation

Thus far, five phosphorylation sites of HDAC5 have been described to participate during its phosphorylation-dependent nuclear export mechanism. Compelling evidence have established that HDAC5 nucleocytoplasmic shuttling is highly regulated by its phosphorylation at serine 259 (S259) and serine 498 (S498) to initiate its phosphorylation-dependent nuclear export via binding to 14-3-3 chaperone proteins and unmasking its NES signal (Kee and Kook, 2011; McKinsey et al., 2000b; McKinsey et al., 2001). Accordingly, we and others have reported that neurohumoral stress stimuli coupled to activation of G-protein receptors can trigger downstream effector kinases, such as CaMKII and PKD to phosphorylate HDAC5 at S259 and S498 to mediate its nuclear export in cardiac myocytes (Bossuyt et al., 2011; Bossuyt et al., 2008; Ellis et al., 2003; Harrison et al., 2010; Harrison et al., 2006; Lu et al., 2000b; McKinsey and Olson, 2005; McKinsey et al., 2001).

Moreover, alternative phosphorylation sites of HDAC5 have also been introduced during its phosphorylation-dependent nuclear export. For instance, Carnegie et al. reports that serine 660 (S660) of HDAC5 is another site available for PKA and PKD phosphorylation to trigger nuclear export (Carnegie et al., 2008). In addition, serine 279 within the NLS region of HDAC5 was recently

discovered to be involved in PKA-induced phosphorylation to negatively modulate its nuclear export event (Ha et al., 2010). Threonine 292 (T292) with the NLS of HDAC5 is reported to be phosphorylated by protein kinase C-related kinase (PRK)-2 and PRK1 (Harrison et al., 2010).

Recent proteomics approach encourages further comprehensive study of phosphorylation sites of HDAC5 *in vivo* and reports a total of 17 sites that can or might dynamically regulate HDAC5 translocation and function (Greco et al., 2011). These findings strongly implicate the complexity of the fundamental regulation of HDAC5 trafficking within the cell via phosphorylation and association with other proteins. The dynamic shuttling of HDAC5 between the nucleus and the cytosol has been investigated due to its specific expression and function in cardiac myocytes, yet detailed mechanism of its regulated trafficking remains to be elucidated, especially during activation of multiple stress stimuli.

8. Conclusion

The heart is a complex organ that is structured and designed to serve its contractile function as the pump of the circulatory system by continuously supplying blood throughout the body. Its intrinsic conduction properties allow the synchronization of the electrical activity among the myocytes to produce one contraction after the other. The heart display remarkable adaptive responses to diverse stress stimuli in order to preserve its contractile function. When the heart is subject to any prolonged stress or hindrance to conduct electrical activity, its

function to eject blood will decrease over time.

These alterations to cardiac function will induce development of many cardiac diseases. The inherent compensatory mechanism of the heart to cope with its increased work load is by triggering myocyte growth, thus resulting in cardiac hypertrophy. It is well established that cardiac hypertrophy produces initial compensatory effects, but it deteriorates ventricular function over time and typically progress toward heart failure. With cardiac hypertrophy being the hallmark characteristic of worsening cardiac function, therefore it has become one of the major topics for cardiovascular research for the last fifty years.

With many cell line and neonatal studies laying the ground work of hypertrophic signaling pathways, many questions remain to be addressed in adult cardiac myocytes. Therefore, my dissertation work aims to explore the cellular mechanism of how hypertrophic signaling occurs in adult cardiac myocytes in response to neurohumoral stimuli. I will focus primarily on using fluorescence imaging techniques (confocal and FRET imaging) combined with some biochemical approaches to investigate the roles of PKD activation and HDAC5 translocation during hypertrophic signaling in adult cardiac myocytes.

CHAPTER 2
SPECIFIC AIMS

Aim 1: To determine the spatiotemporal activation of PKD by hypertrophic agonist, such as ET-1 and PE, in adult cardiac myocytes.

Hypertrophic agonists, ET-1 and PE, act on GPCR and activate phospholipase C- β , which produces two important 2nd messengers, IP₃ and DAG. PKD, along with CaMKII, has been identified as important mediators for hypertrophy and heart failure via the regulation of transcription repressor, HDAC5 (Bucks et al., 2008; Harrison et al., 2010; Harrison et al., 2006; McKinsey and Olson, 2005; Wu et al., 2006). Previous work by Bossuyt et al. showed that both CaMKII and PKD are upregulated in both expression and activity during heart failure (Bossuyt et al., 2008). In order to better understand the role of PKD activation during Gq-driven hypertrophic signaling and its potential as a therapeutic target, it is critical to examine its fundamental spatial and temporal activation within the myocytes.

My overall hypothesis is that ET-1 and PE induce different spatiotemporal patterns of PKD activation which may influence the role of PKD during hypertrophic signaling. We plan to evaluate intracellular PKD activation using multiple approaches. Since HDAC5 is a known target of PKD

and its nuclear export parallels fetal gene activation in adult myocytes, we will first measure HDAC5 nuclear export in response to ET-1 and PE stimulation. To further investigate the role of PKD in mediating Gq-driven HDAC5 nuclear export, we will use a number of pharmacological inhibitors (e.g Gö6976 and PKDI) to block PKD activity prior to ET-1 or PE treatment. To date, canonical PKD activation mechanism describes its phosphorylation by upstream PKC at S744 and S748 sites followed by PKD autophosphorylation at its S916 residue. Using immunoblotting and phospho-specific antibodies, we will also assess the level of its phosphorylation at S744 and S748 (PKC sites) and its auto-phosphorylation at S916 in response to ET-1 and PE stimulation.

To test whether PKD activation by ET-1 and PE results in its translocation within the myocyte, we will generate an adenoviral construct of wild type (WT) PKD1-green fluorescence protein (GFP) to measure its intracellular translocation using confocal microscopy. Specifically, we will focus on PKD translocation to the sarcolemma, where it is postulated to be phosphorylated by upstream PKC isoforms and to the nucleus, where it can phosphorylate and mediate HDAC5 nuclear export.

In parallel, we will also use a FRET-based sensor, D Kinase Activity Reporter (DKAR), to measure the PKD activity in response to ET-1 and PE treatment. The DKAR biosensor produces a decrease in FRET signal when a conformational change occurs due to active PKD phosphorylating its pseudo substrate peptide flanking the cyan fluorescence protein (CFP) and yellow

fluorescence protein (YFP). After we have measured the spatial dynamics of PKD activation in confocal translocation studies, we can assess its localized activity using targeted DKAR, such as the sarcolemmal DKAR (DKAR-MyrP) and nuclear DKAR (DKAR-NLS) to measure its activity near the plasma membrane and in the nucleus respectively.

Overall, aim 1 will investigate the spatiotemporal activation of PKD in response to hypertrophic agonist, ET-1 and PE in adult cardiac myocytes. Global PKD activation will be assessed by its phosphorylation level at S744/S748 and at S916, its auto-phosphorylation site. Confocal translocation study will provide spatial dynamic data of real time PKD activation within the myocytes. Results of FRET biosensor, DKAR, will measure its global activity during ET-1 and PE stimulation. Specifically, we will contrast its global activation with its local activation signals obtained in two particular compartments, the sarcolemma and the nucleus, where PKD can be activated by upstream PKC and can mediate HDAC5 nuclear export respectively. Understanding the fundamental spatiotemporal activation of PKD within the myocyte will improve future therapeutic strategy in treating hypertrophy and heart failure.

Aim 2: To examine the cross-interaction of Gq and β -AR signaling in adult cardiac myocytes at the level of HDAC5 shuttling between the nucleus and the cytosol

Previous work from our lab (Bossuyt et al., 2011; Bossuyt et al., 2008; Wu et al., 2006) shows that Gq-signaling triggers HDAC5 nuclear export during hypertrophy. However, the role of PKA signaling in HDAC5 localization in adult cardiac myocytes remains unclear. There is evidence suggesting that PKA activation may facilitate hypertrophic signaling of PKD and HDAC5 nuclear export (Carnegie et al., 2004; Carnegie et al., 2008) while some report that PKA signaling exerts an anti-hypertrophic effect to inhibit HDAC5 nuclear export (Ha et al., 2010; Sucharov et al., 2011). During hemodynamic stress, catecholamines and neurohumoral stimuli may induce co-activation of Gq-coupled receptors and β -AR leading to cardiac remodeling. Dynamic regulation of HDAC5, a transcriptional repressor, is crucial during stress signaling due to its role in epigenetic control of fetal gene markers. Little is known about its regulation during acute and chronic β -AR stimulation and its cross interaction with Gq-signaling in adult cardiac myocytes.

My overall hypothesis is that β -AR signaling regulates the Gq-driven transcriptional control at the level of HDAC5 nucleocytoplasmic shuttling in adult cardiac myocytes. First, we will use confocal microscopy to determine HDAC5 translocation under Gq-agonist, ET-1 and PE, and β -AR/PKA activation using isoproterenol (ISO) and forskolin. The results of β -AR and PKA signaling

on HDAC5 translocation will be compared to the previous work from our lab where Gq-agonist mediates its nuclear export. S259/S498 residues of HDAC5 have been well characterized to be the major targets of CaMKII and PKD to mediate its phosphorylation-dependent nuclear export. We plan to create a mutant with alanine substitution at these two sites and test its response to β -AR/PKA and Gq-signaling. While the S279 site of HDAC5 has been recently identified to be phosphorylated by PKA *in vitro* (Ha et al., 2010), we will generate a non-phosphorylatable mutant (S279A) and a phosphomimetic mutant (S279D) to test its translocation under β -AR and Gq-activation.

In addition, we will also pretreat myocytes with ISO and forskolin for 20 min prior to Gq-stimulation by ET-1 or PE to examine the effect of PKA activation on Gq-driven HDAC5 nuclear export. To complement the results of the confocal HDAC5 translocation, we will also use western blots to determine the level of endogenous HDAC5 S498 phosphorylation and co-immunoprecipitation (co-IP) to probe its binding to chaperone protein, 14-3-3, under Gq-agonist treatment alone or pre-incubation with ISO or forskolin.

To further dissect the mechanism of PKA action on hypertrophic signaling, we will also study the effects of acute vs. chronic treatment of forskolin on Gq-mediated HDAC5 nuclear export. Understanding the acute vs. chronic PKA stimulation is important in terms of cardiac physiology regulation. Acute PKA signaling in short term sympathetic tone, such as during exercise or fight or flight response, can increase cardiac output without triggering transcriptional changes.

In contrast, it is also well documented that chronic β -AR signaling alters cardiac function and results in maladaptive changes that could lead to hypertrophy and heart failure. Therefore, it is important to compare the difference between acute vs. chronic PKA signaling on cardiac (patho)physiology regulation. Lastly, we will examine the effects of Gq and β -AR/PKA signaling using heart failure (HF) rabbit myocytes to better understand the cellular mechanism of HDAC5 trafficking between control and HF adult myocytes. Since β -AR response is well known to be downregulated during HF, any differential regulation of HDAC5 trafficking between control and HF myocytes will represent an important physiological epigenetic control by PKA via HDAC5 shuttling.

In summary, this aim will investigate the cross-interaction between Gq and β -AR signaling at the level of HDAC5 translocation between the nucleus and the cytosol. In particular, we will contrast any differences between regulation of acute vs. chronic PKA signaling on Gq-driven transcriptional control via HDAC5 shuttling.

Aim 3: To determine the modulatory role of β -AR/PKA activation on PKD activation and its consequent effects on hypertrophic signaling

Neurohumoral signaling can activate PKD and CaMKII to mediate cardiac hypertrophic responses. These two kinases have been previously reported to have increased expression and activity in HF (Bossuyt et al., 2008). CaMKII and PKD can both phosphorylate the transcriptional repressor, HDAC5, to mediate its phosphorylation-dependent nuclear export (Bucks et al., 2008; Bossuyt et al., 2011; Harrison et al., 2010; Harrison et al., 2006; Wu et al., 2006). We have recently uncovered that β -AR/PKA activation alone can trigger nuclear accumulation of HDAC5 and also blocks Gq-driven HDAC5 nuclear export if followed by ET-1 or PE treatment (unpublished data). The data suggest that acute β -AR/PKA signaling is protective against hypertrophic response by Gq-mediated transcriptional activation. One possible mechanism by which β -AR/PKA might inhibit HDAC5 nuclear export is by inhibiting upstream PKD activation to block HDAC5 phosphorylation-dependent export.

My overall hypothesis is that β -AR signaling or PKA activation prevents HDAC5 nuclear export in part by limiting upstream PKD activation by Gq-agonist, ET-1 and PE. The role of PKA in PKD activation in adult cardiac myocytes is still unclear. There is evidence in immortal cell lines demonstrating that PKA activation potentiates PKD activation via AKAP-Lbc complex (Carnegie et al., 2004; Carnegie et al., 2008). Conversely, other reports indicate a counter regulatory role of PKA on PKD activation, describing an

inhibitory effect of PKA (Haworth et al., 2011; Haworth et al., 2007; Sucharov et al., 2011). There is little information regarding this PKA-PKD crosstalk in adult ventricular myocytes.

First, we will determine if β -AR or PKA activation has any effect on PKD activation in adult myocytes using GFP-tagged PKD1 adenoviral construct since canonical PKD activation is characterized by its initial sarcolemmal translocation followed by nuclear translocation (Bossuyt et al., 2011). In addition, we will also probe for its S916 auto-phosphorylation using a S916 phospho-specific antibody to measure PKD activation under β -AR/PKA or Gq-agonist stimulation. We will also use various DKAR constructs (general, membrane-targeted and nuclear) to determine global and local PKD activity in response to β -AR/PKA activation and Gq-agonist stimulation. We will also compare any difference in PKD activation between Gq-agonist alone with pre-activation of β -AR/PKA.

Secondly, *in silico* analysis suggests that there exists a potential consensus PKA phosphorylation site within the PKD sequence at serine 427 (S427), and this site was also recently reported to be a direct PKA phosphorylation target using a peptide screening array (Smith et al., 2011). To further dissect the mechanism of PKA modulation on PKD activation, we will create a mutant with an alanine substitution (S427A) or a glutamate substitution (S427E) to test if PKA modulation on PKD activation is altered due to presence of a non-phosphorylatable or phospho-mimetic mutation.

First, we will examine whether GFP-tagged PKD mutants, S427A or

S427E, have altered translocation in response to ET-1 and PE with or without ISO and forskolin pretreatment. The non-phosphorylatable mutant, S427A, would abolish any PKA effects on PKD translocation if this residue was indeed the PKA phosphorylation site. Conversely, S427E phosphomimetic mutant should recapitulate PKA phosphorylation effect on PKD translocation in response to ET-1 or PE in the absence of ISO or forskolin pretreatment.

Moreover, to study the cross-interaction of PKA-PKD signaling, I plan to infect adenoviral constructs of GFP-tagged WT PKD, S427A and S427E variants in adult cardiac myocytes and treat with ISO or forskolin. Using a GFP pull-down method, I will compare the levels of PKA phosphorylation across all three variants using two different PKA substrate phospho-antibodies. This assay will reveal any difference in PKA phosphorylation of PKD in adult myocytes in response to ISO or forskolin. If we observe any significant difference in PKA substrate phosphorylation levels between the WT or mutant variants, the results will suggest that S427 is the major PKA target site. Conversely, if we do not detect any appreciable difference between the WT or the mutant, the data would indicate that there could be alternative or additional PKA target sites on PKD. In addition, PKA could also modulate PKD activity in an indirect manner via another molecular partner, such as AKAP-Lbc, which has been reported to be a critical scaffolding complex to play a role in multiple signaling pathways (Appert-Collin et al., 2007; Carnegie et al., 2004; Carnegie et al., 2008; Diviani et al., 2011).

In conclusion, the results of this study will provide information on PKA

modulation on spatiotemporal activation of PKD in adult cardiac myocytes. The crosstalk between PKA-PKD signaling pathways can be critical in future therapeutic design for hypertrophy and heart failure treatment where PKA response is downregulated (Bristow et al., 1982; Choi and Rockman, 1999) and PKD expression and activity are increased (Bossuyt et al., 2008).

CHAPTER 3

MATERIALS AND METHODS

The overall molecular cloning techniques and laboratory procedures are described in this chapter. Critical experiments pertinent to the results of each major chapter are included in individual chapters.

1. Site-Directed Mutagenesis

We obtained the wildtype (WT) human PKD1 DNA plasmid from Dr. Eric Rozengurt (University of California – Los Angeles). The adenovirus was kindly generated by Dr. Jody Martin (Loyola University Chicago). General D kinase activity report (DKAR) was purchased from Addgene (plasmid 14879). Our collaborator, Dr. Alexandra Newton (University of California – San Diego), generously provided us with the targeted DKAR plasmid for sarcolemmal and nuclear DKAR. We inserted the DKAR into pShuttle-CMV vector to generate adenovirus (figure 4 and 5).

We obtained both the GFP-tagged WT HDAC5 and HDAC5 SS259/498AA adenovirus from Dr. Eric Olson (University of Texas – Southwestern). MEF2 luciferase reporter adenovirus was a gift from Dr. Jeff Molkenin (University of Cincinnati – Children’s Hospital).

We created the HDAC5 S279A and S279D mutants by site-directed mutagenesis using Quik Change II XL Lightning kit (Agilent, catalog 200521). The primers used for HDAC5 S279A and S279D are included in the table below. We excised the plasmid from its pcDNA backbone and inserted into psh-CMV using Hind III and Xba I (Figure 6).

HDAC5 primers	
S279A-F	5'-ctgagcggagaagcgctcccctcctgcgtc-3'
S279A-R	5'-gacgcaggaggggagcgcttctccgctcag-3'
S279D-F	5'-ctgagcggagaagcgatcccctcctgcgtc-3'
S279D-R	5'-gacgcaggaggggatcgcttctccgctcag-3'

2. Bacterial Transformation

For DNA amplification, the plasmid DNA was transformed into chemically competent DH5 α bacterial cells (Invitrogen). The bacterial cells were thawed on ice. 5 μ l of plasmid DNA was added into 50 μ l of bacterial cells and incubated on ice for 30 min. The mixture was heat shocked at 37°C for 45 s immediately followed by addition of 350 μ l of S.O.C medium (Invitrogen). The tubes were placed on mechanical shaker at 225 rpm in 37°C warm room for 1 hr. 100 μ l of bacterial mixture was plated on agar plate with ampicillin (100 μ g/mL) or kanamycin (50 μ g/mL) near an open flame and incubated at 37°C overnight.

3. Bacterial Plasmid Isolation

a) *Small plasmid isolation (Promega PureYield™ Plasmid Miniprep System)*

The small DNA plasmid was isolated according to the manufacturer's protocol. 1.5 mL of overnight culture was centrifuged for 1 min, and cell pellet was re-suspended with 250 μ l of cell resuspension solution. 250 μ l of cell lysis solution was added to each sample and inverted 4 times to mix. 10 μ l of alkaline protease solution was added to each sample and inverted 4 times to mix. The samples were incubated for 5 min at room temperature. 350 μ l of neutralization solution was added and mixing by inverting 4 times. The mixture was centrifuged at top speed for 10 min at room temperature. Vacuum adapters were attached to manifold port and spin columns were inserted into adapter. Cleared lysates were decanted into the column followed by applying vacuum to pull liquid through column. 750 μ l of wash solution as added and this step was repeated with 250 μ l of wash solution. The columns were dried by applying vacuum for 10 min. To elute the DNA, the columns were transferred to a 1.5 mL eppendorf tubes. 50 μ l of nuclease-free water was added to the column and centrifuged at top speed for 1 min at room temperature. The column was discarded and plasmid DNA was stored at -20°C.

b) *Large Plasmid Isolation (Promega PureYield™ Plasmid Midiprep System)*

The large plasmid isolation was prepared according to the manufacturer's protocol. 100 mL of overnight bacterial culture was pelleted at 5,000 x g for 10 min. The cell pellet was re-suspended with 3 mL of cell resuspension solution. 3 mL of cell lysis solution was added and tubes were inverted 3 times to mix. The mixture was incubated for 3 min at room temperature. 5 mL of neutralization solution was added and tubes were inverted 5 times to mix. The cell lysates were centrifuged at 15,000 x g for 15 min at room temperature. A column stack was assembled by placing a blue PureYield™ clearing column on top of a white PureYield™ binding column and attached to a vacuum manifold. The cleared lysate was poured into the clearing column followed by applying vacuum to pull the liquid through both columns. The vacuum was released from the filtration device and clearing column was removed, leaving the binding column behind. To wash the DNA, 5 mL of endotoxin removal wash was added to the binding column and vacuum was applied to pull the solution through. 20 mL of wash solution was added to the binding column and allowed to pull through using the vacuum. The vacuum was applied until the membrane was dried and binding column was removed the vacuum manifold. To elute the DNA, 1.5 mL microcentrifuge tube was inserted into the base of the Eluator™ vacuum elution device (Promega, catalog A1071) and the device was assembled onto the vacuum manifold with the binding column stack on top. 500 µl of nuclease-free water was added to the DNA binding membrane in the binding column followed by applying vacuum for 1 min or until all the liquid was pulled through the column. The microcentrifuge was removed from the device and DNA was stored at -20°C.

4. Adenoviral Generation

Phase I: Subcloning into pShuttle-Cytomegalovirus (psh-CMV)

DNA plasmid and pShuttle-CMV were digested with restriction enzymes (New England Biolabs). The digested DNA fragment was inserted into the multiple cloning site (MCS) of linearized pShuttle-CMV in a ligation reaction incubated at 16°C overnight. The next day, the ligation product was transformed into DH5 α chemically competent cells. 100 μ l of bacterial mixture was plated on agar plate with kanamycin (50 μ g/mL) and incubated at 37°C overnight. The next day, colonies were picked to grow mini-cultures in 2 mL of Luria-Bertani (LB) media with kanamycin (50 μ g/mL) at 37°C overnight. Small plasmid DNA was isolated from the bacterial cultures using PureYield™ Plasmid Miniprep System (Promega) according to manufacturer's instructions. The eluted DNA was screened using Pac I enzyme (New England Biolabs) and cut at 37°C for 1.5 hr. The digest was loaded with 6x loading dye (Promega) and run on a 0.8% agarose gel at 130 V for 45 min. Positive samples should consist of two bands: a 3 kb band and a larger band (= 4.5 kb + insert size).

Phase II: Generation of Adenoviral DNA

Once subcloned into pShuttle-CMV, positive construct (2 μ g) was linearized with Pme I (New England Biolabs) at 37°C for 1.5 hr. The DNA was run on a 0.8% agarose gel to confirm linearity. The linear band was excised from agarose gel and purified using Wizard SV Gel and PCR Clean up System

(Promega). Once gel-purified, the DNA was transformed into BJ5183-AD-1 electroporation-competent cells (Invitrogen) using an electroporator (200 Ω , 2.5 kV, 25 μ F) (Bio-Rad). The next day, the plate should yield in varying sizes of colonies with large, medium and small colonies. Since recombination occurs at a low rate, the tiny colonies were chosen to grow mini-cultures, and bacterial plasmid DNA was isolated using Qiagen PREP SPIN miniprep kit (catalog 27106). The plasmid DNA was screened using Pac I and should yield in two bands with a 3 kb band and a larger band (4.5 kb + insert). The recombinant adenoviral DNA was transformed into chemically competent DH5 α cells and screened with Pac I.

5. Adenoviral Plaque Generation and Amplification

The recombinant adenoviral DNA (10 μ g) was digested with Pac I at 37°C for 2 h and cleaned using Wizard SV Gel and PCR Clean up System (Promega). To begin generation of adenoviral plaques, 100 μ l of plain D-glucose Minimal Essential medium (DMEM) was added to the Pac I-digested adenoviral DNA followed by adding 50 μ l of polyfect (Qiagen, catalog 301107). The mixture was perturbed by tapping gently and incubated at room temperature in the culture hood for 5-10 min. The total 200 μ l solution mixture was added drop by drop to the 80% confluent human embryonic kidney (HEK) cells in 6 cm dishes. The dishes were incubated at 37°C incubator. The next day, media was aspirated and replaced with 3 mL of DMEM with 2% FBS and 5% penicillin/streptomycin. The plaques with cytopathic effect (CPE) should become visible after 5 days in the

monolayer of HEK cells using a phase-contrast microscope. The 6 cm HEK cells should lyse completely after approximately 14 days. The dish was scraped and transferred onto 10 cm dish to begin amplifying the adenoviral DNA. Once the 10 cm dish was completely lysed, the mixture was collected in 50 mL falcon tubes and clarified by centrifugation at 2,000 rpm for 5 min. The supernatant was added to thirty 10 cm dishes of 80% confluent HEK cells in DMEM with 2% fetal bovine serum (FBS) and 5% penicillin/streptomycin. The HEK cells were harvested after 2-3 days and pelleted at 2,000 rpm for 5 min. The virus pellet was collected into one tube using 4 mL of 1x phosphate buffered saline (PBS) and flash-frozen in liquid nitrogen. The excess adenoviral solution (50 mL) was kept at -20°C for future amplification.

6. Adenoviral DNA Purification

The adenovirus pellet was extracted using 3 freeze/thaw cycles and resuspended using a vortex mixer. The mixture was pelleted at 4,000 rpm for 5 min, and 1 mL of supernatant was aliquoted into eppendorf tubes. The supernatant was clarified further by centrifuging at 14,000 rpm for 10 min at 4°C to separate the cell debris from the adenoviral particles. The adenoviral DNA was purified using a cesium chloride (CsCl) gradient method. To begin gradient purification in Beckman centrifuge tube, 1.2 g/mL CsCl was added first to the bottom, followed by addition of 1.45 g/mL CsCl to the bottom of the tube without disturbing the top layer. The volume ratio of 1.2 g/mL to 1.45 g/mL should be a little less than 2:1.

Once complete, there should be a grey ring formed between the two density layers of CsCl. The adenoviral supernatant was added to the top of CsCl gradient leaving 0.5 inches on the top. The Beckman centrifuge tubes were balanced before inserting into Beckman ultra centrifuge tubes and centrifuged at 22,000 rpm for 2.5 hr at 4°C. The mature adenovirus was collected by extracting the particles using a 22G needle and injected into Slide-A-Lyzer Dialysis Cassette (with molecular weight (M.W.) cutoff at 10,000 kDa). The cassette was placed in 1x PBS dialysis buffer at 4°C overnight followed by replacement of fresh 1xPBS for additional 2 hr the next day to remove the CsCl. The adenovirus was extracted from the cassette using a 22G needle once dialysis was complete and kept at -80°C with 10% glycerol.

7. Rat Cardiomyocyte Isolation

We isolated ventricular myocytes from rat (Sprague Dawley purchased from Charles River Laboratories) as described previously (Bassani et al., 1994). All animal procedures performed were in accordance to Institutional Animal Care and Use Committee regulations at University of California – Davis. The rat cell isolation was performed by our technicians, Khanhha Dao and Matthew Stein. The rat was injected with 0.3 mL (1,000 units) heparin subcutaneously 30 min prior to 1.4 mL Nembutal (50 mg/mL). The enzyme and 0 Ca²⁺ minimal essential medium (MEM) solutions were pre-warmed in 37°C water bath. 5 mL of the enzyme with MEM solution was set aside in a beaker in the shaker bath. 15 mL of 0 Ca²⁺ MEM solution and 0.2 mL heparin in each of three 20 mL beakers and 5 mL of 0

Ca^{2+} MEM solution in a syringe on ice. A connector attached to the cannula was affixed to the perfusion set-up. The suture was loosely fit around the cannula in preparation of tying the aorta. The perfusion apparatus was primed with 0 Ca^{2+} MEM solution to eliminate air bubbles. Before beginning surgery, rat's eyes and footpad reflexes were checked and tested. The chest area was soaked with ethanol followed by pinching and cutting away the skin. The ribs were opened with reinforced titanium scissors to create a flap. The heart was removed by cutting the aorta and washed by sequentially transferring it through 0 Ca^{2+} MEM solution beakers on ice (3x). The heart was quickly cannulated and perfused with 0 Ca^{2+} MEM solution for 3-5 min. The heart was perfused with enzyme solution. After first minute of enzyme perfusion, the enzyme solution was collected and recycled. Total enzyme perfusion is about 12 min. The heart (ventricles only) was cut to be released from the cannula leaving the atria behind. The ventricles were placed in a petri dish with 15 mL of enzyme solution and triturated carefully. 20 mL of bovine serum albumin (BSA) solution was added and cell pellets were re-suspended for 1 min using a wide-bore Pasteur pipette. The cell mixture was filtered through 210 μm mesh in three 15 mL falcon tubes. The solution was centrifuged for 1 min and supernatant was removed. The remaining pellet was re-suspended in a mix of BSA solution with final wash solution (1:1 ratio) and filed through 210 μm mesh in two 15 mL falcon tubes (if necessary). The filtrate was allowed to rest for 10 min. The supernatant was removed and re-suspended in 7 mL of final wash solution. The cells were gravity settled for 5 min (2x). The supernatant was removed the re-suspended in 8 mL of final wash solution before

plating.

