Compartmentalized Phospholipase C Signaling as a Hub That Mediates β-adrenergic Receptor Crosstalk in Cardiac Hypertrophy

by

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Dedication

This work is dedicated to my family-

My parents, Ziyun Wei and Chunhua Wei

My uncle and auntie, Wencai Wu and Xia Li

My sister, bothers and many friends

This work will not be possible without their support and constant love.

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Table of Contents

List of Figures

Abstract

β-adrenergic receptors are G protein coupled receptors that are critical regulators of cardiac output during sympathetic stimulation. Chronic stimulation of the adrenergic system of the heart by sympathetic nerves or circulating catecholamines under conditions of cardiac stress leads to cardiac hypertrophy and ultimately heart failure. One of the first line therapies for the treatment of heart failure is β-blockers to prevent the activation of β-adrenergic receptors. β-blockers ameliorate the symptoms of heart failure but still have side effects. Therefore, novel insights for designing selective therapies will provide substantial therapeutic impact.

Previously, our laboratory characterized one isoform of phospholipase C, PLCε, and showed that it is tightly associated with the pathogenesis of cardiac hypertrophy. Further investigations revealed a novel pro-hypertrophic pathway where PLCε at the nuclear envelope/Golgi interface, hydrolyzes Golgi phosphatidylinositol 4-phosphate (PI4P), generates local diacylglycerol (DAG), mediates nuclear protein kinase D (PKD) activation and hypertrophic gene expression. This pathway can be activated by multiple upstream signals including, EPAC-selective cAMP analog, cpTOME or adenylyl cyclase activator forskolin. However, stimulation of β-ARs with membrane impermeant agonist isoproterenol does not activate this pathway despite strongly raising intracellular cAMP. It was reported that β1ARs are present at Golgi in HeLa cells and can generate cAMP there. These studies lead to our overall hypothesis that compartmentalized PLCε activity is regulated distinctly by discrete spatially distributed βARs in cardiomyocytes. In my dissertation research, we tested the role of internal β1ARs in the stimulation of Golgi PLCε in neonatal ventricular myocytes. By utilizing a fluorescent biosensor that recognize an active form

of β-ARs, we detected a pre-existing pool of Golgi-localized β1AR in cardiomyocytes that can be activated by the permeant agonist, dobutamine, or the endogenous ligand, norepinephrine (NE) through an organic cation transporter in cardiomyocytes. The activation of Golgi PLCε is uniquely regulated by Golgi-β1-AR stimulation but not cell surface β1AR stimulation. Importantly, blockade of Golgi β1ARs prevents NE induced cardiomyocyte hypertrophy. This defines a functional role of Golgi-β1ARs in a highly biologically relevant system.

I further investigated other mechanisms associated with Golgi PLCε in the regulation of cardiac hypertrophy. β1-AR and β2-ARs are two major subtypes of β-ARs present in the heart. However, the two β-AR subtypes elicit different or opposite effects on cardiac function and hypertrophy. We showed that β2-AR protects against hypertrophy through inhibition of hypertrophic Golgi PLCε signaling. Using pharmacological approaches, we found that endosomal Gβγ released by internalized β2ARs inhibited the activition of Golgi PLCε by angiotensin II and Golgi-β1-ARs, ultimately resulting in decreased PKD and histone deacetylase 5 (HDAC5) phosphorylation and protection against cardiac hypertrophy. This supports a novel potential mechanism for β2-AR antagonism of the PLCε pathway that may contribute to the known protective effects of β2-AR signaling on the development of heart failure.

We used a genetically-encoded Golgi-targeted β1AR inhibitor to study the functional role of Golgi-β1ARs in animals. By analysis of the heart tissues expressing Golgi-β-blocker or negative control undergoing transaortic constriction induced heart failure, we found promising statistical trends supporting our overall hypothesis that Golgi-β1ARs is involved in the promotion of pathological cardiac hypertrophy.

Together this study provides evidence of functionally active subcellular βARs that regulate distinct compartmentalized signaling in cardiac muscle cells. Our work informs a novel therapeutic

approach of combining a β2 agonist with hydrophobic β1 selective blocker in the treatment of heart failure.

Chapter 1 General Introduction

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The sympathetic nervous system (SNS) has a fundamental role in maintaining cardiac output by regulating heart rate, cardiac contractility, and vascular resistance. The activity of SNS is mainly regulated by catecholamines including epinephrine (Epi) and norepinephrine (NE). These sympathetic neurotransmitters bind to β-adrenergic receptors (βARs) in the heart. βARs are G protein coupled receptors (GPCRs) and have been extensively studied as paradigms for overall GPCR function, trafficking, and structure, they are critical regulators of cardiac physiologic functions and diseases and are important therapeutic targets for cardiovascular diseases. In this chapter, I will provide an overview of GPCR signaling and discuss βAR signaling in physiological and pathological regulation of functions within the cardiac myocyte, highlighting the spatiotemporal features of βAR signaling at intracellular compartments, and the emerging role of spatiotemporal regulation of cardiovascular diseases. Also, I will discuss signaling mechanisms associated with phospholipase C that regulates cardiac functions.

1.1 GPCRs and GPCR Signaling

G protein coupled receptors (GPCRs) are the largest family of transmembrane receptors, regulating an array of physiological processes(1). These receptors are encoded by over 800 known

mammalian genes(1), and they are critical and frequent drug targets (approximately 35% of approved drugs target GPCRs). Classically, GPCRs are thought to be located at the cell surface where they initiate signaling cascades upon binding to the extracellular ligands including circulating hormones, chemokines, neurotransmitters, and other stimuli(2, 3). Activated receptors signal through heterotrimeric G-proteins at the inner surface of the membrane(2, 4-6) promoting an exchange of guanosine diphosphate (GDP) bound to Gα subunit for guanosine triphosphate (GTP), leading to dissociation of activated Gα subunits from the receptors and Gβγ subunits, initiating activation of intracellular signaling pathways(2, 4, 7). Activated and G-protein free receptors are then ready to stimulate the next Gα subunit.

Gα subunits can be divided into four families: Gαs, Gαi, Gαq and Gα12/13 based on sequence identity and signaling properties(8, 9). It is known that different receptors can couple to the same Gα protein and same receptors can couple to different Gα subtypes as well. Canonical effectors and signaling pathways downstream of different G protein subtypes are well-defined. Specifically, Gαs stimulates adenylyl cyclase (AC) converting adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP)(3, 4). cAMP in turn activates protein kinase A (PKA)(10), EPAC (exchange protein directly activated by cAMP)(11), and cAMP-mediated ion channels. Whereas Gαi inhibits AC proteins, and it has been shown to interact with c-Src Kinase and Hck tyrosine kinases(12). Our laboratory demonstrated that Gαi1 and Gαi3 can interact and strongly activate PDZ-RhoGEF (PRG)(13). Gαq is involved in the activation of the β-isoforms of phosphoinositide-specific phospholipase C (PLCβ) which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2), generating second messengers: diffusible inositol trisphosphate (IP3) and membrane bound diacylglycerol (DAG)(14, 15). IP3 diffuses from the plasma membrane to the endoplasmic reticulum (ER) and mediates the activation of an IP3 regulated calcium channel to

stimulate calcium release. DAG, on the other hand, can recruit and activate protein kinase C (PKC) and initiate other subsequent cellular events (15, 16). Gα12/13 regulates cellular functions through Gα12/13-RhoGEF-Rho signaling axis(8). Gβγ interacts with a variety of downstream effectors including the inwardly rectifying K+ channels(17), PLC-βs(18, 19), and adenylyl cyclases(20, 21). Gαi is thought to be the primary source of free Gβγ released upon activation by a GPCR that mediates downstream signaling pathways.

1.2 GPCR Desensitization

GPCRs desensitization and signal turn off is a well-documented phenomenon. The universal mechanism involves in the G-protein-coupled receptor kinase (GRK)-arrestin pathway. In response to prolonged exposure to an agonist, free Gβγ released upon G-protein activation enables recruitment of GRK2/3 to the plasma membrane through binding to the pleckstrin homology (PH) domain of GRK(22, 23). Other GRK isoforms are also involved with other GPCRs that are constitutively membrane localized and do not require Gβγ-dependent membrane recruitment. Plasma membrane localized GRK phosphorylates the carboxy terminal tail of domains of GPCRs(24). β-arrestin interacts with the phosphorylated receptors and occupies the heterotrimeric G protein binding site of the receptors, therefore, preventing G protein signaling even in the presence of an agonist(25-27). β-arrestin also facilitates receptor internalization via clathrin-coated pits (CCPs)(28, 29), which then can be sorted to lysosomes for degradation, or recycling back to the plasma membrane(30).

1.3 Phospholipase C and compartmentalized PLC signaling.

Phospholipase Cs (PLC) are a family of enzymes that cleave the phosphodiester bond on the head group of phosphatidylinositol 4,5-biphosphate (PIP2) leading to the generation of two

important second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) that control numerous crucial signaling events(31, 32). The PLC family can be divided into six isoforms: β, γ, δ, ε, η and ζ(31). They share a conserved core structure that is composed of a pleckstrin homology (PH) domain, four tandem EF hand domains, a TIM barrel (consists of X region and Y region and the linker between them), and a C2 domain(33). TIM barrel is involved in the enzyme activity including the catalytic residues and a calcium binding site. The proximal and distal CTD domain and the X-Y linker contribute to the autoinhibition of PLCs(34, 35). Though deletion of the X-Y linker activates PLCs, these constitutively activated PLCs still respond to further stimulation, suggesting other mechanisms are involved in its activation. Gαq can interact with PLCβ, and positions it on the membrane to overcome autoinhibition and expose the catalytic core(35). PLCs can also respond to Gβγ, $Ca^{2+,}$ protein tyrosine kinases, and small G proteins, and act as signaling hubs for multiple upstream inputs(32).

Among PLCs, PLCε is the largest PLC that contains a CDC25 domain at its N-terminus and two Ras association homology domains (RA domains) at the C-terminus in addition to the conserved core domains for most PLCs. The unique structure characteristics of PLCε confers the potential to be activated downstream of many proteins including Ras family (RA2 domain), Gα12/13, and Gβγ (RA2 and CDC25 domains). Our laboratory has been investigating the role of PLCε in cardiac myocytes. Initial studies from our laboratory suggest that PLCε plays a critical role in the regulation of contractile force and Ca^{2+}-induced Ca^{2+} release (CICR) by Iso(36, 37). This process involves βARs-cAMP-EPAC-Rap-PLCε signaling axis to enhance intracellular calcium release in cardiac myocytes. In addition to contractile responses, our laboratory found that consistent with cardiomyocyte data in vitro, inducible cardiomyocyte-specific deletion of PLCε after development prevents cardiac hypertrophy in response to pressure overload in mice (38). It

is worth mentioning that mice with global knockout of PLCε are more susceptible to the development of cardiac hypertrophy by chronic Iso treatment(39). This discrepancy is very likely due to the compensatory pathways that are upregulated by global PLCε knockout from birth. We further investigated the signaling pathways that are associated with PLCε and have reported that PLCε can physically interact with muscle-specific A kinase anchoring protein (mAKAP), a nuclear scaffolding protein, through RA1 domain of PLCε and SR1 domain of mAKAP(40). Disruption of their interaction inhibits the development of cardiac driven by ET-1. One of the signaling consequences of nucleus envelope localized PLCε is to drive nuclear PKD activation which is critical in the regulation of cardiac hypertrophy(38). We hypothesized that DAG-dependent PKD activation is localized, in part, due to the fact that DAG is a membrane bound lipid and not diffusible through cytoplasm. And PKD activated at PM is not sufficient to maintain nucleus PKD activity. Thus, DAG produced in proximity to nuclear envelope through PLCε is a major source to drive nuclear PKD activation(41).

As discussed above, PLCε can receive multiple stimuli and function as a signaling nexus. The EPAC selective cAMP analog, cpTOME, or adenylate cyclase stimulator, forskolin, activates nuclear envelope PLCε(38). As will be discussed in Chapter 2, localized cAMP generated downstream of Golgi-β1ARs mediates the activation of EPAC/PLCε/mAKAP complex.(42) Interestingly, different cAMP pools have distinct effects on the PLCε activity(43). One pool of cAMP controlled by PDE3 activates EPAC/PLCε, the other pool of cAMP controlled by PDE2 and PDE9A inhibits nuclear PLCε activity dependent on PKA. We have also shown that endosomal β2ARs inhibit nucleus PLCε activity through Gi-Gβγ-ERK signaling (unpublished and is the subject of chapter 3 of this thesis).

1.4 Phosphatidylinositol metabolism

Phosphatidylinositol (PI) is the parent lipid of phosphoinositides which is crucial in membrane trafficking and cell signaling. The 3,4, and 5 positions of the inositol head group can be reversibly phosphorylated, generating seven different phosphoinositide derivatives including PI3P, PI4P, PI5P, PI(3, 4)P2, PI(4, 5)P2, PI(3, 5)P2, and PI(3, 4, 5)P3. They are distributed at different membrane compartments limited by the phosphoinositide kinases and phosphatases. Among these, PI(4,5)P2 is the most abundant phosphoinositide and it is mostly enriched at the plasma membrane and inside the nucleus. PI(4,5)P2 is synthesized from plasma membrane PI by two kinases, PI4-kinase (PI4K, converting PI into PI4P) and PI4P5K (converting PI4P into PI(4,5)P2)(44). Breakdown of PI(4,5)P2 can be regulated by dephosphorylation by PI(4,5)P2-5-phosphatase family or PLCs to generate diffusible IP3 (regulates calcium release from SR) and membrane bound DAG (activates PKC/PKD)(32).

PI4P can be synthesized by phosphorylation at the 4' position of the inositol head group or dephosphorylated from PI(4,5)P2 and PI (3,4)P2. It has been suggested that PI4P is found at plasma membrane and the Golgi apparatus(45). We have reported that Golgi PI4P is hydrolyzed by PLCε to generate inactive IP2 and bioactive Golgi membrane bound DAG(38). Golgi PI4P is an alternative substrate for PLCε at the Golgi owing to the low abundance of PI(4,5)P2 at intracellular compartments(38).

PI3P is essential in membrane dynamics and tracking. PI3P is enriched in the early endosomal autoantigen (EEA1)-positive vesicles(46). It can recruit proteins that contain PI3P-binding domains including the FYVE domain or the PX (Phox homology) domain(47). PI(3,4,5)P3 has been implicated in the regulation of cell proliferation, migration, growth, and cancer. It can recruit PH domain-containing proteins to regulate cell migration. PTEN phosphatase, which

dephosphorylates PI(3,4,5)P3 on the 3' position to generate PI(4,5)P2, is studied extensively in cancer(48).

Phosphoinositide binding domains fused to a fluorescent protein are utilized to visualize phosphoinositide changes in living cells. The PLCδ-PH-GFP detects PI(4,5)P2 at PM but no other intracellular compartments(49). PLCδ-PH-GFP recognizes the head group of PI(4,5)P2, therefore, IP3 can compete with PI(4,5)P2 for binding to the sensor resulting in its change in the membrane localization(50). Therefore, it does not necessarily reflect the lipid level at plasma membrane. Tubby domain also detects PI(4,5)P2(51) and a mutant form of the Tubby domain has been shown to be less sensitive to IP3 changes(52, 53). For PI4P detection, several PH domains have been discovered such as OSBP (oxysterol binding protein) and FAPP (four-phosphate-adaptor protein)(54). They bind to PI4P at the Golgi apparatus and their binding requires both ARF1-GTP at the Golgi apparatus and PI4P(55). More recently, a new unbiased PI4P biosensor P4M has been used to reveal the cellular distribution of PI4P including PM, Golgi, and Rab7-positive late endosomes(56). There is no absolute quantitative way to measure the activity of PLCs at specific compartments, so we utilize FAPP-PH-GFP that detects Golgi-PI4P as a readout of PLCε's activity in living cardiomyocytes.

1.5 β-Adrenergic Receptor Subtypes in the Heart

GPCRs participate in nearly all physiological activities and are widely expressed in cardiovascular systems including a variety of cell types such as cardiomyocytes, cardiac fibroblasts, and endothelial cells. β-adrenergic receptors (β-ARs) are extensively studied members of the Gs coupled receptor family and they are predominant GPCRs in regulating cardiac physiologic functions and diseases. There are three subtypes of βARs present in the human heart, β1AR, β2AR, and β3AR. The healthy human heart is comprised of 80% β1ARs and 20% β2ARs,

with lower expression of β3ARs(57, 58). The major focus here will be on the β1 and β2ARs that have been most extensively studied in the heart. β1ARs and β2ARs share high homology (57% full-length identity)(59), and both stimulate Gαs and cAMP production. Epi and NE bind to βARs in the heart with different affinities with NE targeting β1ARs with approximately 10-fold higher selectivity relative to β2ARs whereas Epi is non-selective for β1- and β2ARs(60). Catecholamine-induced stimulation of βARs acutely improves cardiac performance in healthy human hearts by increasing heart rate (chronotropy) and contractility (inotropy). The simplistic view is that these receptors act at cell surface generating second messengers that diffuse throughout the cell to regulate cardiac cell physiology. This view has come under scrutiny in recent years. Cardiac myocytes have unique structural features that require a more detailed understanding of subcellular locations and functions of these GPCRs to establish a mechanistic picture of how they are involved in cardiac regulation and pathophysiology.

1.6 β-adrenergic Receptor Subtypes in Cardiac Contraction and Hypertrophy

1.6.1 Human Heart Failure

Heart failure is a complicated syndrome where the ability of the heart to pump blood declines over time due to chronic stress resulting from hypertension or left ventricular dysfunction and is eventually unable to meet the metabolic demands of the body. It is the end point of many cardiovascular diseases. It is well-recognized that hyperactivated adrenergic signaling, due to prolonged elevation of catecholamines and other neurohumoral factors, leads to the progression of maladaptive cardiac hypertrophy, myocyte apoptosis, fibrosis, and eventually heart failure, likely accounting, at least in part, for the effectiveness of β-blockers in treatment of heart failure. In failing hearts, β1ARs are reduced from 80% of total βAR population to approximately 50% depending on disease severity, whereas the β2AR population remained unchanged(61).

1.6.2 Mouse Models of Cardiac Function and Failure

The first transgenic mice with cardiac-specific overexpression of β2ARs were created by Milano et al. in 1994(62). These mice exhibited enhanced atrial contractility and left ventricular function even in the absence of agonists, possibly due to an increased level of receptors with basal constitutive activity. Later studies confirmed this observation(63-65), however, there is still some debate since studies have shown that high level over-expression of β2ARs, 300-fold over endogenous receptors, has detrimental effects on cardiac performance in the long term(66). These long-term adverse consequences require β2ARs to be present at a high level while the positive inotropic effects from β2ARs possibly involve low to moderate level of β2ARs overexpression(67). By contrast, studies on β1ARs consistently demonstrate that mice with increased (15-45 fold) cardiac-specific expression of β1ARs had enhanced cardiac contractility at young age but developed significant myocyte hypertrophy and reduced contractility as they aged(68, 69).

β1AR knockout mice, β2AR knockout mice or β1/β2AR double knockout mice have also been generated. Most mice lacking β1ARs die prenatally, but those that survive have blunted chronotropic and inotropic responses in response to isoproterenol (Iso), despite normal expression of β2ARs(70). β2AR knockout mice, on the other hand, had normal resting heart rate and blood pressure, no prenatal death, and the chronotropic response to Iso was normal(71). These studies indicated that β1ARs play a predominant role in regulating heart rate and contractility. Dual βAR knockout prevented increases in inflammatory cytokine production, fibrosis, and cardiac hypertrophic responses in a pressure overload transverse aortic constriction model (TAC)(72). Zhao et al.(73) confirmed that TAC-induced cardiac hypertrophy was abolished in the dual knockout mice. Interestingly, they also found distinct regulation by these subtypes in pressure

overload-induced hypertrophy. Specifically, mice with β1AR deletion had similar hypertrophic responses compared to wild type, whereas β2AR deletion led to development of exaggerated hypertrophy. This indicated that coordination of signaling between these two subtypes is essential in mediating cardiac remodeling. It is clear that β1ARs and β2ARs elicit markedly different outcomes in cardiac pathology; however, definitive answers as to the molecular mechanisms underlying these phenomena have not been fully elucidated.