8. Rabbit Myocyte Isolation

We isolated ventricular myocytes from adult New Zealand white rabbit (purchased from Charles River Laboratories) as described previously (Bassani et al., 1994). All animal procedures used were in accordance to Institutional Animal Care and Use Committee regulations at Loyola University Chicago and University of California – Davis. The cell isolation procedure was performed by technicians Charles Wilkerson, Khanha Dao and Matthew Stein. The Langendorff perfusion system was rinsed with Baxter purified water (3x) and 37°C water bath was turned on. Adult New Zealand white rabbit was retrieved and gently placed in restrainer. The rabbit was heparinized with 0.16 c.c of heparin (50,000 units/mL) subcutaneously for 30 min. During this period, the following items were prepared as follows: 40 mL of initial wash solution was poured into three 100 mL beakers, and two 60 mL syringes were also filled with initial wash solution and chilled on ice for 30 min prior to surgery. The remaining initial wash solution was poured into the Langendorff system, and oxygen was turned on. After 30 min of heparin incubation, 3.6-4.2 mL of Nembutal/sodium pentobarbital was obtained in a 5 mL syringe and attached to a 27G butterfly needle. The drug was injected intravenously into the rabbit ear. The rabbit was checked for vital response (by pinching toe on the footpad) and placed with a gas mask of 100% O₂ to maintain oxygenation of the blood if it was unconscious. The chest area of the rabbit was sprayed with 70% ethanol immediately followed by opening of the chest with

scissors. Once the chest was opened, the diaphragm was cut to remove the beating heart from the pericardial sac. The heart was quickly rinsed in beakers containing chilled initial wash solution. The heart was promptly cannulated and secured tightly. The cannulated heart was flushed using 60 mL syringes with chilled initial wash solution. Prior to clamping, pooled blood within the heart was removed using a transfer pipette. Any opening vessels were clamped close while allowing initial wash to run through. Collagenase enzyme was added and allowed to digest the heart tissue for approximately 28 min. During the enzyme digestion, the heart should remain pink, soft but firm. It was observed for any changes in texture. Once the digestion was complete, the heart was taken down, cut into pieces and carefully triturated. The BSA solution was poured into the diced heart mixtures. Any loose cells were removed and more BSA was added. The cells were incubated in warm water bath for 2 more min. This was repeated one more time. The remaining BSA was used to triturate the residual tissue to extract extra cells. The myocytes were spun down using a hand centrifuge for 5 min. The supernatant was filtered to remove excess fat tissue. The cell pellet was resuspended in 20 mL of final wash solution and extra BSA. The cells were allowed to gravity settle for 10 min, and the supernatant was removed. The pellet was resuspended further in 20 mL of final wash and gravity settled for 5 min. The supernatant was removed and remaining cell pellet was resuspended in final wash before plating on petri-dishes.

9. Heart Failure Animal Model

Heart failure was induced in adult New Zealand White rabbit as described previously (Pogwizd S et al. 1999). Briefly, HF was induced by two sequential survival surgeries by first inducing aortic insufficiency and after 2-8 weeks post-op monitoring, followed with aortic constriction. Each rabbit was monitored with echocardiogram to determine the severity of the HF status (typically 4-6 months) and scheduled for isolation procedure accordingly.

10. Cell Culturing

Acutely isolated adult ventricular myocytes were collected in a falcon tube and allowed gravity settled. The supernatant was aspirated and replaced with warm PC-1 medium (Lonza) with 5% penicillin/streptomycin and supplement. 2-well Borosilicate coverglass chambers (Fisher Scientific, catalog 155380) were coated with laminin mixture and allowed to air-dry. Myocytes were re-suspended using fresh PC-1 medium, and 1.5 mL of cell resuspension was added into each well. The myocytes were incubated at 37°C incubator for attachment for 45 min. The cells were gently washed and incubated with fresh PC-1 medium before addition of adenovirus (multiplicity of infection, MOI = 10-100) and incubated at 37°C overnight.

11. Confocal Imaging

2-well Borosilicate coverglass chamber was mounted on an inverted microscope (Zeiss, LSM5 Pascal or Olympus) equipped with 40x water

immersion object lens. Argon laser (3-5%) was used to excite GFP at 488 nm, and emission wavelength was collected with long-pass filter above 505 nm. A schematic of confocal LSM 5 is illustrated in figure 7. PC-1 medium was replaced with normal tyrode solution (10 mM glucose, 140 mM sodium chloride (NaCl), 1 mM magnesium chloride (MgCl₂), 4 mM potassium chloride (KCl), 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) containing 1.8 mM Ca²⁺ before each experiment. Cells were imaged before and after treatment with agonists.

National Institute of Health (NIH) Image J software was downloaded from its website: <http://rsbweb.nih.gov/ij/download.html> and was used for image analysis. A similar analysis method was used for measuring sarcolemmal and nuclear translocation of PKD1-GFP and nucleocytoplasmic shuttling of HDAC5. For example, the nucleus was traced with region of interest (ROI) to measure its fluorescence intensity and divided by the average of three randomly selected ROIs of equal size in the cytoplasm to compare the nuclear vs. cytosolic signal. Each $F_{\text{nuc}}/F_{\text{cyto}}$ ratio was normalized to initial time point (time 0) for comparison before and after drug treatment and between different data groups.

12. Immunoblotting

The samples were loaded onto 4-20% gradient SDS-PAGE gels (Biorad) and subject to electrophoresis at 90V for 2 h. Upon completion, the gels were transferred onto nitrocellulose membranes in 1 L of transfer buffer (125 mL of electroblot stock, 20% methanol and 675 mL of cold water) at 20 V in 4°C cold

room overnight. Once transfer was complete, the nitrocellulose membrane was rinsed with water (2x) followed by 1x tris buffered saline (TBS) (2x) for 5 min. The membrane was incubated with blocking solution (0.2% Tween, 8% milk, 1x TBS) for 1 hr and rinsed with 1x TBST (tris buffered saline tween) (3x). The membrane was cut and incubated with primary antibody in 1x TBST at optimal concentration at 4°C overnight. The antibody used for each experiment was included in each chapter. The primary antibody was washed off using 1x TBS (5 min), 1x TBST (2 x 10 min), 1x TBS (5 min) and 1x TBST (10 min). The membrane was then incubated with appropriate secondary antibody (goat anti-rabbit Immunoglobulin-G (IgG) or anti-mouse IgG conjugated with horseradish peroxidase (HRP)) at 1:5000 dilution with 0.2% milk in 1x TBST at room temperature for 1 hr. The secondary antibody was washed off using 1x TBS (5 min), 1x TBST (2 x 10 min), 1x TBS (5 min) and 1x TBST (10 min). The membrane was developed using West Dura (Pierce, catalog 34076) for 5 min and imaged with ImageReader LAS 4000. Densitometry analysis was performed using Image J software (NIH).

13. Statistical Analysis

Pooled data are represented as mean \pm standard error of measure (SEM). Statistical significance was determined using either Student's t-test (unpaired) or one way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. * p < 0.05 was considered statistically significant.

DKAR & Targeted- DKAR in pcDNA3.1 (+)

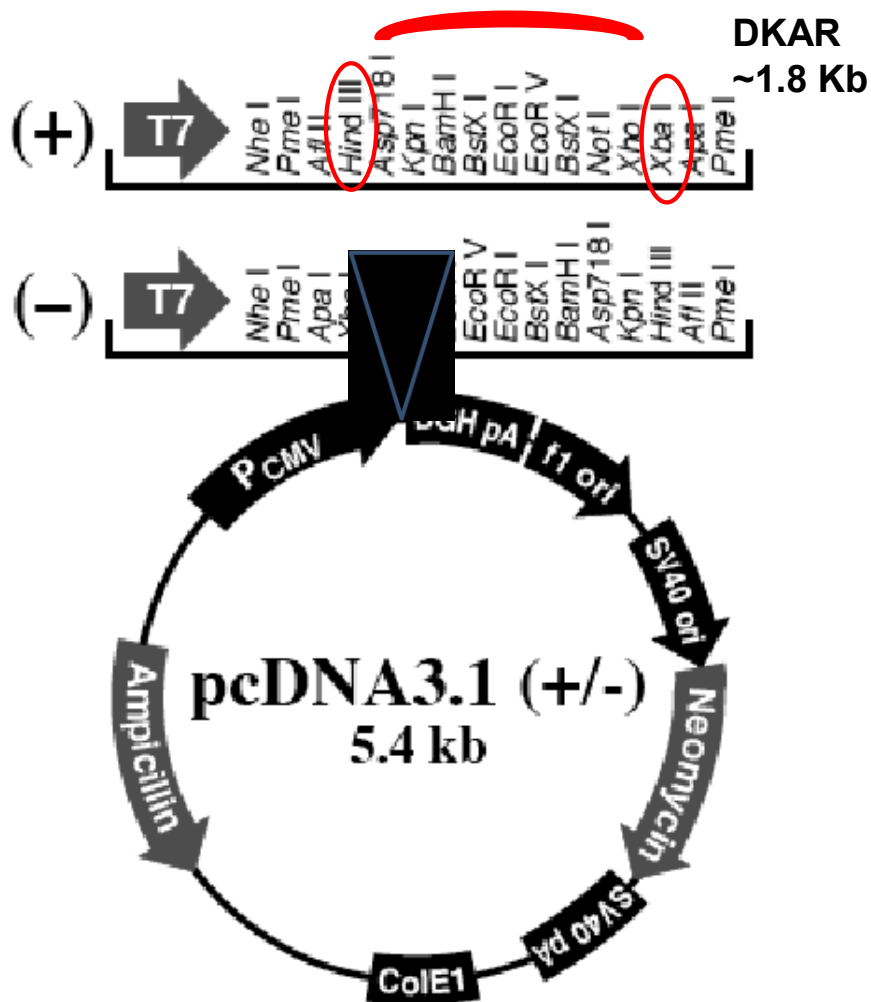
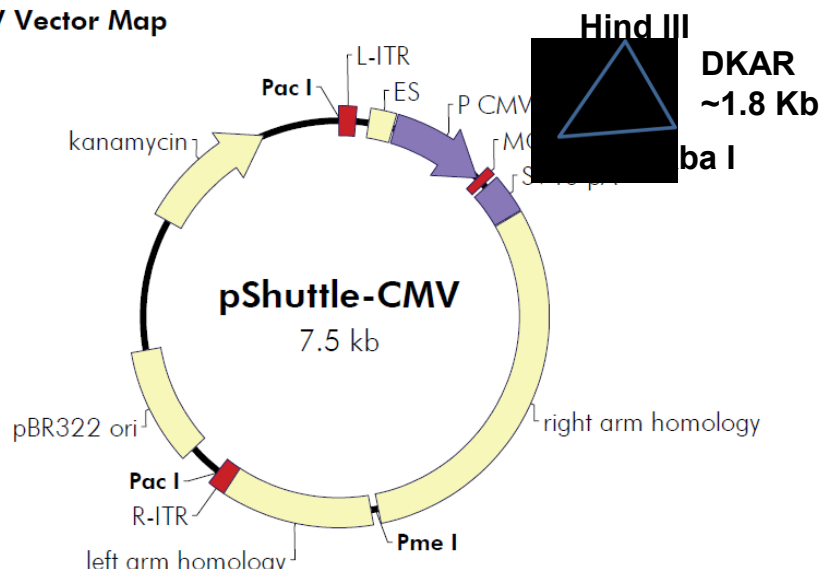


Figure 4. Map of DKAR in pcDNA. Construct map of DKAR in pcDNA vector.

DKAR & Targeted- DKAR into pshCMV (DAM-)

pShuttle-CMV Vector Map



pShuttle-CMV Multiple Cloning Site Region (sequence shown 888–1031)

Forward primer binding site

GGTCTATATAAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAG...

...AGATCTGGTACCGTCGACGCGGCCGCTCGAGCCTAAGCTTCTAGATAAGATATC...

Reverse primer binding site

...CGATCCACCGGATCTAGATAACTGATCATAATCAGCCATACCAC

Figure 5. Map DKAR in psh-CMV vector. We inserted DKAR into psh-CMV using Hind III and Xba I restriction enzymes.

HDAC5-S279A/D into psh-CMV (Dam-)

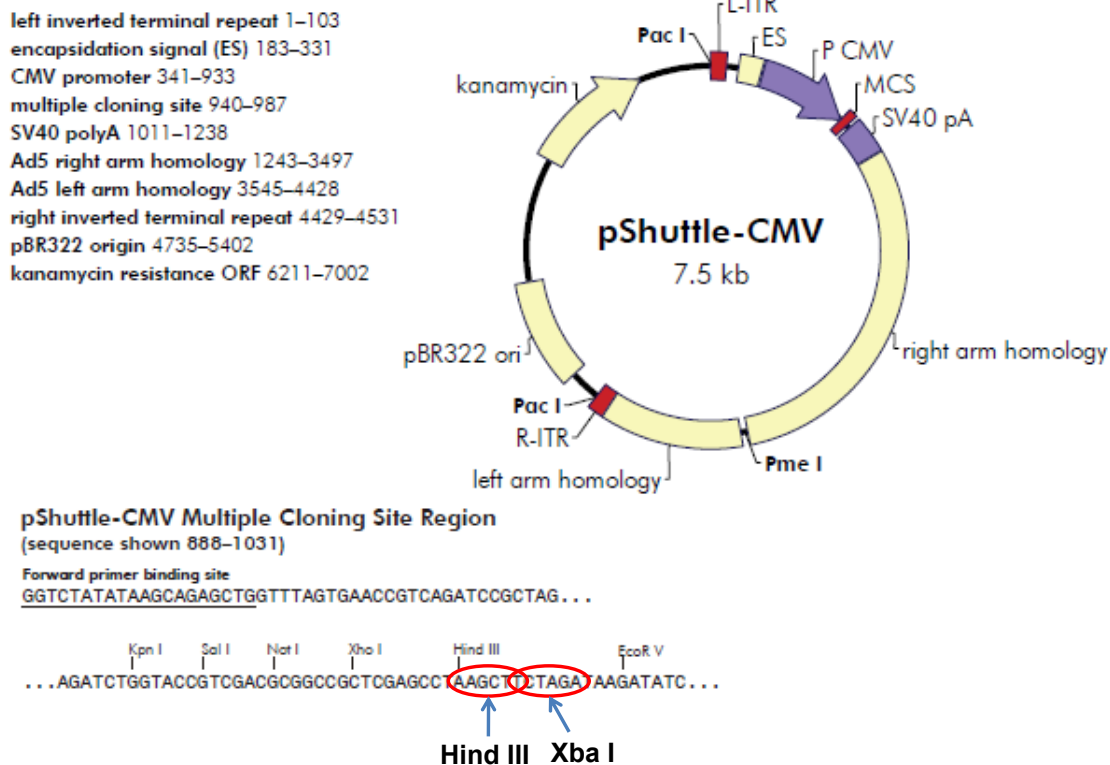


Figure 6. Map of HDAC5-S279A/D in psh-CMV. We used Hind III and Xba I to insert HDAC5-S279A and HDAC5-S279D mutant into psh-CMV vector.

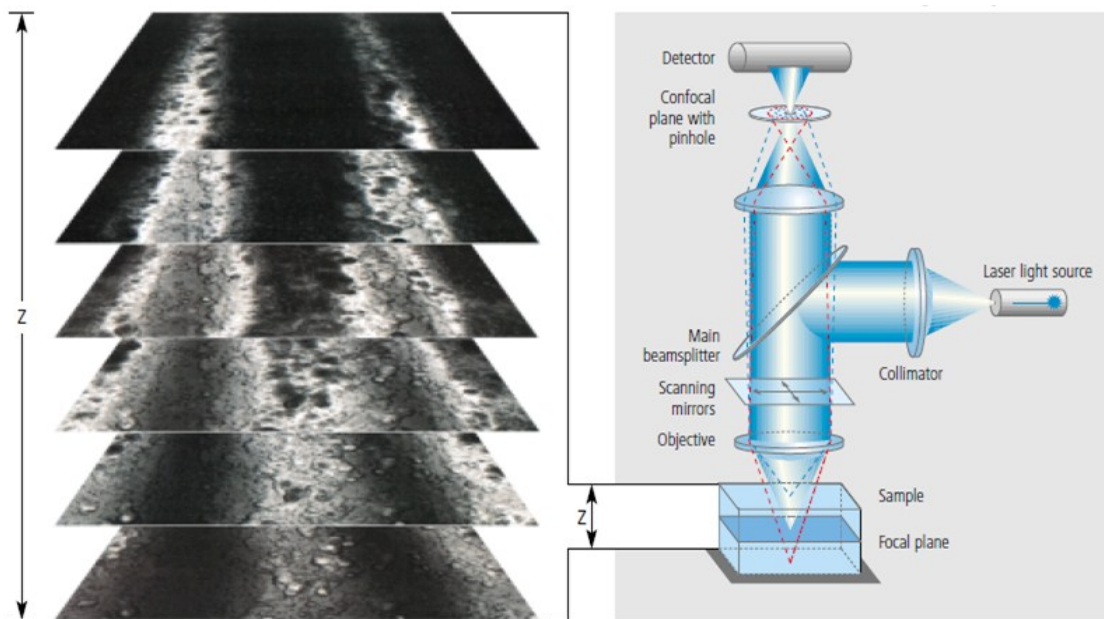


Figure 7. Schematic of beam path in the confocal LSM 5 PASCAL Laser Scanning Microscope. Image taken from Zeiss LSM 5 PASCAL manual.

CHAPTER 4

AIM 1

“Spatiotemporally Distinct Protein Kinase D Activation in Adult Cardiomyocytes in Response to Phenylephrine and Endothelin”

Introduction

Cardiac hypertrophy and remodeling can be induced by diverse stress stimuli ranging from mechanical stress, pressure overload, oxidative stress to neurohumoral signaling (Frey and Olson, 2003; McKinsey, 2007). The remodeling process exhibits initial beneficial functional effects, but eventually triggers maladaptive changes that deteriorate the heart to pump blood effectively. The remodeling process becomes pathological and typically progresses towards heart failure. To induce cardiac hypertrophic growth, the process needs to be initiated by epigenetic re-programming to encourage protein synthesis (Avkiran et al., 2008). The transcriptional activation during such genetic re-programming has been linked to a family of transcriptional repressors, called class II HDACs (Bucks et al., 2008; Bertos et al., 2001; Yang and Gregoire, 2005; Zhang et al., 2002).

In particular, HDAC5 has been reported to regulate cardiac hypertrophy

via its association with transcription factor, myocyte enhancer factor 2 (MEF2) family (Chang et al., 2004; Lu et al., 2000a; Lu et al., 2000b; McKinsey et al., 2000a). The functional role of HDAC5 serves to repress target gene expression through condensation of chromatin structure by removal of acetyl groups from histone tails (Backs and Olson, 2006; McKinsey and Olson, 2005). Both CaMKII and PKD have been reported to phosphorylate HDAC5 to mediate its nuclear export (Backs et al., 2008; Ellis et al., 2003; Harrison et al., 2010; Harrison et al., 2006; Wu et al., 2006), and emerged as crucial transducers of extracellular stress stimuli to HDAC5 signaling. Moreover, CaMKII and PKD are found to have increased expression and activity in failing myocardium supporting their potential as therapeutic targets in the future (Bossuyt et al., 2008; Harrison et al., 2006).

Although PKD was initially discovered as a member of the PKC family, it was later re-categorized into the CaMK superfamily based on its enzymatic function and substrate specificity (Avkiran et al., 2008; McKinsey, 2007). PKD is a serine/threonine kinase that is involved in diverse cellular processes, such as signal transduction, cellular proliferation, ischemia/reperfusion, immune regulation, cardiac hypertrophy, contraction and cancer (Bardswell et al., 2010; Fu and Rubin, 2011; Ghanekar and Lowe, 2005; Liu et al., 2007; Matthews et al., 2006; Xiang et al., 2011). PKD family contains three isoforms of PKD (PKD 1, 2 and 3), which share relatively conserved sequence homology and domain function (Avkiran et al., 2008; Wang, 2006)

While PKD1 is the predominant cardiac isoform, it has also emerged as a

key protein in cardiovascular research since PKD has been involved in regulating cardiac pathophysiology in multiple ways. For example, it plays a role in phosphorylating HDAC5 to mediate its nuclear export during hypertrophic signaling (Bossuyt et al., 2008; Harrison et al., 2010; Harrison et al., 2006; Vega et al., 2004), and cardiac troponin I to modulate myocyte contraction via altering its Ca²⁺ sensitivity (Bardswell et al., 2010; Cuello et al., 2007; Goodall et al., 2010; Haworth et al., 2004). Transgenic mice with overexpression of constitutively active PKD1 showed enlarged heart (Harrison et al., 2006). Similarly, cardiac-specific deletion of PKD1 effectively reduced cardiac hypertrophy induced by aortic constriction (Fielitz et al., 2008). Thus, all these data strongly support the regulatory role of PKD1 in cardiac hypertrophic signaling and demands further understanding of its fundamental activation mechanism in adult myocytes.

PKD consists of two cysteine-rich regulatory domains (C1a and C1b) near the N-terminus, a pleckstrin-homology domain in the middle and a C-terminal catalytic domain (Hayashi et al., 1999; Sturany et al., 2001; Valverde et al., 1994). The C1 domains of PKD share similar function to those in PKC, to mediate reversible plasma membrane translocation in response to DAG produced by GPCR activation or phorbol esters (Newton and Johnson, 1998). To date, the canonical mechanism of PKD activation depicts initial upstream PKC phosphorylation at S744/S748 within the catalytic domain, followed by PKD autophosphorylation at S916 residue (Matthews et al., 1999; Rey et al., 2004;

Rozengurt et al., 2005; Waldron and Rozengurt, 2003; Wang, 2006). A possible Ca^{2+} -dependent mechanism of PKD activation is also recently reported (Kunkel et al., 2007; Wu et al., 2006). While PKD was found to be the major HDAC5 kinase in cultured neonatal rat myocytes (Vega et al., 2004), however, its expression in adult myocyte is known to be significantly reduced compared to neonates (Haworth et al., 2000). This raises the question of PKD role in regulating HDAC5 translocation in adult myocytes.

We have previously identified an IP_3 -CaMKII pathway for the ET-1 induced HDAC5 nuclear export in adult myocyte (Wu et al., 2006). In this study, we examine and contrast the spatiotemporal PKD activation in response to two common neurohumoral stimuli, ET-1 and PE in adult myocytes. PE is an α -adrenergic agonist, which also has been reported to induce cardiac remodeling (Vega et al., 2004). Our current data show subcellular local activation of PKD is differentially regulated by ET-1 and PE although these two Gq-agonists induce comparable global PKD activation and HDAC5 nuclear export. PE-mediated PKD activation is found to be independent of IP_3 -CaMKII signaling in contrast to the PKD activation induced by ET-1. Moreover, PE induces rapid, transient PKD translocation to the sarcolemma followed by quick nuclear import of active PKD to phosphorylate HDAC5 in the nucleus. Conversely, ET-1 exhibits PKD translocation that stays bound to the sarcolemma longer and results in slower and smaller nuclear PKD activation. This is consistent with our previous study where IP_3 -CaMKII signaling is the major pathway involved in ET-1 mediated

HDAC5 nuclear export (Wu et al., 2006). Our current study provides a critical evaluation of dynamic spatiotemporal PKD activation in both global and local environments. This study indicates that Gq-agonists, ET-1 and PE, diverge in their signaling to preferentially activate CaMKII and PKD respectively to regulate HDAC5 translocation in adult myocytes.

Materials and Methods

Myocyte isolation and adenoviral infection

All animal and biohazard protocols were approved by the appropriate committees at the University of California, Davis. Rabbit ventricular myocytes were isolated as previously described (Bassani et al., 1994) and plated on laminin-coated culture inserts. Culture media was PC-1 media (Lonza) supplemented with 5% penicillin-streptomycin. Myocytes were infected for 2 hr (MOI = 10-50) with recombinant replication-deficient adenovirus expressing HDAC5-GFP, PKD1-GFP or DKAR variants, with subsequent culture for 20-30 hr. GFP fluorescence (CFP/YFP for DKARs) indicated infection and localization. For non-targeted DKAR experiments, ventricular myocytes were also infected with adenovirus encoding PKD1. To ensure adequate infection of myocytes (lack of GFP expression) protein expression of PKD was assessed by Western blotting. After 24 hr, myocytes were exposed for 1 hr to 100 nM ET-1, 10 μ M PE, 200 nM PDBu or vehicle as indicated. These experiments were performed with or without 1 μ M KN-93, 10 μ M BisI, 2 μ M 2-APB or 10 μ M Gö6967 pretreatment for 20 min.

Immunoblotting and Immunocytochemistry

Isolated myocytes were rinsed in PBS and treated as indicated before lysing in ice-cold buffer containing in mM: 150 NaCl, 10 Tris (pH 7.4), 2 EGTA, 50 NaF, 0.2 NaVO₃, 1% Triton X-100, and protease and phosphatase inhibitor cocktail III (Calbiochem). Cell lysates were flash-frozen and stored at -80°C.

Proteins were size-fractionated on 8% SDS-PAGE before transferring to a 0.2 micron nitrocellulose membrane. Immunoblots were blocked with 8% milk in TBST. The blots were then incubated overnight at 4°C with primary antibody: PKD, PKD-pS916 or PKD-pSS744/748 (1:1000; Cell Signaling). After incubation with the HRP-labeled secondary antibody, blots were developed using enhanced chemiluminescence (Pierce Supersignal). All signals were recorded using a UVP-EpichemII darkroom imaging system for quantification and captured on film for representation. Equal protein loading was ensured by reprobing for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000; Abcam). All experiments were performed in duplicate. For immunostaining myocytes seeded on glass coverslips were fixed with 4% para-formaldehyde in PBS. After incubation in PBS with 0.1% glycine, cells were permeabilized with 1% Triton X-100 and incubated with anti-PKD (1:50, overnight, 4°C). This was visualized with anti-rabbit (Alexa488).

Confocal measurements

GFP-HDAC5 and PKD1-GFP signals were measured by confocal microscopy with argon laser excitation at 488 nm and emitted fluorescence (F) at LP 500. Image J software (<http://rsb.info.nih.gov/ij/>) was used for analysis with the intensity of the regions of interest normalized to area. Fluorescence intensities were also corrected for background signal. For quantification of recruitment to Z-lines, a plot profile was fitted to a sinusoidal equation and the amplitude was taken as the Z-line signal.

Fluorescence resonance energy transfer (FRET) measurements

FRET was measured 2 ways. (1) a non-destructive, ratiometric technique was used to monitor time-dependent FRET changes (and this also helped to verify that the DKAR signal was not saturated under the conditions used). In this method CFP and YFP emission are measured upon CFP excitation (N.B. relative abundance of donor and acceptor is not an issue for DKAR) (Van Munster et al., 2005). The following protocol was used: 100 ms acquisitions of CFP image (CFP excitation and emission), 100 ms acquisitions of YFP image (YFP excitation and emission), and finally 100 ms acquisition of FRET image (CFP excitation but YFP emission). This is then followed by the second approach, the acceptor photobleach method, where FRET efficiency was measured quantitatively as the increase in donor (CFP) fluorescence upon YFP photobleaching (which eliminates energy transfer from the donor to YFP) (Van Munster et al., 2005). Here, YFP was progressively photobleached using 100 ms acquisitions of CFP image, 40 ms acquisitions of YFP image, followed by repeated 10 s exposure to YFP-selective photobleach (504/12nm excitation). This protocol has been validated with FRET-standard samples (Kelly et al., 2008).

Fluorescence imaging utilized an inverted microscope equipped with a 1.49NA objective, and a back-thinned CCD camera (iXon 887, Andor Technology). Image acquisition and acceptor photobleaching was automated with custom software macros in MetaMorph that controlled motorized excitation/emission filter wheels (Sutter Instrument Co.) with filters for CFP (Ex 427/10, Em 472/30 nm) and YFP (Ex 504/12 nm, Em 542/27) (Semrock).

Statistical analysis

Data are expressed as mean \pm SEM. Statistical discriminations were performed with Student's t test (paired when appropriate), and ANOVA with $p < 0.05$ was considered significant.

Results

Role of PKD in the regulation of HDAC

ET-1-induced HDAC5-GFP nuclear export and excitation-transcription coupling in adult cardiac myocytes is entirely dependent on local InsP₃-induced Ca²⁺ release and CaM, and depends equally on CaMKII and PKD phosphorylation of HDAC5 (Figure 8) (Wu et al., 2006). Figure 9 shows that in adult rabbit ventricular myocytes PE (another Gq-coupled receptor hypertrophic agonist) produces very similar HDAC5-GFP nuclear export to ET-1 (34±2% vs. 35±2% at 60 min). Control studies confirmed that the ET-1 and PE concentrations used are maximally activating. However, PKC inhibition (with Bis I) virtually abolished PE-induced HDAC5 nuclear export, without altering that induced by ET-1 (Figure 10). Also PKD blockade (with Gö6976, a PKC/PKD inhibitor) caused ~50% inhibition of ET-1-induced HDAC5 translocation, and again completely blocked PE-induced effect. Conversely, pretreatment with the CaMKII inhibitor KN93 or the InsP₃R blocker 2-APB did not significantly alter the PE response, but potently inhibited the ET-1-induced HDAC5 nuclear export (Figure 11). Parallel effects were observed for PE- vs. ET-1-induced MEF2 transcriptional activation, using a MEF2-luciferase reporter construct in adult rabbit ventricular myocytes (not shown). These experiments indicate that PKC-dependent PKD activation is required for PE-induced HDAC5 nuclear export (independent of InsP₃-dependent Ca²⁺ release or CaMKII). This contrasts with the ET-1-dependent pathway, which requires InsP₃-sensitive Ca²⁺ stores and both CaMKII plus PKD, but not PKC. Thus, each Gq-coupled receptor agonist

(ET-1 and PE) activates divergent signaling pathways in adult cardiac myocytes. This also indicates that ET-1-induced PKD activation might be PKC independent while that by PE requires PKC. Notably, inhibition of PLC by U73122 blocks PKD activation by both ET-1 and PE, consistent with DAG-dependent activation of PKD that is independent of PKC, as suggested by previous work (Harrison et al., 2006; Haworth et al., 2000).

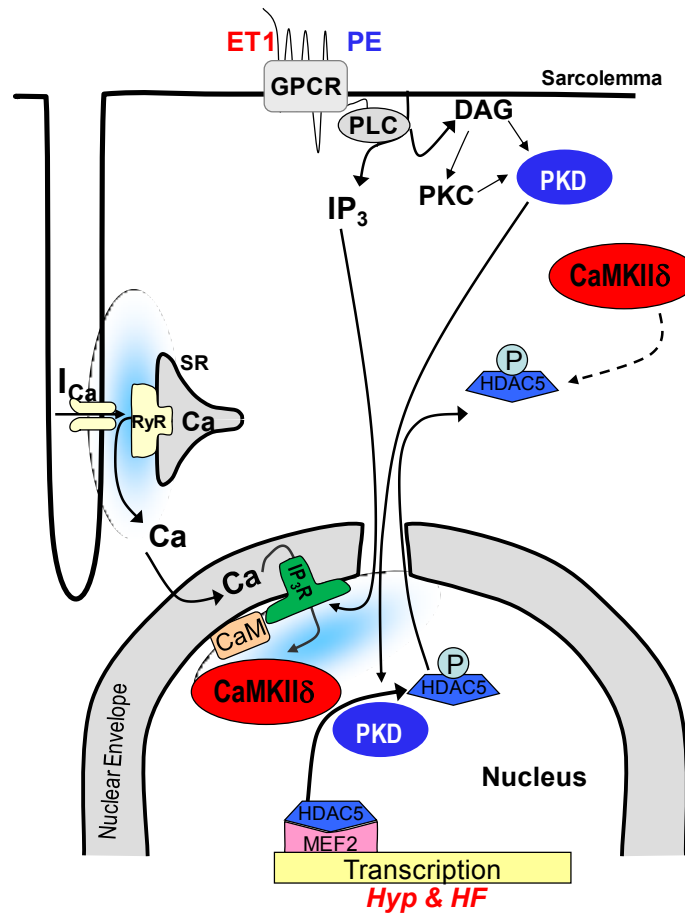


Figure 8. Agonist-dependent differential signaling of HDAC5. Schematic diagram of ET-1 and PE signaling to HDAC5 in adult cardiac myocytes. ET-1 signaling triggers activation of CaMKIIδ to mediate HDAC5 nuclear export while PE differentially activates PKD to induce HDAC5 nuclear export.

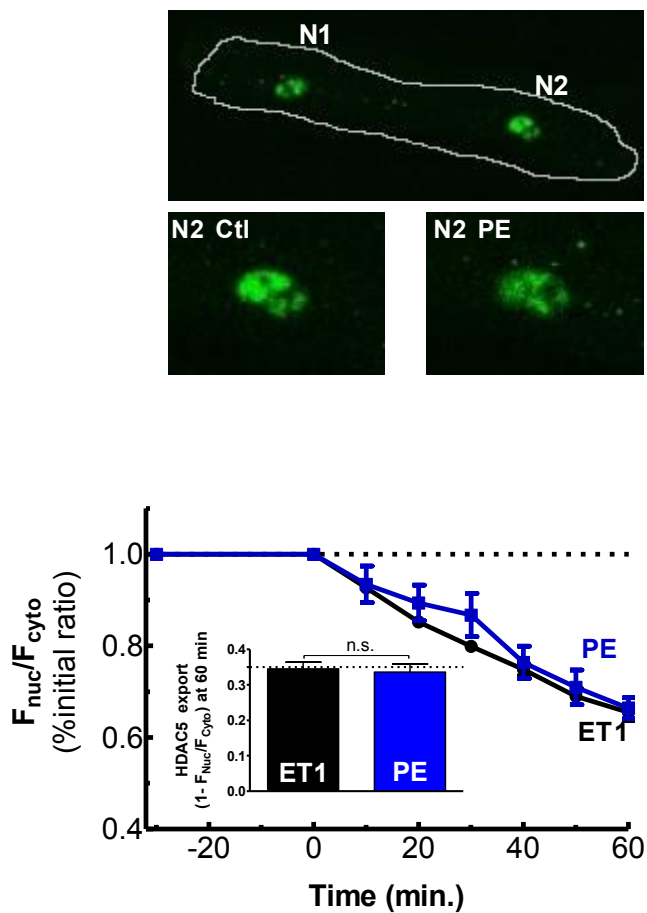


Figure 9. ET-1 and PE mediate nuclear export of HDAC5. Top: Confocal imaging of WT HDAC5-GFP expression in adult cardiac myocyte. Example of PE-induced nuclear export of HDAC5-GFP vs. control is shown. Bottom: ET-1 (100 nM) and PE (20 μ M) trigger comparable nuclear export of HDAC5 at 1 hr post-treatment.

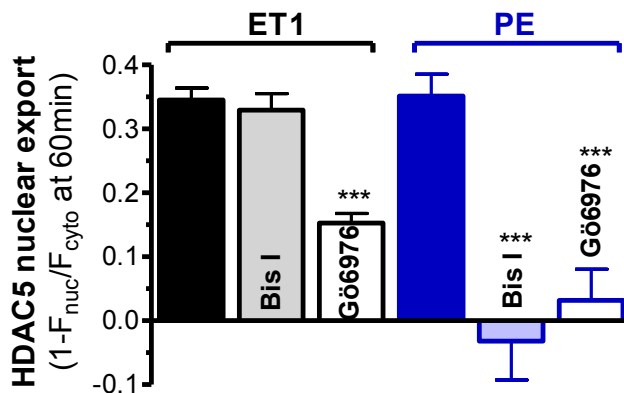


Figure 10. ET-1 and PE exhibit different PKC and PKD signaling dependence on mediating HDAC5 nuclear export. Cultured adult myocytes were incubated with Bis I (10 μ M) or Gö 6976 (10 μ M) for 20 min before ET-1 (100 nM) or PE (20 μ M) treatment.

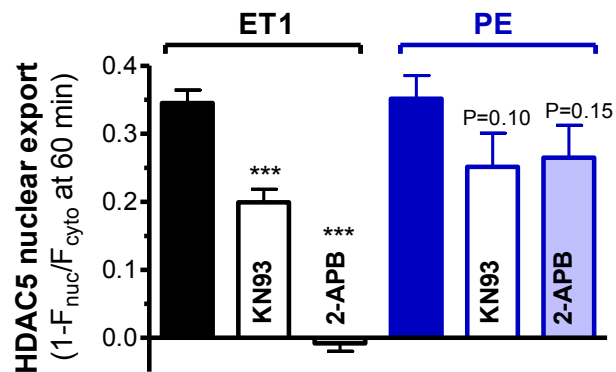


Figure 11. ET-1 and PE signaling demonstrate differential activation of IP₃-CaMKII in triggering HDAC5 nuclear export. Cultured adult myocytes were pretreated with CaMKII inhibitor, KN93 (1 μ M), or IP₃ receptor blocker, 2-APB (2 μ M), for 20 min prior to ET-1 (100 nM) or PE (20 μ M) stimulation.

PE and ET-1 differ in their ability to activate PKD1

Figure 12 shows that exposure to PE, ET-1 or the phorbol ester phorbol 12,13-dibutyrate (PdBu) cause a similar 5-fold increase in PKD autophosphorylation levels at S916 (often used as a read-out of PKD1 activity). PE and PdBu both strongly activated phosphorylation of the activation loop SS744/748 sites on PKD1 (by ~4-fold), but ET-1 was much less effective in causing phosphorylation at SS744/748. Since PKC is known to phosphorylate these sites (Rey et al., 2004; Rozengurt et al., 2005), this is consistent with the more pronounced PKC dependence for PE-induced HDAC5 nuclear export (vs. ET-1; Figure 10).

To more directly measure PKD activity and activation time course in adult cardiac myocytes, we used the FRET-based PKD activation reporter (DKAR) (Kunkel et al., 2007). DKAR includes a PKD-specific substrate and phospho-amino acid binding domain linking CFP and YFP, such that upon phosphorylation by PKD, FRET is reduced (Figure 13). Monitoring the CFP/YFP ratio, we found that PE and ET-1 activated global PKD activity to a similar degree in adult myocytes (Figure 14). We also used an additional DKAR FRET measurement, i.e. donor fluorescence enhancement upon acceptor photobleach (figure15). This quantitative method also showed similar PKD activity after 20 min exposure to PE, ET-1 and PDBu. These results are consistent with our *in vitro* surrogate measure of PKD activity (phospho-S916 PKD) in Figure12. These changes in DKAR FRET were reversible upon agonist withdrawal and blocked by pretreatment with Gö6976 (not shown).

The similar PKD activation by PE and ET-1 (Figure 12 to Figure 15) does not explain how these two Gq-coupled receptors differently affect HDAC5 nuclear export and transcriptional activation (i.e. why is PKD much more critical for PE vs. ET-1 signaling to the nucleus?). It is possible that PKD localization and translocation differ for PE and ET-1, as PKD can redistribute among intracellular targets in other cell types (Matthews et al., 2000; Rey et al., 2001). We explored this using PKD1-GFP fusion proteins expressed in adult rabbit ventricular myocytes using an adenoviral vector.

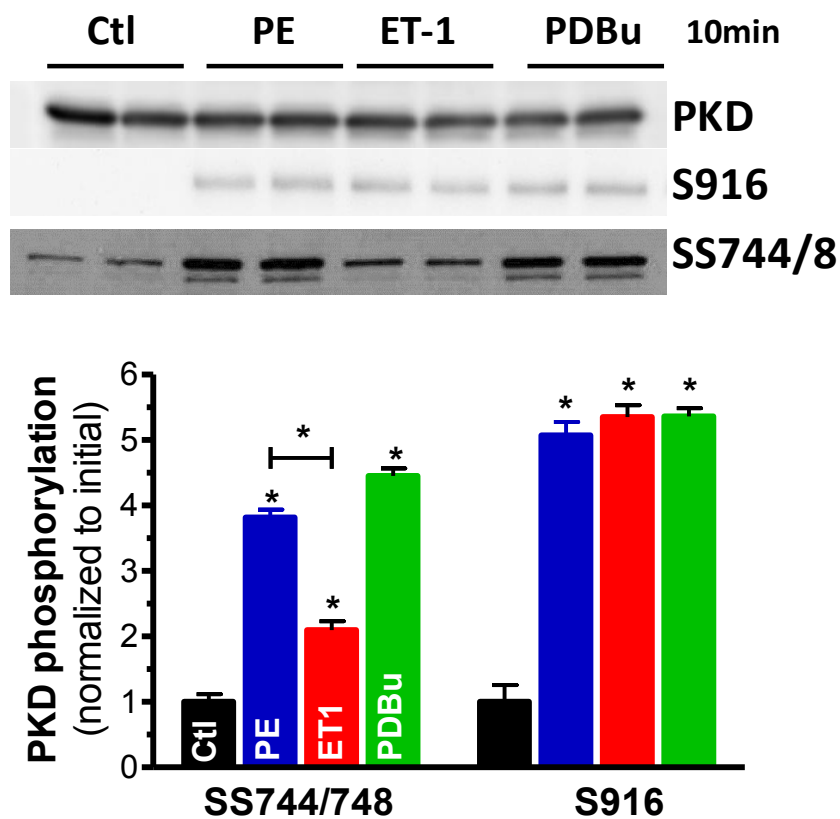


Figure 12. PKD activation by Gq-agonists. Top: Representative immunoblots of PKD activation in adult rabbit myocytes using PKD antibody, S916 phospho-specific antibody and SS744/748 phospho-specific antibody. Bottom: Quantification of PKD activation measuring SS744/748 and S916 phosphorylation in response to 10 min treatment with Gq-agonists, ET-1 (100 nM), PE (20 μ M) and PdBu (200 nM). * $p < 0.05$. $n = 6$.

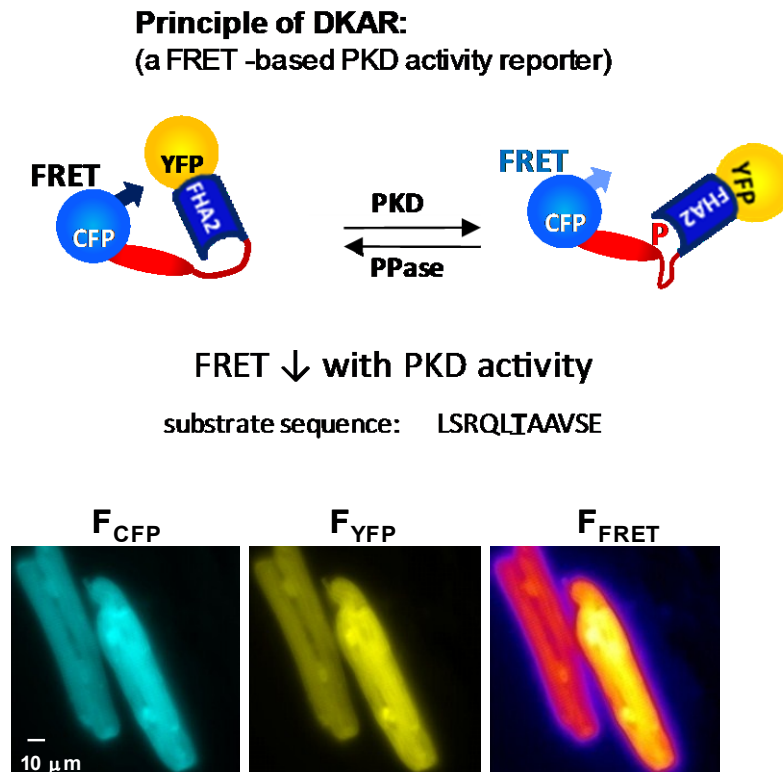


Figure 13. D Kinase Activity Reporter (DKAR) measuring PKD activity. Top: Modified illustration of DKAR principle 40 where its FRET signal decreases with active PKD phosphorylating its pseudo-substrate resulting in conformational shift between CFP and YFP. Bottom: Expression of DKAR sensor in adult myocyte is shown with selective excitation of CFP, YFP or FRET (excitation of CFP, detection of YFP emission).

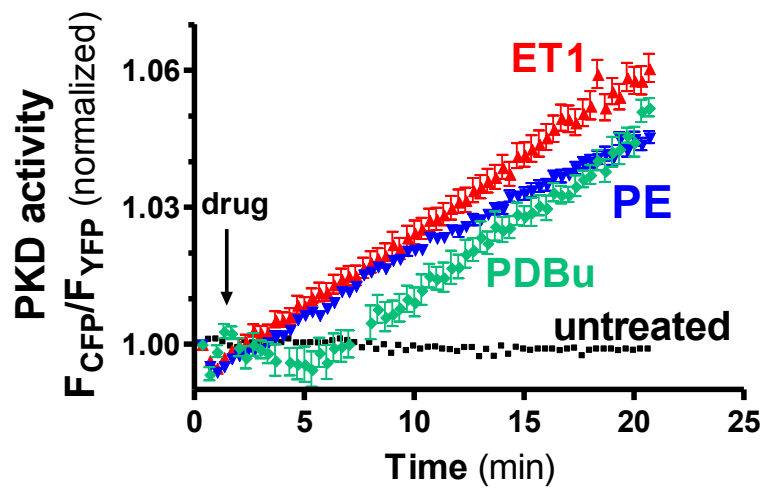


Figure 14. Agonist-dependent real-time ratiometric DKAR measurements.

Cultured adult myocytes (expressing both DKAR and PKD1) were treated with ET-1 (100 nM), PE (20 μ M) or PdBu (200 nM) for 20 min. $n = 6$.

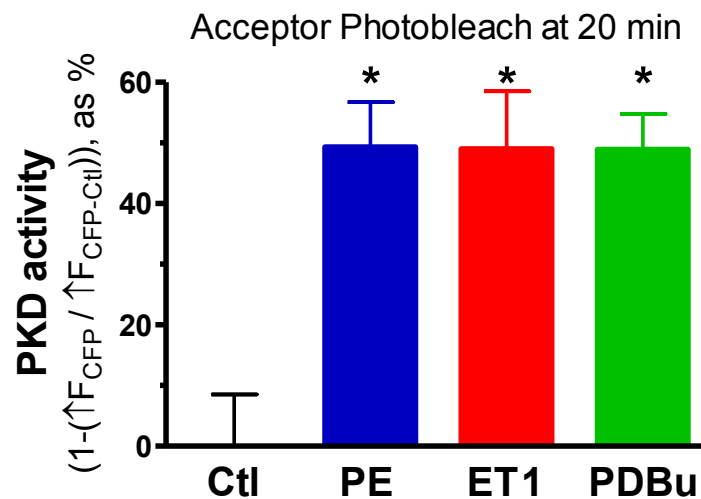


Figure 15. Acceptor photobleach-induced enhancement of CFP in response to agonist. Cultured adult myocytes were treated with agonist, ET-1 (100 nM), PE (20 μ M) or PdBu (200 nM) for 20 min followed by photobleaching the acceptor YFP to measure increase in CFP (correlates with PKD activity). Data were represented as % change in FRET signal. n = 6.

Spatiotemporal dynamics of PKD1 localization in response to PE and ET-1

Confocal imaging of adenovirally expressed PKD1-GFP fusion protein and of immunostained endogenous PKD1 revealed that at rest, PKD1 expression is relatively uniform and cytosolic (slightly higher at Z-line/transverse tubule region), and largely non-nuclear (Figure 16) ($F_{\text{nuc}}/F_{\text{cyto}}$ is 0.5 ± 0.03 and 0.68 ± 0.06 , respectively). Application of ET-1 caused rapid, sustained PKD recruitment to the plasma membrane or sarcolemma (SL) (SL, $39\pm 4\%$ increase), with only a modest and much delayed nuclear import ($9\pm 3\%$; or 23% of the SL increase). In striking contrast, PE only slightly increased membrane PKD ($7\pm 2\%$), but much more strongly increased nuclear translocation ($25\pm 2\%$; or 357% of the SL increase) (Figure 16 and Figure 18). Note also that the smaller rise in nuclear PKD1 concentration with ET-1 happens with a clear delay and as the SL starts to decline. These findings indicate that ET-1 drives SL recruitment of PKD1, while PE drives rapid nuclear recruitment of PKD1 (consistent with a more prominent role in nuclear HDAC5 phosphorylation). Similar results were obtained with immunostaining of endogenous PKD1 in adult rat myocytes (data obtained by Linda Lee) (Figure 17), validating the use of PKD1-GFP as a tracer for the endogenous protein (not all data shown). PKD1 is also recruited to the T-tubular membrane (SL invaginations in cardiac myocytes), as indicated by the sharpening of the fluorescence intensity at the Z-line/T-tubule location vs. the mid-sarcomere region (Figure 19). Moreover, the relative strength of agonists (PdBu > ET-1 > PE) causing T-tubule concentration was the same as that for surface SL (Figure 18).

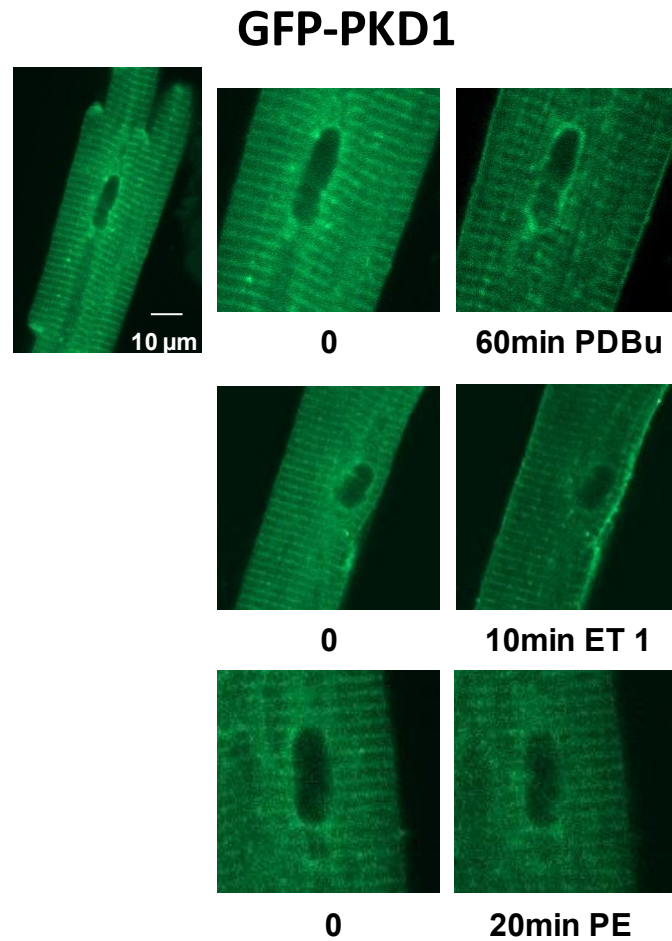


Figure 16. Agonist-induced GFP-PKD1 translocation. Examples of confocal imaging showing cultured adult myocytes treated with ET-1 (100 nM), PE (20 μ M) or PdBu (200 nM).

Endogenous PKD1

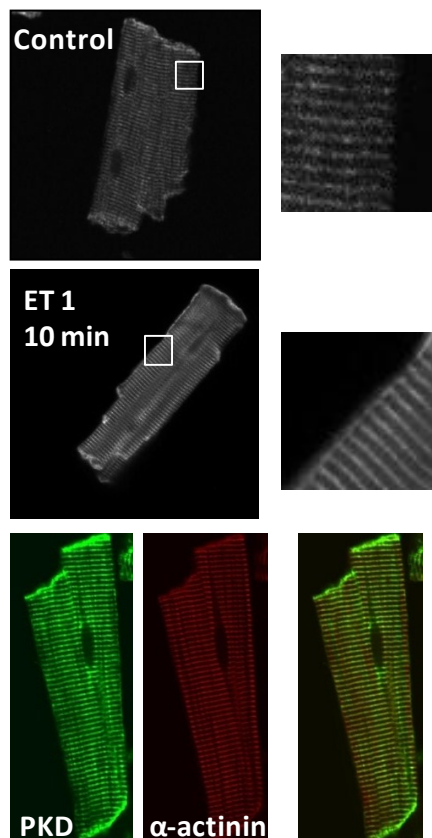


Figure 17. Endogenous PKD1 localization in isolated adult myocytes. Top: Immunostaining of endogenous PKD1 localization using PKD1 specific antibody. Middle: Example of endogenous ET-1 induced PKD1 translocation to the sarcolemma. Bottom: Immunostaining of PKD1 co-localization with α -actinin.

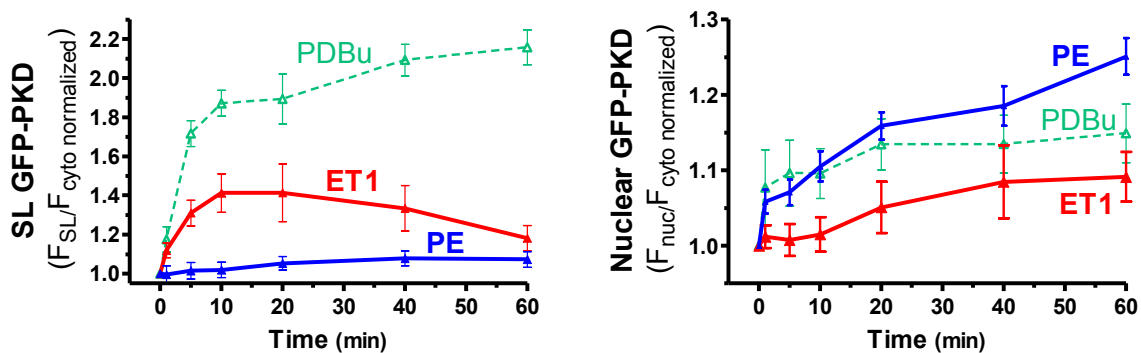


Figure 18. Sarcolemmal and nuclear translocation of PKD1 in adult myocytes. Left: Confocal measurements of GFP-PKD1 sarcolemmal translocation in response to ET-1 (100 nM), PE (20 μ M) or PdBu (200 nM). Right: Nuclear translocation of GFP-PKD1 after treatment with ET-1 (100 nM), PE (20 μ M) or PdBu (200 nM).

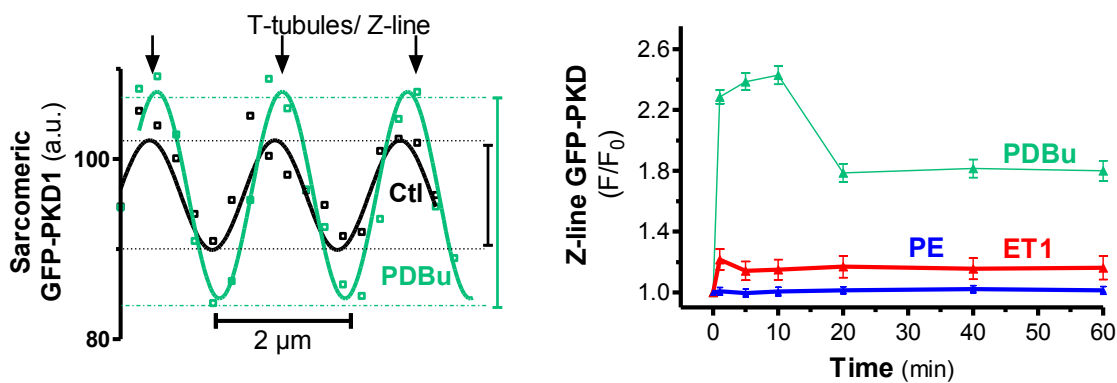


Figure 19. PKD1 translocation in T-tubules. Left: Example of confocal signal of GFP-PKD1 translocation to the T-tubular membrane after PdBu treatment (200 nM). Right: Confocal measurements of GFP-PKD1 translocation to the T-tubules in response to ET-1 (100 nM), PE (20 μM) or PdBu (200 nM).

Spatiotemporal dynamics of PKD1 activity in response to PE and ET-1

We also used targeted PKD activity reporters to measure its localized activation at the sarcolemma (DKAR-SL) and the nucleus (DKAR-Nuc) respectively. All three agonists elicited a similar increase in PKD activity at the SL (FRET was reduced $31\pm 5\%$, $31\pm 4\%$ and $24\pm 5\%$ for PDBu, ET-1 and PE respectively; based on acceptor bleach induced donor enhancement) (Figure 20). In contrast PE was much more potent in activating nuclear PKD than either ET-1 or PDBu (Figure 21). This was also apparent in the real-time, ratiometric FRET measurements of nuclear and sarcolemmal PKD activity. PE and ET-1 triggered similar responses at the sarcolemma, whereas ET-1 caused a small, slow rise in nuclear PKD activity while PE elicited a faster and larger response (Figure 22). These findings are consistent with a more pronounced nuclear role for PKD1 upon PE than ET-1 stimulation.