1.7 Different β1 and β2 adrenergic Receptor Signaling, Internalization, and Locations

The marked differences in physiological and pathological outcomes downstream or either β1 or β2ARs occurs despite the fact that they stimulate the same core signaling pathway (Gαs-AC-cAMP) and share overall sequence and structural homology. A large body of research has sought to decipher their different or even opposite effects on cardiac functions. Possible mechanistic explanations include differences in β1AR or β2AR sarcolemmal microdomain distribution in cardiac myocytes, subcellular trafficking and location, scaffolding interactions, and signal transduction. Some of these data are described below.

1.7.1 β1 and β2AR-dependent Signaling Pathways and Contractility

cAMP signaling downstream of β1AR stimulation increases Ca2+ transient amplitudes, at least in part, through protein kinase A (PKA)-dependent phosphorylation of a variety of intracellular proteins such as the type 2 ryanodine receptor (RyR2), phospholamban (PLB), cardiac myosin binding protein-C (cMyBP-c) and Cav1.2 (L-type) Ca2+ channels. Our laboratory and others identified roles of EPAC proteins in adrenergic regulation of contractility, independent from PKA(37, 74). EPAC is an exchange factor for the small GTPase Rap, activated by directly binding cAMP(11). Ours and other studies indicated that EPAC-activated Rap directly binds to, and

stimulates, a specific phospholipase (PLC) isoform, PLCε, leading to PKC activation, DAG production and activation of Ca2+/calmodulin kinase II (CaMKII)(75) ultimately resulting in phosphorylation of RyR2(37, 74) and potentially other proteins to modulate Ca2+ transient amplitudes. Work by another group found that this pathway regulates SR Ca2+ leak(74).

Compared with β1ARs, β2AR activation elicits a significantly smaller effect on cardiac inotropy(76, 77). Both receptors stimulate cAMP production although with different characteristics. β2AR-stimulated cAMP is highly compartmentalized whereas β1AR-cAMP signaling is more far-reaching and diffuse in cardiac myocytes(78) (Fig. 1.1A). One hypothesis is that the cAMP produced downstream of β2AR cannot access PKA and EPAC to regulate the machinery involve in contraction. The relatively minor β2ARs-dependent increases in intracellular Ca2+ transient amplitudes are independent from cAMP-mediated protein phosphorylation or EPAC activation.

Another significant signaling difference between β2 and β1ARs is that β2ARs couple to both Gs and Gi proteins whereas β1 ARs couple only to Gs. Studies in neonatal rat cardiac myocytes (NRVMs) indicated the initial transient contractile response to β2 activation is suppressed by a switch to Gi coupling(79). Pertussis toxin (PTX) treatment revealed a sustained contractile response in response to β2AR stimulation(79, 80). Activation of β1ARs in the same system resulted in sustained signaling that was not affected by PTX treatment. Thus, differences in signaling properties (ie, coupling to different G proteins and pathways) of the receptors themselves could be responsible for some of the observed physiological differences.

1.7.2 Different Internalization of β1 vs. β2ARs

In transfected cells, β2 adrenergic receptors robustly internalize, whereas β1ARs also do but to a lesser extent(81). Initial studies in NRVMs isolated from β1/β2 AR double knockout mice

transduced with either flag epitope tagged-β1 or β2 ARs indicated that β2ARs robustly internalize into endosomes but β1ARs do not(78, 82). This difference was demonstrated to result from differential scaffolding by PSD-95/Discs large/Zonula occludens-1 interacting proteins at the C-termini of βARs. Subsequent studies presented conflicting results in NRVMs where flag-β1ARs were observed to internalize(83). Both studies explored the role of scaffolding by PSD-95/Discs large/Zonula occludens-1 ligands at the C-terminus.

Neonatal myocytes are a very useful model system for studying cardiomyocyte function but do not have the complex subcellular architecture of mature adult myocytes. In particular, mature myocytes have a highly organized network of T-tubules that are extended invaginations of the sarcolemma. This network of tubules contains L-type Ca2+ channels directly opposed to Ryr2 in the SR where excitation-contraction coupling occurs. A recent study examined the location of β1ARs in adult ventricular myocytes (AVMs) with adenoviral mediated expression of flag-β1ARs(84). Here, under non stimulated conditions β1ARs were broadly expressed at the cell surface but not in t-tubules. Upon stimulation with a high concentration of isoproterenol (Iso) (10 μM), flag-β1 ARs redistributed to tubules without any visible receptor internalization into endosomes (Fig.1.1C). β2ARs were not examined in this study. More recently, studies were conducted with a novel fluorescent derivative of the βAR antagonist carazolol to label βARs expressed at native levels in mature cardiac myocytes(85). Current antibodies against β1ARs are not reliable in the detection of endogenous receptors. Using sophisticated image analysis, it was shown that endogenous β1ARs are present at the cell surface and in T tubules. On the other hand, β2ARs are present only at the T tubules consistent with a restricted pool of cAMP generated at this location. Owing to the nature of ligand used for these studies, agonist- dependent changes in receptor location could not be monitored. Interestingly the results with native receptors had

important differences from those with expressed receptors where expressed β2ARs were observed at both the cell surface and in T tubules emphasizing the importance of examining endogenous receptors where possible.

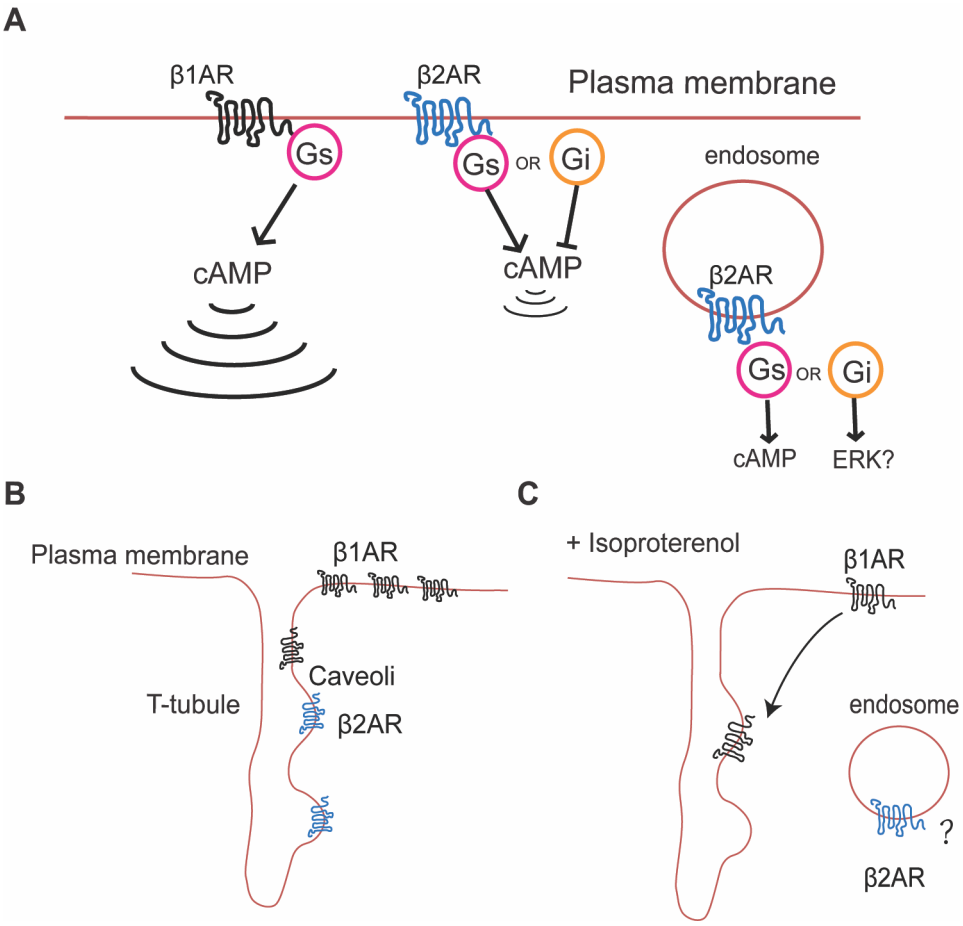

Figure 1-1 Discrete cAMP signals and microdomain distributions of βARs.
A) β1AR-induced cAMP signaling is far-reaching, whereas β2AR-induced cAMP signaling is locally confined and Internalized β2ARs may continue to signal in myocytes. B) In healthy cardiomyocytes, β1ARs distribute widely across the cell surface[33,34], while β2ARs are localized to T-tubules and caveoli. C) Stimulation with a βAR agonist induces a redistribution of β1ARs into T-tubules[32] and possible internalization into endosomes, although this has not been directly confirmed in mature cardiac myocytes.

1.7.3 βARs in Caveolae

Caveolae are microdomains in the plasma membrane enriched in sphingolipids and cholesterol and caveolin. These microdomains can promote interactions between signaling proteins that sort into these domains based in part on their lipid modification. Cardiac myocytes are enriched in caveolae and adrenergic receptor subtypes differentially sort into these domains with β2ARs selectively partitioning into caveolae, while the majority of β1ARs excluded from these domains(86, 87). Experimental manipulations that disrupted caveolae led to stronger coupling of β2ARs to cAMP production, suggesting that residence in caveolae limits cAMP signaling from β2ARs(87).

1.7.4 Signaling by Internalized βARs from Endosomes.

Although internalization of GPCRs has been observed for many years, it had been assumed that the purpose of internalization was to desensitize the receptor. Development of genetically encodable nanobody-based sensors(88) or engineered Gα subunits (fluorescent protein tagged miniG proteins)(89) has enabled direct monitoring of the activation state of βARs in living cells at a subcellular level. Using nanobody 80 fused to GFP (Nb80-GFP), which detects activated βARs, and Nb37-GFP, which detects active Gαs, Irannejad et al. found activate β2ARs coupled to Gαs activation at early endosomes(81). Using inhibitors of receptor internalization, it was shown internalized receptors contributed to the overall cAMP response to an agonist in some cell types(88). Following up on this work, it was found that signaling from internal receptors was selectively associated with expression of a subset of genes(90). The Vilardaga laboratory(91) also identified roles for intracellular GPCRs in regulation of sustained cAMP generation downstream of parathyroid hormone receptors. The existence or role of signaling by internalized activated β2ARs has not been investigated in cardiac myocytes, or in the heart.

1.7.5 Resident Intracellular β1ARs

Nuclear Envelope. Initial evidence for intracellular localization of βARs in cardiomyocytes was reported by Boivin et al. in 2006(92). Using immunocytochemistry in adult cardiomyocytes, they showed that in addition to cell surface and t-tubules localizations, β1ARs but not β2ARs are localized on the periphery of nuclear envelope. These intracellular β1ARs activated AC and stimulated RNA synthesis in isolated nuclear fractions possibly through Gαs or other signaling pathways including ERK1/2 and p38(93). Interestingly, these investigators also identified β3ARs on the nuclear envelope, where β3 but not β1ARs regulated NO production(94). Other GPCRs have been found at the nuclear envelope in CMs including endothelin and a1-adrenergic receptors and have been demonstrated to stimulate phospholipase C activation and subsequent nuclear Ca2+ increases(95, 96). One possible caveat to some of these studies is that it is difficult to differentiate Golgi and NE via light microscopy in cardiac myocytes.

Golgi apparatus. In 2017, Irannejad et al(81) found a functional reserve pool of β1ARs at the Golgi apparatus that is not delivered via receptor endocytosis in HeLa cells. However, β2ARs were not detected at this location. This suggests an additional possible mechanism underlying the divergence in βAR subtype functions in the heart. Activation and signaling from Golgi-β1AR was independent from receptors at the cell surface. In my thesis work, our laboratory demonstrated activation of a signaling pathway downstream of Golgi resident β1ARs(42) (Fig. 1.2). This will be discussed in detail in Chapter 2.

Sarcoplasmic reticulum. A recent study showed that a population of β1ARs was localized to the SR, and regulated contractility through local PKA activation (discussed below) and phospholamban phosphorylation(97). It was also shown that β1ARs could coimmunoprecipitate with SERCA2 supporting an SR location, although it is possible these interactions could be in

other compartments or between compartments. Here, OCT3 knockout animals had blunted contractile responses to Iso and NE supporting the idea that OCT3 transporters have physiological significance beyond neurotransmitter clearance.

1.7.6 Organic Cation Transporters Provide Access of catecholamines to Intracellular Receptors

For intracellular adrenergic systems to operate physiologically, they must be accessed by endogenous neurotransmitters. The endogenous catecholamines Epi and NE, are ligands of βARs but they are membrane impermeant. How these physiologically relevant ligands access their intracellular targets in the Golgi and elsewhere must be explained. A particular organic cation transporter subtype, OCT3, has been shown to facilitate the uptake of catecholamines across the plasma membrane (PM)/sarcolemma, and is also found in the Golgi, and nuclear membranes of cultured cardiac cells(96). An OCT3-dependent mechanism has been previously reported to be involved in the uptake of NE into cardiac myocytes for the regulation of a-adrenergic receptors located at the nuclear envelope(96). In Chapter 2, I explored the role of OCT3 in intracellular Golgi β1ARs activation in cardiac myocytes.

The data in this thesis and other published data support a physiological relevant role of intracellular roles βARs and provide a mechanism by which catecholamines can cross the membranes to act on the intracellular receptors. Interestingly, corticosterone, released by adrenal gland, blocks OCT3 activity. It is possible that corticosterone might exert direct effects in cardiomyocytes in vivo by inhibiting OCT3 activity(98), thereby regulating intracellular β-AR-related cardiac functions. Supporting this view, clinical evidence suggests that adrenal insufficiency is associated with cardiac dysfunction(99, 100).

1.8 Compartmentalized cAMP Signaling in Cardiac Myocytes.

1.8.1 cAMP Microdomains.

The accumulating evidence discussed above and in this thesis support the emerging concept of functional pools of βARs at various subcellular compartments that contribute to cAMP compartmentalization to optimally exert physiological cardiac outcomes. There is ample evidence cAMP is tightly regulated at a nanodomain level, rather than simply diffusing from the site of generation, to optimally regulate different downstream effectors. Early in 1980s, the working hypothesis of cAMP compartmentalization and subcellular pools of cAMP-dependent protein kinases downstream of β-AR activation in cardiomyocytes was proposed(101). This concept became generally accepted when the optical probes (fluorescence resonance energy transfer, FRET-based reporters) were exploited to visualize distinct subcellular cAMP pools(102, 103). By targeting the sensors to specific subcellular locations in living cells or even in living animals, intracellular cAMP levels could be measured in a real-time, quantitative, and spatiotemporal manner. This tool is extremely useful especially in the architecturally complicated cells such as ventricular cardiac myocytes. In one series of experiments, a modified FRET sensor named "CUTie" was targeted to the different sites known to regulate cardiac excitation-contraction coupling (ECC) including sarcolemma, SR, and myofilaments in isolated cardiac myocytes. They found distinct regulation of cAMP pools at these sites with significantly smaller and delayed response at myofilaments compared to sarcolemma and SR(104). Importantly, they also showed that when the cAMP compartmentation was abolished using phosphodiesterase (PDE) inhibitors, the inotropic response was blunted in cardiomyocytes indicating the importance of the precise control of local cAMP level in contractility(104).

These approaches were also employed to study cAMP generation by βARs located at the SR. In these experiments blockade or knockout of OCT3 inhibited NE-dependent cAMP generation detected by SR-targeted PKA regulated FRET reporter (SR-AKAR). This implied, but did not directly show, that βARs localized to the SR are responsible for local cAMP production at the SR. Targeting the βAR inhibitor, NB80, to the SR would more directly address a role for βARs at the SR. Similar studies have not yet been used to monitor cAMP production downstream of Golgi localized βARs.

1.8.2 PDEs Control cAMP Microdomains

Where cAMP signaling is terminated is a major contributor to restricting microdomains of cAMP. The enzymes responsible for cAMP degradation, cAMP-phosphodiesterases (PDEs), consist of 11 members (PDE1-11). Different PDE isoforms have unique subcellular locations, substrate specificity and regulatory mechanisms, therefore, localization of specific PDE isoforms to specific myocyte subdomains shapes cAMP signaling locally leading to specific biological outcomes without broadly altering cAMP concentration within cells. PDEs 1-5 and 8-10 are reported to be expressed in the heart(105). PDEs 1,2,3, and 10 hydrolyze both cAMP and cGMP, and PDE 4 and 8 are cAMP-specific. Also, it has been shown that the sizes of cAMP pools are quite small and PDEs can shape nanometer-scale domains of cAMP signaling. Dysregulation of PDE isoform expression level, subcellular localization and activation have been implicated in cardiac diseases.

PDEs are selectively anchored to signalosomes via anchoring proteins including A kinase anchoring proteins (AKAPs), which also bring together PKA, adenylyl cyclase (AC), and other cAMP-effectors in many cell types including the heart. For example, mAKAPβ, scaffolds PDE4D3, AC5, PKA, EPAC, PLCε, protein kinase (PKD), and other proteins, at the nuclear

membrane, to regulate cardiac hypertrophy(106-108). AKAP12 scaffolds PDE4D and β2ARs(109, 110). β2AR-dependent cAMP production is limited by PDE4, since blockade of PDE4D results in sustained cAMP production(111). PDE5 also binds to β2ARs under conditions of diabetic cardiomyopathy(112). β1AR-dependent cAMP signaling has also been shown to be regulated by through scaffolding to PDE4D(113). Thus, PDE scaffolding to βARs modulates cAMP production downstream of both receptor subtypes, but likely does so to different extents. Additionally, PDE3A and PDE 3B have different intracellular localizations with PDE3A localized to the SR with AKAP18, PKA, and SR Ca2+ ATPase 2 (SERCA2) and PLB to regulate contractile function(114), while PDE3B co-localizes with caveolin-3, a caveolae protein on or near T-tubule membrane(115). PDE2A2 located at mitochondria controls the local cAMP generated at plasma membrane to regulate cardiomyocyte death(116). Interestingly, different PDE isoform-associated signals have been reported to be synergistic or opposing. PDE1 and PDE5 have been shown to have a synergistic effect on the promotion of cardiac hypertrophy(117).

1.8.3 PDEs Limit Access of cAMP Generated at the Cell Surface to the mAKAP/EPAC/PLCε Signaling Pathway at the Golgi-nuclear Envelope Interface

Treatment of cells with the cell impermeant agonist Iso is unable to stimulate activation of mAKAP/EPAC/PLCε at the Golgi/nuclear envelope interface(43). This indicates that something must be restricting the access of cAMP to this complex. We identified PDE3 as a key PDE preventing access of cAMP to the mAKAP/EPAC/PLCε complex in cardiac myocytes (Fig. 1.3). Inhibition of PDE3 revealed activation of PLCε at the NE/Golgi interface in the absence of ligand stimulation and led to development of cardiomyocyte hypertrophy in NRVMs. Treatment with Iso in combination with PDE3 inhibitor led to an enhanced response. A second pool of cAMP controlled by PDEs 2 and 9A opposed activation of PLCε at the Golgi through a PKA-dependent

mechanism, which would be predicted to oppose hypertrophy (Fig. 1.2). This evidence again emphasizes the importance of balanced cAMP compartmentalized signaling in cardiac functions and shows that cAMP signaling can be either detrimental or protective depending on where or how it is regulated in the heart. Though there are clinically available PDE inhibitors, their limitations include the PDE isozymes selectivity within the same family, effects in non-cardiac tissues, or lack of precise targeting within cells. For example, targeting inhibitors to specific subcellular locations to disrupt specific PDEs in signalosomes may avoid some unwanted effects.