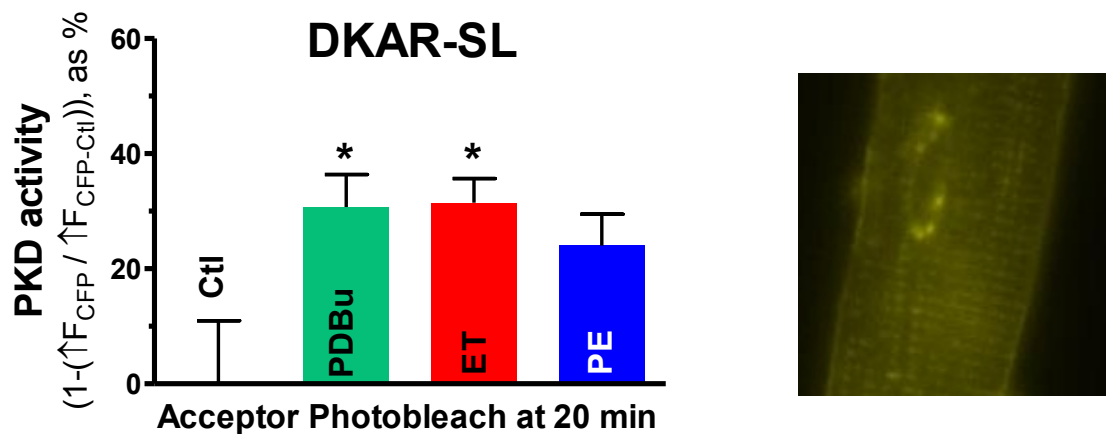


Figure 20. Agonist-dependent activation of sarcolemmal PKD activity. Left: Membrane-targeted DKAR measurement of PKD activity after treatment with PdBu (200 nM), ET-1 (100 nM) or PE (20 μ M). Right: Representative DKAR-SL expression in adult myocyte with pronounced membrane appearance. * $p < 0.05$ vs. control.

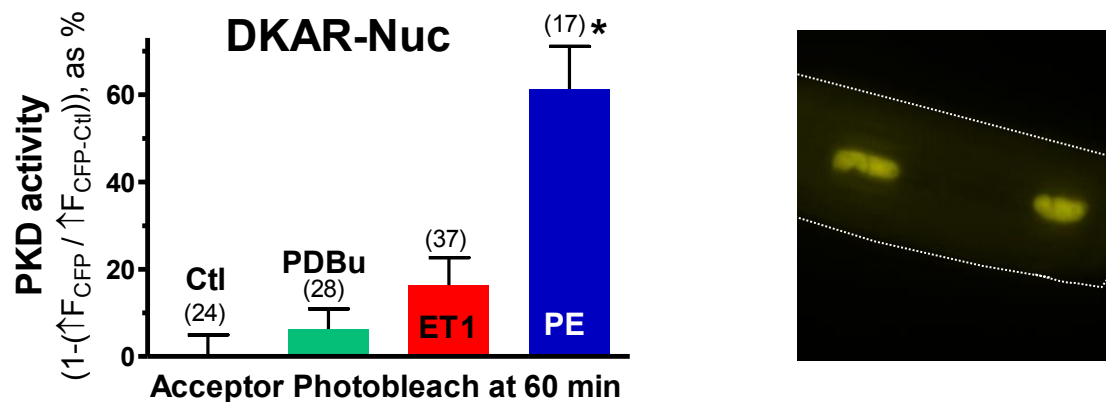


Figure 21. Agonist-dependent nuclear PKD activity. Left: Nuclear-targeted DKAR measurement of PKD activity after treatment with PDBu (200 nM), ET-1 (100 nM) or PE (20 μ M). Right: Representative DKAR-Nuc expression in adult myocyte with pronounced nuclear localization. * $p < 0.05$ vs. control.

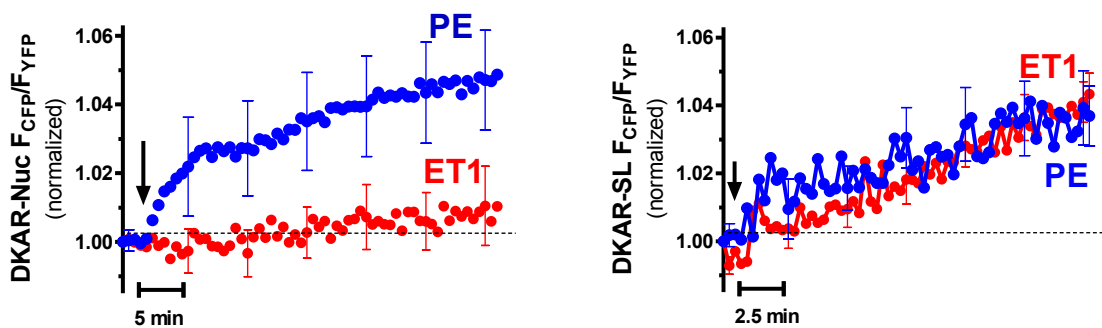


Figure 22. Differential PKD activation by ET-1 and PE at the nucleus and the sarcolemma. Left: DKAR-Nuc measurements reflected that PE induced a stronger PKD nuclear activation vs. ET-1. Right: DKAR-SL showed comparable membrane activation of PKD by ET-1 and PE.

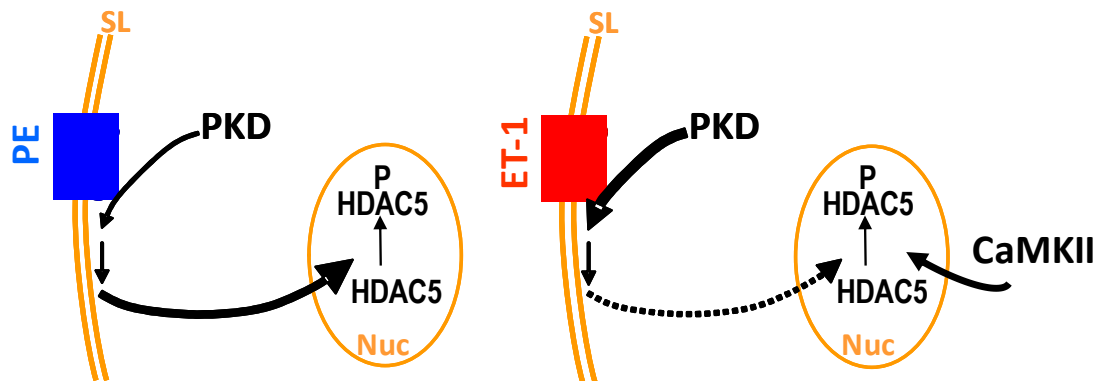


Figure 23. Working hypothesis of PKD activation. Left: PE recruits PKD to the sarcolemma where it is activated and rapidly translocates to the nucleus, indicating a fast membrane-cytosolic movement. Right: ET-1, similar to PdBu, causes sarcolemmal recruitment and activation of PKD. PKD remains membrane-bound for a longer period of time, thus resulting in slower and smaller nuclear translocation.

Discussion

PKD and CaMKII are crucial mediators of cardiac hypertrophic signaling and remodeling, thus are desirable pharmacological drug candidates for cardiac therapy (Harikumar et al., 2010; McKinsey and Olson, 2005; Meredith et al., 2010a; Meredith et al., 2010b; Monovich et al., 2009; Monovich et al., 2010). Although both kinases can be activated by common neurohumoral stimuli, ET-1 and PE, the differential signaling mechanism between the two pathways remain to be elucidated in adult cardiac myocytes. In this study, we demonstrate for the first time that ET-1 and PE have distinct signaling pathways that allow divergent activation of CaMKII and PKD to phosphorylate their mutual downstream target, HDAC5. Our findings suggest that there is redundant hypertrophic signaling by these two Gq-agonists to provide differential epigenetic modulations via HDAC5 translocation in adult cardiac myocytes.

Extensive work by Olson's group in cell lines and neonatal myocytes indicates that HDAC4 nuclear export is facilitated by CaMKII phosphorylation while HDAC5 is phosphorylated by PKD (Backs et al., 2008; Vega et al., 2004). However, expression of PKD is notably reduced in adult myocytes compared to the neonates (Haworth et al., 2000), thus the impact of PKD on HDAC5 translocation in adult myocytes may differ. Indeed, we previously demonstrated that ET-1 hypertrophic signaling is predominantly dependent on local IP₃-induced Ca²⁺ release and independent of PKC isoforms (Wu et al., 2006). We also showed that ET-1 mediated HDAC5 nuclear export was contributed equally by CaMKII and PKD activation (Wu et al., 2006). Here, we report that α -agonist, PE,

induces comparable HDAC5 nuclear export to ET-1, but triggers a different signaling cascade. PE-induced HDAC5 nuclear export is largely driven by PKC and PKD activation, but is independent of IP₃-CaMKII activation. This prompts the question of how both Gq-agonists can induce hypertrophic signaling via nuclear export of HDAC5, but diverge in their activation pathway of two parallel cytosolic kinases. To dissect the difference in ET-1 and PE mediated signaling mechanism, we examine in detail the spatiotemporal activation of PKD by these two Gq-agonists in adult myocytes.

Both ET-1 and PE trigger comparable global PKD activation based on S916 auto-phosphorylation and FRET-based DKAR measurements. Nevertheless, the two Gq-agonists differ in their activation mechanism indicated by reduced SS744/748 phosphorylation (PKC sites) during ET-1 stimulation compared to PE (Figure 12). The western blot suggests that ET-1 is capable of activating PKD without upstream PKC activation. This is consistent with our previous observation where ET-1 driven HDAC5 nuclear export is partially PKD-dependent, but independent of PKC isoforms (Wu et al., 2006).

The classical PKD activation mechanism depicts a PKC-dependent activation in two sequential phases (Jacamo et al., 2008; Rykx et al., 2003). The first phase describes initial PKD recruitment to the plasma membrane, along with upstream PKC, to be phosphorylated by active PKC isoforms at its SS744/748 site. The second phase denotes its subsequent auto-phosphorylation at S916 once PKD itself is catalytically active. Our translocation data (Figure 16 to Figure 19) confirm that both ET-1 and PE are capable of activating PKD in adult cardiac

myocytes. Notably, ET-1 induced plasma membrane recruitment of PKD is more prominent and more stable than PE, yet there is higher nuclear PKD translocation inducible by PE than ET-1. This could be explained in part by the notion that ET-1 can activate PKD without upstream PKC (Wu et al., 2006) and results in more stable membrane-bound PKD that associate to other membrane protein partners. Moreover, the TIRF data collected by Dr. Julie Bossuyt show similar initial sarcolemmal recruitment rate of PKD by both agonists. While PE recruits a smaller amount of PKD to the membrane (Bossuyt et al., 2011), its peak recruitment to the plasma membrane occurs faster than those inducible by ET-1 and PdBu (Bossuyt et al., 2011). These translocation data are consistent with the notion that PKC phosphorylation at SS744/748 (as seen during PE stimulation) facilitates the reverse membrane translocation of PKD to other intracellular compartments, such as the nucleus (Newton and Johnson, 1998). Such reverse translocation is unavailable during ET-1 stimulation since PKC phosphorylation at SS744/748 sites is not required (Figure 12) (Wu et al., 2006), thus results a more stable membrane-bound PKD.

Recent publication by Wright et al. reports that α -adrenergic receptors are mostly perinuclear (Wright et al., 2008), which can provide an alternative interpretation of our data where PE-induced lower sarcolemmal recruitment of PKD is due to the location of α -adrenergic receptors. Nevertheless, we did not observe distinguishable perinuclear PKD localization upon PE stimulation during our confocal study. In addition, our TIRF data supports the theory that PE, like ET-1, initiates PKD activation at the sarcolemma, thus is able to produce a rapid

reverse membrane translocation to the nucleus as seen in our FRAP study (Figure 4 and 5 from (Bossuyt et al., 2011)). Overall, these data describes a mechanism of PKD activation where α -adrenergic agonist, PE, initiates PKD activation near the sarcolemma followed by its rapid translocation to the nucleus. However, we cannot exclude that some PKD activation upon PE stimulation may occur near the nuclear envelope.

Our data above also explains why HDAC5 phosphorylation and nuclear export is predominantly PKD-dependent for PE and not for ET-1. On the other hand, ET-1 mediated HDAC5 phosphorylation and nuclear export require IP₃-CaMKII activation. This is supported by our observation of a slow and smaller nuclear PKD activation when ET-1 is applied. Although our study has greatly improved the overall mechanistic understanding of PKD activation in response to ET-1 and PE in adult myocytes, further study will be needed to answer the following questions. First, what are the molecular protein partners near the sarcolemma to allow rapid reverse translocation of PKD during PE stimulation or to encourage membrane-associated PKD in response to ET-1? What is the PKC-independent mechanism of PKD activation during ET-1 treatment (in contrast to PE)? Other studies have previously reported some PKC-independent activation of PKD (Jacamo et al., 2008; Rozengurt et al., 2005; Wang, 2006), but they exhibit a different temporal scale. Lastly, since PE can trigger IP₃ and DAG production similarly to ET-1, what prevents the PE-induced IP₃ from reaching intracellular IP₃ receptors to induce CaMKII activation? Although there are questions remain to be answered, our study provides valuable information

regarding the agonist-specific differential activation of CaMKII and PKD to phosphorylate and mediate HDAC5 nuclear export by ET-1 and PE respectively.

An important aspect of our findings point to the difference in global vs. local PKD activation. For example, all three hypertrophic agonists, ET-1, PE and PdBu induce global PKD activation via S916 phosphorylation and DKAR measurements. Targeted DKAR at the membrane also demonstrate similar near maximal PKD activity upon ET-1, PE and PDBu stimulation. This suggests that even with a small portion of PKD recruitment at the membrane, PE is able to elicit comparable PKD activation here (compared to stronger membrane-associated PKD seen with ET-1 and PDBu treatments).

Moreover, nuclear PKD import and activation is faster and larger under PE treatment than ET-1 based on our confocal and Nuc-DKAR experiments. Yet, a moderate amount of nuclear activation PKD is sufficient to phosphorylate nuclear HDAC5 and mediate its export during ET-1 stimulation when CaMKII is inhibited (Figure 15). Furthermore, PKD may have additional functions in the cytosol to re-phosphorylate HDAC5 and keep it cytosolic longer (as shown for CaMKII) (Zhang et al., 2007).

In conclusion, our current study highlights the differential dynamic spatiotemporal activation of PKD in response to important Gq-agonists, ET-1 and PE. The findings here support the notion that PKD, as an emerging cardiac kinase, is subject to cellular fine-tuning regulatory mechanism. Specifically, its global activation can differ greatly from its local activation in many intracellular compartments, such as the plasma membrane and the nucleus. Therefore,

inhibition of PKD activity may be an attractive therapeutic target in the future due to its role in modulation of transcriptional repressor, HDAC5, in adult cardiac myocytes.

Chapter 5

AIM 2

“Acute β -adrenergic Activation Triggers Nuclear Import of Histone Deacetylase 5 and Delays Gq-induced Transcriptional Activation”

Introduction

Histone deacetylases (HDACs) are transcriptional repressors crucial to the regulation of gene transcription and key transducers of extracellular stimuli that impact genetic programming (Bacs and Olson, 2006; Johnson and Turner, 1999). As epigenetic regulators, HDACs interact with multitude of transcriptional factors to regulate gene transcription or repression, thus have important implications in disease and development (Haberland et al., 2009; Martin et al., 2007). The functional role of HDAC is typically achieved by removing acetyl groups from histone tails to suppress transcriptional activation, thereby antagonizing the action of histone acetyl transferases (HAT), which promotes transcription via acetylation (Bacs and Olson, 2006; Bush and McKinsey, 2009; McKinsey and Olson, 2005).

To date, with 18 identified mammalian isoforms, the HDAC superfamily is divided into 4 distinct classes (class I, II, III and IV) with a relatively conserved

catalytic domain (Haberland et al., 2009; Parra and Verdin, 2010; Yang and Gregoire, 2005) Compared to other subtypes, class II HDACs (4, 5, 6, 7, 9 and 10) exhibit tissue-specificity with predominant expression in skeletal muscles, heart, brain and T-cells while class I and III are found more ubiquitously expressed in other cell types (Bertos et al., 2001; Calalb et al., 2009; Parra and Verdin). Like other class IIa members, HDAC5 contains a catalytic deacetylase domain in the C-terminal half between a NLS at the N-terminus and a NES near the C-terminus. Many *in vivo* studies have examined the functional role of HDAC5 in the heart and its consequent stress-induced exacerbated cardiac hypertrophy in knockout mice with a loss-of-function phenotype (Chang et al., 2004; Haberland et al., 2009; Kee and Kook), highlighting the importance of its cellular localization and function in regulating cardiac physiology and pathology. Specifically, fundamental regulation of HDAC5 is crucial to cardiac remodeling and hypertrophy at the cellular level, where activation of the “fetal gene program” is initiated by relieving transcriptional inhibition to activate MEF2 controlled genes (Lu et al., 2000b; Martin et al., 2009; McKinsey et al., 2000a; McKinsey et al., 2000b; McKinsey et al., 2002).

Compelling evidence has established that HDAC5 nucleocytoplasmic shuttling is highly regulated by its phosphorylation at S259 and S498 to initiate its phosphorylation-dependent nuclear export via binding to 14-3-3 chaperone proteins and unmasking its NES signal (Ellis et al., 2003; Grozinger and Schreiber, 2000; Harrison et al., 2004; McKinsey et al., 2000b; McKinsey et al.,

2001; McKinsey et al., 2002). Accordingly, we and others have reported that neurohumoral stress stimuli coupled to activation of G-protein receptors can trigger downstream target kinases, such as CaMKII, PKC and PKD to phosphorylate HDAC5 at these sites and mediate its nuclear export in cardiac myocytes (Bossuyt et al.; Bossuyt et al., 2008; Harrison et al.; Harrison et al., 2006; Vega et al., 2004; Wu et al., 2006). The dynamic shuttling of HDAC5 between the nucleus and the cytosol has been investigated due to its specific expression and function in cardiac myocytes, yet detailed mechanism of its regulated trafficking remains to be elucidated, especially during activation of multiple stress stimuli.

Neurohumoral signaling has uncovered several class II HDAC kinases that can mediate de-repression of MEF-2 dependent genes via nuclear export of HDAC5. There is little information about the cross interaction of neurohumoral signaling with β -AR activation, a major pathway involved in sympathetic stimulation that can improve cardiac functions acutely but can also trigger pathological cardiac remodeling and heart failure under chronic activation (Chidsey et al., 1963; Movsesian and Bristow, 2005). There are several studies investigating the role of PKA activation on hypertrophy signaling, but the pathway is unclear. For example, PKA has been reported to facilitate PKD activation and mobilize the consequent hypertrophic signaling due to their common association with scaffolding protein, AKAP-Lbc, in Cos7 cells and neonatal rat ventricular myocytes (NRVM) (Appert-Collin et al., 2007; Carnegie et al., 2004; Carnegie et

al., 2008). Alternatively, PKA has recently been identified to modulate MEF-2 regulated gene expression by promoting proteolysis of HDAC4 and phosphorylating HDAC5 directly exerting anti-hypertrophic effect against stress stimuli in HEK cells, Cos7 cells and NRVM (Backs et al., 2011; Ha et al., 2010; Sucharov et al., 2011). These biochemical and enzymatic experiments show results that are mostly described in immortal cell lines and neonatal myocytes, both provide very different cellular environment from adult myocytes. Thus, the role of PKA activation on hypertrophic signaling remains to be clarified, especially in adult cardiac myocytes.

In this study, we investigate the effects of β -AR signaling and PKA activation on regulation of nucleocytoplasmic HDAC5 shuttling and its cross interaction with Gq-driven hypertrophic signaling in adult ventricular myocytes. We focus on contrasting the effects of acute vs. chronic β -AR stimulation since chronic β -AR stimulation also triggers maladaptive changes leading to hypertrophy and is associated with decreased cardiac function (Chidsey et al., 1963; Movsesian and Bristow, 2005). We report that acute ISO and forskolin treatments promote strong nuclear HDAC5 accumulation in contrast to the nuclear export during Gq-agonist stimulation. Thus, acute PKA activation protects against Gq-driven HDAC5 nuclear export and hypertrophic signaling. Prolonged forskolin treatment results in further enhancement of nuclear HDAC5 signal, yet permits the subsequent Gq-mediated HDAC5 nuclear export. Heart failure rabbit myocytes exhibit a more cytosolic HDAC5 distribution with an

absence of ISO-induced HDAC5 nuclear accumulation, demonstrating blunted β -AR sensitivity in heart failure cells and reduced gene repression control. In summary, this study provides insights to cross-interaction between β -AR and Gq signaling pathways at the level of gene transcriptional control via regulating HDAC5 shuttling between nucleus and cytosol in adult cardiac myocytes.

Materials and Methods

Adenoviral Generation

WT HDAC5, S279A and S279D variants were subcloned into a pShuttle-CMV vector by restriction enzyme digestion and ligation. Using the AdEasy Adenoviral Vector System (Agilent), HDAC5-GFP adenoviral DNA was generated and prepared by Wizard Plus Miniprep DNA Kit (Promega). HEK cells plated at 90% confluence on 6 cm culture dishes was transfected with GFP-tagged HDAC5 adenoviral DNA (10 μ g) using Polyfect transfection reagent (Qiagen) in 2 mL of growth medium supplemented with 3% penicillin/streptomycin and 2% FBS (CellGro). After 2 weeks of plaque formation, lysed cells were harvested and centrifuged at 2,000 rpm for 3 min. The supernatant was then transferred onto 10 cm culture dishes plated with HEK cells at 90% confluency. Upon completion of cell lysis, adenovirus DNA was further amplified by transferring to thirty 10 cm dishes. Cells were harvested, lysed by freeze-thaw cycles (3 times), and purified using a cesium chloride gradient method. The mature adenovirus particles were then extracted and dialyzed in Slide-A-Lyzer Dialysis cassette (Thermo Scientific) in 1x PBS at 4°C overnight. The next day, the purified adenovirus was aliquoted and stored at -80°C.

Cell Isolation and Culturing

We isolated ventricular myocytes from adult New Zealand white rabbit (male, average age of 3.5 months, average weight of 2.2 kg and purchased from Charles River Laboratories) as described previously (Bassani et al., 1994). All

animal procedures were performed in accordance to Institutional Animal Care Use Committee regulations at University of California-Davis. Isolated myocytes were allowed to settle by gravity and re-suspended in PC-1 medium (Lonza) supplemented with 5% penicillin/streptomycin. 2-well glass coverslips were coated with laminin (Invitrogen) and allowed to air-dry prior to plating. 2 mL of cell resuspension was added to each well of the coverslip and incubated at 37°C for 45 min for attachment. Cells were gently washed and incubated with fresh PC-1 medium immediately before adenoviral infection with GFP-tagged constructs of WT HDAC5, SS259/498AA, S279A and S279D at 37°C overnight (multiplicity of infection at 10-100).

Confocal Imaging

Coverslip was placed on an inverted microscope (Zeiss, LSM5 Pascal or Olympus) equipped with 40x water immersion object lens. Argon laser (3-5%) was used to excite GFP at 488 nm, and emission wavelength was collected with long-pass filter above 505 nm. PC-1 medium was replaced with normal tyrode solution containing 1.8 mM Ca^{2+} before each experiment. Cells were imaged before and after treatment with agonists, such as ET-1 (100 nM), PE (20 μM), ISO (100 nM) or forskolin (10 μM) for 1 hr. NIH Image J software was used for image analysis. The nucleus was traced with ROI to measure its fluorescence intensity and divided by the average of three randomly selected ROIs of equal size in the cytoplasm to compare the nuclear vs. cytosolic signal. Each $F_{\text{nuc}}/F_{\text{cyto}}$ ratio was normalized to initial time point for comparison between different groups.

Heart Failure Animal Model

Heart failure was induced in adult New Zealand White rabbit as described previously (Pogwizd et al., 1999). Briefly, HF was induced by two sequential survival surgeries by first inducing aortic insufficiency and followed with aortic constriction after 2-8 weeks post-op monitoring. Each rabbit was monitored with echocardiogram to determine the severity of the HF status (typically 4-6months) and scheduled for isolation procedure accordingly.

Co-immunoprecipitation (Co-IP)

Isolated adult rabbit myocytes were infected with GFP-tagged WT and S279A constructs of HDAC5 and cultured overnight in PC-1 media (Lonza) (multiplicity of infection = 10-100). Next day, myocytes were treated with or without ISO (100 nM) for 20 min prior to addition of ET-1 (100 nM) for 1 hr. Cells were scraped, pelleted at 2,000 rpm for 3 min and supernatant was removed. Control and treated pellets were frozen in liquid nitrogen and then were lysed with IP buffer (pH = 7.4 containing 25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1% NP-40, 1 mL phosphatase inhibitor cocktail set III (Calbiochem), 1 mL protease inhibitor cocktail set III (Calbiochem)). Cell lysates were re-suspended and incubated on ice for 30 min. Cell debris was pelleted using a hand centrifuge for 1 min, and supernatant was collected to determine protein concentration using BCA kit (Thermo Scientific). 200 µg of protein from each sample was incubated end over end with GFP antibody conjugated with agarose

beads (Santa Cruz Biotechnology, sc-9996 AC) in 3 mL of IP buffer at 4°C overnight. The next day, samples were centrifuged at 2,000 rpm for 3 min and washed 3 times with washing buffer (pH = 7.4 containing 25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1 mL phosphatase inhibitor cocktail set III (Calbiochem), 1 mL protease inhibitor cocktail set III (Calbiochem)). IP pellets were collected for probing levels of 14-3-3 binding to WT HDAC5-GFP or S279A-GFP using immuno-blots.

Western Blotting

Adult rabbit or rat ventricular myocyte samples added with Laemmli sample buffer were heated at 95°C for 5 min prior to loading onto SDS-PAGE gels (Biorad), and subject to electrophoresis at 90V for 2 hr. Proteins were transferred onto nitrocellulose membrane overnight at 20V for 12 hr. The next day, nitrocellulose membranes were blocked with 8% milk in Tris-buffered saline solution (TBS) with 0.2% Tween followed by incubation with primary antibody overnight at 4°C. The primary antibodies used for immunoblotting were diluted in TBST as follows: rabbit GFP antibody (1:10,000) from Abcam, rabbit pan 14-3-3 antibody (1:1000) from Santa Cruz Biotechnology, rabbit HDAC5 total (1:2000) from Signal Antibody Technology, rabbit phospho-HDAC5 Ser498 (1:2000) from Signal Antibody Technology and mouse GAPDH (1:30,000) from Abcam. The next day, membranes were washed five times with TBS/TBST and incubated with secondary goat anti-rabbit or anti-mouse antibody (1:2000 + 0.2% milk) (Thermo Scientific) for 2 hours at room temperature. After five repetitive washing,

membranes were developed using Super Signal West Dura (Thermo Scientific) and digitally captured with ImageReader LAS-4000. Densitometry analysis was performed using NIH Image J software.

Luciferase Assay

Isolated rabbit ventricular myocytes were infected with myocyte enhancer factor-2 (MEF2) luciferase reporter and cultured at 37°C overnight. The next day, cells were treated with ET-1, PE, ISO or forskolin for 2 hr. Some myocytes were also pretreated with ISO or forskolin for 20 min prior to treatment with ET-1 or PE for 2 hr. The cells were collected, pelleted at 2,000 rpm for 2 min and supernatant was aspirated. The Dual-Luciferase Reporter Assay (Promega, catalog E1910) was used for this assay. All reagents were prepared according to manufacturer's instructions. 100 µl of 1x passive lysis buffer (Promega) was added to each tube to resuspend the pellet by vortexing. The cell lysates were transferred to 1.5 mL of eppendorf tubes and centrifuged at 25,000 rpm for 5 min. To begin luciferase assay, 20 µl of passive lysis buffer lysate/well of each sample was added to the 96-well white costar plate with opaque bottom. (Each sample was prepared as duplicates for luciferase assay.) 40 µl of both luciferase substrate with buffer and Stop & Glo substrate with buffer were required for each sample. Total amounts of both reagents were calculated and prepared in separate falcon tubes covered with aluminum foil. The 96-well plate, luciferase reagent and renilla reagent were all placed in the Veritas luminometer. The assay was performed using Veritas software to measure firefly luciferase activity

followed by renilla luciferase activity. Cardiac myocytes do not possess endogenous renilla reporter, thus the renilla luciferase readouts are used and subtracted as background fluorescence. All treatments were normalized to amount of protein content and control (untreated sample) for each experiment.

Statistical Analysis

Pooled data are represented as mean \pm SEM. We performed student's t-test or one-way analysis of variance (ANOVA) with Bonferroni post hoc test when applicable and $p < 0.05$ was considered statistically significant.

Results

β -AR signaling via PKA induced HDAC5 nuclear accumulation in contrast to Gq-mediated signaling

To study the intracellular translocation of HDAC5 in isolated adult rabbit ventricular myocytes, we used a GFP-tagged HDAC5 construct displaying predominant nuclear expression (Figure 24). We compared the ET-1 mediated nuclear export of HDAC5 and ISO induced nuclear accumulation of HDAC5 at 1 hour. As shown in Figure 31, β -AR agonist, ISO, induced a strong accumulation of HDAC5-GFP in the nucleus ($F_{\text{nuc}}/F_{\text{cyto}} = 1.33 \pm 0.03$) in contrast to the nuclear depletion mediated by Gq-agonists, ET-1 ($F_{\text{nuc}}/F_{\text{cyto}} = 0.76 \pm 0.01$) or PE ($F_{\text{nuc}}/F_{\text{cyto}} = 0.79 \pm 0.01$) at 1 hour (Figure 25). Since PKA is a known downstream effector of β -AR signaling, we tested the hypothesis that the effect of HDAC5 nuclear accumulation is mainly triggered by PKA. Thus, we used forskolin, an adenylyl cyclase activator to activate PKA, and this resulted in increased nuclear HDAC5 accumulation similar to ISO, but to a slightly lesser degree ($F_{\text{nuc}}/F_{\text{cyto}} = 1.23 \pm 0.04$). Figure 26 summarizes the results of HDAC5-GFP translocation at 1 hour post-treatment in response to various stimuli. ISO and forskolin increased nuclear accumulation of HDAC5 by $31\% \pm 2.5\%$ and $17\% \pm 3.5\%$ respectively, while Gq-agonists, ET-1 and PE, mediated comparable nuclear export of HDAC5 ($F_{\text{nuc}}/F_{\text{cyto}} = 0.68 \pm 0.03$ for ET-1; $F_{\text{nuc}}/F_{\text{cyto}} = 0.71 \pm 0.04$ for PE). Furthermore, figure 27 shows that 20 min pre-inhibition of PKA with H89 (10 μM) or PKI (36 μM) effectively blocked the PKA-mediated

nuclear accumulation under ISO treatment. ISO-induced nuclear accumulation of HDAC5 was significantly reduced by $81.1\% \pm 6.3\%$ with H89 pretreatment ($p < 0.05$) and by $82.6\% \pm 2.1\%$ with PKI ($p < 0.05$). The inhibition of ISO-induced effect on HDAC5 accumulation in the nucleus by these two PKA inhibitors confirms that PKA is the major kinase involved in the β -AR signaling induced HDAC5 nuclear import.

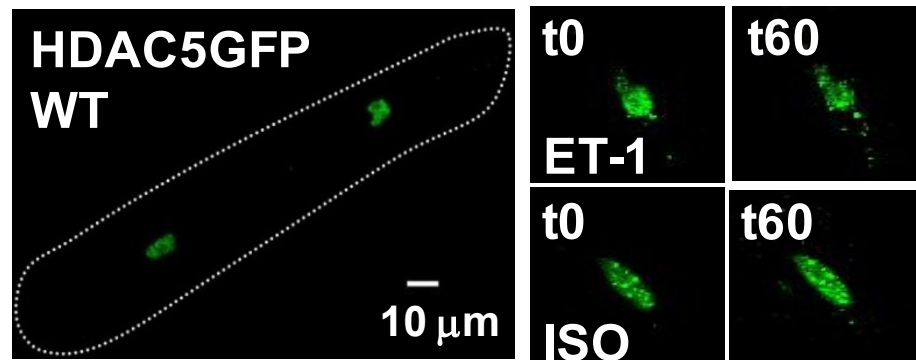


Figure 24. ET-1 induces WT HDAC5-GFP nuclear export while ISO triggers nuclear import. Representative confocal images of HDAC5-GFP expression in rabbit adult ventricular myocytes at baseline, pre- and post- treatments with ET-1 (100 nM) or ISO (100 nM) after 1 hr.

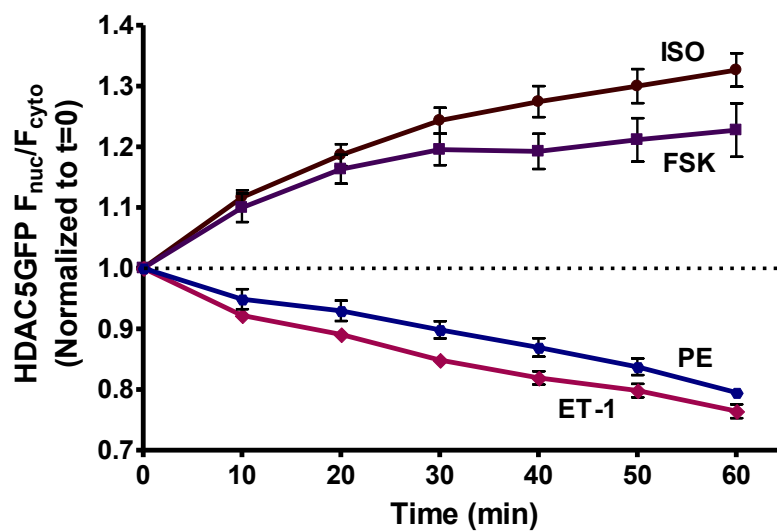


Figure 25. β -AR signaling induces nuclear import of HDAC5 in contrast to Gq-mediated export. HDAC5-GFP translocation data expressed as normalized F_{nuc}/F_{cyto} in response to ISO (100 nM), forskolin (10 μ M), ET-1 (100 nM) and PE (20 μ M) over the course of 60 min.

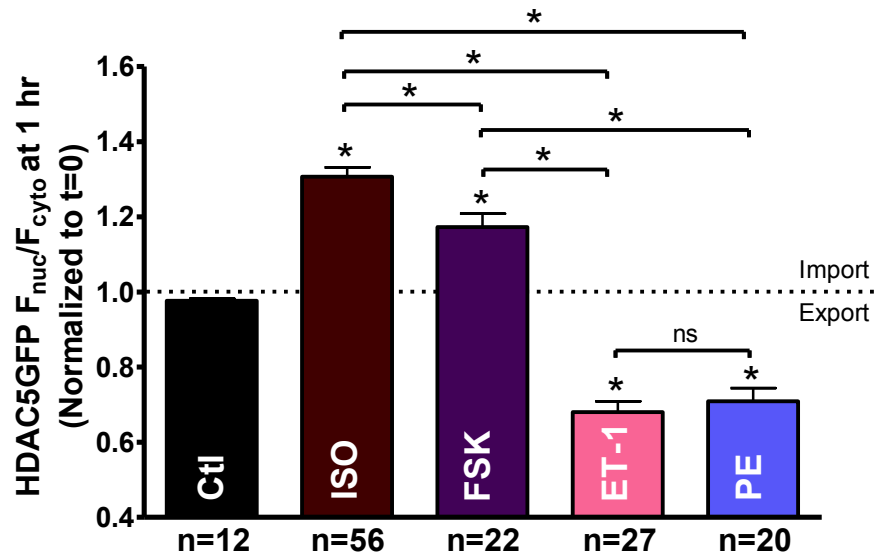


Figure 26. β -AR signaling induces nuclear import of HDAC5 in contrast to Gq-mediated export. Bar graph summarizes HDAC5-GFP translocation at 1 hr post-treatment with ISO (100 nM), forskolin (10 μ M), ET-1 (100 nM) and PE (20 μ M). * $p < 0.05$.

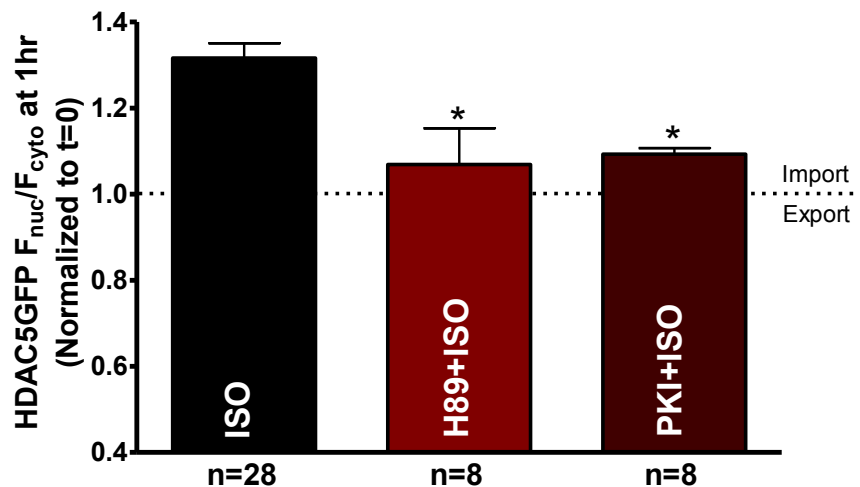


Figure 27. ISO induced nuclear import of HDAC5 is mainly triggered by PKA activity. WT HDAC5-GFP translocation induced by ISO (100 nM) treatment alone or with 20 min pre-incubation of PKA inhibitors, H89 (10 μ M) or PKI (36 μ M). * $p < 0.05$.

14-3-3 chaperone binding sites are crucial for Gq-mediated but not for β -AR or PKA-induced HDAC5 translocation

Several studies have established that serine 259 and 498 (S259/S498) of HDAC5 are phosphorylated by CaMKII, PKC or PKD to trigger its phosphorylation-dependent nuclear export during hypertrophic signaling (Bossuyt et al., 2008; Harrison et al., 2006; Harrison et al., 2004; Vega et al., 2004; Wu et al., 2006). Phosphorylation at these two residues is important for binding with chaperone protein, 14-3-3, to initiate its nuclear export (Figure 28) (Ellis et al., 2003; Grozinger and Schreiber, 2000; Harrison et al., 2004; McKinsey et al., 2000b; McKinsey et al., 2001). We tested whether these two serine residues are also involved in nuclear accumulation of HDAC5 induced by β -AR signaling or PKA activation. We used a GFP-tagged HDAC5 mutant with alanine substitution at these residues (SS259/498AA) to study the importance of these sites in β -AR/PKA signaling and HDAC5 translocation. The SS259/498AA mutant also displayed predominant nuclear localization although baseline level of HDAC5 nuclear localization was $31\% \pm 8\%$ higher compared to the WT (Figure 29). This higher basal nuclear expression was likely due to the total absence of serine phosphorylation of the SS259/498AA mutant by CaMKII or PKD, thus disrupting the basal HDAC5 shuttling between the nucleus and the cytosol and preventing its phosphorylation-dependent nuclear exports at baseline. The implication is that physiologically, there may be some finite level of baseline phosphorylation at these sites.

The non-phosphorylatable mutant SS259/498AA significantly reduced the HDAC5 nuclear export induced by ET-1 or PE compared to WT (Figure 30). Conversely, the SS259/498AA mutant did not perturb the ISO or forskolin-induced nuclear import (Figure 30), suggesting that the PKA-induced nuclear accumulation effect of HDAC5 must occur at site(s) different from S259 and S498. Taken together, the 14-3-3 chaperone binding sites are crucial for the Gq-mediated nuclear export mechanism, but are not important for the β -AR or PKA-induced HDAC5 accumulation in the nucleus.

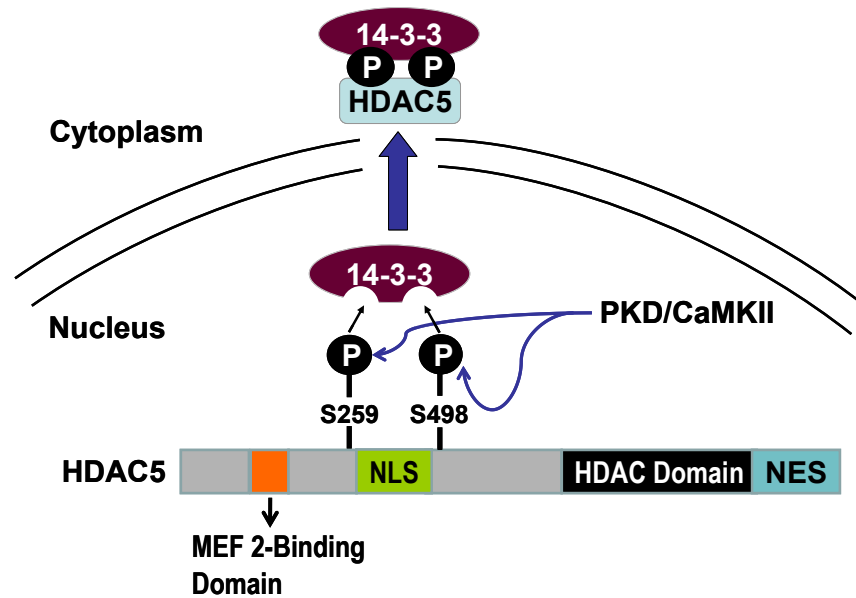


Figure 28. S259 and S498 sites of HDAC5 are crucial for Gq-mediated phosphorylation and consequent 14-3-3 nuclear export. Schematic diagram of HDAC5 domains and its phosphorylation sites by CaMKII and PKD for 14-3-3-dependent nuclear export mechanism.

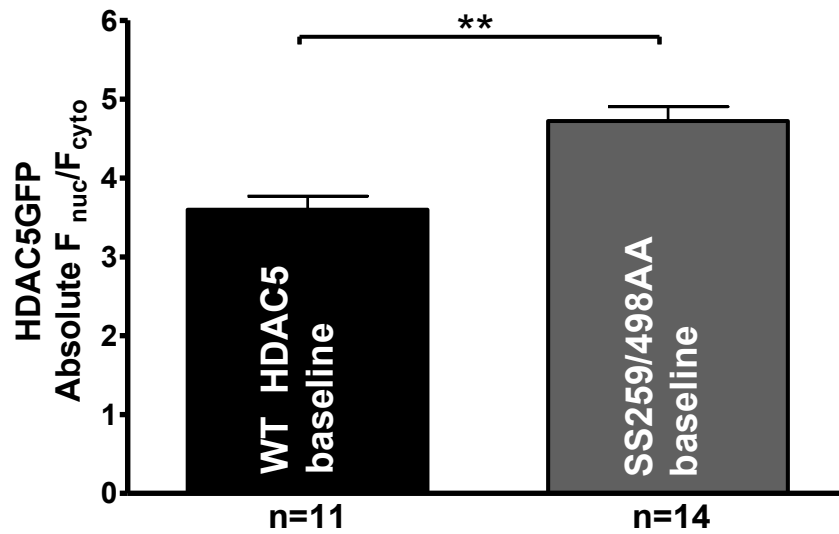


Figure 29. Non-phosphorylatable SS259/498AA mutant results in higher baseline HDAC5 nuclear localization. Comparison of absolute baseline localization F_{nuc}/F_{cyto} of WT HDAC5 vs. SS259/498AA mutant overexpression in adult cardiac myocytes. ** $p < 0.01$.

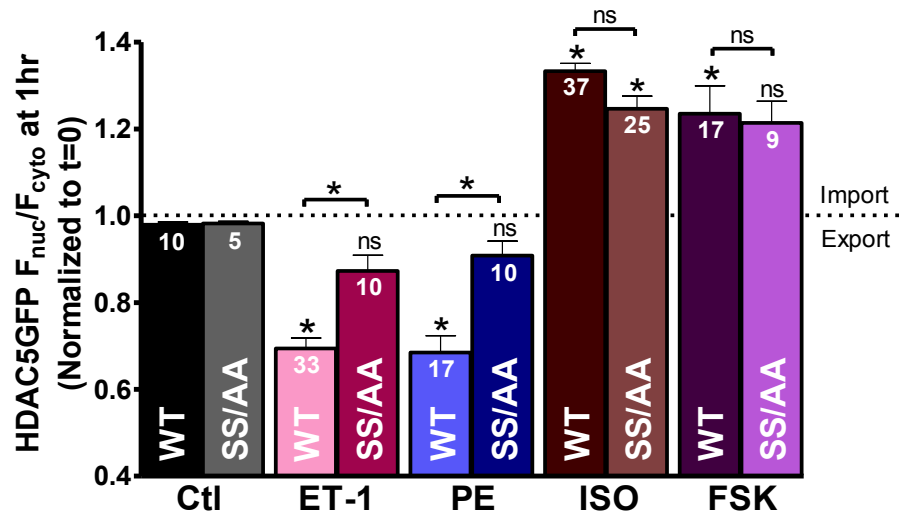


Figure 30. 14-3-3 chaperone binding sites are crucial for Gq-mediated but not for β -AR or PKA-induced HDAC5 translocation. Bar graph comparison of GFP-tagged HDAC5 SS259/498AA mutant translocation vs. WT HDAC5 at 1 hr post-treatment with indicated agonists (ET-1 = 100 nM, PE = 20 μ M, ISO = 100 nM, FSK = 10 μ M). * $p < 0.05$.

S279 phosphorylation by β -AR and PKA stimulation induce HDAC5 nuclear accumulation

Recently, serine 279 (S279) within the NLS region of HDAC5 was identified to be directly phosphorylated by PKA (Ha et al., 2010). To test that β -AR or PKA activation mediates its inhibitory effects on HDAC5 nuclear export predominantly via phosphorylation of S279, we designed an adenoviral construct of GFP-tagged HDAC5 mutant with alanine substitution at S279 (S279A) to study its translocation under β -AR/PKA signaling. Baseline expression of the GFP-tagged HDAC5 S279A mutant showed similar nuclear targeting compared to WT (Figure 31), indicating that baseline nucleocytoplasmic shuttling of S279A remained intact. This result was significantly different from the basal expression observed with the SS259/498AA mutant where basal shuttling was disturbed by preventing the phosphorylation at S259 and S498 sites, thus favoring nuclear retention of HDAC5 (Figure 29). So basal phosphorylation at S279 site may be negligible.

The GFP-tagged S279A mutant of HDAC5 completely ablated the ISO and forskolin-induced nuclear import while export remained intact under ET-1 and PE stimulation over 1 hour (Figure 32). Grouped data summarize S279A translocation at 1 hour post-treatment, where ET-1 or PE-mediated nuclear export was comparable to WT, while ISO or forskolin induced nuclear accumulation was completely abolished (Figure 33). These data suggest that S279 serves as a major site for PKA phosphorylation during β -AR signaling induced HDAC5 nuclear import in adult cardiac myocytes.

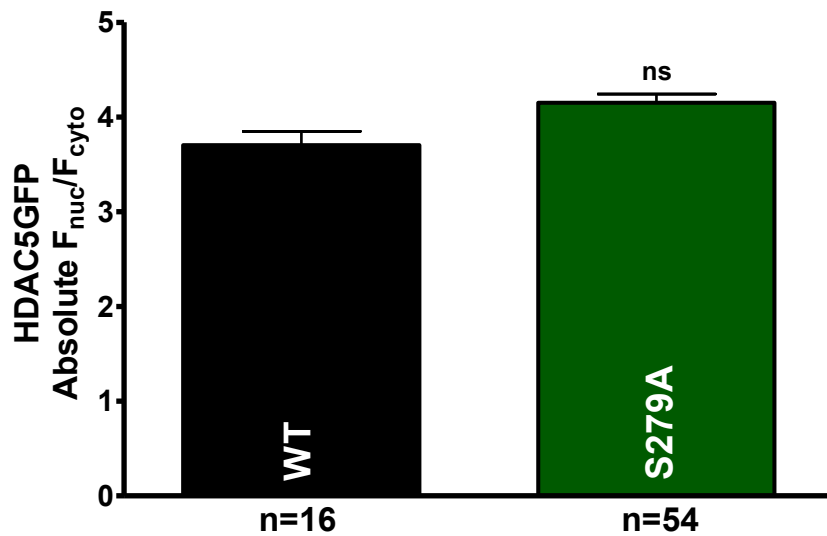


Figure 31. S279A mutant has similar baseline localization compared to WT HDAC5. Comparison of absolute baseline F_{nuc}/F_{cyto} of HDAC5 between WT vs. S279A variant overexpression in adult cardiac myocytes.

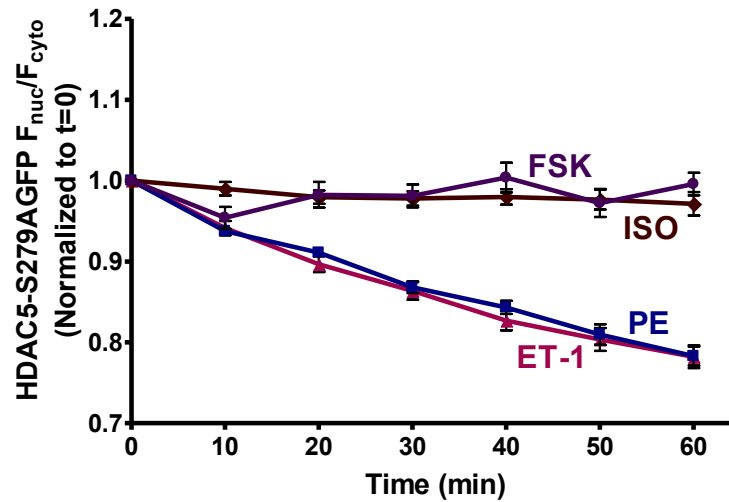


Figure 32. S279A mutant blocks nuclear import induced by β -AR or PKA activation but responds to Gq-mediated export. Response of GFP-tagged HDAC5 S279A translocation in response to ISO (100 nM), forskolin (10 μ M), ET-1 (100 nM) and PE (20 μ M) over 60 min.

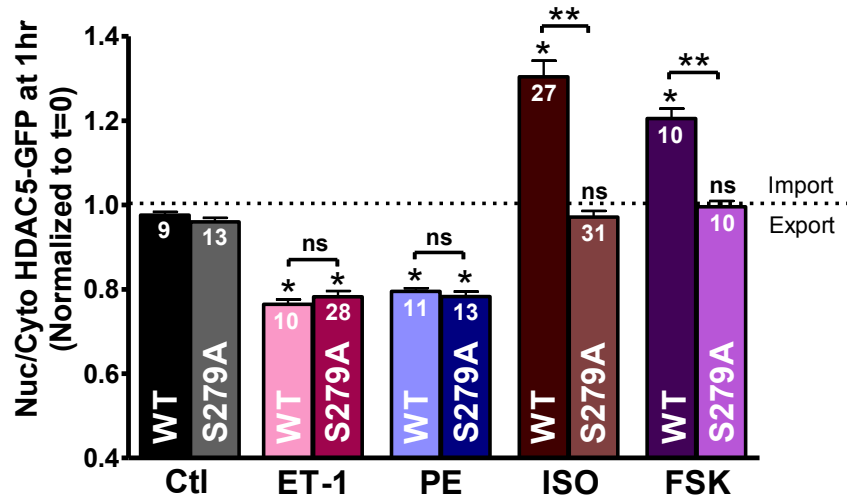


Figure 33. HDAC5 S279A mutant completely ablates the β -AR or PKA-mediated HDAC5 nuclear import. Bar graph summary of GFP-tagged HDAC5 S279A mutant at 1 hr post-treatment with indicated agonists (ET-1 = 100 nM, PE = 20 μ M, ISO = 100 nM and forskolin = 10 μ M) in comparison to WT HDAC5. * $p < 0.05$. ** $p < 0.01$.

S279 phospho-mimetic mutant inhibits HDAC5 translocation

To test the hypothesis that phosphorylation at S279 prevents nuclear export inducible by ET-1 or PE, we designed a phosphomimetic HDAC5 mutant with an aspartic acid substitution at S279 (S279D). Notably, the GFP-tagged S279D phosphomimetic HDAC5 resulted in $49.9\% \pm 8.7\%$ increase in basal $F_{\text{nuc}}/F_{\text{cyto}}$ ratio compared to WT. This indicates that the S279D was an effective phosphomimetic mutant at baseline, giving similar $F_{\text{nuc}}/F_{\text{cyto}}$ ratio as the maximal ISO or forskolin induced nuclear accumulation of WT HDAC5 (Figure 34).

The GFP-tagged S279D phosphomimetic mutant also completely prevented ET-1 or PE triggered nuclear export, suggesting that phosphorylation at S279 site reduces the amount of nuclear export inducible by Gq-agonists (Figure 35). Moreover, forskolin had no further effect on S279D mutant HDAC5 translocation, indicating that this phosphomimetic mutant was able to completely abolish the PKA-effects on HDAC5 nuclear accumulation (Figure 35-36). Intriguingly, we still observed slight nuclear import of HDAC5 by 10% upon ISO stimulation compared to forskolin. This minor increase suggests that there could be additional PKA-independent effects on HDAC5 translocation during β -AR stimulation by ISO. In summary, the phosphomimetic mutant significantly reduced the nuclear export induced by ET-1 or PE, and the HDAC5 nuclear import induced by ISO and PKA (Figure 36). Therefore, β -AR or PKA mediates HDAC5 nuclear accumulation predominantly via phosphorylation of S279, and S279 phosphorylation of HDAC5 effectively attenuates the Gq-induced nuclear export.

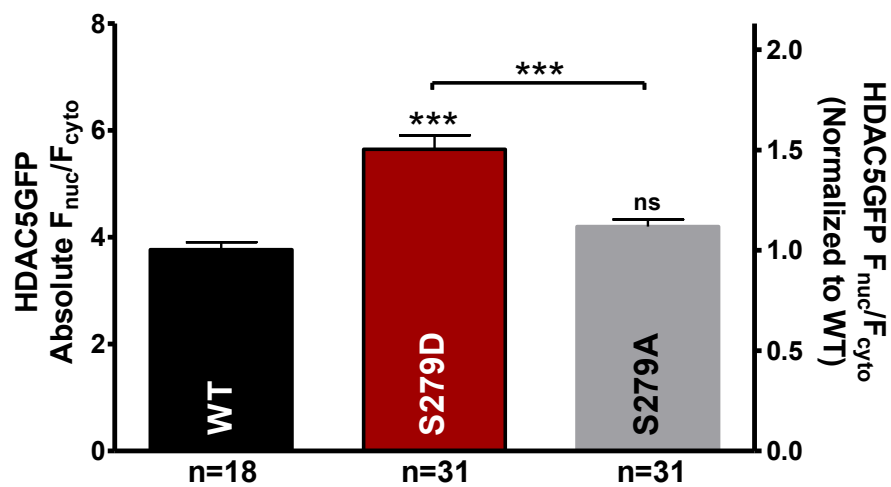


Figure 34. S279D phosphomimetic mutant results in higher baseline nuclear localization than WT HDAC5. Comparison of absolute baseline $F_{\text{nuc}}/F_{\text{cyto}}$ between of WT HDAC5, S279D and S279A variants.

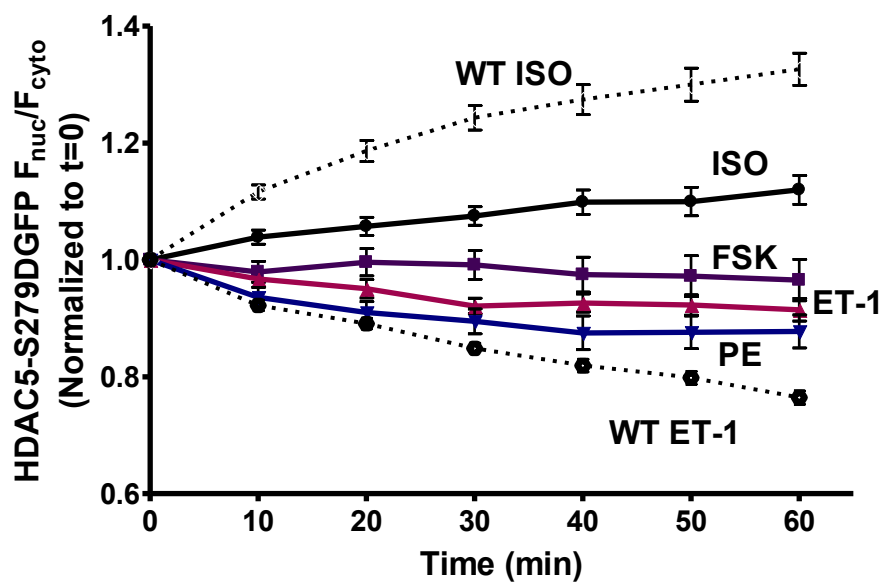


Figure 35. Phosphomimetic S279D mutant significantly reduces HDAC5 translocation triggered by both β -AR/PKA or Gq signaling. Response curves of GFP-tagged HDAC5-S79D translocation in response to ISO (100 nM), forskolin (10 μ M), ET-1 (100 nM) and PE (20 μ M) over 60 min.