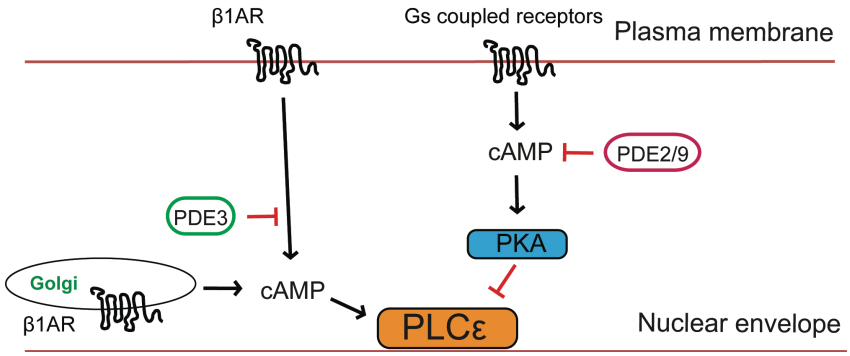

Figure 1-2 Balancing pools of cAMP signaling to PLCε by PDE isoforms.
Distinct cAMP pools are differentially controlled by PDEs 2/9 and PDE3. PDE3 prevents cAMP diffusion to the nuclear envelope/Golgi limiting activation of PLCε at that location. A distinct pool of cAMP regulated by PDEs 2/9 at plasma membrane opposes the activity of PLCε through a PKA dependent mechanism.

1.9 Compartmentalized Gβγ signaling.

Upon activation of Gα subunits, Gβγ subunits are released that mediate a plethora of signaling processes downstream of GPCRs under physiological or pathological conditions. Instead of targeting GPCRs, direct Gβγ targeting is a potential alternative strategy for many human diseases such as analgesia, chronic inflammation, cancer cells proliferation, heart failure, and fibrosis(118). Gβγ inhibitors including C-terminal tail of G protein coupled receptor kinase 2 (GRK2CT), a Nanobody (NB5)(119), and the small molecule inhibitors, Gallein, M119 have been shown to specifically inhibit Gβγ signaling without having effects on Gα signaling(118, 120).

The canonical view is that Gβγ exerts its function via signaling at the plasma membrane, however, recent studies have shown that Gβγ subunits also are activated via GPCRs at various intracellular compartments including, the Golgi apparatus, endoplasmic reticulum, endosomes, nucleus, and mitochondria(121). The Golgi-Gβγ activation has been shown to be released from Gi3 coupled to GPRC5A at the TGN or downstream of S1PR translocated from the PM to the TGN dependent on GRK2 and β-arrestin-2(122, 123). In addition, Gα-interacting vesicle-associated protein (GIV), a non-receptor guanine nucleotide exchange factor (GEF), has been shown to localize to the Golgi and activate heterotrimeric G proteins to release Gβγ(124). Gβγ can also be activated at Golgi through translocation from plasma membrane either alone(121) or in complex with Gα subunit(125). It has been suggested that the mechanisms of Gβγ translocation to intracellular compartments involve in the diffusion of Gβγ in the cytosol upon their dissociation from the PM and active transportation such as endocytosis(121).

One of the functional outcomes downstream of Gβγ is the regulation of Golgi fragmentation causing fission of vesicles from the TGN to PM(126). The signaling mechanism involves in the recruitment and activation of protein kinase D (PKD) through DAG production at

TGN and Golgi localized protein kinase C. Irannejad et al. showed that phospholipase C and PKD dependent vesiculation relies on Gβγ at the Golgi apparatus, but not other places(127). Considering the importance of phospholipase C and PKD activities in the regulation of cardiac hypertrophy, our laboratory has been investigating the role of Golgi Gβγ in cardiomyocyte hypertrophy. Our laboratory has shown that Gβγ directly interacts with PLCε(128). In cardiomyocytes, PLCε is found at nuclear envelope/Golgi interface through its interaction with mAKAP(40). Perinuclear PLCε can hydrolyze PI4P, rather than PIP2, to generate local DAG and mediate nuclear PKD activation therefore regulating cardiac hypertrophy(38). Our studies also provide evidence that Golgi Gβγ is critical in ET-1 dependent PLCε activation and downstream PI4P hydrolysis and cardiomyocyte hypertrophy(129). More details will be discussed later.

Gβγ also plays an important role in the activation of MAPK. It has been implicated that Golgi Gβγ can activate ERK1/2 in a process dependent on PI3Ky p110y-p101 heterodimers(130). Like Gβγ signaling, ERK is highly spatiotemporal regulated. Recently, it has been reported that activation of ERK signaling downstream of β2ARs is mediated by endosomal Gαs downstream β2ARs, rather than those at PM, to regulate gene expression in HEK cells(131). In our study, as shown in chapter 3, endosomal Gβγ is critical in the regulation of ERK signaling and PLCε activity. The development of a location specific Gβγ inhibitor would be valuable for dissecting the mechanisms of spatiotemporal regulation of Gβγ signaling.

1.10 Conclusions and Future Directions

It is well established that under both normal and pathologic conditions, βARs play an important role in initiating and regulating signaling pathways involved in cardiac function. Owing to the inhibitory effects of β-blockers on myocardial contractility, they were initially considered as contraindicated in the treatment of heart failure. In mid 1970s, small-scale trials were conducted

to show an improved outcome of β-blockers in patients with cardiac dysfunction(132, 133). Today, β-blockers such as carvedilol, bisoprolol, and metoprolol along with other medications are among first-line treatment used in patients with stable, mild, moderate, and severe heart failure, although the exact mechanism of action of β-blockers remain incompletely understood. Studies have shown several potential mechanisms of β-blockers that contribute to their beneficial effects on heart failure. The mechanisms include but are not limited to antagonizing neurohormonal stimulatory effects of hyper-βAR-mediated hypertrophic and proapoptotic effects on cardiomyocytes, slowing heart rate, lowering blood pressure, reducing myocardial oxygen consumption, and restoring the ratio of β1ARs and β2ARs(134, 135). A large body of evidence shows favorable effects of β-blockers in heart failure and reversal of cardiac remodeling. However, not all β-blockers showed the same beneficial effects. Currently available β-blockers are divided based on their different interactions with β1 and β2 subtypes. Bisoprolol, metoprolol, and nebivolol are β1-selective blockers while carvedilol is a non-selective β-blocker. In addition to subtype selectivity, a new principle for functional selectivity could be based on the accessibility to the subcellular locations. Bisoprolol, carvedilol and metoprolol which show beneficial effects in heart failure tend to be hydrophobic whereas sotalol, a membrane impermeant β-blocker, is not used to treat heart failure. Hydrophobicity might be an important factor for a clinically effective β-blocker based on the discovery of functional roles of intracellular βAR signaling in cardiac contractility, hypertrophy, and subsequent heart failure. Furthermore, elucidation of the distinct signaling properties of βAR signaling at the cell surface versus the interior of the cell, and the balance between cardiac protective versus toxic effects could provide critical insights into the development of new strategies for heart failure.

A genetically encoded, and nanobody based β-AR blocker, Nb80, has been recently utilized to specifically target βARs intracellularly by fusing different targeting sequences to its N terminus(42, 81). This approach combined with adeno-associated virus (AAV) based gene delivery system opens the possibility of targeting select subcellular pools of receptors with a high specificity in specific cell types. Investigation of βARs at various compartments and their common or unique physiological roles will provide substantial and comprehensive information of the roles of βARs in cardiac functions.

1.11 Thesis overview

Despite decades of investigations of therapies for heart failure and deep studies of GPCR signaling, treatments such as β-blockers or calcium channel blockers, ameliorate the symptoms of heart failure but still have significant side effects including exercise intolerance and poor circulation. New approaches to improve the β-blocker therapy would have substantial therapeutic impact. β-adrenergic receptors are a class of Gs coupled receptors that are traditionally thought to reside at the plasma membrane to mediate a plethora of cardiomyocyte signaling through production of cAMP. Studies from recent years have shown that GPCRs at specific intracellular compartments are functionally active and can initiate local cAMP accumulation to precisely control cellular function especially in architecturally complicated cells such as cardiomyocytes. β2ARs are well-known to be internalized after initial stimulation and continued to signal at endosomes. Though the recent data indicates that β1ARs reside at the Golgi apparatus in HeLa cells and contribute to cAMP production, their roles in cardiomyocytes, highly physiologically relevant cells, have not been explored. Another recent study showed that β1ARs are located at sarcoplasmic reticulum and can also generate local cAMP to regulate contractility in a PKA-dependent phospholamban phosphorylation manner. The goal of this dissertation was to determine

the functional roles of subcellular βARs in cardiac myocytes and therefore provide insights to the design of an optimal therapeutic strategy for heart failure. In this thesis, I will describe our studies of the signaling pathways that are downstream of Golgi-β1ARs and endosomal β2ARs to regulate the activities of PLCε, an enzyme that is tightly involved in cardiac hypertrophy.

In chapter 1, I provided an overview of βAR signaling in physiological and pathological regulation of functions within the cardiac myocyte, highlighting the spatiotemporal features of βAR signaling at intracellular compartments. Also, I discussed signaling mechanisms associated with phospholipase C that regulate cardiac functions. In chapter 2, I provided evidence of a functionally active pre-existing pool of Golgi-localized β1AR in cardiomyocytes and its role in the activation of nuclear envelope Epac/mAKAP/PLCε complex to regulate cardiac hypertrophy. In chapter 3, I demonstrated a mechanism behind the protective effects of β2-AR activation against hypertrophy through inhibition of hypertrophic PLCε signaling at the Golgi apparatus. The mechanism for β2AR-mediated PLC inhibition requires internalization of β2AR, activation of Gi and Gβγ subunit signaling at endosomes and ERK activation. In chapter 4, I described a Golgi-targeted β-adrenergic receptor blocker, eNOS (1-33)-mApple-NB80, that we developed to deliver the β1AR inhibitor, nanobody 80 (NB80), specifically to the Golgi apparatus. I verified its function in vitro, successfully delivered it into animal hearts and added further evidence of Golgi-β1ARs in the activation of PLCε in AVMs. I showed promising data with statistical trends that support our overall hypothesis that Golgi-β1ARs is involved in the pathogenesis of cardiac hypertrophy. In chapter 5, I discussed the overall implications of our findings and future directions.

Chapter 2 Golgi Localized β1-Adrenergic Receptors Stimulate Golgi PI4P Hydrolysis by PLCε to Regulate Cardiac Hypertrophy

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2.1 Abstract

Hyperactivated adrenergic tone, owing to chronically elevated neurohormonal factors, is a key mechanism involved in the progression of heart failure. The effectiveness of β-blocker therapy partially results from antagonizing hyper-βAR-mediated hypertrophic effects on cardiomyocytes. Emerging studies have demonstrated intracellularly localized βARs, although the biological significance is not defined. This chapter showed a pre-existing pool of Golgi-localized β1AR in cardiomyocytes that can be activated by the permeant exogenous agonist, dobutamine, or the physiological relevant ligand, norepinephrine through an OCT3 organic cation transporter. The hydrolysis of Golgi, prohypertrophic, phosphoinositide is uniquely regulated by Golgi-β1-AR stimulation but not cell surface β1AR stimulation. Blockade of intracellular β1ARs prevents cardiomyocyte hypertrophy. This defines a pathway activated by intracellular β1ARs in a highly biologically relevant system and has implications for the development of β-blocker therapies.

2.2 Introduction

Pathological cardiac hypertrophy contributes to a higher risk of developing cardiac dysfunction, such as heart failure, one of the major causes of morbidity or mortality in the western world. Chronic stress such as hypertension or myocardial infarction will result in the increase level of neurohumoral factors such as catecholamines, endothelin, and angiotensin II further driving cardiac hypertrophy and remodeling, ultimately leading to heart failure. G protein coupled receptors (GPCRs) are the main targets for these hormones(136-138). Today, the first-line treatments for patients with heart failure are β-blockers such as carvedilol, bisoprolol, and metoprolol along with other medications. Though the mechanism is unclear, the efficacy of β-blocker therapy is in part by antagonizing chronic βAR stimulation(139, 140).

We and others showed that Gs or Gq-mediated phospholipase C activation is involved in the regulation of cardiac hypertrophy. The endothelin-1 (ET-1) receptors or cell surface a-adrenergic receptors have been implicated in cardiac remodeling and one of the underlying mechanisms is dependent on Gq/PLC-β/DAG/PKC signaling pathway(141, 142). Additionally, our laboratory has identified a critical role of PLCε in the regulation of pathological cardiac hypertrophy(38, 40). PLCε is poised as a signaling hub for multiple regulators including Gβg subunits, EPAC, and small GTPases(32, 129, 143-145). In cardiac myocytes, PLCε scaffolds with muscle-specific A kinase anchoring protein (mAKAP)(106) at the nuclear envelope in the close proximity to the Golgi apparatus. Activation of PLCε at Golgi/nuclear envelope interface in cardiac myocytes induces the hydrolysis of Golgi phosphatidylinositol-4-phosphate (PI4P) leading to the generation of diacylglycerol (DAG) to facilitate protein kinase D phosphorylation and subsequent hypertrophic signals(38). Previous work from our laboratory found that activation of adenylate cyclases by forskolin or the EPAC-selective cAMP analog, cpTOME, induces PLCε-

dependent Golgi PI4P hydrolysis in cardiac myocytes. However, the βAR agonist isoproterenol (Iso), does not stimulate Golgi PI4P hydrolysis despite the massive cAMP generation(43). A model we proposed for this apparent paradox is that PLCε-dependent Golgi PI4P hydrolysis is regulated by two functionally distinct pools of cAMP controlled by different PDE isoforms. Iso is relatively membrane impermeant and its downstream cAMP generation near cell surface cannot access the EPAC/ mAKAP/PLCε complex at nuclear envelope/ Golgi interface unless PDE3 is inhibited.

It is now well established that subcellular localized pools of cAMP at nanometer scale generated by GPCRs at different intracellular compartments have distinct signaling outputs(81, 88, 146-149). For example, βARs internalized into endosomes continue to stimulate cAMP accumulation, initiating a set of signaling and transcriptional control events distinct from those at the plasma membrane(90). Moreover, β1ARs rather than β2ARs preexist at the Golgi apparatus in HeLa cells and Golgi β1ARs will induce G protein activation once being activated by a membrane permeant agonist, dobutamine, or by the physiological hormone, epinephrine though the organic cation transporter, OCT3(81). These ideas led to our hypothesis that in cardiac myocytes, β1ARs reside at the Golgi apparatus could generate a local pool of cAMP that has privileged access to the Epac/mAKAP/PLCε complex at the nuclear/Golgi interface, thereby yielding a set of signals divergent from those generated by the cell surface βARs. In this study, we demonstrate that endogenous intracellular β1ARs are required to stimulate hypertrophic Epac/PLCε-dependent Golgi PI4P hydrolysis in cardiac myocytes and they can be accessed by physiological neurotransmitters and synthetic β-blockers and agonists. These data provide a potential therapeutic strategy for treating heart failure patients through deliberately targeting of internal β1ARs in cardiac myocytes.

2.3 Materials and Methods

2.3.1 Materials.

Compounds and antibodies: Butanedione monoxime (Sigma, 112135); Isoproterenol (Sigma, 1351005); Dyngo (Abcam, Ab120689); Sotalol (Sigma, S0278); HJC0726 (gift from Xiaodong Cheng, UT Houston Health Science Center); Brefeldin A (Biolegend, 420601); Gallein (Sigma, 371708); Corticosterone (Tocris, 3685); Metoprolol (Sigma, M5391); Dobutamine (Tocris, 0515); Abacavir (Tocirs, 4148); Norepinephrine (Sigma, A0937); Collagenase Type II (Worthington, CLS-2); rabbit anti-ANF (Millipore, AB5490); goat anti-rabbit 568 secondary antibody (Invitrogen, A11011)

Plasmids and adenoviral constructs: NES-Venus-mini-Gs (gift from Nevin Lambert, Augusta University, GA); Flag-β1-adrenergic receptor (Addgene_14698); CFP-Giantin; FAPP-PH-GFP; CFP-NB80-FRB; FKBP-mApple-GalT.

2.3.2 Methods.

Isolation of neonatal cardiac myocytes and adenoviral transduction

Briefly, hearts were excised from 2 to 4-day old Sprague-Dawley rats, ventricles separated and minced thoroughly before digestion with Collagen type II (Worthington) in Hanks buffered saline solution (HBSS) without Ca2+ or Mg2+. Following digestion, cells were collected by centrifugation into Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine and 2 µg/mL vitamin B12. Contaminating cells were removed by preplating cells onto tissue culture plastic for a minimum of 1 h at 37 °C. NRVMs were then plated onto either glass-bottom tissue culture plates

or 12 well plates coated with 0.2% gelatin and cultured in DMEM (composition as above, with additional 10 μM cytosine arabinoside). 48 h later, cells were transferred into media supplemented with 1% FBS. For transduction with adenovirus, 50 MOI of indicated adenovirus was added overnight upon transfer to 1% FBS containing media. For fluorescent adenovirus constructs, expression was confirmed the next day by epifluorescence microscopy.

Isolation of adult ventricular myocytes

Adult myocytes were isolated from 2-4 month-old wild type C57BL/6J mice as previously described (150). Briefly, mice were anesthetized with ketamine (100mg/kg body weight) and xylazine (5 mg/kg body weight) i.p.. Hearts were cannulated and perfused with perfusion buffer via the aorta. Subsequently, the hearts were perfused with digestion buffer consisting of: collagenase type-II (773.48 U/mL), trypsin (0.14 mg/mL), and calcium chloride (12.5 μM) in the perfusion buffer (pH 7.46). The atria were removed, and the ventricles were minced in stop buffer containing 10% FBS and 12.5 μM calcium chloride in perfusion buffer. After calcium was added back to a final concentration of 1 mM, cells were plated onto laminin-coated 20-mm glass bottom dishes in minimum essential medium supplement with 0.35 g/L sodium bicarbonate, 2.5% FBS, and 10mM 2,3 butanedione monoxime (BDM).

Transduction of AVMs with adenovirus

After AVMs were adhered to the laminin-coated 20 mm dishes for 1-hour, plating media was removed and infected cells with adenoviruses expressing FAPP-PH-GFP (100 MOI) in BDM-free media for 2-3 hours. The virus was then removed, and BDM was added to the culture media. 18-24 hours later, cells were imaged by confocal microscopy with 40X oil-immersion lens for

measurement of FAPP-PH-GFP fluorescence. EGFP was excited at 488 nm and images were acquired with 25 ms exposure at 2.5 min intervals.

NES-Venus-mini-Gs imaging

NRVMs were plated into gelatin-coated 20 mm glass bottom cell culture dishes. Cells were transfected the following day with plasmids (500-800 ng of β1-ARs and 250-400 ng of NES-Venus-mini-Gs per dish) using lipofectamine 3000. Media was changed to 1% FBS the next day and transduced with adenovirus-expressing CFP-Giantin overnight. Cells were imaged in confocal mode with a Leica DMi8 equipped with a Crest-optics X-light V2 confocal unit and a 100x 1.4 NA oil-immersion lens. Venus was excited at 515 with an X-Cite Xled1 light source, and emission monitored imaged on a backlit CMOS Photometrics Prime 95B camera.

Measurement of PI4P hydrolysis

Measurements of PI4P hydrolysis were made as previously described (43, 151, 152). After preparation and culture of myocytes, cells were transduced with adenovirus (50 MOI) expressing GFP-FAPP-PH overnight. The following day, expression was confirmed by epifluorescence microscopy. Time lapse video fluorescence Imaging of GFP-FAPP-PH fluorescence was performed at room temperature or 37C, where indicated, on a LEICA DMi8 with a 20x air lens in confocal mode. EGFP was excited at 488 nm with an X-Cite Xled1 light source and emission monitored imaged on a backlit CMOS Photometrics Prime 95B camera. Images were acquired with 50 ms exposure times at 1 min intervals to minimize photobleaching. Analysis of fluorescence intensity changes from the videos was performed using NIH Image J unless otherwise stated. Analysis was performed by subtracting background fluorescence intensity from a region of

interest intensity at all time points measured. Data is presented as percentage of fluorescence remaining after agonist stimulation when compared to cells prior to stimulation.