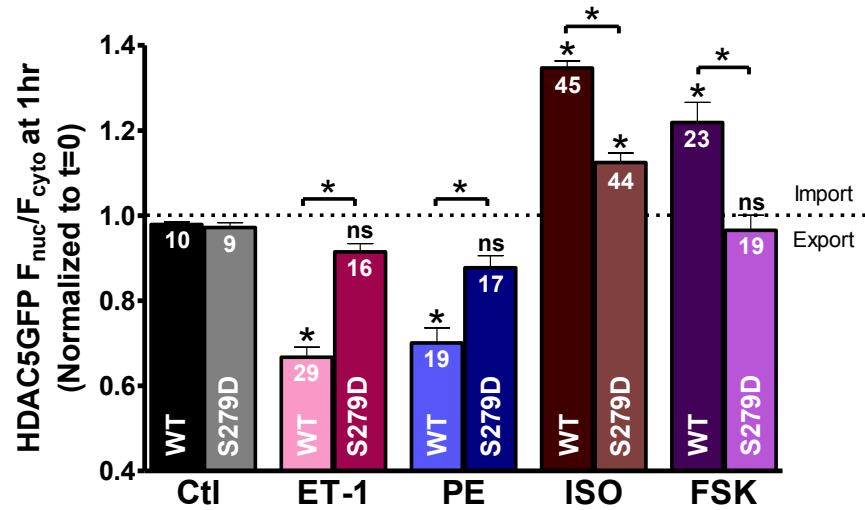


Figure 36. S279D phospho-mimetic mutant inhibits HDAC5 translocation.

Bar graph summary of HDAC5 S279D mutant at 1 hr post-treatment with indicated agonists compared to WT. * $p < 0.05$.

Acute β -AR or PKA activation blocks Gq-mediated HDAC5 nuclear export

Since PKA seems to drive HDAC5 import via S279 phosphorylation and Gq activation (via PKD and CaMKII) drive nuclear export via phosphorylation at S259 and S498, it is of interest to see which direction dominates HDAC5 translocation upon co-activation. Based on our mutant studies, we hypothesized that the PKA effect will dominate. To test our hypothesis, we examined the effects of β -AR signaling on Gq-mediated HDAC5 translocation between the nucleus and the cytosol. Acute PKA pre-stimulation by forskolin for 20 min abolished the nuclear export of HDAC5 induced by ET-1 or PE treatment (Figure 37). Acute β -AR activation with ISO followed by Gq-agonists also effectively blocked the HDAC5 nuclear export that is inducible by ET-1 or PE. Additionally, ISO caused ET-1 and PE to trigger slightly more nuclear accumulation. This again points to the notion that β -AR may have a PKA-independent effect to favor HDAC5 nuclear localization (vs. forskolin). Therefore, acute pre-stimulation of ISO or forskolin completely prevented the subsequent Gq-agonists mediated effects on HDAC5 shuttling, indicating that acute β -AR stimulation via PKA may suppress the Gq-driven HDAC5 nuclear export.

To further test whether the β -AR stimulation or PKA activation can prevent or limit HDAC5 S498 phosphorylation in response to Gq activation, we probed for S498 phosphorylation of endogenous HDAC5 in adult rat myocytes. We used rat instead of rabbit myocytes for this experiment to minimize non-specific detection of the primary rabbit polyclonal HDAC5 antibody that is commercially available. Our results showed that hypertrophic agonists, ET-1, PE and phorbol 12,13-

dibutyrate (PdBu) all enhanced S498 phosphorylation compared to untreated control (Figure 38). Conversely, HDAC5 S498 phosphorylation was reduced under ISO or forskolin stimulation, and 20 min pretreatment of forskolin effectively abolished endogenous HDAC5 S498 phosphorylation induced by ET-1, PE and PdBu. Additionally, co-immunoprecipitation with GFP antibody enables us to detect the levels 14-3-3 binding with HDAC5-GFP during β -AR/PKA and Gq activation (Figure 39). ET-1 increases the amount of 14-3-3 associated with WT HDAC5-GFP, but this effect is abolished by 20 min ISO pretreatment. Notably, ISO treatment alone slightly reduced 14-3-3 association with HDAC5-GFP, in agreement with the higher nuclear localization seen in this condition. In contrast, when the PKA resistant S279A HDAC5-GFP was used, ISO did not perturb basal 14-3-3 association and failed to prevent ET-1 induced 14-3-3 binding.

We also examined the effects of β -AR/PKA on MEF2-dependent transcriptional activity in adult cardiac myocytes to test whether the β -AR/PKA effects on HDAC5 translocation resulted in transcriptional regulation. Figure 40 shows that Gq-agonists, ET-1 and PE, promoted MEF2 luciferase activity, while ISO and forskolin failed to alter basal luciferase activity. Pre-incubation of ISO and forskolin, however, effectively abolished Gq-agonist induced increases MEF2 activity. This parallels our confocal studies where β -AR/PKA pre-activation blocks subsequent Gq-driven HDAC5 nuclear export.

The data suggest that ISO and forskolin treatments diminish basal S498 phosphorylation of endogenous HDAC5, an indicator of its nuclear export shuttling. Moreover, acute PKA activation reduces S498 phosphorylation of

HDAC5 during hypertrophic agonist stimulation, thereby promoting HDAC5 nuclear retention. Co-immunoprecipitation data confirm that the ET-1 enhanced WT HDAC5 interaction with 14-3-3, and this interaction was prevented by pre-incubation of ISO. HDAC5 S279A mutant, lacking the PKA phosphorylation site, relieves the ISO inhibitory effect on ET-1-induced 14-3-3 binding. MEF2-dependent luciferase activity is promoted by Gq-agonist, but this transcriptional activation can be effectively abolished with pretreatment of ISO or forskolin. Therefore, β -AR and PKA activation can modulate Gq-mediated HDAC5 translocation via S279 phosphorylation, thus reducing phosphorylation at S498 and precluding binding to 14-3-3 chaperone. Consequently, the Gq-driven MEF2 transcriptional activity can be blocked with acute β -AR/PKA activation, indicating that HDAC5 nuclear export is necessary for relieving the HDAC5 repression on MEF2-dependent transcriptional control in adult cardiac myocytes.

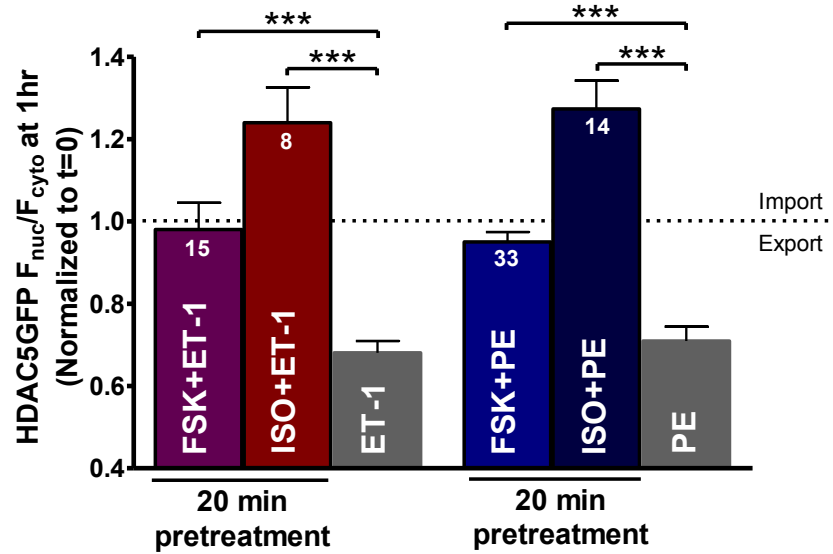


Figure 37. Acute pre- β -AR stimulation and PKA activation blocks Gq-mediated HDAC5 nuclear export. Comparison of normalized HDAC5GFP F_{nuc}/F_{cyto} at 1 hr post-treatment with ET-1 (100 nM) or PE (20 μ M) alone or with 20 min pre-incubation of forskolin (10 μ M) or ISO (100 nM). *** $p < 0.0001$.

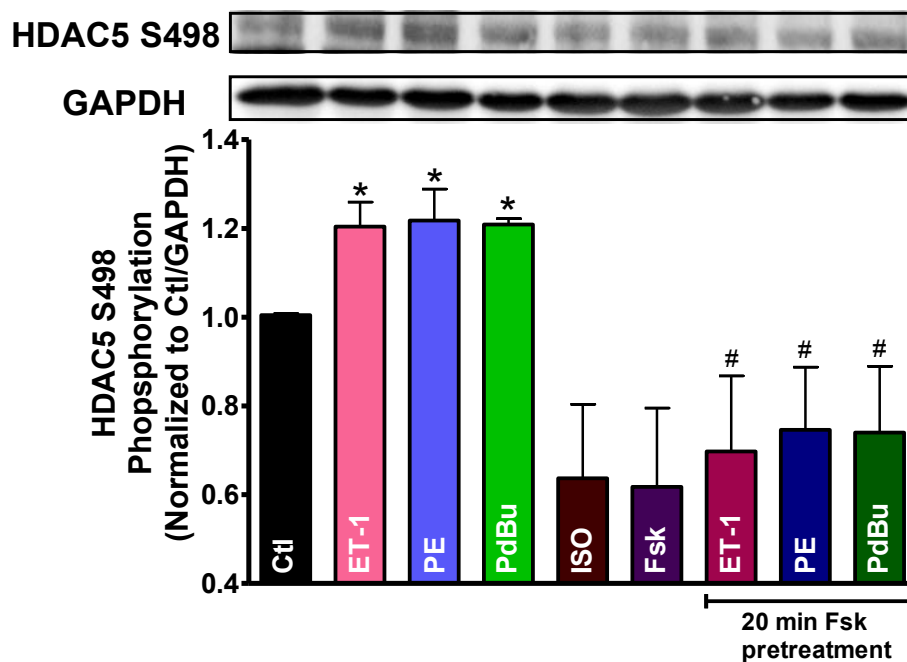


Figure 38. Endogenous HDAC5 S498 phosphorylation is increased by hypertrophic Gq-agonists, but decreased by β -AR/PKA activation or pretreatment. Representative immunoblot of endogenous HDAC5 S498 phosphorylation levels in adult rat ventricular myocytes. Isolated myocytes were treated with indicated agonists alone for 1 hour or with pre-incubation of forskolin (10 μ M) for 20 min at 37°C. * $p < 0.05$. (n=3)

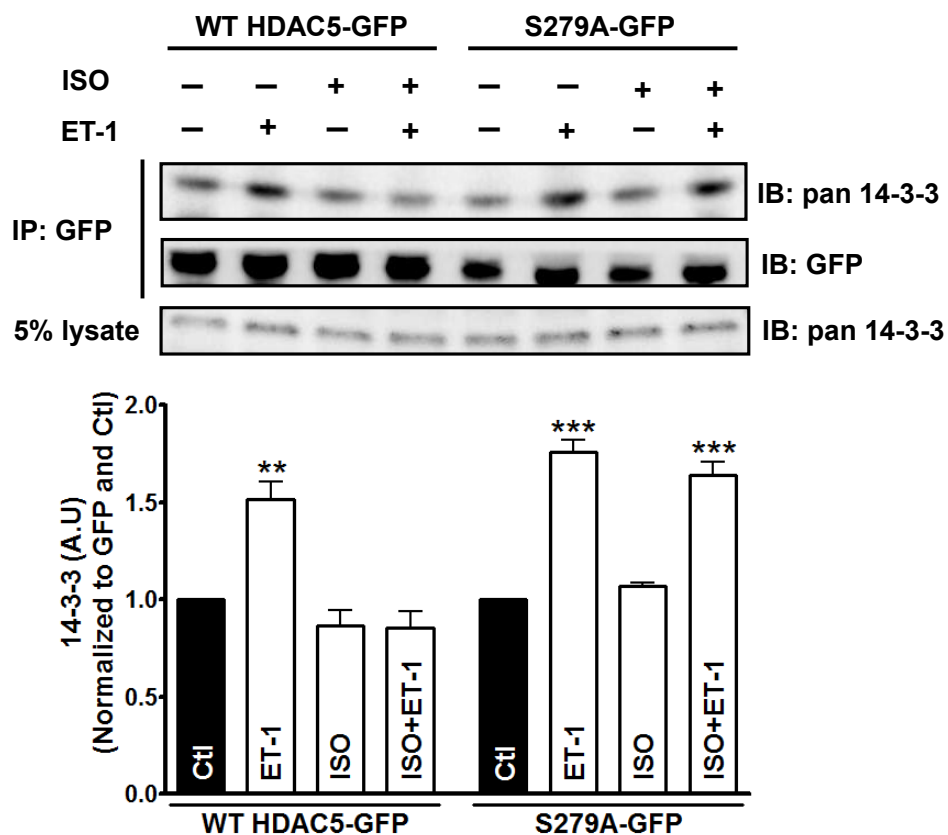


Figure 39. β -AR signaling inhibits WT HDAC5 binding to 14-3-3 via its S279 phosphorylation. Co-immunoprecipitation detecting endogenous 14-3-3 binding to WT HDAC5-GFP or S279A-GFP in adult rabbit myocytes treated with indicated drugs. ** p<0.01. *** p<0.001. (n=3)

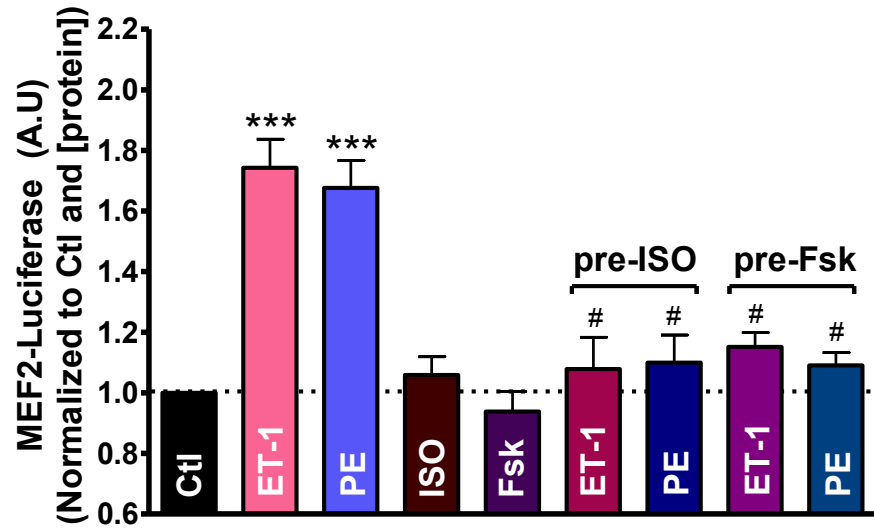


Figure 40. β -AR or PKA pre-activation inhibits Gq-agonist-induced hypertrophic MEF2 transcriptional activation. MEF2-dependent luciferase assay measuring myocyte transcriptional activity in response to agonist treatment. ***<0.001. (n=5).

Chronic forskolin exposure enhances HDAC5 nuclear accumulation

Chronic β -AR activation is associated with hypertrophy and cardiac remodeling, whereas we observe that acute β -AR and PKA stimulation prevent the hypertrophic agonist-induced HDAC5 shuttling. To further study the mechanism of acute vs. chronic PKA signaling, we studied the effects of 24-hr forskolin exposure on baseline HDAC5 nucleocytoplasmic shuttling. Confocal images of HDAC5-GFP expression after 24-hr treatment with forskolin are illustrated in figure 41. Figure 42 shows that prolonged forskolin exposure increased the nuclear accumulation of HDAC5 by 1.6-fold ($F_{\text{nuc}}/F_{\text{cyto}} = 10.27 \pm 0.89$ vs. $F_{\text{nuc}}/F_{\text{cyto}} = 6.33 \pm 0.31$, $p < 0.0001$ after 1 hour forskolin exposure). Our acute studies suggest that ISO and PKA-induced HDAC5 nuclear import is mediated predominantly via S279 phosphorylation. To test whether HDAC5 S279 phosphorylation is also crucial to the prolonged forskolin-mediated nuclear accumulation, we evaluated the effects of 24-hr forskolin exposure on the nucleocytoplasmic shuttling of WT HDAC5, S279A and S279D. Figure 43 showed that prolonged forskolin treatment caused significant further increase of WT HDAC5 in the nucleus while the S279A and S279D mutants both completely eliminated the increase, suggesting that prolonged forskolin-induced continuous nuclear import of HDAC5 also occurs in a S279 phosphorylation-dependent manner.

Next, we assessed if chronic forskolin treatment can inhibit Gq-induced nuclear export in myocytes treated with forskolin for 24 hr prior to addition of ET-1 or PE. Results in figure 44 showed that 24-hr forskolin pretreatment did not

block the HDAC5 nuclear export triggered by either ET-1 or PE, opposite to that seen for acute β -AR/PKA stimulation. Our data provide evidence that prolonged forskolin exposure causes further increase of nuclear HDAC5-GFP signal that is dependent on its S279 phosphorylation. However, chronic forskolin pretreatment does not block the Gq-mediated HDAC5 nuclear export, in contrast to the inhibitory effect by acute β -AR and PKA activation.

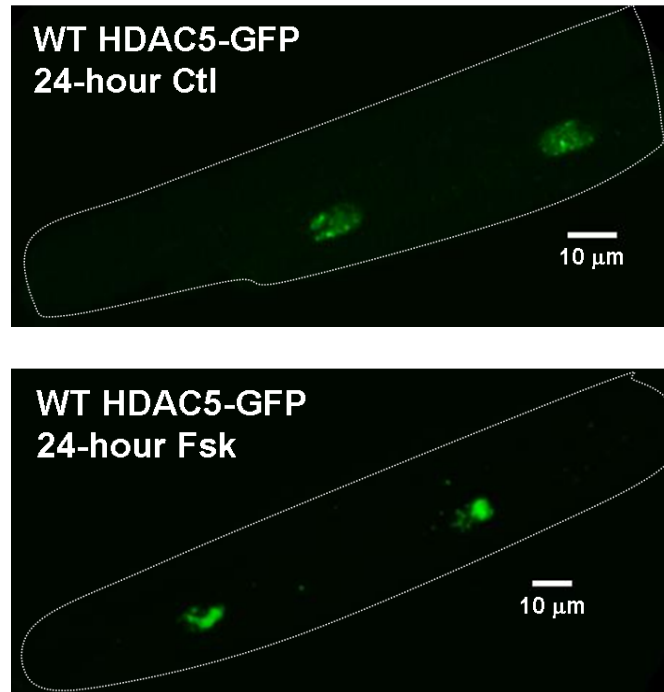


Figure 41. Chronic forskolin treatment results in further increase in nuclear WT HDAC5 localization. Representative confocal images of cultured adult rabbit ventricular myocytes expressing WT HDAC5-GFP as control or treated with forskolin for 24 hr.

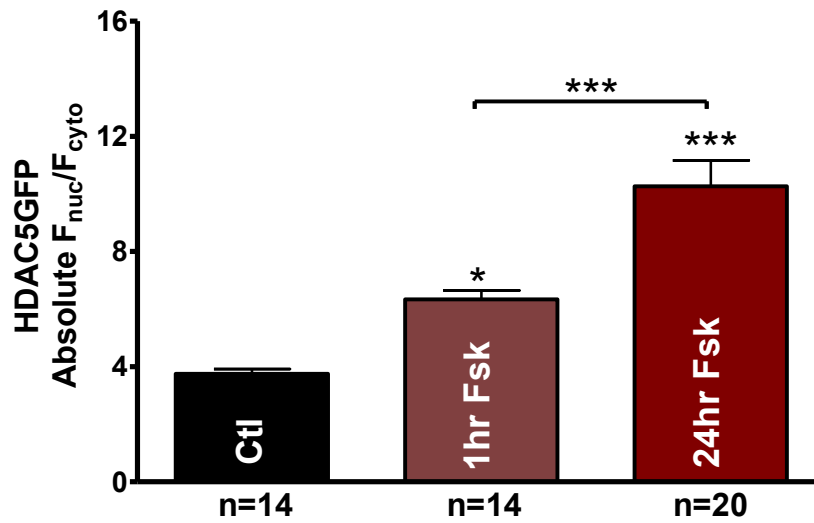


Figure 42. Chronic 24-hour forskolin treatment results in further increase of nuclear WT HDAC5 localization compared to 1-hour treatment or control. Comparison of absolute baseline F_{nuc}/F_{cyto} of WT HDAC5-GFP after 1-hr or 24-hr exposure to forskolin (10 μ M).

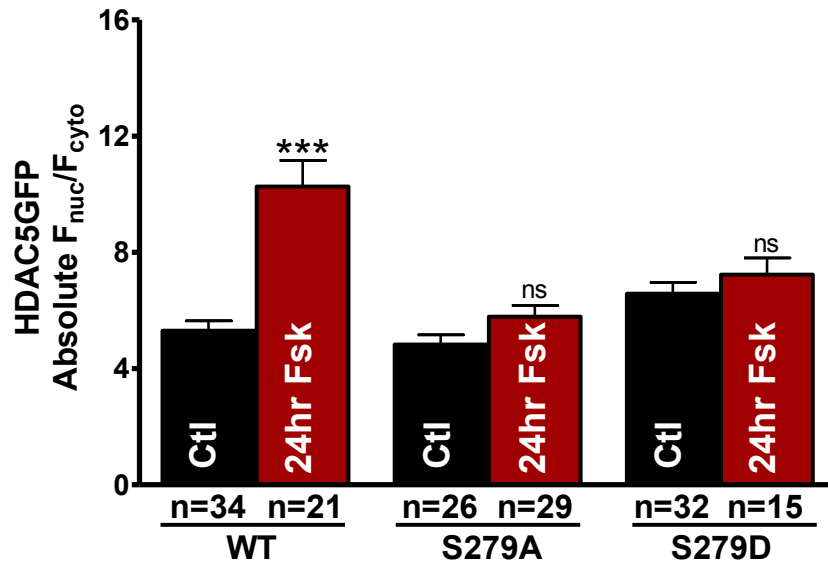


Figure 43. 24-hr forskolin treatment enhances HDAC5 nuclear accumulation in a S279-dependent manner. Comparison of cultured myocytes under 24-hr forskolin (10 μ M) treatment using GFP-tagged WT HDAC5, S279A and S279D.

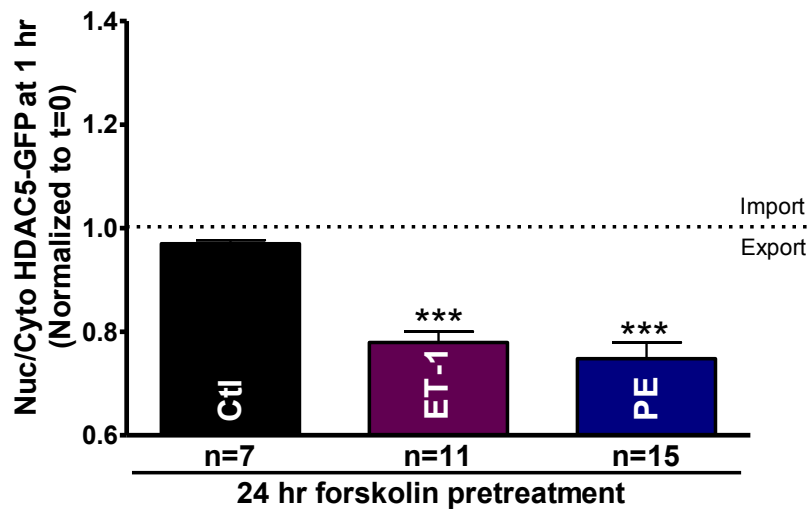


Figure 44. Chronic forskolin pretreatment does not inhibit subsequent Gq-agonist induced nuclear export of WT HDAC5. Results of GFP-tagged WT HDAC5 translocation in cultured rabbit myocytes incubated with forskolin (10 μ M) for 24 hr followed by ET-1 (100 nM) or PE (20 μ M) treatment for 1 hr. *** $p < 0.0001$.

Absence of ISO-induced HDAC5 nuclear import in HF rabbit myocytes

Our current study demonstrates that acute β -AR or PKA activation can counter Gq-driven transcription activation by preventing nuclear export of HDAC5. We postulate that the acute ISO/PKA-induced HDAC5 nuclear accumulation is altered in HF because of the well-documented hyper-adrenergic state in HF (Port and Bristow, 2001). To validate our hypothesis, we examined the HDAC5 translocation in HF rabbit myocytes using adenoviral construct of GFP-tagged WT HDAC5. HF myocytes showed a more cytosolic HDAC5 expression resulting in lower baseline $F_{\text{nuc}}/F_{\text{cyto}}$ ratio as previously reported (Bossuyt et al., 2008), indicating a decrease in nuclear HDAC5 localization and gene repression (Figure 45). Treatment with ISO for 1 hr did not trigger HDAC5 nuclear accumulation in these HF myocytes (vs. control) while ET-1 and PE still induced modest nuclear export (Figure 46). Moreover, pre-incubation of ISO did not block the following HDAC5 export mediated by ET-1 or PE in HF myocytes (Figure 47), consistent with data in Figure 44 where prolonged forskolin treatment did not prevent subsequent Gq-induced HDAC5 export. In summary, the HDAC5 nuclear import induced by acute β -AR stimulation is abolished in HF myocytes, along with a loss of protective effects against Gq-mediated hypertrophic response (as was seen for 24-hr forskolin).

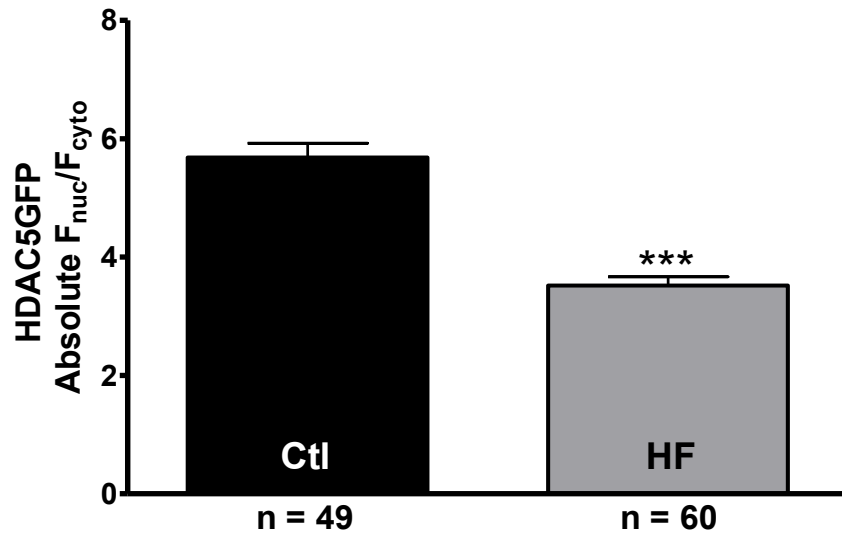


Figure 45. HF myocytes have increased cytosolic localization compared to control myocytes. Comparison of absolute baseline F_{nuc}/F_{cyto} in adult ventricular myocytes between aged-match controls and HF rabbits.

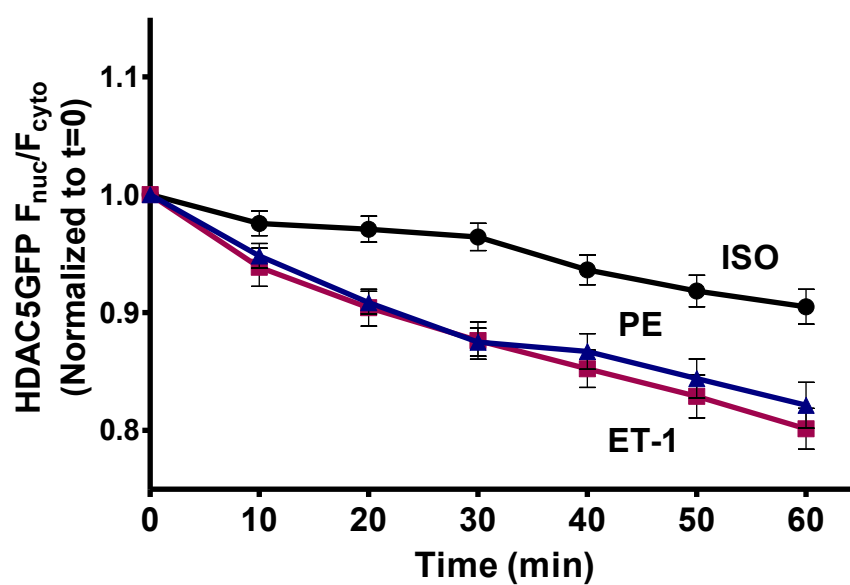


Figure 46. Absent of ISO-induced HDAC5 nuclear accumulation in HF rabbit myocytes. GFP-tagged WT HDAC5 translocation in response to ISO (100 nM), ET-1 (100 nM) and PE (20 μ M).