Measurement of myocyte cell area

NRVMs were plated into gelatin-coated 12 well plates or glass-bottom 96 well plates and allowed to grow overnight. The following day, cells were infected overnight with adenovirus expressing YFP. Subsequently, NRVMs were stimulated with dobutamine (100 nM) or norepinephrine (10μM) for 48 h, in the presence of antagonists or transduction with FRB-CFP-Nb80 and FKBP-mApple-GalT, as indicated. Following stimulation, cells were fixed in 4% (w/v) paraformaldehyde. Fluorescent images were taken at 10 x magnification and cell area measured using NIH Image J from over 500 cells from at least 3 separate experiments.

Immunocytochemistry for ANF induction

NRVMs were plated into gelatin-coated 8 chamber glass slides or glass-bottom 96 well plates and allowed to grow overnight. The following day, cells were serum starved for 24 hours. Subsequently, NRVMs were stimulated with dobutamine (100 nM) or norepinephrine (10μM) for 48 h, in the presence of antagonists or transduction with FRB-CFP-Nb80 and FKBP-mApple-GalT, as indicated. Cells were washed with PBS and fixed with 4% PFA for 15 minutes and then incubated with 10% normal goat serum in phosphate buffered saline containing 0.1% Triton X100 (PBS-T) for 1 hour at room temperature. Primary antibody was incubated at a dilution of 1:1000 in 2 % goat serum in PBS-T overnight at 4°C. After three washes with PBST, cells were incubated with secondary antibody (Goat anti-rabbit Alexa Fluor 568) at a dilution of 1:1000 in PBS-T for 1.5 hours at room temperature. After three washes with PBS-T, DAPI was added at a dilution of

1:500 in PBS and incubated for 30 minutes. Fluorescence images were captured at 10 x magnification and fluorescence intensity corresponding to ANF staining surrounding the nucleus was quantified using either NIH Image J.

Statistical Analysis: All graphs are presented as the mean ± SE of the results from independent preparations of cells (ie. N=3-4 as indicated in the figure legends). Agonist treatments were compared to vehicle control performed on the same day and were added where indicated by the arrow. All data was analyzed by two-way unpaired ANOVA with Sidak's post-hoc test unless otherwise indicated. * $p<0.05$ ** $p<0.001$ *** $p<0.0001$ **** $p<0.00001$ using GraphPad Prism 7.0.

2.4 Results

2.4.1 A membrane permeant βAR agonist, dobutamine induces Golgi PI4P hydrolysis.

We have previously shown that stimulation with a membrane impermeant βAR agonist, Iso, does not induce Golgi PI4P hydrolysis in NRVMs despite it is capable of producing cAMP(43). To test our hypothesis of the activation of intracellular β1ARs is required to generate a specific pool cAMP with privileged access to the Epac/mAKAP/PLCε complex where the cell impermeant agonist iso cannot reach to, we utilized a membrane permeant β1AR agonist, dobutamine (Dob)(81). To access the activity of PLCε, we monitor its downstream Golgi PI4P hydrolysis by utilizing a Golgi PI4P biosensor, FAPP-PH-GFP. NRVMs were transduced with an adenovirus expressing FAPP-PH-GFP and Golgi associated FAPP-PH-GFP fluorescence was measured over time. In contrast to Iso, Dob (100nM) stimulated rapid and sustained PI4P hydrolysis suggesting intracellular βARs could be involved in mediating the activation of PLCε.

To confirm that dobutamine stimulated Golgi PI4P hydrolysis is not unique to neonatal myocytes, we tested the ability of Dob to stimulate PI4P hydrolysis in acutely isolated murine adult ventricular myocytes (AVMs). Infection of AVMs for 24 hours with FAPP-PH-GFP labels the Golgi surrounding the nucleus as we have previously reported. Stimulation of AVMs with Dob induces PI4P depletion in the Golgi as was seen in NRVMs. To further solidify it, we preincubated with either metoprolol, a membrane permeant βAR antagonist, or sotalol, a membrane impermeant βAR antagonist in NRVMs. Addition of metoprolol but not sotalol, blocked Dob-stimulated Golgi PI4P hydrolysis. This indicates the stimulation of βARs at intracellular compartments rather than those at plasma membrane is required for Dob-mediated Golgi PI4P hydrolysis.

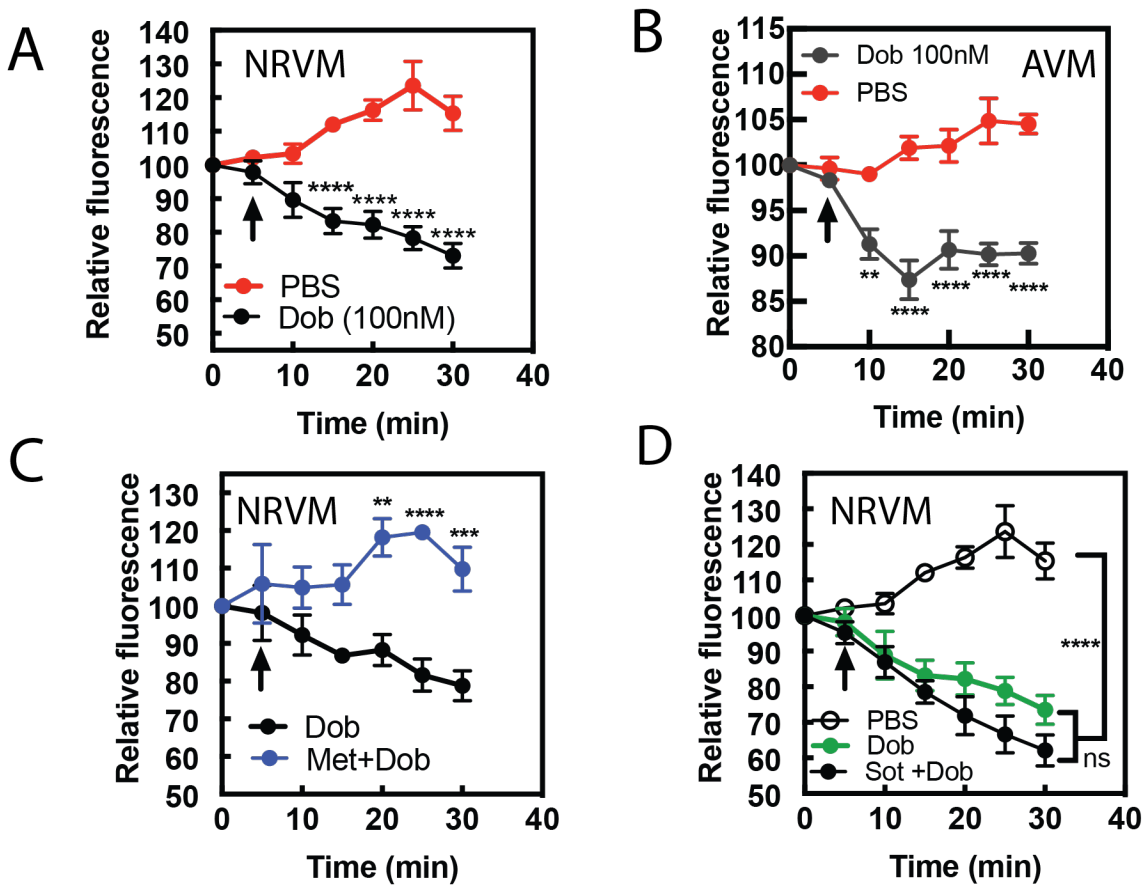
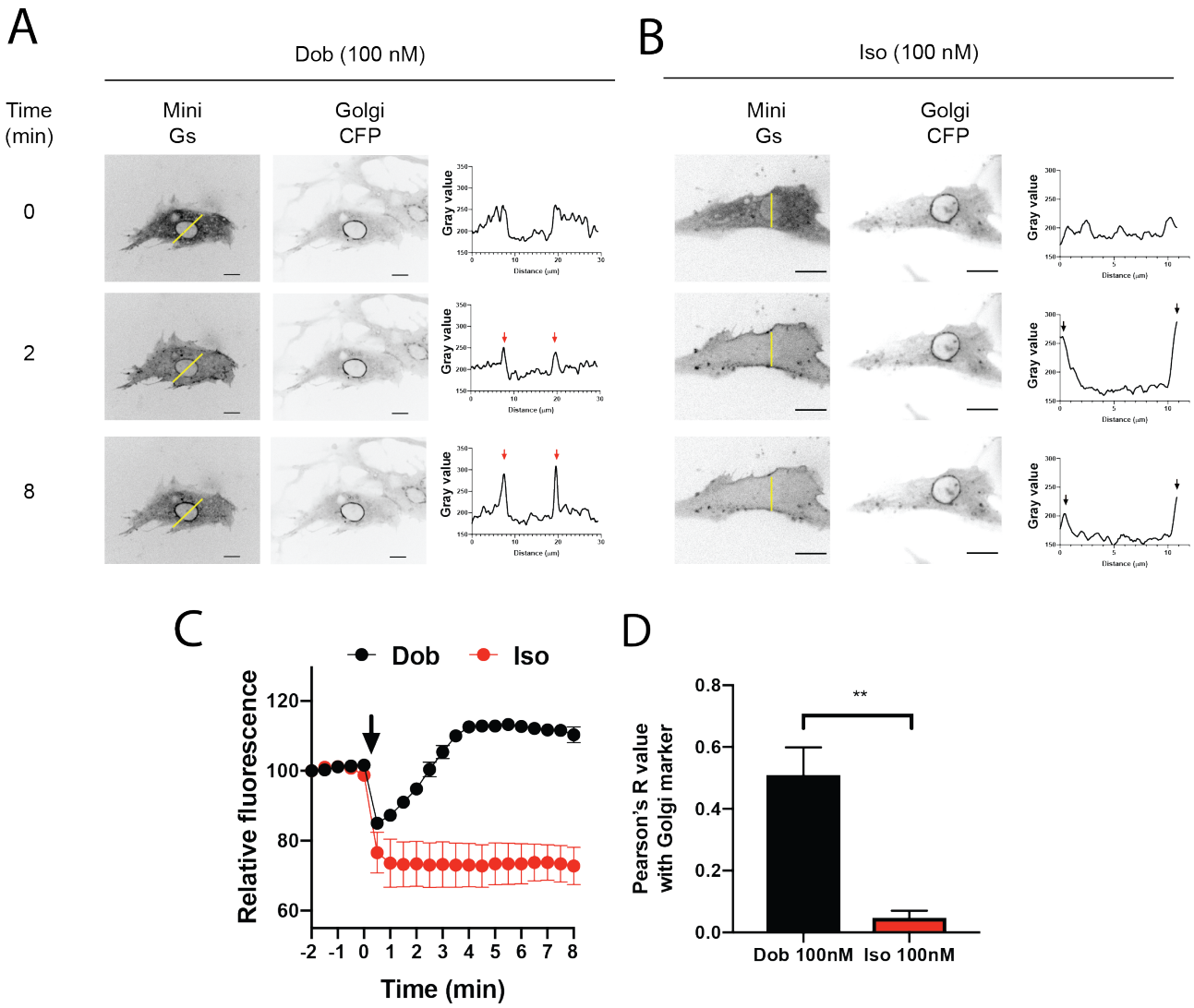


Figure 2-1 Dobutamine induces PI4P hydrolysis through the activation of internal βARs.

(A)NRVMs and (B) AVMs were transduced with FAPP-PH-GFP and stimulated as indicated. Cells were stimulated with dobutamine (100nM) at the arrow and remaining fluorescence intensity at the Golgi apparatus was monitored over time. (C and D) NRVMs were stimulated with either dobutamine alone or in the presence of metoprolol (100μM, C) or sotalol (5mM, D). Data are from at least 4 cells for each N. Data on time course graph are presented as mean +/- standard error from at least N=4 independent preparations of myocytes. Agonist was added by the arrow.

2.4.2 Dobutamine activates β1ARs at the Golgi apparatus.

To visualize β1AR activation at the Golgi apparatus, we utilized a translocatable Venus-based sensor of Gs coupled receptor activation, NES-Venus-miniGs(89). Upon the activation of a Gs coupled receptor, NES-Venus-minGs binds to the activated receptor, which is then visualized as the translocation from the cytoplasm to membranes where the activated receptor is located, providing information on receptor activation at subcellular compartments. We could not detect translocations of NES-Venus-miniGs to the endogenous receptors, so, NRVMs were co-transfected with β1ARs and NES-Venus-miniGs and locations of miniGs were monitored. In unstimulated cells, miniGs were distributed throughout the cytoplasm. The addition of Dob caused a rapid clearance of cytoplasmic fluorescence and followed by accumulated fluorescence at PM and punctate structures, possibly corresponding to microtubules. This was followed by relatively slower translocation of miniGs to the perinuclear region corresponding to a Golgi marker, CFP-Giantin. Iso also caused a rapid translocation of miniGs to the PM, but in contrast to Dob, we didn't observe fluorescence accumulation at intracellular compartments. To further confirm dob-mediated translocations of miniGs to Golgi, cells were treated with Dob for 8 min to cause miniGs translocations to the perinuclear region and followed by treatment with Brefeldin A to disassemble Golgi apparatus. Brefeldin A treatment significantly disrupted miniGs association with the perinuclear region, confirming Golgi location of miniGs after addition of Dob. Together, these data suggest that Golgi-resident βARs can be activated by exogenous membrane permeant agonists and stimulate Gs, leading to PLCε dependent PI4P hydrolysis.


A
Dob (100 nM)
Time (min)
Mini Gs
Golgi CFP
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Iso (100 nM)
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Golgi CFP
C
Dob
Iso
Relative fluorescence
Time (min)
D
Pearson's R value with Golgi marker
**
Dob 100nM
Iso 100nM
E
Time (min)
BreA added
0
8
30
Mini Gs YFP
Golgi CFP

Figure 2-2 Dobutamine but not isoproterenol mediates β1AR activation at the Golgi apparatus.
(A) NRVMs were co-transfected with β1-ARs and NES-Venus-miniGs and followed by adenoviral transduction with CFP-Giantin. Representative confocal fluorescence images of NES-Venus-miniGs recruitment by dobutamine (A) and isoproterenol (B), (100nM, left), CFP-Giantin (center) and histogrtam of representative NES-Venus-mini-Gs recruitment (right). Red arrow=perinuclear region, black arrow=sarcolemma. The yellow line indicates where the histogram data was captured. Scale bar=10 μm. (C) Mean data of fluorescence intensity of NES-Venus-mini-Gs at the perinuclear region corresponding to the Golgi +/- SEM from at least 5 cells. (D) Pearson's correlation coefficient for overlap of YFP-Mini-Gs and CFP-Giantin images. (E) Representative images of dobutamine-mediated NES-Venus-mini-Gs recruitment (100 nM, Upper panels) and CFP-Giantin Golgi marker (lower panels). Dobutamine was added at 2 min and Brefeldin A (5μg/ml), was added at 8 min just after the 8 min image above was captured. NES-Venus-mini-Gs recruitment continually monitored. Scale bar, 10 μm. All symbols on time course graphs are presented as mean ± standard error from N=4 or more independent preparations of myocytes.

2.4.3 Dobutamine induced Golgi PI4P hydrolysis requires Epac.

Previous data from our laboratory showed that the Epac/mAKAP/PLCε complex is responsible for cAMP mediated PI4P hydrolysis at Golgi in NRVMs(38, 43). To determine if Dob stimulated Golgi PI4P hydrolysis is dependent on Epac, we incubated cells with the Epac-selective inhibitor, HJC0726(153). HJC0726 inhibited Golgi PI4P hydrolysis by Dob however, the Gβγ inhibitor Gallein had no effect suggesting Dob mediated Golgi PLCε activation relies on cAMP/Epac rather than Gβγ.

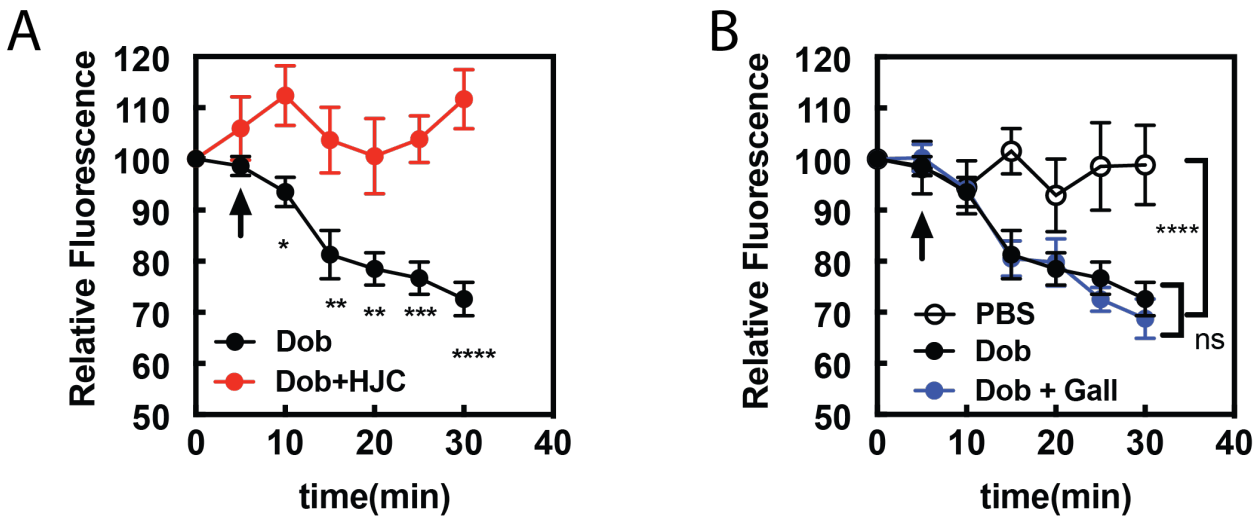


Figure 2-3 Dobtamine-mediated PI4P hydrolysis is dependent on EPAC.
(A) NRVMs were transduced with FAPP-PH-GFP and Golgi associated fluorescence was monitored with stimulation over time. The Epac inhibitor HJC0726 at 1μM was added to NRVMs 20 min before imaging and dobμmtamine (100nM) was added at the arrow. (B) The Gβγ inhibitor, galleon (10μM) was added 20 min before dobutamine addition (indicated by the arrow). Images were taken from at least 4 cells for each separation of NRVMs (N=3).

2.4.4 Norepinephrine (NE) induces β1AR activation at intracellular compartments and PI4P hydrolysis in cardiac myocytes.

Thus far we have shown that the synthetic β1AR selective agonist, Dob, can enter cells through passive diffusion, activate intracellular βARs and stimulate PLCε activation at nuclear envelope which has pharmacological implications but may not be relevant to physiological cardiac regulation. For this reason, we determined if the endogenous sympathetic neurotransmitter norepinephrine (NE) could stimulate Golgi β1AR activation and PLCε-dependent PI4P hydrolysis. To detect if NE could enter cells and activate β1ARs at the Golgi, we monitored NES-Venus-miniGs translocations in response to NE. NRVMs co-transfected with β1ARs and NES-Venus-miniGs were stimulated with NE at 37C. NE initially caused NES-Venus-miniGs recruitment to the PM as was observed for Dob, followed by a slower accumulation of NES-Venus-miniGs to the Golgi. Then we sought to determine if receptor internalization is involved for NE-mediated Golgi β1AR activation, an inhibitor of dynamin-mediated receptor internalization, Dyngo, had no effect on the recruitment of NES-Venus-miniGs to the Golgi apparatus by NE. To further determine the effects of NE on PLCε activation, cells were transduced with adenovirus containing FAPP-PH-GFP and were stimulated with NE at 37C. NE caused delayed but robust PI4P hydrolysis and dyngo has no effect suggesting NE mediated Golgi β1ARs and PLCε activation is independent of receptor internalization.