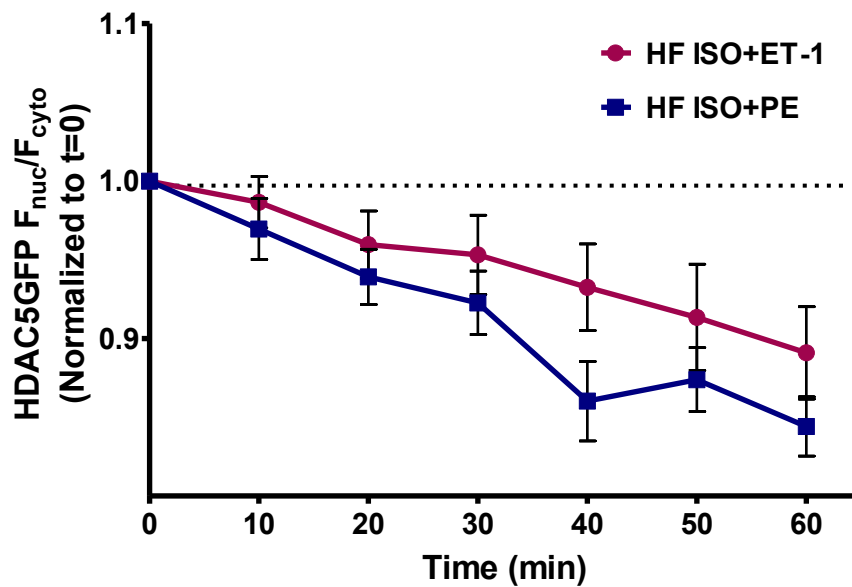


Figure 47. Inhibitory effect of ISO on Gq-mediated nuclear export is absent in HF myocytes. GFP-tagged WT HDAC5 translocation at 1 hr post-treatment with 20 min pre-incubation of ISO (100 nM) followed by addition of ET-1 (100 nM) or PE (20 μ M) for 1 hr.

Discussion

The current study highlights the physiological cross interactions between β -AR and Gq-driven signaling at the level of HDAC5 shuttling in adult cardiac myocytes which is important because hemodynamic stress often induces co-activation of both systems. We report that acute β -AR stimulation or PKA activation promotes nuclear accumulation of HDAC5 in contrast to the Gq-induced nuclear export of HDAC5. In addition, acute pretreatment of ISO or forskolin effectively prevents the Gq-induced HDAC5 nuclear export and would prevent or delay transcriptional activation in adult cardiac myocytes.

It has been well characterized that S259 and S498 residues of HDAC5 are the major phosphorylation targets by CaMKII and PKD to initiate the 14-3-3 dependent nuclear export in neonatal and adult myocytes during Gq-driven hypertrophic signaling (Bossuyt et al., 2008; Grozinger and Schreiber, 2000; Harrison et al., 2006; McKinsey et al., 2000a; McKinsey et al., 2000b; Vega et al., 2004). On the other hand, there are conflicting data regarding the role of PKA activation during hypertrophic signaling, and its pathway remains unclear in adult myocytes. Moreover, the fundamental impact of HDAC5 phosphorylation by PKA in adult myocytes is not well defined.

We present here that the non-phosphorylatable HDAC5 mutant, S279A, ablates the ISO or forskolin induced HDAC5 nuclear import, but does not perturb Gq-driven export. Conversely, mimicking phosphorylation at S279 site resulted in constitutive HDAC5 nuclear import and effectively blocked Gq-induced nuclear export of HDAC5. Moreover, the constitutive nuclear accumulation of the S279D

mutant could not be further enhanced by PKA activation. The data suggest that β -AR and PKA activation mediated nuclear translocation of HDAC5 is predominantly S279 phosphorylation-dependent. Our confocal study of HDAC5-GFP translocation in adult myocytes complements recent publications where an *in vitro* kinase assay identified PKA as a novel kinase to directly phosphorylate HDAC5 at S279 in Cos 7 cells and neonatal rat ventricular myocytes (Ha et al., 2010), and PKA activation negatively regulates α_1 -adrenergic mediated HDAC5 phosphorylation in neonatal rat ventricular myocytes (Sucharov et al., 2011).

Furthermore, our immunoblot data indicate that forskolin pretreatment significantly reduces the level of S498 phosphorylation of endogenous HDAC5 inducible by Gq-agonist in adult rat myocytes. This observation is supplemented by our co-immunoprecipitation results detecting a decreased level of endogenous 14-3-3 binding to WT HDAC5-GFP in the presence of β -AR activation with Gq-agonist, ET-1. Together, we show that β -AR or PKA activation prevents Gq-induced phosphorylation at S498 and thus attenuates the consequent 14-3-3 association to prevent its nuclear export. In contrast, Carnegie et al. has reported that an AKAP-Lbc complex allows PKA activation to potentiate PKD activation *in vitro*, thereby facilitating downstream HDAC5 nuclear export in HEK cells (Carnegie et al., 2004; Carnegie et al., 2008). The discrepancies could be due to the nature of different cell types and the respective 14-3-3 isoforms present to associate with HDACs (Ellis et al., 2003; Faul et al., 2007; Margariti et al., 2010) Additionally, substrate specificity of cAMP/PKA signaling is achieved by compartmentalization of localized cAMP gradient via

phosphodiesterase isoforms or beta arrestins and by association with various AKAP scaffolding subtypes in different cell types (Berthouze et al., 2011; Diviani et al., 2011; Dodge-Kafka et al., 2010; Dodge-Kafka et al., 2006; Dodge-Kafka et al., 2005; Fischmeister et al., 2006; Leroy et al., 2008; Mika et al., 2012; Mongillo et al., 2004; Mongillo and Zaccolo, 2006; Nikolaev et al., 2006; Perry et al., 2002; Redden and Dodge-Kafka, 2011). Our study provides real time observation of HDAC5-GFP translocation in adult myocytes to more accurately reflect the fundamentally dominant endogenous signaling pathway in adult ventricular myocytes.

The confocal data are further validated by our experiment of MEF2-dependent transcriptional regulation in adult cardiac myocytes. The MEF2-dependent transcriptional activity can be upregulated upon Gq activation to promote hypertrophic signaling. However, acute β -AR/PKA activation significantly blocks the Gq-driven MEF2 activity, indicating that short term β -AR/PKA signaling counters the transcriptional activation induced by neurohumoral stimuli.

Collectively, our data suggest that acute β -AR or PKA activation is protective against the hypertrophic stimulation during Gq-activation, by preventing nuclear export of HDAC5 in adult myocytes. This has significant implications in the regulation of cardiac physiology where short term sympathetic stimulation, such as exercise or fight or flight response, encourages beneficial compensations of increased cardiac output without triggering acute transcriptional changes. The protective effect of β -AR/PKA activation provides a short term window for cardiac myocytes to adjust quickly to external stimuli

before attempting to re-program its genetic control that is otherwise necessary during more severe long-term stress conditions.

Chronic catecholamine stimulation is associated with decreased cardiac function and pathological cardiac remodeling, leading to hypertrophy and heart failure. While we observe that acute β -AR/PKA activation protects against neurohumoral-induced transcriptional activation, we show that the HDAC5-GFP nuclear signal is substantially elevated after a 24-hour exposure to forskolin (1.6 fold over 1 hour treatment). While this enhancement of nuclear HDAC5 is also dependent upon S279 phosphorylation, prolonged forskolin treatment does not prevent the nuclear HDAC5 export induced by Gq-agonist. Our findings suggest that S279 phosphorylation by PKA is still important during chronic forskolin induced elevation of nuclear HDAC5, yet the mechanism of allowing subsequent Gq-drive nuclear export remains unclear. Although Ha and colleagues identified PKA as a novel HDAC5 kinase to directly phosphorylate at its S279 site using *in vitro* kinase assay, we importantly show the dynamics of this regulation in adult cardiac myocytes. An additional mechanistic issue remains as to how intracellular PKA localizes to HDAC5 during the acute or chronic β -AR stimulation to alter HDAC5 translocation and transcriptional activity. One possibility is that a PKA anchoring proteins (AKAP) could be involved, and that has been suggested (Carnegie et al., 2004; Carnegie et al., 2008; Diviani et al., 2011). It will be of future interest to test whether a specific AKAP association is altered during the acute vs. chronic β -AR activation or perhaps the neighboring proteins associated with PKA are differentially partnered with the AKAP.

We speculate that chronic PKA activation may recruit additional effects (e.g. PKA-dependent phosphorylation at additional HDAC5 sites), which restore the ability of PKD and CaMKII to phosphorylate S259 and S498 to drive HDAC5 nuclear export. This impact of temporal dynamic phosphorylation of HDAC5 on functional systemic regulation is further supported by a parallel study in rodent striatum where reduced S279 phosphorylation of HDAC5 during acute cocaine injection is restored after 24-hr exposure to cocaine (Taniguchi et al., 2012). Moreover, a recent proteomic study indicates that HDAC5 contains approximately 17 conserved serine/threonine sites available for *in vivo* phosphorylation (Greco et al., 2011). The impact of additional phosphorylation sites could alter potential interaction with other protein partners, such as kinases, phosphatases or scaffolding complex. Hence, we cannot exclude the possibility that chronic S279 phosphorylation may facilitate phosphorylation at S259/S498 or other sites to permit HDAC5 nuclear export after chronic PKA activation in cardiac myocytes.

While the mechanistic details of this acute vs. chronic PKA regulation of HDAC5 translocation will require further study, it represents a novel and elegant switch that fulfills a key physiological control point. That is, in short-term fight or flight responses, transcriptional activation by Gq-HDAC5 signaling is held in check, whereas in chronic PKA-related stress, such repression is relieved. Our findings in HF myocytes further support this time-dependence regulation of transcriptional activation where protection against Gq-induced HDAC5 nuclear export occurs only during acute β -AR or PKA activation, and not during sustained

activation of PKA.

To the extent that HF constitutes a hyper-adrenergic state, one might expect HDAC5 F_{nuc}/F_{cyto} ratio to be elevated. However, in HF, that ratio is reduced vs. control. This may have two causes: 1) the level of chronic PKA activation is likely much less in HF than we used experimentally (and downregulation of β_1 -AR and elevated phosphatases function could also limit the PKA effect) and 2) HF is known to be accompanied by elevated levels of circulating Gq agonists (norepinephrine, ET-1 and angiotensin II). Moreover, ET-1 and PE can and do trigger nuclear HDAC5 nuclear export in HF (Figure 46), possibly attributed to the increased PKD and CaMKII activity in HF (Bossuyt et al., 2008). The net result is that in HF there is reduced repression by nuclear HDAC5 of hypertrophic gene transcription and retained sensitivity to neurohumoral regulation via Gq-coupled receptor agonists. The dynamic interplay and opposing effects of β -AR vs. Gq-coupled receptors on HDAC5 translocation may be important to consider in the context of β -blockers, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) in HF. For example, it is possible that acute β -blockade alone could exacerbate Gq-agonist effects in HF and coordinated block of Gq and β -AR signaling (or Gq block first) could most effectively dampen this maladaptive signaling pathway.

In conclusion, our study demonstrates the physiological interaction between β -AR and Gq-coupled signaling pathway and their regulation on HDAC5 nucleocytoplasmic shuttling in adult cardiac myocytes. We report that acute β -AR

and PKA activation favors HDAC5-dependent transcriptional repression by blocking neurohumoral activation effects (Figure 48) and provides a time window for myocytes to adjust to stress stimuli before resorting to epigenetic regulation. Accordingly, chronic stress such as HF permits neurohumoral signaling mediated transcriptional activation by relieving HDAC5 transcriptional inhibition. Our findings illustrate the importance of acute vs. chronic β -AR regulation in adult cardiac myocytes where acute sympathetic signaling is protective against Gq-driven hypertrophy while chronic stress promotes pathological cardiac remodeling via HDAC5-dependent transcriptional regulation.

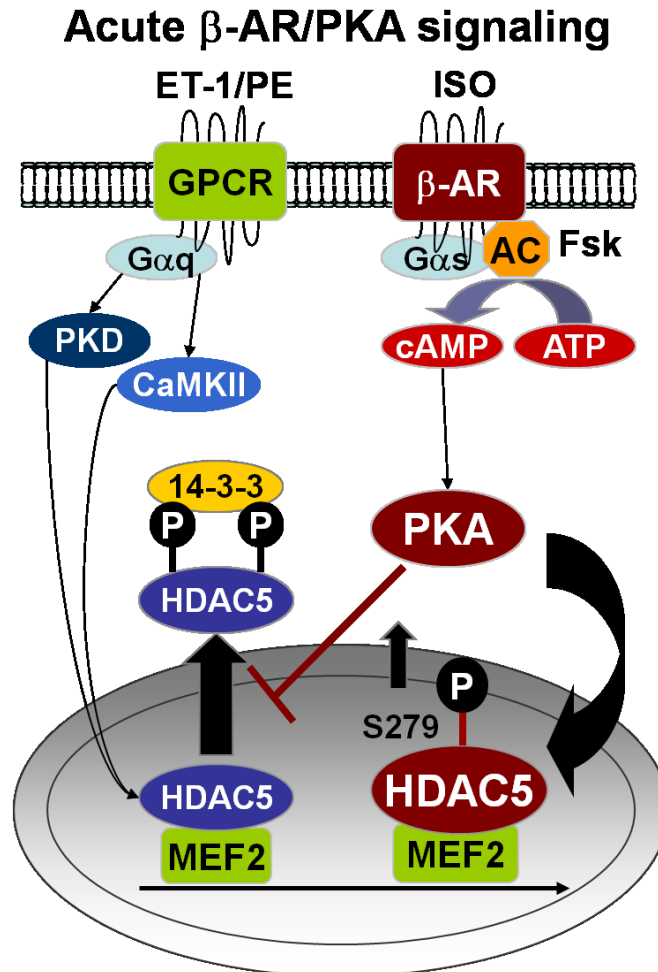


Figure 48. Working hypothesis in adult cardiac myocytes. Acute β -AR/PKA signaling activates PKA to promote HDAC5 nuclear accumulation and prevent Gq-driven HDAC5 nuclear export by S279 phosphorylation.

CHAPTER 6

AIM 3

Introduction

The functional role of PKD in hypertrophic signaling has become increasingly important due to its involvement in many aspects of cardiac physiology. For instance, PKD is found to alter cardiac contractile function via its ability to alter Ca^{2+} sensitivity of myofilament protein phosphorylation (Bardswell et al., 2010; Cuello et al., 2007; Goodall et al., 2010; Haworth et al., 2004). Moreover, PKD is also implicated in non-contractile regulation of cardiac myocytes, such as cardiac remodeling and hypertrophy via HDAC phosphorylation and shuttling (Bossuyt et al., 2011; Bossuyt et al., 2008; Harrison et al., 2010; Harrison et al., 2006; McKinsey, 2007; Vega et al., 2004). PKD can also participate in the cardiac immune response by activation of transcriptional factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) (Avkiran et al., 2008; Fu and Rubin, 2011; Rozengurt, 2011; Rozengurt et al., 2005). In particular, constitutively active PKD can trigger cardiac hypertrophy (Harrison et al., 2006) while cardiac specific PKD knockout mice exhibit reduced amount of cardiac remodeling in response to TAC-induced pressure overload (Fielitz et al., 2008). These findings substantiate the

importance of PKD in modulating cardiac pathophysiology and its potential as a therapeutic target for cardiovascular disease.

The regulatory role of PKD during hypertrophic response and cardiac remodeling is attributed to its association with transcriptional repressor, HDAC5. PKD, along with CaMKII, has been reported to mediate direct phosphorylation of HDAC5 and relieve the transcriptional inhibition on MEF2-dependent genes (McKinsey and Olson, 2005). This epigenetic modification is absolutely fundamental to the initiation of “fetal gene program” in adult cardiac myocytes. This molecular reprogramming occurs as an adaptive response to increased cardiac demand by neurohumoral or hemodynamic stress to encourage growth in the terminally differentiated cardiac myocytes. Even with brief initial functional benefits, the remodeling process eventually deteriorates the cardiac phenotype and can trigger maladaptive changes in the heart if stress stimuli are prolonged.

Recent interest has been dedicated to study the intracellular activation mechanism of PKD to explore its potential in future therapy for cardiac disease due to its unequivocal role in epigenetic re-programming of adult cardiac myocytes. However, the potential crosstalk interaction between PKD and PKA signaling pathways remains unclear due to contradicting reports regarding the effects of PKA signaling on PKD activation. Carnegie et al. and colleagues demonstrate the positive regulatory role of PKA signaling on PKD activation via AKAP-Lbc association in cell lines and neonatal myocytes (Carnegie et al., 2004; Carnegie et al., 2008). In contrast, others studies report a negative modulatory

role for PKA activity on PKD activation in adult rat myocytes (Haworth et al., 2011; Haworth et al., 2007). Therefore, the crosstalk interaction between these two signaling pathways demands further clarification, especially in adult myocytes.

Accordingly, we and others have recently uncovered the protective effect of acute β -AR/PKA signaling on Gq-driven transcriptional activation via nuclear accumulation of HDAC5 (Ha et al., 2010; Sucharov et al., 2011). Although PKA is shown to directly phosphorylate HDAC5 and blocks its nuclear export (Ha et al., 2010), we hypothesized that an additional mechanism to inhibit HDAC5 nuclear export is by limiting upstream PKD activity. For instance, PKA can also directly phosphorylate PKD and alter its translocation and activation patterns. To this end, we propose to examine the spatiotemporal activation of PKD in adult myocytes using fluorescent techniques, such as confocal and FRET imaging, in combination with biochemical approach to compare any differences in β -AR/PKA activation vs. Gq-agonist stimulation. Our initial findings indicate a negative modulatory role of β -AR/PKA signaling in Gq-induced PKD translocation and activation in adult myocytes.

Results

β -AR and PKA activation negatively modulates Gq-mediated PKD translocation

We have recently uncovered that β -AR and PKA activation can trigger nuclear accumulation of HDAC5 by directly phosphorylation of S279 and that this also blocks the Gq-driven HDAC5 nuclear export if followed by ET-1 or PE treatment (unpublished data). Our data suggest that β -AR/PKA signaling is protective against hypertrophic response to Gq-mediated transcriptional activation. An additional indirect mechanism by which β -AR/PKA might inhibit HDAC5 nuclear export is by limiting upstream PKD activation to block the HDAC5 phosphorylation-dependent export. Therefore, we hypothesized that β -AR and PKA activation play a negative modulatory role in PKD activation in adult myocytes.

To test this concept, we first investigated the effects of β -AR and PKA activation on PKD translocation in adult cardiac myocytes since classic PKD activation is characterized by its initial sarcolemmal translocation followed by its nuclear translocation (Rozenfurt et al., 2005; Wang, 2006). To study intracellular PKD translocation, we cultured adult rabbit myocytes with GFP-tagged PKD1 adenovirus overnight to observe its translocation in response to ISO or forskolin treatment. Results in figure 49 show that ISO or forskolin treatment did not induce any baseline PKD translocation to the sarcolemma. This is opposite to what we had previously observed for ET-1 and PE induced response (Bossuyt et

al., 2011). Notably, we did detect a minor recruitment of PKD to the nucleus (Figure 50) in response to ISO and forskolin. This is unexpected since we and others have reported that PKD nuclear import usually follows its sarcolemmal translocation under Gq-agonist stimulation (Bossuyt et al., 2011). However, since PKD has been reported to participate in AKAP complexes (Carnegie et al., 2004; Carnegie et al., 2008), it is possible that PKA at that locus could preferentially phosphorylate PKD without the normal sarcolemma recruitment. This suggests that β -AR/PKA activation mediates nuclear import of PKD independent of its translocation to the sarcolemma, where PKD can be activated by upstream novel PKC isoforms during the canonical PKD activation pathway (Rey et al., 2004; Rey et al., 2001; Waldron and Rozengurt, 2003; Wang, 2006).

Since β -AR and PKA activation did not drive PKD translocation to the sarcolemma, we postulated that pre-stimulation of β -AR will block the Gq-mediated PKD translocation in adult myocytes. Figure 51 shows that the prominent sarcolemmal translocation under ET-1 stimulation was effectively blocked with ISO pretreatment, suggesting a negative regulation by β -AR activation of the Gq-induced PKD translocation. Indeed, ISO pretreatment markedly diminished the ET-1 and PE induced PKD translocation to the sarcolemma (Figure 52). In parallel, Gq-agonist mediated nuclear import of PKD was also significantly reduced by pre-incubation of ISO (Figure 53). This could be partially explained by the fact that basal ISO-induced PKD translocation to the nucleus already occurs (Figure 50), thus we did not observe further increase in

PKD nuclear translocation upon ET-1 or PE treatment. These findings suggest that the sequential pattern of PKD translocation to the sarcolemma followed by the nuclear recruitment under Gq-agonist stimulation is altered by β -AR or PKA activation.

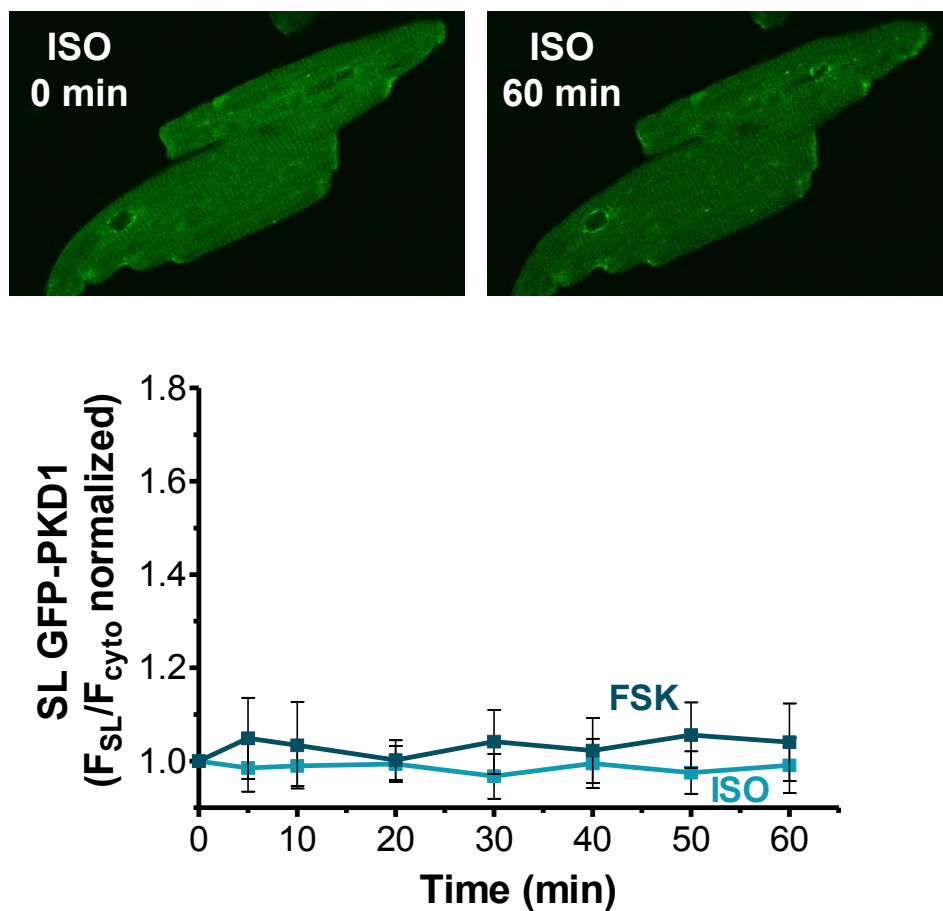


Figure 49. β -AR or PKA activation induced negligible sarcolemmal PKD1-GFP translocation in adult myocytes. Top: Representative confocal image of ISO-induced PKD1-GFP translocation before and after 1 hr treatment. Bottom: Isolated adult rabbit myocytes with PKD1-GFP overexpression were treated with ISO (100 nM) or forskolin (10 μ M) for 1 hr.

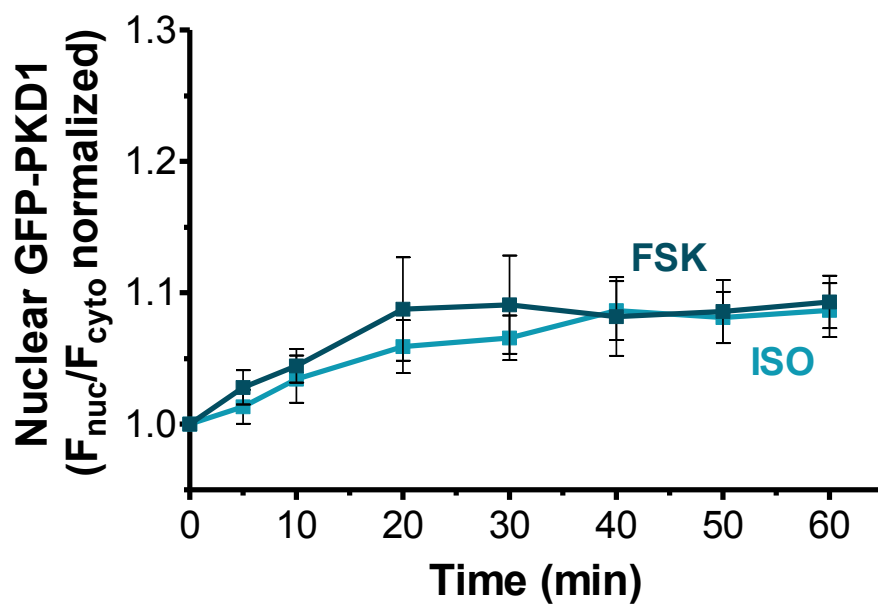


Figure 50. β -AR or PKA activation mediated minor PKD1-GFP nuclear translocation in adult myocytes. Isolated adult rabbit myocytes with PKD1-GFP overexpression were treated with ISO (100 nM) or forskolin (10 μ M) for 1 hr.

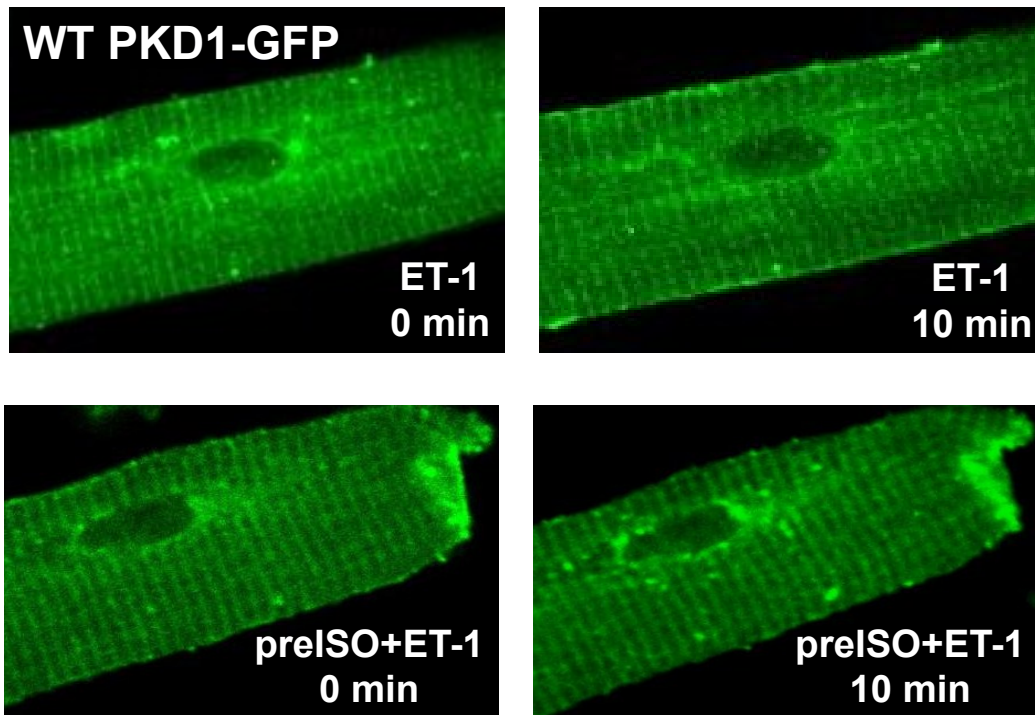


Figure 51. Confocal images of PKD1-GFP in response to ET-1 alone or with β -AR pre-stimulation. Cultured adult myocytes were treated with ET-1 (100 nM) alone or pretreated with ISO (100 nM) for 20 min.