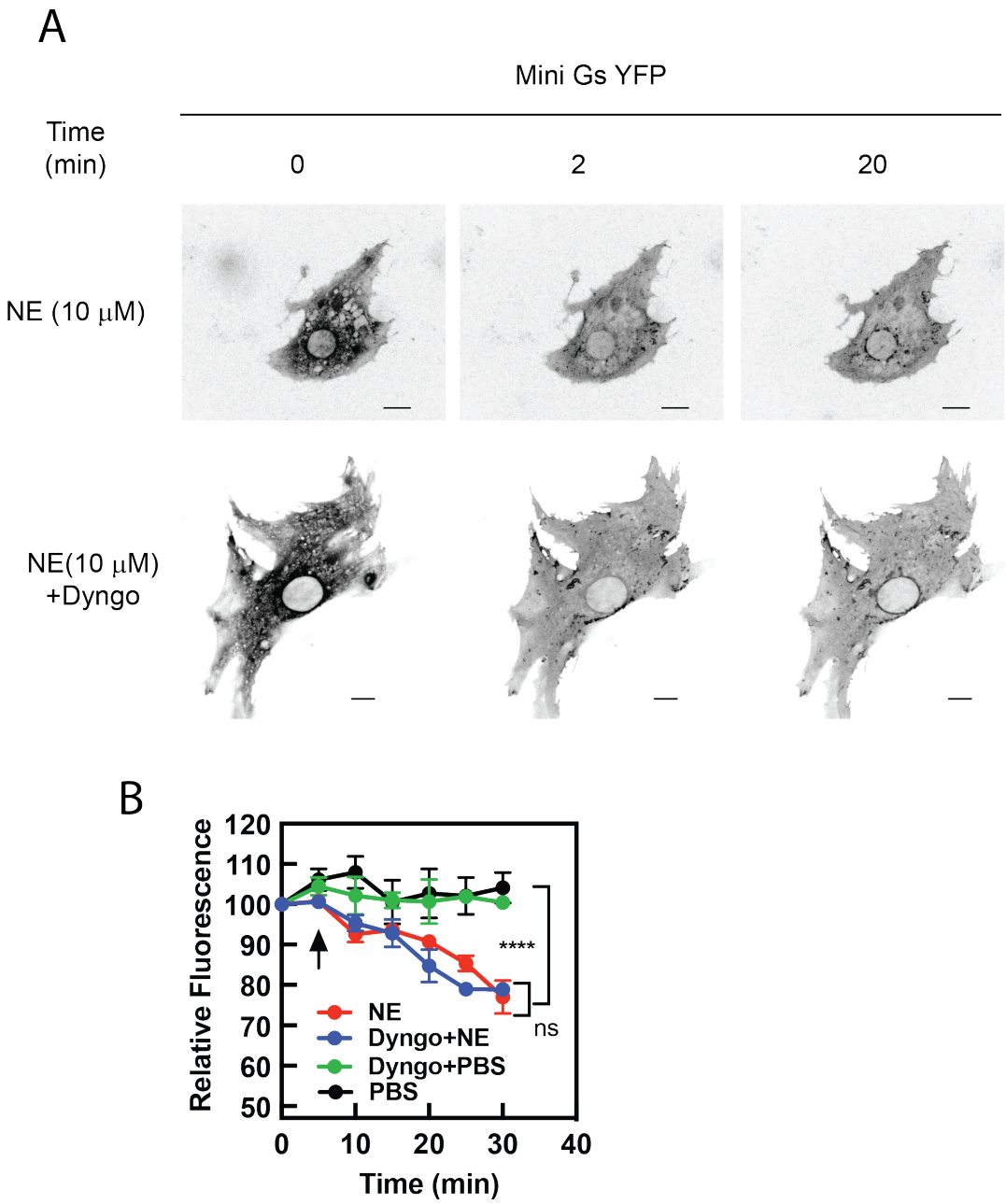


Figure 2-4 The physiological neurotransmitter, norepinephrine, induces internal β1AR activation and PI4P hydrolysis independent from receptor internalization.
(A) NRVMs were co-transfected with β1ARs and NES-Venus-miniGs and followed by adenoviral transduction with CFP-Giantin. Representative images of NES-Venus-miniGs recruitment mediated by norepinephrine (10μM, Upper panel) alone or in the presence of a dynamin inhibitor, Dyngo (40μM, bottom panel). (B) NRVMs were transduced with FAPP-PH-GFP and stimulated with norepinephrine (10μM) either alone or in the presence of Dyngo (40μM). Symbols on time course graphs are presented as mean ± standard error from N=4 or more independent preparations of myocytes

2.4.5 Inhibition of the membrane cation transporter, OCT, prevents norepinephrine induced PI4P hydrolysis.

Studies have demonstrated that norepinephrine or epinephrine mediated activation of internal receptors requires organic cation transporter family proteins (OCT proteins) in adult cardiac myocytes and HELA cells. The non-selective cation transporter, OCT3 has been shown to be responsible for the transport of NE into cells(96), therefore we utilized the OCT3 inhibitor, abacavir, to determine if OCT3 is required for NE-stimulated intracellular PLCε activation and PI4P hydrolysis in adult ventricular myocytes (AVMs). Stimulation with NE induced PI4P depletion at Golgi, which was blocked by preincubation with the OCT inhibitor, abacavir indicating NE transport into AVMs is required for the activation of nuclear envelope PLCε activation.

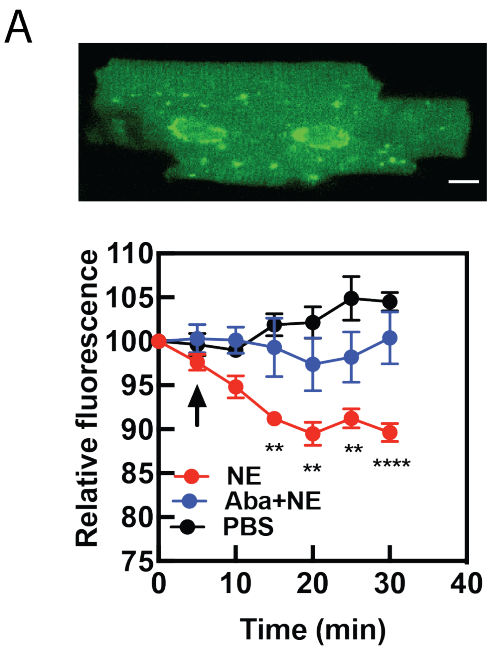


Figure 2-5. Norepinephrine requires OCT transporters to access internal receptors and stimulate PI4P hydrolysis. Representative images of AVMs transduced with FAPP-PH-GFP (upper panel) and transduced AVMs were stimulated with norepinephrine (10μM) either alone or in the presence OCT inhibitor Abacavir (10μM, bottom panel). Data were analyzed as means from three separated preparations of AVMs.

2.4.6 Cardiac hypertrophy induced by dobutamine is inhibited by a membrane permeant antagonist.

To determine if internal βARs are involved in driving cardiac hypertrophy, NRVMs were stimulated with Dob for 48 hours either alone or in the presence of the cell permeant antagonist, metoprolol, or the cell impermeant antagonist, sotalol. Two measurements of hypertrophy were assessed, a direct measurement of cell area and the hypertrophic marker, atrial natriuretic factor (ANF) expression. Dob stimulated a significantly increase in cell area and ANF expression which was strongly blocked by co-incubation with metoprolol but not sotalol. These data, taken together with the PI4P hydrolysis data, suggest that internal βARs are involved in mediating cardiomyocyte hypertrophy.

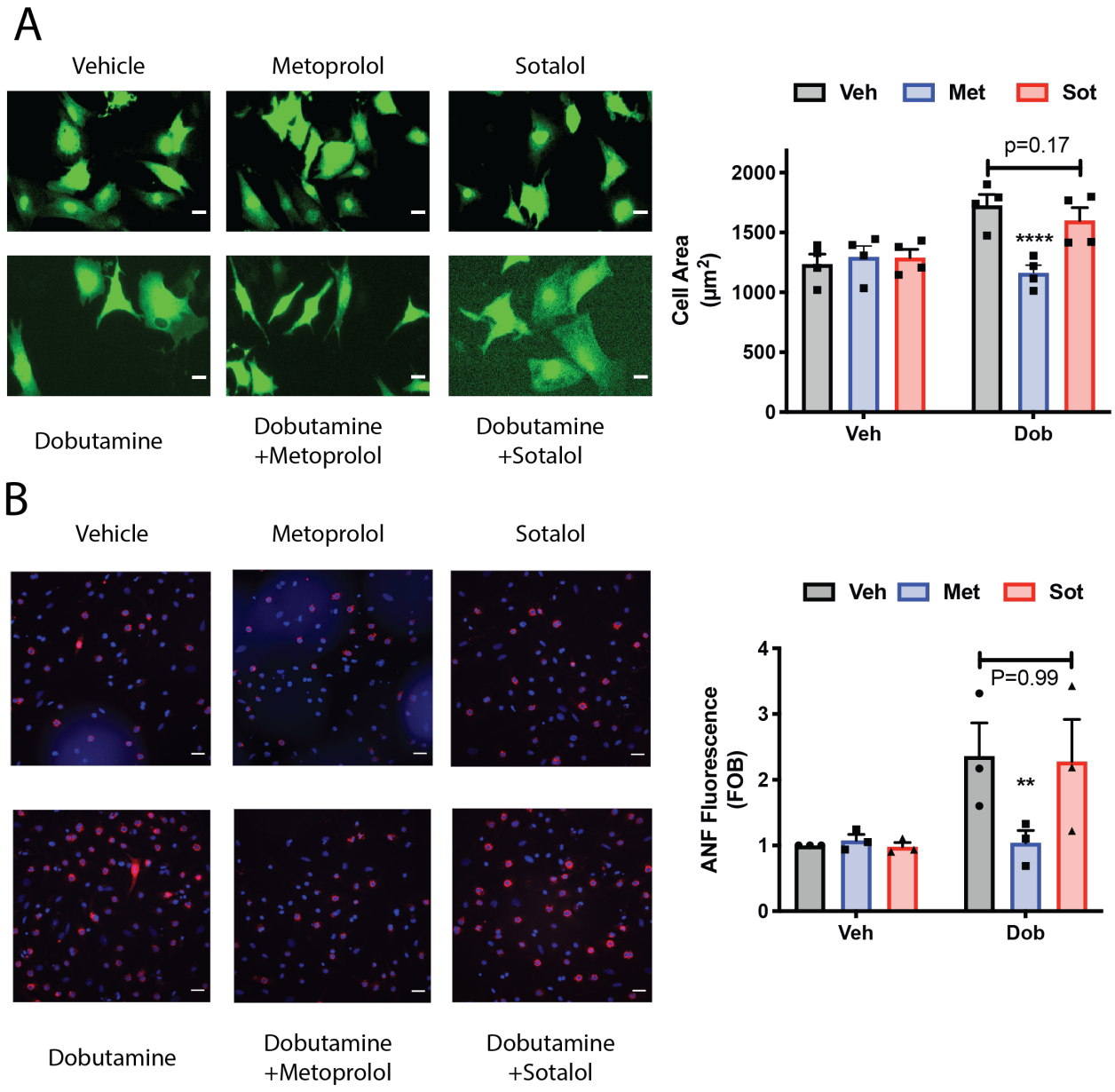


Figure 2-6 Dobutamine induced cardiomyocyte hypertrophy requires intracellular βARs.
(A) Dobutamine induces internal-receptor dependent increases in cell area. NRVMs were transduced with YFP virus prior to stimulation for 48 hours with dobutamine in the presence of the indicated antagonists or vehicle control. Following fixation, cell area was measured using image J. Representative images are on the left with mean data (fold over basal, FOB) on the right (N=4 separate preparations of NRVMs), analyzed with a paired 2 way ANOVA. **** P<0.0001 vs. Dob and Dob+sotalol. Scale bar 10 μm. B) Dobutamine induces an increase in ANF expression via an internal receptor-dependent mechanism. NRVMs were stimulated with dobutamine in the presence or absence of the indicated antagonists or vehicle control for 48 hours before fixation and staining for ANF. Fluorescence of ANF rings was then captured by confocal microscopy, followed by fluorescence intensity analysis with Image J. Representative images are on the left with mean data (fold over basal, FOB) on the right (N=3 separate preparations of NRVMs), analyzed with a paired 2 way ANOVA. **P=0.007 vs. Dob and Dob+sotalol. The total number of cells analyzed was greater than 200 cells for each.

2.4.7 NE-stimulated NRVM hypertrophy requires membrane transport by OCT3 and Golgi resident βARs.

NE is an endogenously produced catecholamine involved in mediating hypertrophy and heart failure and stimulation of NRVMs with NE stimulates hypertrophy. To determine the role of intracellular NE in hypertrophy, NRVMs were treated with NE in the presence or absence of corticosterone to block Oct3 cation transporters for 48h. Treatment with NE stimulated an increase in cell size (Figure 2.7A) and ANF expression (Fig. 2.7B) that was blocked by corticosterone indicating that transport of NE into the myocyte is required to mediate hypertrophy. Treatment with dobutamine also induced hypertrophy but this was not blocked by Oct3 supporting the idea that dobutamine access to internal receptors does not require Oct3 and confirms the specificity of the Oct3 blockade.

To determine if NE and dobutamine driven NRVM hypertrophy requires Golgi βARs NRVMs were transduced with adenovirus containing CFP-Nb80-FRB and FKBP-mApple-GalT for 24 h followed by treatment for 48h with NE or dobutamine with and without co-treatment with rapamycin. In the absence of rapamycin, NE and dobutamine stimulated increases in cell area and ANF expression (Figure 2.7C and D). Co-treatment with rapamycin to translocate Nb80 to the Golgi blocked NE and dobutamine stimulated hypertrophy, demonstrating that Golgi βARs are required for stimulation of hypertrophy by these agonists.

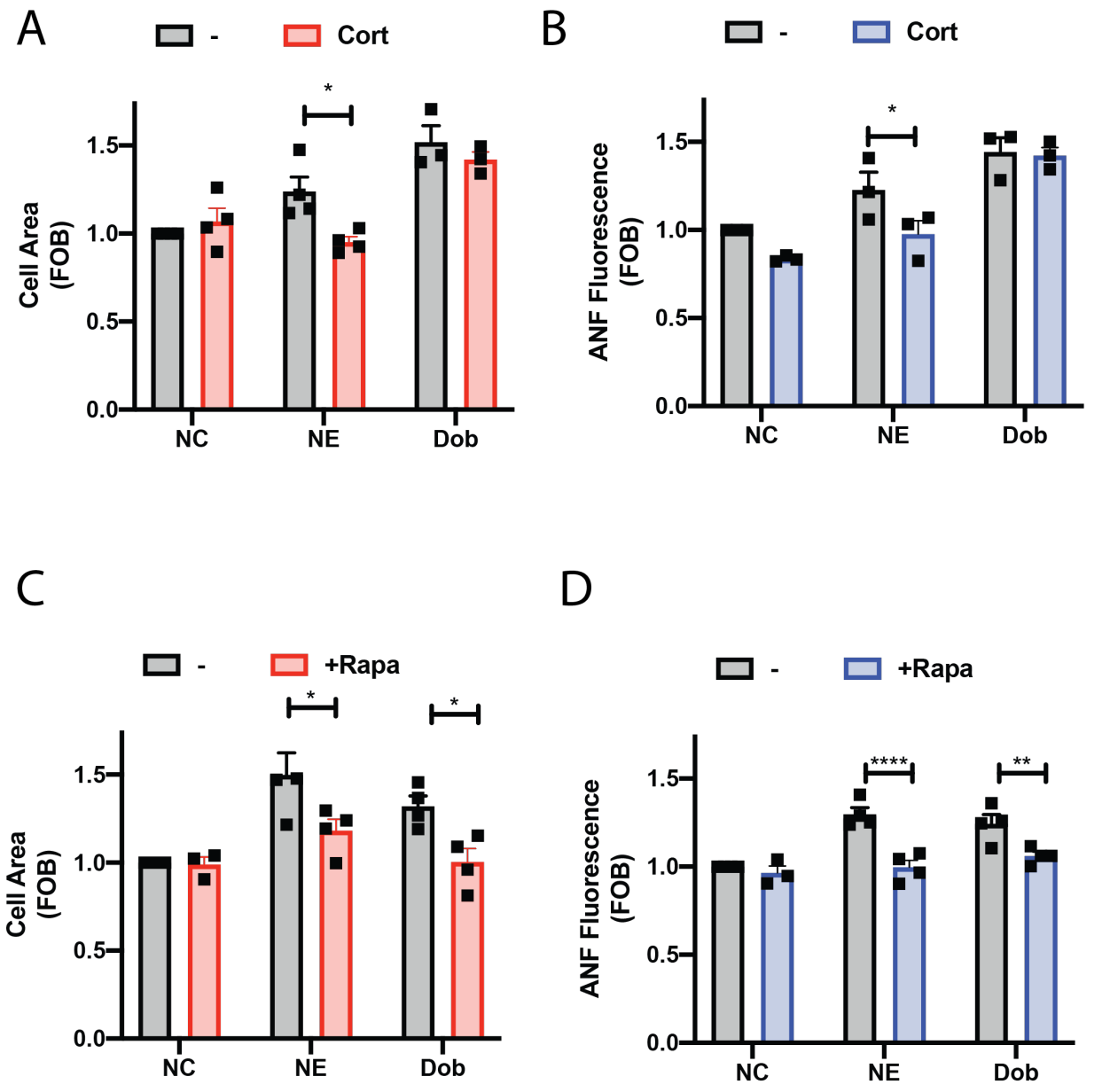


Figure 2-7 Dobutamine and norepinephrine induced cardiomyocyte hypertrophy requires Golgi-localized βARs. Norepinephrine induced hypertrophy also requires agonist internalization. Norepinephrine induces internal-receptor dependent increases in cell area (A) and ANF expression (B). NRVMs were stimulated for 48 hours with norepinephrine (10 μM) or dobutamine (100 nM) in the presence of Corticosterone (100μM) or vehicle control. Following fixation, cells were stained for ANF and using CellTracker Deep Red. Images were captured using a Thermo Fisher Cell Insight and analyzed by Cell Profiler. Both NE and Dobutamine significantly increase cell size (NE, $p<0.05$ and Dob $p<0.001$) and ANF expression (NE, $p<0.01$ and Dob $p<0.001$) in control conditions. Dobutamine and Norepinephrine require Golgi localized βAR to induce increase in cell area (A) and ANF expression (B). NRVMs were transduced with FRB-CFP-Nb80 and FKBP-mApple-GalT for 24 hours before stimulation. 15 minutes before agonist addition, Rapamycin or (1μM) or vehicle control was added. Cells were then stimulated with either dobutamine (100 nM) or norepinephrine (10 μM) for 48 hours. Following fixation, cells were stained for ANF and using CellTracker Deep Red. Images were captured using a Thermo Fisher Cell Insight and analyzed by Cell Profiler. Both NE and dobutamine significantly increase cell size (NE, $p<0.001$ and Dob $p<0.01$) and ANF expression (NE, $p<0.001$ and Dob $p<0.001$) in control conditions. All data is from at least 1200 cells from 3 separate preparations of NRVMs. Data were analyzed as means from N=3 experiments

2.5 Discussion

It is now clear that different subcellular cAMP pools exert distinct signaling outcomes. In our previous study, our laboratory found that elevated levels of cAMP with forskolin treatment in NRVMs induced Golgi PI4P hydrolysis, but not with Iso treatment. This led to the question that how the physiological cAMP pools, generated by Gs coupled receptors, regulate the activity of the Epac/mAKAP/PLCε complex. One mechanism could be that with chronic elevated agonist exposure, the organization of PDEs in the cardiac myocytes are altered, such that Epac/mAKAP/PLCε at nuclear envelope/Golgi interface could be accessed by global cAMP changes. Or that intracellular βARs, after being activated by physiological agonists, generate localized cAMP with privileged access to the Epac/mAKAP/PLCε complex. Here we demonstrate that activation of Golgi β1ARs induces the pro-hypertrophic Epac-PLCε-PI4P hydrolysis pathway upon stimulation with a membrane permeant agonist Dob, or the physiological neurotransmitter, NE. Also, the stimulation of internal βARs is required for the promotion of cardiomyocyte hypertrophy.

2.5.1 Compartmentalized GPCR signaling.

A body of emerging studies indicates that GPCRs can be found at multiple intracellular compartments(154). GPCRs including glutamate receptors, dopamine D1 receptors (D1R), β1ARs, endothelin receptors, and a1-ARs have been characterized on the nuclear envelope or the Golgi apparatus in neurons and cardiac myocytes and signaling mechanisms have been proposed(92, 155-157). Though, β1ARs have been found on other intracellular compartments including the Golgi apparatus (81)and sarcoplasmic reticulum(97), but the functional outcomes for β1ARs at the Golgi are not well defined. It is not fully clear that whether these GPCRs at Golgi are trafficked from endoplasmic reticulum (ER) where they are synthesized through the Golgi on their way to

the cell surface or are delivered to Golgi through endosomal retrograde trafficking of internalization from the PM. It is also not fully understood that GPCRs at the Golgi are fully functional or have a signaling output. Recent work by Calebiro laboratory demonstrated that the thyroid stimulating hormone (TSH) receptor activates Gs at the Golgi in mouse thyroid cells through a mechanism that relies on endosomal retrograde trafficking of internalized receptors(158). In contrast, Iranejad et al. showed that a pre-existing pool of β1ARs can be driven to an active conformation and activate Gs after addition of exogenous ligands and this process is independent from receptor internalization(81). Here we've showed that in cardiac myocytes, and as reported by Irannejad et al. in HELA cells, where activation of Golgi β1ARs does not involved in receptor internalization but rather relies on pre-existing Golgi resident receptors and transport of ligands across the cell membrane via OCT3.

We have previously demonstrated a signal transduction pathway that is critical in the regulation of cardiac hypertrophy. This pathway involves a nuclear envelope scaffolded complex of Epac/PLCε and mAKAPβ(38, 40, 43, 129). cAMP dependent activation of Epac acts as an exchange factor for the small GTPase Rap which directly stimulates PLCε enzymatic activity toward Golgi PI4P, resulting in local production of DAG and subsequent nuclear PKD activation. Here we elucidate a possible physiological function for Golgi-resident β1-ARs by mediating this process (Fig. 2.7). Missing from this analysis is the demonstration of the presence of adenylyl cyclase (AC) at the Golgi. Due to the inadequacy of AC antibodies and the likely low levels of this enzyme, localization of AC by immunocytochemical analysis is not possible. Nevertheless, binding of AC5 to mAKAPβ in cardiac myocytes has been previously demonstrated indicating that AC is likely locally available to be activated by Golgi β1ARs and Gs(107).

How are Golgi-resident β1-ARs accessed by the endogenous neurotransmitters? The endogenous β1-AR ligands, including NE and Epi, are membrane impermeant. Therefore, the mechanism for the activation of β1AR at intracellular compartments under physiological or pathological conditions is needed. It has been shown that an organic cation transporter subtype, OCT3 is found in hearts, and it facilitates the uptake of catecholamines across the cell surface(159). Importantly, the transporter has also been found at Golgi for these physiologically relevant ligands to access Golgi localized receptors since the carboxyl domains of Golgi localized β1ARs face the cytosol. The known OCT3 inhibitors are corticosterone (released by adrenal gland), abacavir (a drug used to control HIV infections), lamotrigine (a drug used to treat epilepsy) and a few marketed tyrosine kinase inhibitors. Their implications on regulating intracellular βAR activities should also be considered.

Precise and localized signaling is required for cardiomyocytes to perform properly to avoid a global unrestricted response. For example, during acute sympathetic stimulation, increased circulating catecholamines may only access cell surface βARs to phosphorylate PKA and its downstream calcium handling including phospholamban and L-type calcium channels which, in turn, increase cardiac contractility to meet the demands of a sympathetic response. Activation of Golgi-β1ARs requires ligand uptake, which process is slower, and needs to be chronically exposed to ligands under cardiovascular stress conditions and may not be accessed during an acute sympathetic response. Sequestration of β1ARs-Epac-PLCε-PI4P-PKD signaling pathway at the nuclear envelop/Golgi interface from cAMP-PKA activation at the cell surface can prevent inappropriate pathological hypertrophic responses to acute bAR activation.

2.5.2 Design ß-blocker therapies.

As we've discussed, sustained elevated sympathetic activation is one of the major factors that drives the development of pathological cardiac hypertrophy. β-blocker therapy is the first line treatment for patients with heart failure. The mechanisms involved in restoring cell surface βARs after chronic stimulation and desensitization by catecholamines. Also, other mechanisms such as lowering blood pressure, anti-apoptosis of cardiomyocytes, reducing myocardial oxygen consumption, and restoring the ratios of β1ARs and β2ARs have been proposed. However, not all bAR antagonists are clinically effective. In addition to their difference in receptor selectivity, their hydrophobicities are different. To be more specific, clinically effective β-blockers, metoprolol and carvedilol have log P values 1.8 and 4.2 and are thus relatively membrane permeant. Sotalol, on the other hand, has a log P value of -0.85 and is thus membrane impermeant, and is not clinically used for heart failure treatment. This is in consistent with our in vitro hypertrophy data that metoprolol that block internal βARs prevent cardiomyocyte hypertrophy but sotalol which blocks cell surface βARs is not sufficient to prevent hypertrophy. Thus, we speculate that hydrophobicity is an important factor for the effectiveness of β-blockers, allowing these inhibitors to access internal pools of receptor. In the future, a caged β-blocker could also be designed to effectively block the hypertrophic signaling at the Golgi apparatus without affecting the cell surface that potentially involved in contractility.

As we have shown in our in vitro studies, Iso was poorly membrane permeant and does not activate intracellular β1ARs. However, there is some evidence showing Iso is also a substrate for OCT3 and it is possible Iso could be transported into cells through OCT3 and potentially engage internal β1ARs at a high concentration. Also, Iso can possibly internalize together with receptors to reach the receptors at other compartments. This could partially account for its ability to induce cardiac hypertrophy in vivo.

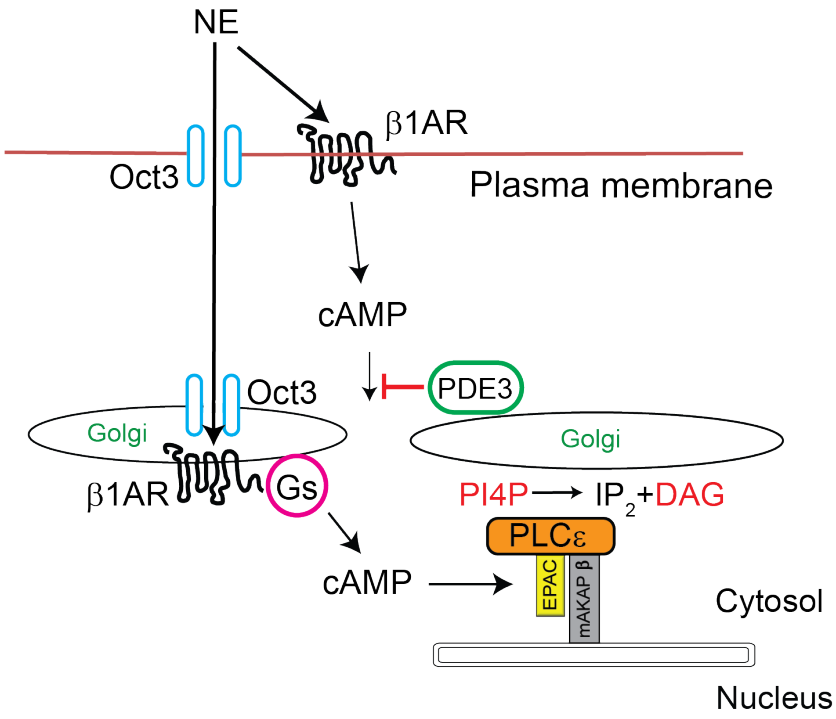


Figure 2-8 Signal transduction by cell surface and Golgi β1ARs.
β1ARs are located on both the plasma membrane and the Golgi apparatus in cardiac myocytes. Stimulation of cell surface β1ARs leads to production of cytosolic cAMP but this cAMP cannot access the Epac/PLCε/mAKAPβ due to PDE3 dependent hydrolysis of cAMP. To access Golgi β1AR, NE crosses the plasma membrane via the Oct3 transporter. We speculate that the Oct3 transporter is also present in the Golgi allowing access to the Golgi lumen and there is evidence for the presence of Oct3 on intracellular membranes as discussed in the text. Once activated in the Golgi, β1AR stimulates Gs and subsequent production of cAMP locally. Missing from this diagram is adenylyl cyclase which we presume is in the Golgi membrane. There is evidence for adenylyl cyclase binding to mAKAPβ in cardiac myocytes. Local cAMP has privileged access to the Epac/PLCε/mAKAPβ complex which leads to PLCε-dependent production of local DAG from PI4P.

2.6 Contributions

Craig A. Nash provided the data for Figure 2.7. Alan V Smrcka provided Figure 2.8 and contributed to conceptualization, editing and preparation of the figures and text.

Chapter 3 The Opposing Effects of β1 and β2-Adrenergic Receptors on Phospholipase C-mediated Cardiac Hypertrophic Signaling

3.1 Abstract

Chronically elevated neurohumoral drive, and particularly elevated adrenergic tone leading to β-adrenergic receptor (β-AR) overstimulation in cardiac myocytes, is a key mechanism involved in the progression of heart failure. β1-AR and β2-ARs are the two major subtypes of β-ARs present in the human heart, however, they elicit different or even opposite effects on cardiac function and hypertrophy. For example, chronic activation of β1ARs drives detrimental cardiac remodeling while β2AR signaling is protective. The underlying molecular mechanisms for cardiac protection through β2ARs remain unclear. Here we show that β2-AR protects against hypertrophy through inhibition of hypertrophic PLCε signaling at the Golgi apparatus. The mechanism for β2AR-mediated PLC inhibition requires internalization of β2AR, activation of Gi and Gβγ subunit signaling at endosomes and ERK activation. This pathway inhibits both angiotensin II and Golgi-β1-AR-mediated stimulation of phosphoinositide hydrolysis at the Golgi apparatus ultimately resulting in decreased PKD and HDAC5 phosphorylation and protection against cardiac hypertrophy. This reveals a novel potential mechanism for β2-AR antagonism of the PLCε pathway that may contribute to the known protective effects of β2-AR signaling on the development of heart failure.

3.2 Introduction

Catecholamines of the sympathetic nervous system (SNS) regulate heart rate, contractility and vascular resistance through activation of adrenergic receptors. Prolonged elevation of circulating catecholamines, including epinephrine and norepinephrine, in response to cardiac injury, vascular disease, or stress is tied closely to the pathogenesis of heart failure (139). These hormones act in part through β-adrenergic receptors (βARs) on cardiac myocytes, where chronic activation drives maladaptive cardiac hypertrophy, apoptosis, and fibrosis, ultimately resulting in heart failure (138). βARs are G protein-coupled receptors (GPCRs) and consist of three subtypes, β1, β2 and β3. β1-adrenergic receptors (β1ARs) comprise of 80% of βARs in the healthy human hearts with the β2ARs comprising the remaining 20% (138). Although β1ARs and β2ARs respond to the same physiologic agents, share high sequence homology (59), and core signaling pathways (both couple to Gαs and stimulate cAMP production), they have distinct or even opposite physiological and pathological roles in the heart (160). Specifically, chronic pathological stimulation by endogenous catecholamines leads to decreased β1AR levels and function in the heart, and mild overexpression of β1ARs results in significant cardiac hypertrophy and heart failure in mice (68, 69). In contrast, the β2AR population remains unchanged during heart failure and moderate levels of β2AR overexpression leads to positive inotropic effects (62-65). Interestingly, β2AR deletion in mice leads to exaggerated cardiac hypertrophy in a pressure overload-induced model, and β1AR deletion mice had comparable responses to wild type, however, in β1AR/β2AR dual knockout mice, cardiac hypertrophy was abolished (72, 73). These observations suggest that coordinated signaling events downstream of these receptors are critical in mediating cardiac hypertrophy and heart failure.

Phospholipase C (PLC) enzymes have important roles in the heart (36-38, 129, 141, 161). PLCs mediate hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP2) downstream of GPCR and receptor tyrosine kinase (RTKs) activation leading to production of inositol triphosphate (IP3) and diacylglycerol (DAG) (31, 32). Classically, PLCβ isoforms of the PLC family are stimulated downstream of GPCRs by Gq and Gβγ subunits from Gi (14). More recently, PLCε has also been shown to be downstream of multiple GPCR families and RTKs due to its ability to be directly activated by a diverse array of upstream regulators including multiple members of the family of small GTPases (Ras, Rho and Rap) and Gβγ subunits, (143, 144, 162, 163).

A pathway that has been extensively studied in cardiac myocytes in our laboratory is regulation of PLCε by Rap after cAMP-dependent stimulation of the Rap GEF, Exchange Protein Activated by cAMP (EPAC) (36-38, 42). We previously identified an essential role of PLCεin regulating cardiac hypertrophy in an animal model of pressure-overload induced cardiac hypertrophy (38). In cardiac myocytes, PLCε scaffolds to muscle-specific A kinase anchoring protein (mAKAP) along with PKA, EPAC, PKD and other hypertrophic regulatory proteins at nuclear envelope (40, 164). Activation of PLCε at this location induces the hydrolysis of phosphatidylinositol-4-phosphate (PI4P) in the closely associated Golgi apparatus, generating inert inositol bisphosphate (IP2) and local DAG to drive the activation of PKD at nucleus and phosphorylation of HDAC leading to the hypertrophic gene expression (38).

While exploring mechanisms for cAMP-dependent stimulation of the Golgi Epac/PLCε pathway we found that stimulation of cell surface β1ARs with Iso was ineffective in regulating this pathway in cardiac myocytes (43). Rather, adrenergic stimulation of the Golgi Epac/PLCε pathway required a pre-existing pool of Golgi localized β1-ARs (42) which stimulate production of a pool of cAMP with privileged access to the Epac/PLCε module at the Golgi/NE interface.

Access of Epi and NE to the intracellular β1-ARs requires an organic cation transporter subtype, OCT3 (96, 159) and blockade of OCT3 attenuated catecholamine-stimulated cardiomyocyte hypertrophy (42) and contractile responses (97). These studies clearly demonstrate a physiologically relevant roles of intracellular β1-ARs, however, how plasma membrane/sarcolemma β-AR signaling coordinates with intracellular β1-AR signaling remains poorly explored.

It is well established that intracellular cAMP pools generated at different intracellular compartments downstream of Gs coupled receptors lead to distinct phenotypic outcomes. In cardiac myocytes, β1-ARs but not β2-AR localize on the periphery of the nuclear envelope (92, 93) or Golgi (42, 81, 97) in addition to cell surface and T-tubule locations of both β1 and β2ARs. Activation or β2-ARs results in internalization into endosomes where they continue to signal, initiating a set of signaling and transcriptional control events distinct from those at the plasma membrane (90). Internalization of activated β2-ARs in cardiac myocytes has been documented (79, 82, 165) but functional roles for these internalized receptors have not been thoroughly examined in cardiomyocytes.

In this study, we demonstrate that activation of plasma membrane β2-ARs inhibits stimulation of the hypertrophic EPAC/PLCε pathway at the Golgi downstream of Golgi-β1-ARs and plasma membrane angiotensin II receptors in cardiac myocytes. We describe a mechanism where activated β2-ARs internalize from the plasma membrane, activate Gi releasing Gβγ subunits leading to ERK activation and inhibition of PI hydrolysis at Golgi apparatus. This prevents PLCε-mediated downstream hypertrophic signaling including nuclear PKD activation and HDAC phosphorylation. These data reveal a new mechanism that could underly the anti-hypertrophic versus hypertrophic signaling balance between β1 and β2-ARs and give insights into novel

strategies for treatment of heart failure by deliberate inhibition of internal β1-ARs and combined with selective β2-AR activation for heart failure therapy.

3.3 Materials and Methods

3.3.1 Materials

Antibodies: mouse anti-a-actinin (Sigma, Cat# A7811), rabbit anti-ANF (Millipore, Cat# AB-5490), mouse anti-GM130 (BD Transduction Laboratories, Cat# 610822), mouse M1 anti-Flag (Millipore Sigma F3040) labelled with Alexa Fluor 488 using Thermofisher labeling kit A20181 (gift from Dr. Manojkumar Puthenveedu), rabbit anti-p-PKD (Cell Signaling Technology, Cat# 2051), rabbit anti-t-PKD (Cell Signaling Technology, Cat#2054), rabbit anti-p-ERK (Cell Signaling Technology, Cat# 4370S), mouse anti-t-ERK (Cell Signaling Technology, Cat# 4696S), rabbit anti-p-HDAC (Cell Signaling Technology Cat# 3433), mouse anti-t-HDAC (Santa Cruz Biotechnology Cat# sc-133225), mouse anti-GAPDH (Invitrogen, Cat# MA5-15738), goat anti-rabbit DyLight 800 (Invitrogen, Cat# SA535571), goat anti-mouse IRDye 680RD (LICOR, Cat# 926-68070), goat anti-mouse 568 secondary antibody (Invitrogen, Cat# A-11031), goat anti-rabbit 568 secondary antibody (Invitrogen, Cat# A-11011), goat anti mouse 488 secondary antibody (Invitrogen, Cat# A-11029).

Compounds: Cytosine Arabinoside (AraC, Sigma); Collagenase Type 2 (Worthington, Cat# LS004176); Gelatin (Sigma), Gallein (Sigma, 371708), Isoproterenol (Sigma, 1351005), Dobutamine (Tocris, Cat# 0515); ICI118551 (Tocris, Cat#0821), butanedione-monoxime (BDM) (Sigma, Cat# 112135), Dyngo (Abcam, Cat# Ab120689), pertussis toxin (Sigma, Cat# P7208-50UG), gefitinib (LC Laboratories, Cat# G-4408), myristoylated PKA inhibitor (Sigma, Cat# 476485), salmeterol xinafoate (Tocris, Cat# 47-1210), angiotensin II (Sigma, Cat# A9525-50mg), PD0325901 (Sigma, PZ0162-5mg), cptome-AM (Fisher scientific, Cat# 4853100U)

Plasmids and adenoviral constructs: FAPP-GFP-PH ((38)), GFP-Sid4M (Addgene plasmid #51472), fyve mApple GRK2ct (created by fusing 2XFYVE domains(166), mApple, and GRK2CT (GRK2 (495-689)), made by twist bioscience), eNos-mApple-Nb80 (created by fusing the first 33 amino acids of enos(167), mApple and Nanobody 80, made by twist bioscience). nDKAR (38, 168)

3.3.2 Methods

Isolation and transduction of neonatal rat ventricular myocytes (NRVMs) and mouse adult cardiac myocytes (ACMs)

Neonatal rat ventricular myocytes were isolated from 2 to 4 day-old Sprague-Dawley rats as described previously(40). Briefly, ventricles were separated from the hearts and minced well before adding digestion buffer (Collagenase type II in Hanks buffered saline solution without calcium). Following digestion, supernatant was collected, and cells were centrifuged at 1200 rpm for 2 min. Fibroblasts were removed by pre-plating cells onto tissue culture plastic for one hour at 37C. Purified NRVMs in the supernatant were transferred onto gelatin-coated glass-bottom dishes or 6 well plates. NRVMs were cultured in DMEM supplemented with 10% FBS, 100U/mL penicillin, 100 ug/mL streptomycin, 2 mg/mL vitamin B12, and 10 mM cytosine arabinoside. 24 hours later, media was changed to 1%FBS or serum free media. For adenovirus transduction, 50 MOI of adenovirus was incubated overnight.

Isolation of adult ventricular myocytes (from 2-5 month-old wild type C57BL/6J mice) was performed following the protocol from Auerbach et al (150). Mice were anesthetized with ketamine (100mg/kg body weight) and xylazine (5mg/kg body weight). Hearts were then removed, cannulated, and perfused with perfusion buffer (10 mM HEPES, 0.6 mM Na2PO4, 113 mM NaCl, 4.7 mM KCl, 12 mM NaCO3, 0.6 mM KH2PO4, 1.2 mM MgSO4, 10 mM KHCO3, 30 mM

Taurine, 10 mM BDM, 5.5 mM Glucose) via the aorta. Subsequently, digestion buffer (collagenase type II (773.48 U/ml), trypsin (0.14 mg/ml), and calcium chloride (12.5μM) in perfusion buffer) was perfused via aorta. Following digestion, hearts were minced in stopping buffer (10% FBS and 12.5 μM calcium chloride in perfusion buffer), debris was allowed to settle, the supernatant was collected, and calcium was added back to a final 1 mM concentration. Cells were then centrifuged at 18g for 3min before plating onto laminin-coated 20mm glass bottom dishes for confocal microscopy imaging. AVMs were maintained in minimum essential medium (MEM) supplemented with 0.35 g/L sodium bicarbonate, 100 U/mL penicillin, 100 U/mL streptomycin, and 10mM BDM. To transduce AVMs with adenovirus, 100 MOI of indicated adenoviruses were added to cells in BDM-free media for 4 hours. Following this, the virus was removed, and the culture media was supplemented with BDM. After 18 hours, cells were imaged by confocal microscopy.