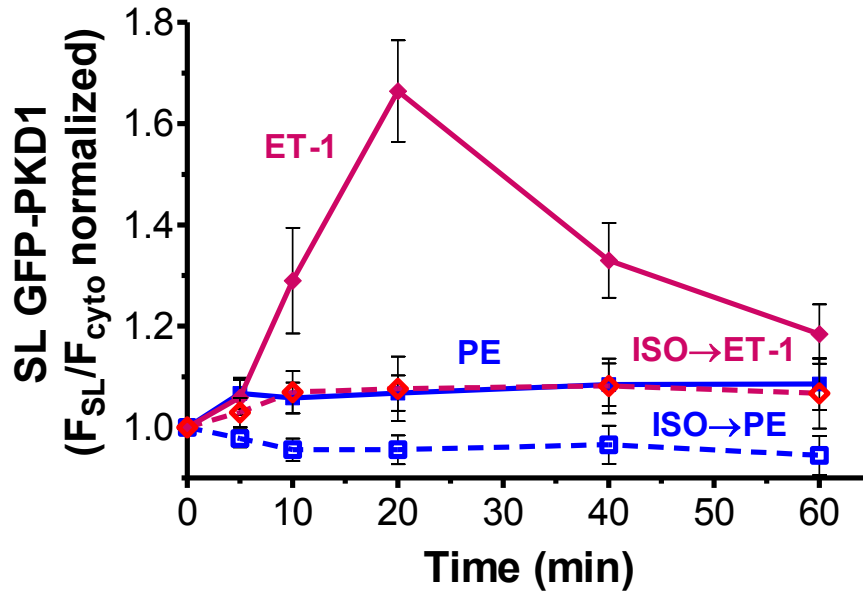


Figure 52. Effects of β -AR pre-stimulation on Gq-agonist induced PKD1-GFP translocation to the sarcolemma. Isolated adult rabbit myocytes were treated with ET-1 (100 nM) or PE (20 μ M) alone for 1 hr or pretreated with ISO (100 nM) for 20 min.

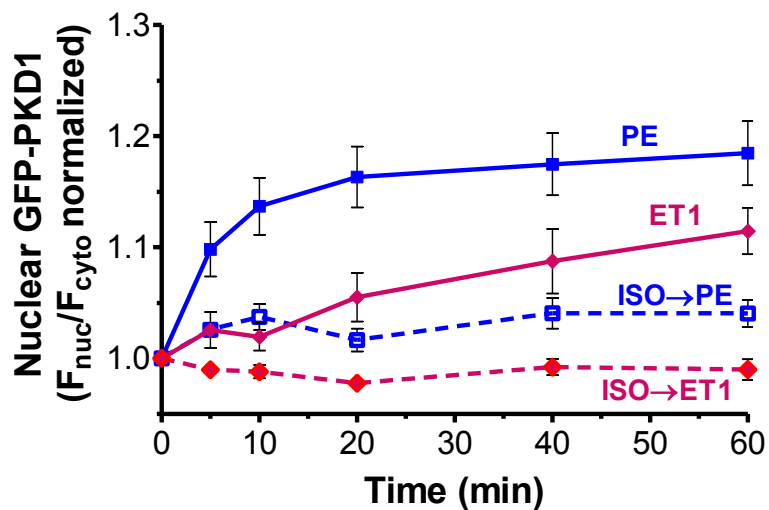


Figure 53. β -AR pre-stimulation negatively modulates Gq-agonist induced PKD1-GFP translocation to the nucleus. Isolated adult rabbit myocytes were treated with ET-1 (100 nM) or PE (20 μ M) alone for 1 hr or pretreated with ISO (100 nM) for 20 min.

β -AR and PKA activation display minor nuclear PKD activation

Our previously publication reports that compartmentalized PKD translocation is associated with its local activation (Bossuyt et al., 2011). Since our data indicated a negative modulatory role of β -AR/PKA activation on Gq-mediated PKD translocation, we postulated that consequently, local PKD activation can also be negatively regulated by β -AR and PKA stimulation. We tested our hypothesis by overexpressing targeted DKAR constructs in cultured adult myocytes to examine PKD activation at the plasma membrane and the nucleus (measured with DKAR-SL and DKAR-Nuc respectively). These FRET-based biosensors produce a decrease in FRET signal due to a conformational change between the CFP and YFP once active PKD phosphorylates its pseudo-substrate (see Figure 13) (Kunkel et al., 2007).

ISO or forskolin treatment alone did not trigger PKD activation at the sarcolemma since we did not observe any PKD activity measured with DKAR-SL (Figure 54). However, consistent with our confocal translocation data in figure 50, we did observe a minor PKD activation signal in the nucleus upon forskolin treatment, but not upon ISO stimulation (Figure 55). These findings imply that basal PKA activation can independently trigger PKD translocation to the nucleus where PKD can be activated without inducing its initial translocation to the sarcolemma. Moreover, ET-1 or PE induced PKD activity at the plasma membrane and the nucleus were both reduced by ISO pretreatment (Figure 56 and Figure 57). The data are consistent our hypothesis that β -AR or PKA

activation negatively regulates the Gq-agonist-induced PKD activation in adult myocytes.

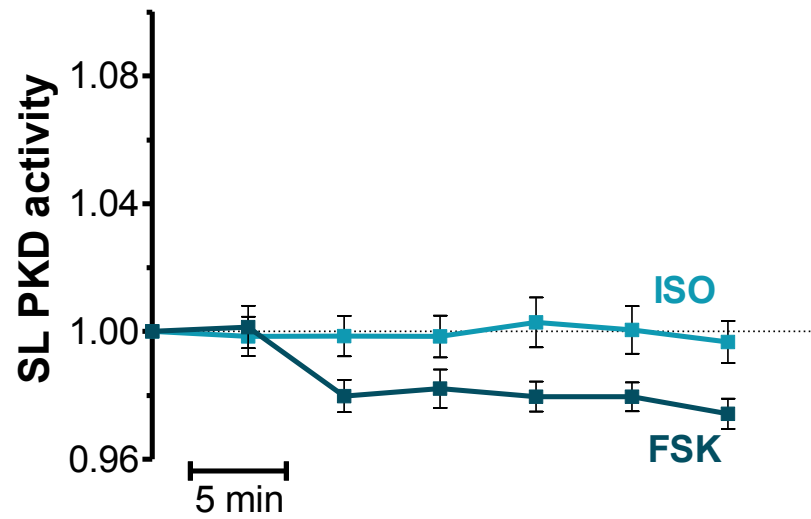


Figure 54. β -AR and PKA activation do not trigger basal sarcolemmal PKD activity. Isolated adult myocytes overexpressing DKAR-SL were treated with ISO (100 nM) or forskolin (10 μ M).

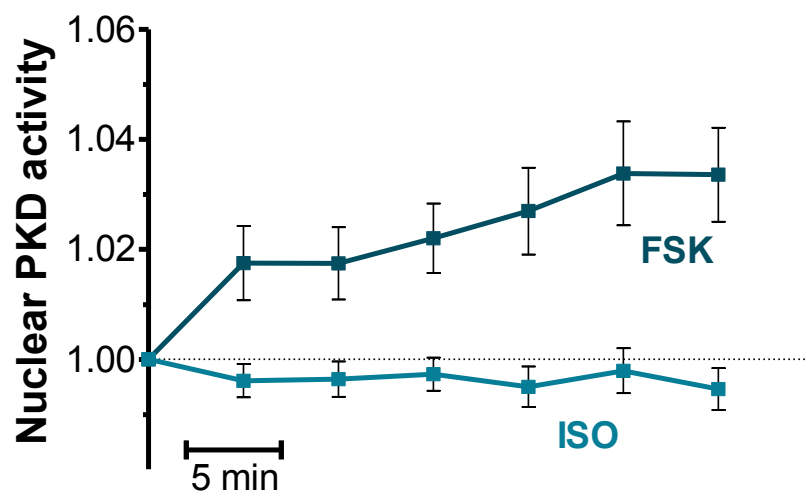


Figure 55. PKA activation triggers nuclear PKD activation. Isolated adult myocytes overexpressing DKAR-Nuc were treated with ISO (100 nM) or forskolin (10 μ M).

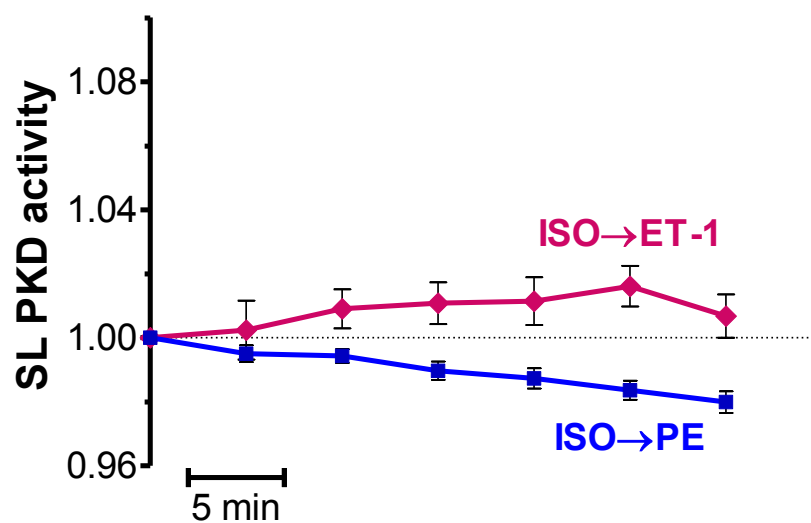


Figure 56. β -AR pre-stimulation reduces PKD activation by ET-1 and PE at the sarcolemma. Isolated adult myocytes overexpressing DKAR-SL were pretreated with ISO (100 nM) for 20 min followed by either ET-1 (100 nM) or PE (20 μ M) treatment for 30 min.

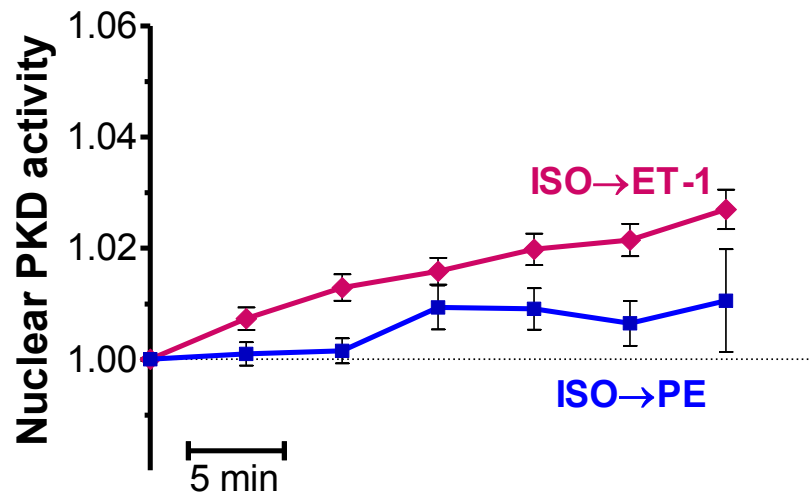


Figure 57. β -AR pre-stimulation reduces PKD activation by ET-1 and PE in the nucleus. Isolated adult myocytes overexpressing DKAR-Nuc were pretreated with ISO (100 nM) for 20 min followed by either ET-1 (100 nM) or PE (20 μ M) treatment for 30 min.

β -AR and PKA stimulation reduces PKD activation at its S916 autophosphorylation site

PKD contains an autophosphorylation site at S916, which is often used as biochemical readout of its activity. Thus, we examined whether endogenous PKD S916 phosphorylation was altered in response to β -AR or PKA activation. Isolated adult rat myocytes were treated with Gq-agonist, ET-1 and PE, or with ISO and forskolin to compare any difference in PKD activation in response to Gq and β -AR signaling. PdBu, a phorbol ester that directly induces maximal PKD activation without Gq-coupling is used as a positive control. Figure 58 shows that hypertrophic agonist, ET-1, PE and PdBu all triggered significant S916 phosphorylation of PKD in adult rat myocytes while ISO and forskolin resulted in reduced PKD activation at S916 compared to control.

In addition, 20 min pretreatment with forskolin markedly decreased the ET-1 and PE mediated S916 phosphorylation. This indicates that PKA activation effectively prevents the subsequent Gq-induced PKD activation at S916 site. S916 phosphorylation of PKD in response to PdBu was not affected by PKA activation because phorbol ester can activate PKD directly, bypassing the Gq-coupled intracellular signaling.

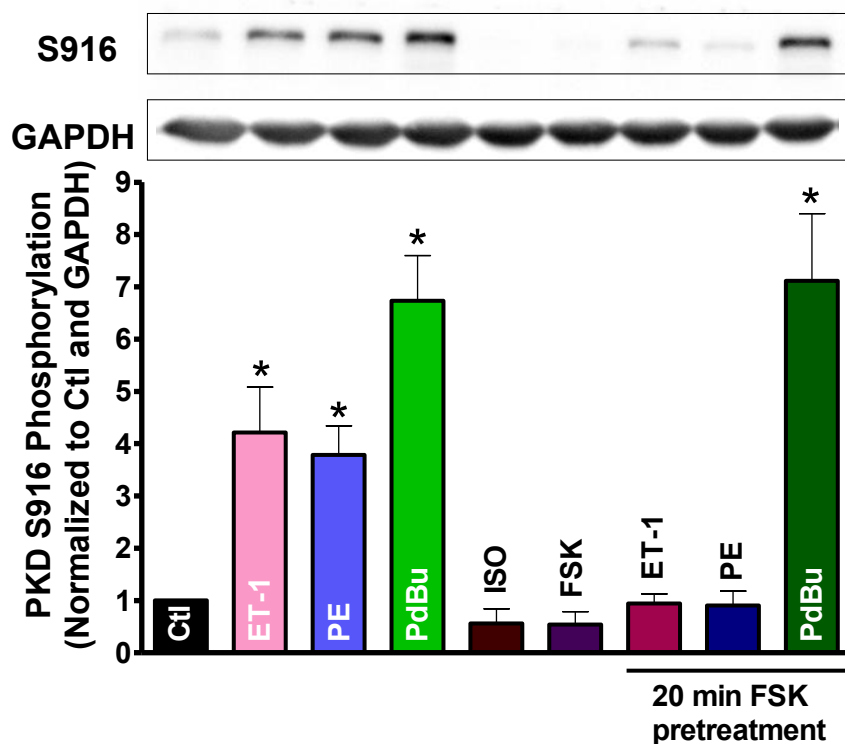


Figure 58. Endogenous PKD S916 phosphorylation in response to Gq or β -AR/PKA activation. Isolated adult rat myocytes were treated with indicated agonist alone for 20 min or with pretreatment of forskolin for 20 min (ET-1 = 100 nM, PE = 20 μ M, PdBu = 200 nM, ISO = 100 nM and forskolin = 10 μ M). We probed for endogenous PKD S916 phosphorylation. * $p < 0.05$. (n = 3).

Mechanism of β -AR/PKA modulation of Gq-induced PKD activation

In silico analysis of PKD sequence reveals that there exists a potential PKA phosphorylation site at serine 427 (S427). To further dissect the mechanism of β -AR/PKA modulation of Gq-induced PKD activation, we aim to create a non-phosphorylatable and a phosphomimetic mutant of PKD at this site by replacing the serine with an alanine (S427A) and a glutamate (S427E) respectively. We have finished making the adenoviral DNA for these two mutations and plan to amplify the mature adenovirus for the following experiments.

We will test our hypothesis that PKA modulation of Gq-induced PKD activation is mediated via PKA phosphorylation at S427 site. If our hypothesis was correct, we anticipate that the non-phosphorylatable mutant will abolish the negative PKA effect on PKD translocation and activation in response to ET-1 and PE. In other words, pretreatment of ISO or forskolin should not alter the Gq-agonist mediated translocation of GFP-tagged PKD-S427A mutant to the sarcolemma and to the nucleus. In addition, ISO or forskolin pretreatment should not affect the Gq-agonist induced PKD activation.

However, we cannot assess PKD activation using the targeted DKAR biosensors because we will need to co-infect with the mutant PKD-S427A with the DKAR construct. Therefore, we plan to culture adult myocytes with GFP-tagged WT-PKD or PKD-S427A mutant and treat them with ET-1 or PE alone and with pretreatment of ISO or forskolin. We will then immunoprecipitate the protein using the GFP antibody to probe for PKD S916 phosphorylation between

the WT PKD and the mutants. If the negative modulation of β -AR/PKA is mediated via S427 phosphorylation, then we anticipate to observe a decrease in PKD S916 phosphorylation in WT PKD when pretreated with ISO or forskolin followed by Gq-agonist exposure. Conversely, the downregulation of PKD S916 phosphorylation by β -AR/PKA activation should be abolished in the PKD-S427A sample due to the unavailability of PKA phosphorylation site.

Parallel to the experiments above, the phosphomimetic PKD-S427E mutant should mimic the PKA phosphorylation at S427 site. We anticipate that the baseline PKD-S427E localization will exhibit slightly higher nuclear localization compared to WT PKD (mimicking our observation in Figure 55 with ISO and forskolin treatment). We will compare the translocation of this mutant to the WT PKD in response to ET-1 and PE treatment alone. If S427 is the PKA modulatory site, then PKD-S427E mutant will block the ET-1 and PE mediated PKD translocation to the sarcolemma and the nucleus as if it was stimulated with ISO or forskolin. Similarly, we will determine the S916 phosphorylation level in myocytes cultured with WT PKD or PKD-S427E variant. The PKD-S427E variant represents a condition where PKA phosphorylation of PKD at S427 was present, and it should exhibit decreased sarcolemmal and nuclear translocation in response to ET-1 or PE treatment alone.

In addition to probing PKD S916 phosphorylation using immunoprecipitation, an alternative approach to detect PKD activity is to perform co-immunoprecipitation (co-IP) of GFP-tagged WT PKD, PKD-S427A and PKD-

S427E from cultured adult myocytes. We will then determine the corresponding PKD activity in the co-IP samples by using a PKD substrate specific phospho-antibody. In parallel, we will also probe for overall PKA phosphorylation levels on PKD by using two different PKA substrate phospho-antibodies. This could provide information as to whether the S427 residue is important for the interaction between PKA and PKD if phosphorylation status itself did not alter the PKD translocation in adult ventricular myocytes.

Alternatively, to investigate whether β -AR/PKA activation drastically alters the binding protein partners for PKD, we can also examine any difference in the associated proteins in a co-IP experiment with or without ISO and forskolin presence. We will cultured the isolated adult myocytes with WT PKD1-GFP and treat them with Gq-agonist alone or pretreat with ISO or forskolin. We will then process the samples to be run on an SDS-PAGE gel and stain with Commassie to detect any visible difference in protein patterns. We plan to determine the molecular identity of any major differential bands between the treatments using proteomics approach. This approach may be an optimal way of discerning changes in PKD association with other proteins via scaffolding complex (e.g AKAP) or microdomain network.

Discussion

This study highlights the PKA modulation on spatiotemporal PKD translocation and activation in adult cardiac myocytes. We do not observe any sarcolemmal PKD translocation upon β -AR and PKA activation, and only minor PKD nuclear recruitment. This is different from the spatiotemporal PKD activation in response to ET-1 and PE which trigger discernible sarcolemma and nuclear PKD mobilization (Bossuyt et al., 2011). Furthermore, pre-activation of β -AR and PKA significantly reduces the following ET-1 and PE mediated PKD translocation and activation compared to Gq-agonist treatment alone. Although the molecular mechanism of such negative PKA modulation on PKD translocation and activation remains to be elucidated, our findings represent an important crosstalk interaction between PKA and PKD signaling pathways in adult myocytes. In particular, our data provide further understanding of heart failure, which is associated with a reduced β -AR/PKA signaling and increased PKD activity.

The functional role of PKD to regulate cardiac disease has been established due to its association with MEF2-dependent transcriptional activation during hypertrophy. In particular, PKD has been reported to be a HDAC5 kinase that mediates the phosphorylation of this transcriptional repressor during its phosphorylation-dependent nuclear export (Bossuyt et al., 2008; Harrison et al., 2010; Harrison et al., 2006; McKinsey, 2011). Activation of PKD is therefore tightly linked to the initiation of “fetal gene program” via its effect to mediate HDAC5 nuclear export, thus relieving the transcriptional inhibition in adult cardiac

myocytes. While we and others report that PKD can be activated in response to neurohumoral stimuli, the interaction of PKD signaling pathway with β -AR/PKA activation remains unclear, especially in adult cardiac myocytes.

Our study examines the direct effect of β -AR/PKA activation on real-time PKD translocation and activation for the first time in adult myocytes. Intracellular PKD activation typically correlates with its translocation upon agonist stimulation. We report that β -AR and PKA activation do not trigger any PKD translocation to the sarcolemma, where PKD can be activated by novel PKC phosphorylation (Rey et al., 2004; Rey et al., 2001; Waldron and Rozengurt, 2003; Wang, 2006). In addition, PKD translocation and activation in response to ET-1 and PE treatment are both attenuated by pretreatment with ISO and forskolin. The data indicate that β -AR/PKA activation plays a negative regulatory role in Gq-mediated PKD translocation and activation.

Although we do not yet understand the mechanism of how PKA activity negatively regulates PKD activation, we speculate this modulation perhaps occurs proximally to Gq activation. Gq-agonist stimulation is known to couple with PLC activation to produce intracellular IP₃ and DAG, which can trigger downstream activation of classical and novel PKC isoforms. Our immunoblot data show that Gq-agonist activation of PKD is effectively reduced by pre-incubation of forskolin while PdBu-induced PKD activation is not altered. Since PdBu is a phorbol ester that can directly activate PKD, we speculate that PKA modulation of PKD activation is dependent on Gq-activation and may occur

upstream of PKD.

For example, previous work by Haworth et al. demonstrated that PKA and PKC ϵ have counter-regulatory roles in PKD activation in response to ET-1 (Haworth et al., 2007). PKC ϵ is identified as the major PKC isoform to facilitate PKD activation, and its effect can be negated by ISO and forskolin pretreatment. This suggests that the acute β -AR/PKA activation dominates the effect of PKC ϵ activation in cardiac myocytes to prevent PKD activation. Their following report in 2011 indicates that basal activity of PDE3 and PDE4 keep PKA activity low, and their combined inhibition is sufficient to mimic PKA activation by forskolin, thus blocking ET-1-induced PKD activation (Haworth et al., 2011). However, the molecular details of PKA countering the functional role of PKC ϵ regulation of PKD activation remain unknown.

Alternatively, there is a potential PKA phosphorylation site within PKD sequence at S427. We propose to mutate this serine to alanine or glutamate to test the hypothesis that PKA can directly phosphorylate PKD, and this phosphorylation negatively regulates the Gq-induced PKD activation. The S427 residue is located right before the PH domain of PKD. Since the PH domain in general is known to mediate binding of intracellular proteins to phosphoinositol phospholipids (Liao and Hung, 2010; Mashanov et al., 2004), we postulate that PKA phosphorylation at S427 site could alter the potential PKD interaction with membrane phospholipids via allosteric hindrance. The canonical PKD activation pathway requires concomitant PKD translocation with PKC toward the plasma

membrane. Any allosteric hindrance of PKD binding to membrane phospholipids will perhaps reduce its translocation and consequently negatively affect its activation. Our future experiments with the non-phosphorylatable and phosphomimetic mutants will help us delineate the mechanism of PKA modulation on PKD translocation and activation.

In addition, we cannot exclude the possibility that PKA activation can mediate PKD nuclear translocation and activity without directing its translocation to the sarcolemma. For instance, there are studies supporting the association of PKA with PKD via AKAP-Lbc in myocytes (Carnegie et al., 2004; Carnegie et al., 2008). The pool of PKD within this scaffolding complex could be positioned near PKA at baseline and could translocate to different intracellular compartments, such as the nucleus or Golgi, upon PKA activation. Alternatively, PKA-dependent phosphorylation of PKD could drive it toward a different signaling pathway, making it less available for its classical nuclear HDAC5 kinase activity. Further studies will be required to explore the alternative pathway for PKA-mediated PKD translocation and function in cardiac myocytes.

Collectively, our findings indicate a crosstalk interaction between PKA and PKD signaling in adult myocytes. Although the molecular mechanism remains to be clarified, our results provide useful understanding of cardiac pathophysiology. For instance, downregulation and desensitization of β -AR response and PKA activity in failing hearts can reduce the PKA suppression of PKD activity triggered by neurohumoral stress. Consequently, PKD may be hyper-active contributing to

progressive cardiac dysfunction. Therefore, insights to PKA regulation of PKD activity can shape the therapeutic designs in the future for heart failure treatment.

CHAPTER 7

CONCLUSION

My dissertation research highlights the importance of two major transducers of extrinsic stress stimuli in adult cardiac myocytes. Both PKD and HDAC5 are attractive therapeutic targets for future cardiovascular drug development due to their implications in cardiac epigenetic regulation. The heart has the unique properties to adapt to biochemical stress by triggering growth in the terminally differentiated cardiac myocytes. The hypertrophic response can be initiated with nuclear export of HDAC5. As a transcriptional repressor, HDAC5 localization and translocation are critical to its functional role as an epigenetic regulator. The shuttling of HDAC5 between the nucleus and the cytosol occurs dynamically and can be modulated by neurohumoral stress. PKD, along with CaMKII, has emerged as key HDAC kinase that mediates its phosphorylation-dependent shuttling. PKD has become a translational interest for cardiovascular drug development since the discovery of its regulatory role in hypertrophic signaling and heart failure.

My work examines the spatiotemporal activation of PKD in isolated adult cardiac myocytes, and how its activation triggers consequent transcriptional activation via HDAC5 nuclear export. Our findings indicate that ET-1 and PE

differentially activate PKD, yet converge at the level of HDAC5 shuttling to trigger hypertrophic signaling in adult myocytes. Hemodynamic stress can often induce activation of both β -AR and Gq-receptors, thus I also explore the crosstalks between the Gq and β -AR signaling pathways regarding their effects on the transcriptional control via HDAC5. The data suggest that acute β -AR signaling via PKA is protective against the Gq-driven transcriptional activation by promoting nuclear accumulation of HDAC5. Furthermore, chronic (24-hour) PKA activation now permits HDAC5 nuclear export in response to Gq-agonist, ET-1 and PE. The contrasting effects of acute vs chronic β -AR/PKA signaling on transcriptional activation triggered by neurohumoral stimuli represent an important regulation in cardiac pathophysiology. This mechanism of transcriptional control is pertinent to exercise or fight-or-flight response where acute sympathetic signaling demands increased cardiac output without triggering maladaptive changes.

Consequently, I postulate a role for PKA modulation in PKD activation and may act as an additional mechanism by which PKA signaling prevents nuclear export of HDAC5 in adult cardiac myocytes. Preliminary data demonstrate that PKA activation alters the classical PKD translocation to the sarcolemma with minor nuclear PKD translocation and activity. While the molecular mechanism remains to be further investigated, the cross interaction between PKA and PKD signaling could have a huge impact on understanding heart failure, where PKA

and β -AR responses are downregulated while PKD expression and activity are increased.

While the cardiac remodeling is a complex process and has many contributing factors, our research has established a critical role for hypertrophic signaling pathway mediated by the activation of PKD and the consequent HDAC5 shuttling in adult myocytes.

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VITA

Chia-Wei “Jenny” Chang was born in Kaohsiung, Taiwan on September 24th, 1982. Jenny finished elementary and middle school education in Taiwan. In 1998, Jenny moved to Belize City, Belize, where she attended Pallotti High School and graduated as the salutatorian in May, 2002. Jenny enrolled at University of Wisconsin – Madison for her undergraduate study, majoring in genetics. Jenny participated in research opportunity with Dr. James L. Kecks to study the oligomerization of RecQ helicase in *E. coli* and received a Hilldale Undergraduate/Faculty research fellowship in 2004. In the summer of 2005, Jenny was a summer undergraduate research fellow (SURF) at Mayo Clinic, Rochester, MN, and worked on lipid trafficking in Dr. Richard Pagano’s lab. Jenny graduated with her bachelor in science with honors from University of Wisconsin – Madison in May, 2006.

In August of 2006, Jenny enrolled as a Ph.D student at Loyola University Medical Center, Maywood, IL. In the spring of 2008, Jenny joined Cell and Molecular Physiology program and the lab of Dr. Donald M Bers to study the hypertrophic signaling in adult cardiac myocytes. Later that year, the lab relocated to University of California – Davis, where Jenny has been working on her dissertation research. In June, 2009, Jenny was awarded a two-year predoctoral fellowship from the American Heart Association (Midwest Affiliate).

Jenny also completed a business development certificate from Graduate School of Management at University of California – Davis in 2011. Jenny plans on pursuing a post-doc position after conferring her Ph.D degree in August, 2012.