Imaging

NRVMs or AVMs were imaged at 37C and 5% CO2 in a stage top incubator 18-24 hours after transduction with indicated adenoviruses in serum free culture media. Live cell imaging was performed on a Leica DMi8 microscope in spinning disc confocal mode (Crest Optics X-light V2) with 40X 1.4-NA (numerical aperture) oil immersion lens. CFP, GFP, and RFP were excited 440, 488 and 555 nm respectively with an 89-North LDI light source and emission was monitored and imaged on a backlit CMOS Photometrics Prime 95B camera.

Measurement of PI4P hydrolysis was previously described (38, 42, 43, 129). NRVMs or AVMs were transduced with adenovirus expressing FAPP-PH-GFP. Transduced cells were identified and imaged on a LEICA DMi8 with 40X oil lens in confocal mode. Timelapse videos

were acquired with 10ms exposure times at 5 min time intervals to minimize phototoxicity and photobleaching. Fluorescence intensity analysis was performed by subtracting background cytosolic fluorescence from the fluorescence intensity corresponding to Golgi using Image J to circle the regions of interest corresponding to Golgi surrounding the nucleus in both AVMs and NRVMs. Agonists were added after 5 min baseline imaging. To measure Golgi GFP-associated fluorescence, regions of Golgi associated fluorescence were circled as regions of interest and quantified with image J software. Fluorescence intensity quantified in each frame of the time course were normalized to the initial fluorescence intensity prior to addition of agonists (F/F0) and shown as a percent. For PI4P measurement in FYVE-mApple-GRK2CT expressing AVMs, cells were excited with GFP and RFP to acquire images before and after 30mins of dobutamine addition with or without salmeterol preincubation. Data is presented as percentage of fluorescence remaining after dobutamine addition at 30min. GFP: excitation: 488nm, emission: 510 nm. RFP: excitation: 555 nm, emission: 583 nm.

Measurement of myocyte cell area and ANF induction.

NRVMs were plated onto gelatin-coated 6-well plates. Cells were then serum starved for 24hours before stimulated with dobutamine (100nM) for 48hours co-incubated with or without salmeterol (100nM). Cells were then fixed with 4% paraformaldehyde for 15min at room temperature and then incubated with 10% goat serum in 0.1% Triton X100 (PBST). a-actinin (1:100) or ANF antibodies (1:1000) were incubated overnight in 2% goat serum in PBST at 4℃. The following day, after three washes with PBST, secondary antibodies (goat anti-rabbit 568 secondary antibody or goat anti mouse 488 secondary antibody) were incubated for 1 hour at room temperature at a dilution of 1:1000 in 2% goat serum in PBST. Fluorescence images was captured

at 10X magnification. The area of the stained myocytes and ANF staining surrounding the nucleus were quantified using Image J software.

AAV9 mediated gene delivery into neonatal mice

AAV9-eNos-mApple or AAV9-eNos-mApple-Nb80 were generated by University of Michigan Viral Vector Core. AAV9-eNos-mApple or AAV9-eNos-mApple-Nb80 were injected into mediastinum of 7 days-old C57BL/6J wild-type mice at a dose of 10^12 viral genomes. Adult myocytes were isolated at 8 weeks of age and expression and localization were confirmed by confocal microscopy.

AngII mini pump

Angiotensin II was dissolved in saline with or without salmeterol xinafoate and were filled into osmotic mini-pumps (Alzet, model 1002). Pre-filled pumps were incubated in sterile saline at 37C overnight. 8-10 week old male C57BL/6J mice were subcutaneously implanted with the mini-pumps to deliver angiotensin at the rate of 1.5mg/kg/day and salmeterol at the rate of 25µg/kg/day for 14 days. Mice were then euthanized and heart weight to body weight ratio was determined. Heart lysates were analyzed by western blotting as indicated.

Western blotting

NRVMs or AVMs were lysed in 1X Laemmli sample buffer, boiled, and loaded onto 4-20% gradient mini-PROTEAN TGX gels (4561094, Bio-Rad). Proteins were then transferred to nitrocellulose membranes overnight at 25mA or 2 hours at 300mA. Membranes were blocked with 4% bovine serum albumin (Fisher BP1600) in TBST (0.1% Tween-20 in Tris-buffered saline) for

2 hours. Primary antibodies were incubated overnight at 4℃ followed by 3X washes with TBST. Secondary antibodies were incubated for 1 hour at room temperature followed by 3X washes with TBST. Western blots were imaged and quantified with a LI-COR Odyssey imaging system.

Nuclear PKD fret reporters

AVMs were transduced with adenovirus expressing nDKAR at 100 MOI. The following day, cells were imaged with LEICA DMi8 microscope at 37C in confocal mode with 40X oil lens. Transduced AVMs were identified with CFP channel and then stimulated with indicated agonists for 30min. FRET was measured as the ratio of YFP emission to CFP emission after CFP excitation. CFP, excitation: 440nm; emission: 480nm. YFP emission: 535nm. Due to the sensitivity of the myocytes to CFP excitation data was collected at 0 and 30 min rather than a full time course.

Internalization of β2-adrenergic receptors.

NRVMs were plated onto glass bottom dishes. After 24 hours, cells were transfected with 500ng Flag-β2-ARs using lipofectamine 3000 for 16 h. Cells were then incubated with M1-Flag-488 antibody for 10min at 37C. Labeled cells were identified in the GFP channel (excitation: 488nm, emission: 510 nm) and then stimulated with salmeterol for 30 min at 37C. NRVMs were imaged in the GFP channel with a LEICA DMi8 microscope in confocal mode with 40X oil lens.

Statistics

Data were presented as mean +/- SEM from independent preparations of cells. Data were analyzed by two way anova with sidak's post-hoc test or one way anova with Tukey's multiple comparison test.

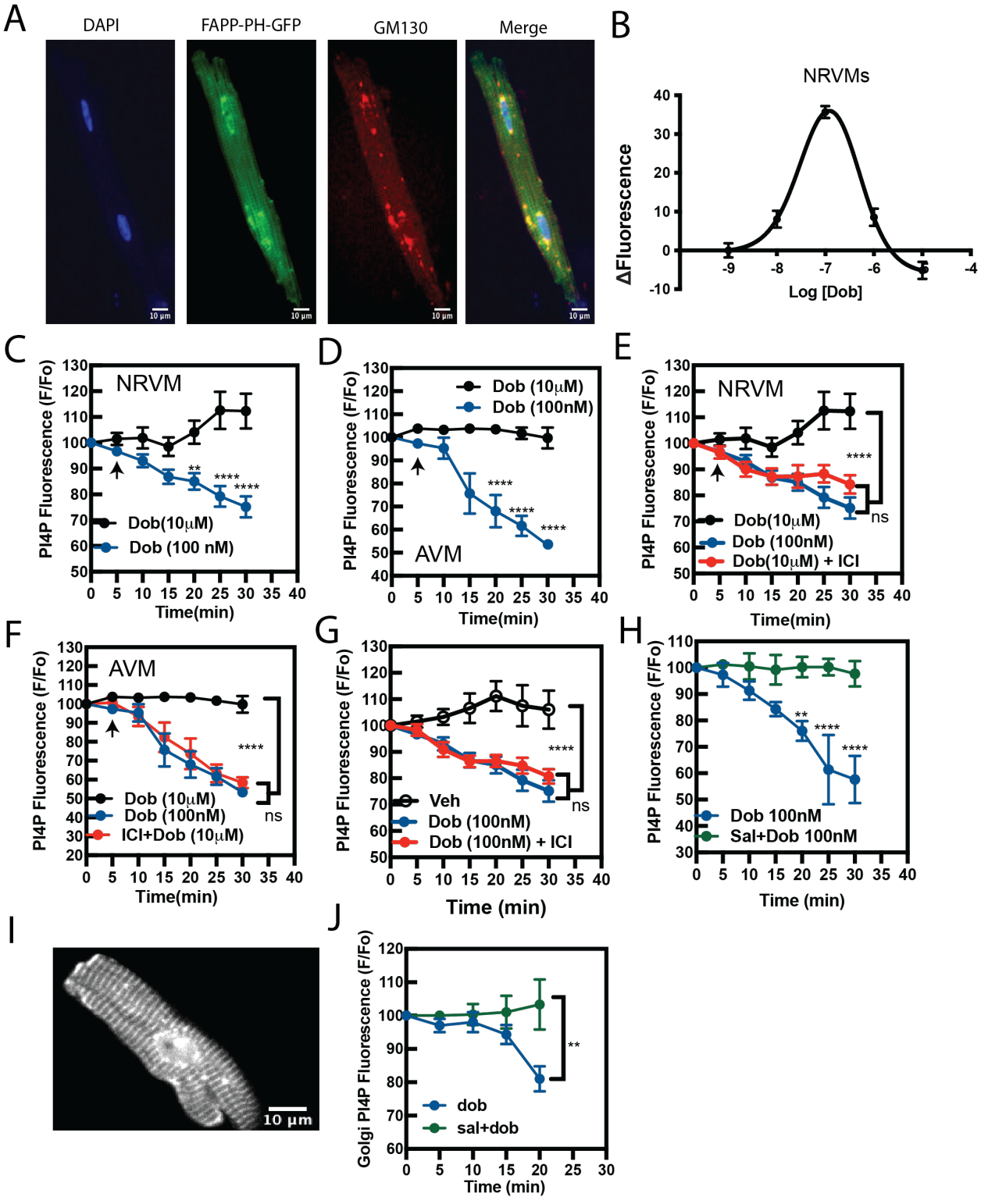
3.4 Results

3.4.1 Activation of β2-ARs opposes Golgi-β1-AR-mediated PLCε activation at the Golgi apparatus.

We previously demonstrated that the cell permeant agonist dobutamine (Dob) stimulates PLCε-dependent PI4P hydrolysis through activation Golgi β1ARs.(42). To assay Golgi PLCε activity we transduce cells with adenoviruses expressing GFP-FAPP-PH which binds selectively to PI4P and measure stimulus-dependent alterations in Golgi associated fluorescence using live-cell time lapse confocal fluorescence imaging in both NRVMs and adult ventricular myocytes (AVMs) (38, 42). Figure 3.1A shows co-localization of the PI4P-specific fluorescent probe with the Golgi marker GM130 in cardiac myocytes. Regions of interest in proximity to the nucleus are quantitated before and after agonist addition.

Unexpectedly, while analyzing the concentration-dependence for Dob-mediated Golgi PLCε activation, we found that 100 nM Dob was optimal, but 10 μM Dob did not stimulate PI4P hydrolysis in NRVMs or AVMs (Fig 3.1B, C, D). Dob is relatively selective β1AR agonist but activates β2ARs at higher concentrations (pKd 5.6 vs 4.8, β1ARs vs β2ARs)(169). To assess whether the inhibitory effect on PI4P hydrolysis at higher concentrations of Dob was due to activation of β2ARs, we pretreated NRVMs or AVMs with the selective β2AR antagonist ICI-118551 prior to Dob addition. Preincubation with ICI-118551 uncovered stimulation of PI4P hydrolysis by 10 μM Dob comparable to that seen with 100 nM Dob in both NRVMs and AVMs (Fig. 3.1E, F). ICI-11851 alone has no effects on 100 nM Dob-stimulated PI4P hydrolysis confirming that Dob-stimulated PI4P hydrolysis is β1AR-dependent (Fig. 3.1G). To extend this observation, we determined if salmeterol (Sal), a selective β2AR agonist, could inhibit PI4P

hydrolysis stimulated by Dob. Preincubation with Sal (100 nM) inhibited PI4P hydrolysis stimulated by 100 nM Dob (Fig. 3.1H). This effect was confirmed utilizing a different PI4P probe, GFP-P4M, which binds to PI4P both at the Golgi and plasma membrane (Fig. 3.1I, J). Taken together, this data indicates that β2AR stimulation opposes PLCε activation downstream of Golgi β1ARs.


A
DAPI
FAPP-PH-GFP
GM130
Merge
10 µm
B
NRVMs
ΔFluorescence
Log [Dob]
C
NRVM
PI4P Fluorescence (F/Fo)
Dob(10µM)
Dob (100 nM)
Time(min)
D
Dob (10µM)
Dob (100nM)
AVM
E
NRVM
Dob(10µM)
Dob (100nM)
Dob(10µM) + ICI
ns
F
AVM
Dob (10µM)
Dob (100nM)
ICI+Dob (10µM)
G
Veh
Dob (100nM)
Dob (100nM) + ICI
Time (min)
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Sal+Dob 100nM
I
J
Golgi PI4P Fluorescence (F/Fo)
dob
sal+dob

Figure 3-1 A pathway downstream of β2ARs suppresses β1AR stimulation of PLCε at the Golgi.
A) AVMs were transduced with adenoviruses expressing FAPP-PH-GFP for 18 hours prior to fixation and staining for GM130, a cis-Golgi marker. B) NRVMs were transduced with FAPP-PH-GFP and imaged by a live cell confocal microscopy. NRVMs were stimulated with dobutamine at either 10 μM or 100 nM at the arrow and remaining fluorescence intensity at the Golgi apparatus was monitored over time. C) Concentration-dependence for dobutamine stimulated PI4P hydrolysis. NRVMs were transduced with FAPP-PH-GFP, the indicated concentrations of dobutamine were added and the change in Golgi associated PI4P fluorescence relative to time=0 was quantified after 25 min of dobutamine addition D) AVMs transduced with FAPP-PH-GFP were stimulated with dobutamine at either 10μM or 100nM at the arrow indicated and Golgi associated fluorescence was monitored over time. NRVMs (E and G) and AVMs (F) were pretreated with a selective β2AR antagonist ICI-118.551 (50 nM) for 30 min before stimulation with dobutamine at the indicated concentrations and Golgi PI4P hydrolysis was measured. H) AVMs were pretreated with either Sal (100 nM) or vehicle control 30 min before imaging and dobutamine was added at the arrow. I) A confocal image showing an AVM expressing GFP-P4M probe. J) AVMs expressing GFP-P4M were pretreated with either Sal (100 nM) or vehicle control 30 min before imaging and dobutamine was added at the arrow. Fluorescence intensity was quantified at the region of Golgi apparatus in each frame of the time course of 20 min and normalized to fluorescence at 0 time point shown as a percent. Images taken for PI4P hydrolysis were collected from n=3-5 cells from at least 3 independent preparations of AVMs and at least n=8 cells from 3 separate preparations of NRVMs. Data was analyzed by a two-way unpaired ANOVA with Sidak's post-hoc test. *$p<0.05$; ** $p<0.001$; **** $p<0.00001$ using GraphPad Prism 9.

3.4.2 β2AR-dependent inhibition of PI4P hydrolysis is at the level of PLCε signaling

Previous studies from our laboratory demonstrated that Golgi β1AR-induced PI4P hydrolysis requires the EPAC activation downstream of βARs and upstream of PLCε in NRVMs (42). To determine if β2ARs inhibit PI4P hydrolysis downstream of β1AR-dependent cAMP accumulation, we stimulated PLCε-dependent PI4P hydrolysis using the EPAC-selective cAMP analog, 8-(4-chlorophenylthio)-2′-O-methyl-cAMP-acetoxymethyl ester, (cpTOME-AM). Pretreatment with Sal prevented cpTOME-AM-stimulated Golgi PI4P hydrolysis indicating that β2ARs inhibit PLCε activation at the level of EPAC or its downstream effectors, not at the level of cAMP generation by Golgi β1ARs (Fig 3.2A, B). We previously demonstrated that a Gq coupled receptor (endothelin 1 A receptor) activates mAKAP associated PLCε through a pathway independent from EPAC. We reasoned that if the inhibitory effect of β2AR activation was at the level of PLCε beyond EPAC/Rap, activation of PLCε by an alternative pathway would also be inhibited (Fig 3.2C). To test this AVMs were treated with Angiotensin II (AngII), which activates ATII receptors that couple to Gq and Gi/o but not Gs and is highly relevant to cardiac pathophysiology. AngII strongly stimulated PI4P hydrolysis which was blocked by Sal pretreatment (Fig 3.2D). This strongly suggests that β2AR-dependent inhibition is at the level of PLCε/mAKAP complex because two independent signaling pathways involving either Rap or signals downstream of Gq converge at the level of PLCε activation at the Golgi.

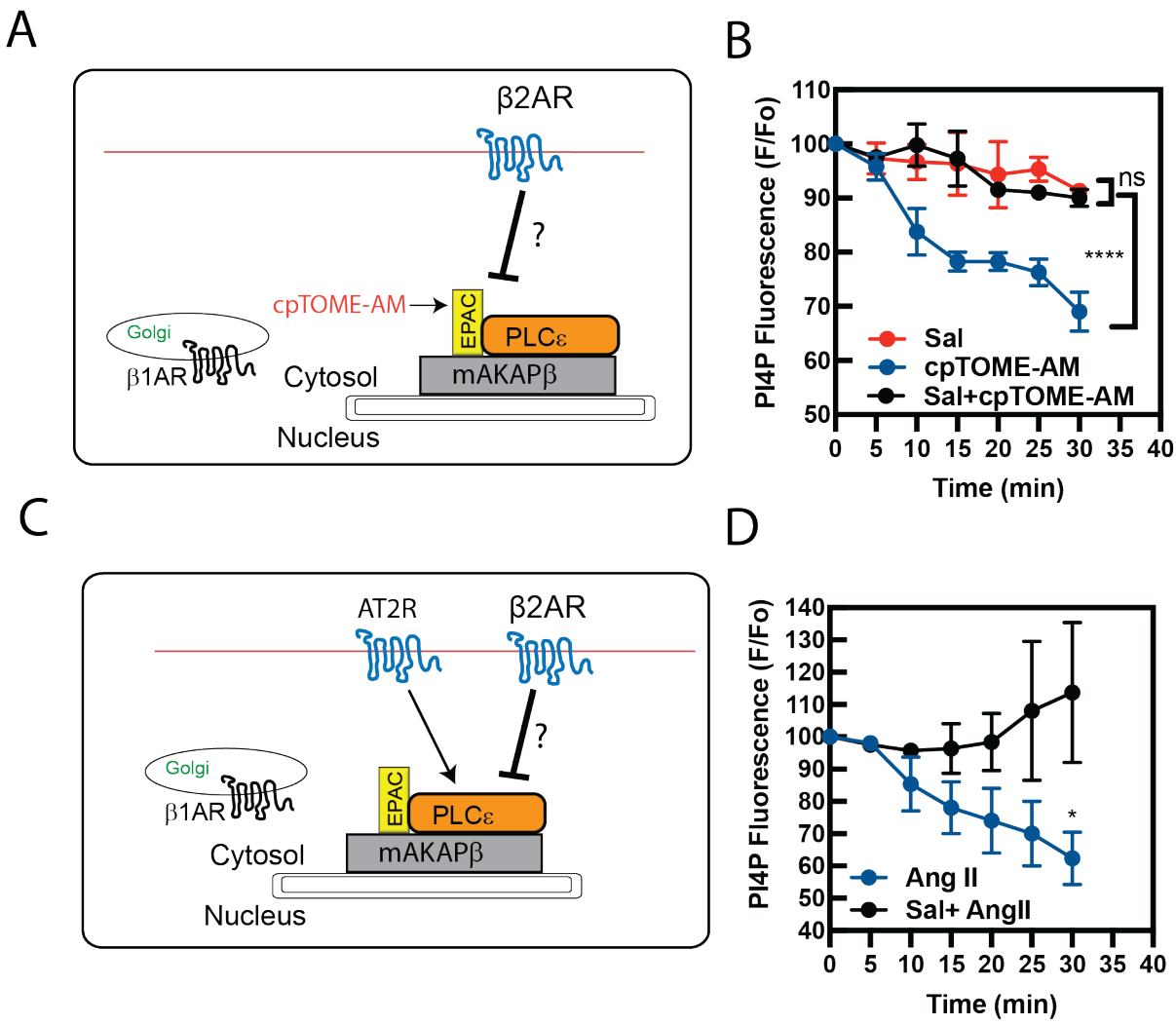


Figure 3-2 PLCε is the likely target for β2AR-dependent inhibition of Golgi PI4P hydrolysis.
A) Diagram of β2AR-dependent blockade of cpTOME-AM mediated activation of EPAC/PLCε. B) AVMs were pretreated with or without Sal (100 nM) before stimulation with cpTOME-AM (10μM) and Golgi PI4P associated fluorescence was monitored with time. C) Diagram of β2AR-dependent blockade of AngII mediated activation of PLCε at the Golgi apparatus. D) AVMs were pretreated with or without Sal (100nM) before stimulation with Angiotensin II (1μM) and Golgi PI4P level was assessed. Images for PI4P hydrolysis were taken from n=3-8 cells from 3 independent preparations of AVMs. Data was analyzed by a two-way unpaired ANOVA with Sidak's pots-hoc test. *p<0.05; ****p<0.00001 using GraphPad Prism 9.

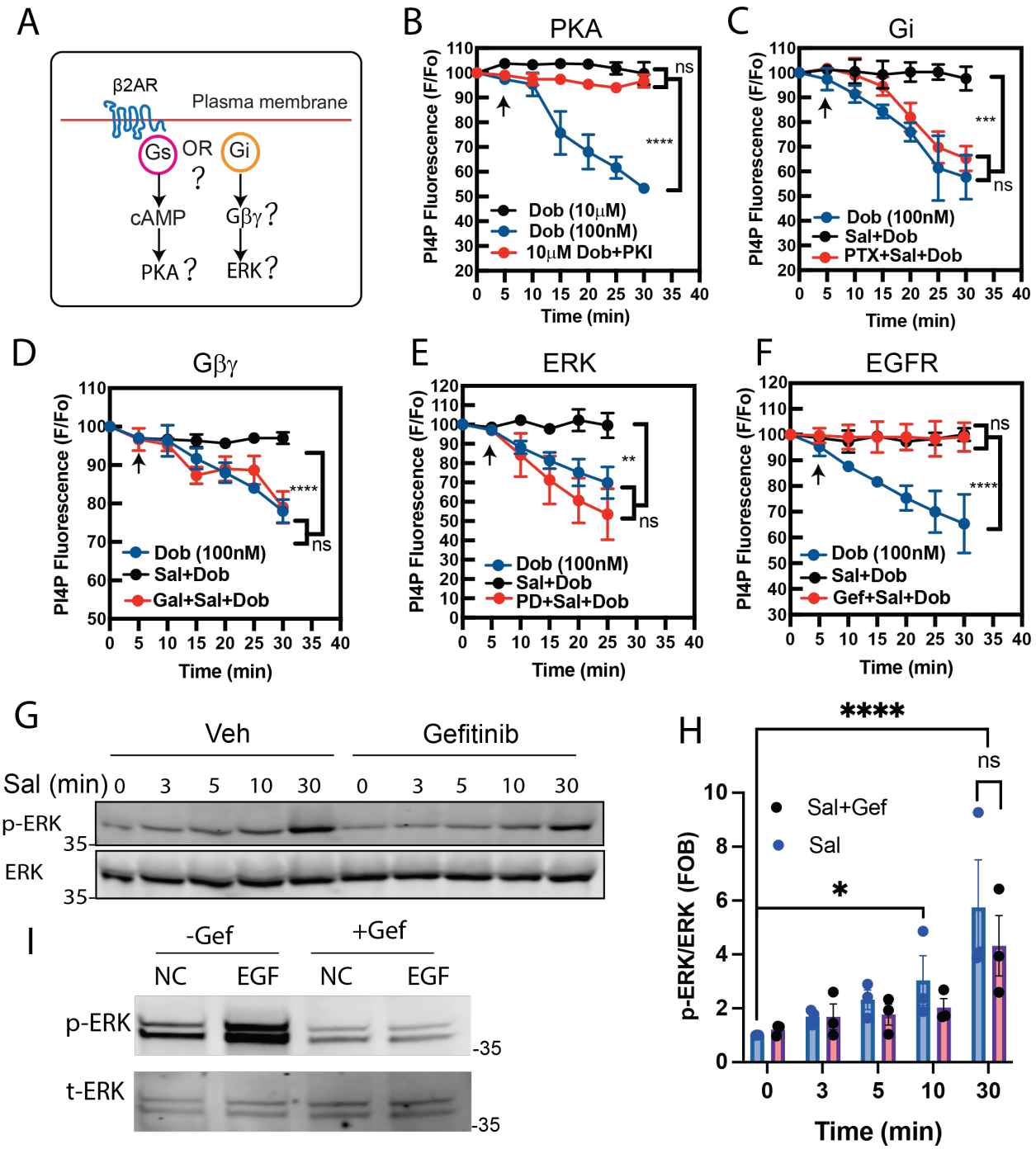
3.4.3 β2AR-dependent inhibition relies on Gi-Gβγ signaling.

Activation of β2ARs stimulates cAMP production and PKA activation. We have reported that PKA activation counters the stimulation of Golgi PLCε by EPAC (43). We tested whether PKA is involved in β2AR-dependent PLCε inhibition (Fig. 3A). Preincubation AVMs with a PKA inhibitor, PKI, did not uncover PI4P hydrolysis upon stimulation with 10 μM Dob indicating that β2AR-mediated inhibition is PKA independent (Fig. 3B). β2ARs preferentially activate Gs, but also couple to Gi/o(79, 80). To test the role of Gi we treated AVMs with the Gi/o inhibitor, pertussis toxin (PTX). PTX treatment abolished Sal- mediated inhibition of PI4P hydrolysis induced by Dob (Fig. 3C). We then tested the role of Gβγ liberated from Gi/o activation downstream of β2ARs in the signaling events. Treatment with gallein, a Gβγ inhibitor (170, 171), prevented Sal-dependent inhibition Dob-stimulated PLCε activation (Fig. 3D).

3.4.4 β2ARs inhibit PLCε activation via ERK signaling

Given that β2ARs can signal through β-arrestin, a nexus for activation of protein kinases such as ERK, we tested whether ERK activation downstream of β2ARs is involved. Pharmacological inhibition of ERK activity with PD0325901 abolished Sal-mediated inhibition of Golgi PI4P hydrolysis (Fig. 3E). Previous studies have reported that β1 and β2ARs can stimulate RTK (EGFR) transactivation, leading to ERK activation (172-174). This pathway requires the release of Gβγ subunits and is PTX sensitive. Additionally, βAR-mediated EGFR transactivation confers protective effects in isolated NRVMs (175). To assess the involvement of β2AR-EGFR transactivation, AVMs were pretreated with an EGFR inhibitor, gefitinib followed by preincubation with or without Sal and 100 nM Dob stimulation. EGFR inhibition did not alter the Sal-dependent inhibition of Dob-mediated PI4P hydrolysis (Fig. 3F, S3). We also examined if β2AR-EGFR transactivation contributes to ERK activation downstream of β2ARs activation.

AVMs were stimulated with Sal over a time course of 0-30 min in the presence with or without gefitinib and ERK phosphorylation was assessed by western blotting. Sal induced ERK activation weakly at 3-10 min, that appears to be inhibited by gefitinib. Sal treatment leads to statistically significant ERK activation at 30min and EGFR inhibition with Gefitinib had no statistically significant effect on this stimulation. (Fig. 3 G, H). These observations favor a model where β2ARs activate Gi and release Gβγ subunits, leading to EGFR-independent ERK activation that antagonizes Golgi PLCε activation.


A
β2AR
Plasma membrane
Gs
OR
Gi
?
cAMP
Gβγ?
PKA?
ERK?
B
PKA
PI4P Fluorescence (F/Fo)
Time (min)
Dob (10μM)
Dob (100nM)
10μM Dob+PKI
ns
****
C
Gi
Dob (100nM)
Sal+Dob
PTX+Sal+Dob
***
ns
D
Gβγ
Dob (100nM)
Sal+Dob
Gal+Sal+Dob
****
ns
E
ERK
Dob (100nM)
Sal+Dob
PD+Sal+Dob
**
ns
F
EGFR
Dob (100nM)
Sal+Dob
Gef+Sal+Dob
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Gefitinib
Sal (min) 0 3 5 10 30 0 3 5 10 30
p-ERK
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p-ERK/ERK (FOB)
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I
-Gef
+Gef
NC
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NC
EGF
p-ERK
t-ERK
-35
-35

Figure 3-3 β2AR-Gi-Gβγ-ERK signaling axis counters activation of PLCε.
A) Diagram of possible signaling pathways downstream of β2ARs. B) AVMs were pretreated with or without the PKA inhibitor, myrPKI (1 μM) 30 min before stimulation with dobutamine at the indicated concentrations and Golgi PI4P hydrolysis was assessed. C) AVMs were pretreated with either PTX (100ng/ml) or vehicle control overnight and followed by pretreatment with Sal 30 min before imaging and dobutamine was added at arrow. D) AVMs were pretreated with the Gβγ inhibitor, gallein (10 μM) or vehicle control for 30 min prior to the pretreatment with Sal for 30 min and dobutamine was added at the arrow. E) AVMs were pretreated with the ERK inhibitor, PD0325901 (100 nM) for 30 min prior to the pretreatment with Sal for 30 min and dobutamine was added at the arrow. F) AVMs were pretreated with or without the EGF receptor inhibitor, gefitinib (10μM) for 2 hours before the pretreatment with Sal for 30 min and dobutamine was added at the arrow. G-H) Acutely isolated adult ventricular myocytes were pretreated or without gefitinib for 2 hours before the treatment with Sal for indicated times followed by western blotting for p-ERK and total ERK. Shown is a representative western blot from three independent preparations of AVMs with quantitation shown in H. I) After 48 hours serum starvation, NRVMs were pretreated with Gef (10μM) for 30min and followed by EGF treatment for 30min. Western blotting was then performed to measure p-ERK/t-ERK. Images for PI4P hydrolysis were taken from n=3-6 from 3 independent preparations of AVMs. Data was analyzed by a two-way unpaired ANOVA with Sidak's pots-hoc test. *p<0.05; **P<0.001; ***P<0.0001; ****p<0.00001; ns, not significant using GraphPad Prism 9.

3.4.5 Internalized β2ARs are required to activate ERK and inhibit PLCε signaling.

β2ARs undergo activation-dependent internalization which has been implicated in ERK activation (131). Inhibition of receptor internalization using a dynamin inhibitor, dyngo-4a markedly reduced the β2AR-mediated blockade of PLCε activation (Fig. 4A) suggesting β2ARs mediate PLCε inhibition from endosomes rather than the plasma membrane. Salmeterol is a partial β2AR agonist for both Gs and β-arrestin recruitment relative to Iso, yet does engage arrestin to some degree (176, 177). To determine if Sal causes β2AR internalization in cardiac myocytes, NRVMs were transfected with flag-β2ARs and internalization was monitored using fluorescent anti-flag M1 antibody and confocal microscopy. Stimulation of cells with Sal led to accumulation of intracellular β2AR associated fluorescence confirming that Sal does cause β2AR internalization in myocytes (Fig 4B). To determine if endocytic blockade affects β2AR stimulation of ERK phosphorylation, freshly isolated AVMs were pretreated with or without dyngo-4a and time-dependent ERK activation stimulated by Sal was measured by western blotting. Dyngo-4a significantly inhibited Sal-elicited ERK phosphorylation at 30 min consistent with a mechanism requiring receptor internalization (Fig. 4C, D). To explicitly examine the involvement of the endosomal Gβγ in the regulation of Golgi PLCε activity, we selectively perturbed endosomal Gβγ using the C-terminus of G protein-coupled receptor kinase (GRK2ct), a highly selective Gβγ inhibitor, fused to a 2XFYVE domain sequence, highly specific to for binding to PI3P enriched endosomes (178), to generate an endosomal targeted GRK2ct (FYVE-mApple-GRK2ct) (Fig. 4E). Expression of FYVE-mApple-GRK2CT in AVMs led to a punctate expression pattern consistent with an endosomal location in AVMs and it is not overlap with Golgi apparatus (Fig. 4E, F). In AVMs co-expressing FAPP-PH-GFP and FYVE-mApple-GRK2CT, Sal was unable block Dob-

induced PI4P hydrolysis (Fig. 4G) indicating that the inhibitory signaling downstream of β2ARs is indeed driven by endosomal Gβγ.

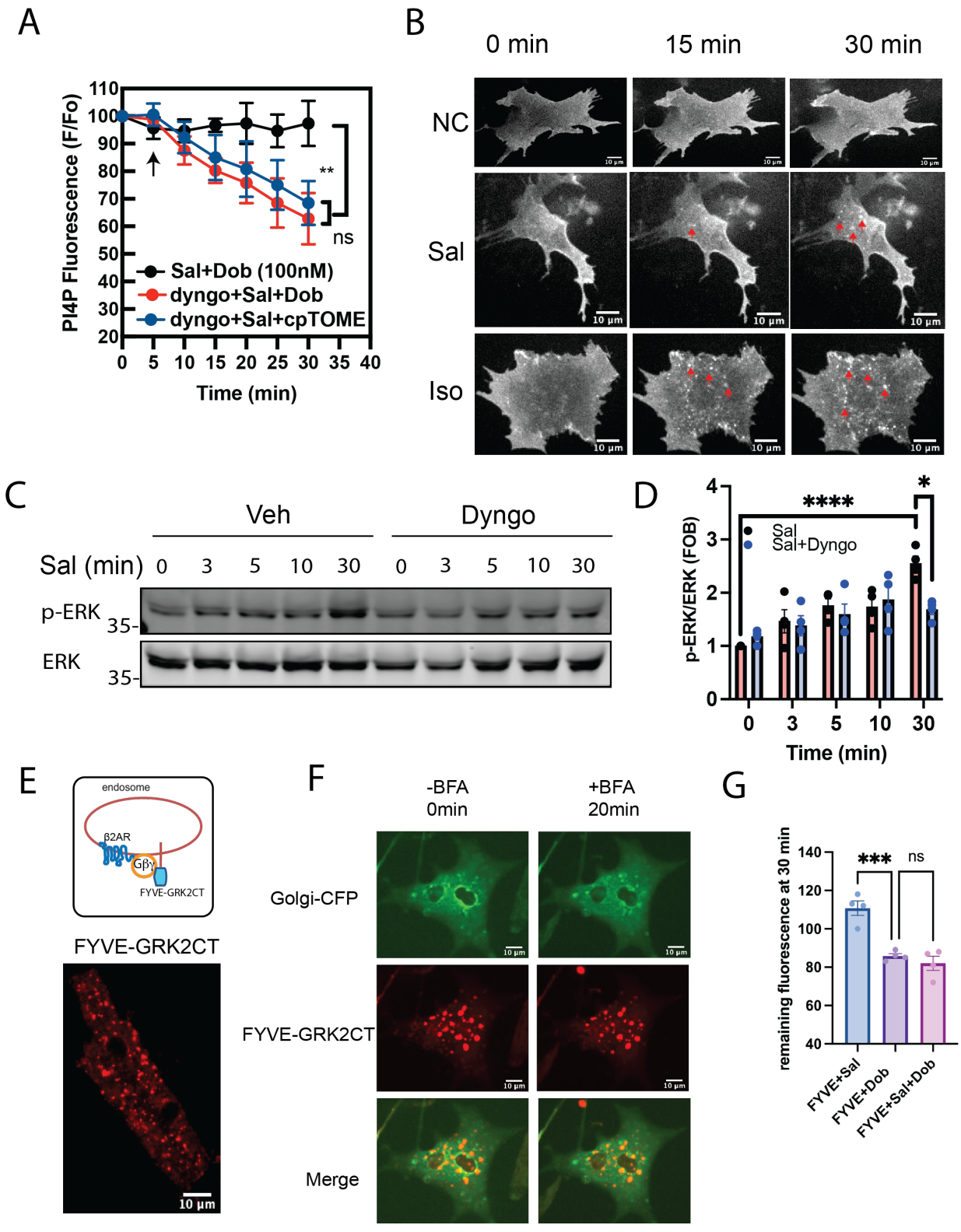


Figure 3-4 β2AR-dependent blockade of PLCε activation relies on endosomal Gβγ.
A) AVMs were pretreated with the dynamin inhibitor Dyngo-4a (40μM) for 30 min prior to pretreatment with Sal for 30 min and dobutamine was added at the arrow. Data was analyzed by a two-way unpaired ANOVA with Sidak's pots-hoc test **p<.001, ns=not significant. B) Representative images showing Sal or Iso mediated β2-AR internalization. NRVMs were transfected with Flag-β2ARs for 24 hours. Cells were then labeled with M1-Flag-488 for 10min at 37C and then treated with negative control, Sal (100nM), or Iso (10μM) at 37C. Images were acquired at the indicated times by confocal microscopy. C-D) Acutely isolated adult ventricular myocytes were pretreated in the presence or absence of Dyngo before the treatment with Sal for indicated times followed by western blotting for p-ERK and total ERK. Shown is a representative western blot from four independent preparations of AVMs with quantitation shown in D. Data was analyzed using an unpaired two-way ANOVA with Sidak's pots-hoc test. *p<0.05; ****p<0.00001. E) Diagram of blockade of Gβγ signaling at endosomal membranes (Top). Representative image of AVMs expressing FYVE-GRK2CT (bottom). (F) NRVMs were infected with FYVE-mApple-GRK2CT and Golgi (Giantin)-CFP treated before and after brefeldin A, an ARF inhibitor to disrupt the Golgi apparatus. G) AVMs were transduced with adenoviruses expressing FYVE-mApple-GRK2CT and FAPP-PH-GFP for 18 hours before imaging. AVMs were stimulated with Dobutamine alone or in the presence of Sal. Golgi associated PI4P fluorescence intensities at 0 and 30 min were measured. Images for PI4P hydrolysis were taken from n=3-9 cells from at least 3 independent preparations of AVMs. Data was analyzed by a one-way ANOVA with Sidak's pots-hoc test. *** p<0.0001 using GraphPad Prism 9.

3.4.6 Activation of β2ARs inhibits nuclear PKD activation.

A signaling event directly downstream of perinuclear PLCε is the activation of nuclear PKD (38). PKD is directly activated by DAG, the principal active product of PLCε activity acting on Golgi PI4P. If Golgi PLCε is inhibited downstream of Sal, we would predict that β2AR activation would inhibit nuclear PKD activation. To determine if activation of β2ARs reduces agonist-dependent PKD activation, NRVMs were pretreated with Sal for 30 min before addition of AngII for 30 min and analysis of PKD phosphorylation by western blotting. Sal treatment eliminated AngII mediated PKD activation (Fig. 5A, B). To more specifically examine nuclear PKD activation downstream of β2AR and Ang II activation, AVMs were transduced with an adenovirus expressing a nuclear-localized fluorescence resonance energy transfer (FRET) PKD activation reporter, nDKAR. (168) (Fig. 5C). AVMs were pretreated with either vehicle or Sal for 30 min and AngII mediated nuclear FRET was measured in the nucleus at 0 and 30 min. AngII caused a significant nuclear PKD activation which was suppressed by β2AR activation (Fig. 5D).

We had previously shown that cardiac specific deletion of PLCε in mice eliminates PKD activation in a TAC model suggesting that PKD phosphorylation can be used as a proxy for PLCε activity in vivo. To extend our observations to mice, we examined PKD phosphorylation in response to the chronic AngII infusion together with Sal by implanting AngII +/- Sal subcutaneous osmotic minipumps into mice for 14 days. Chronic stimulation with Sal significantly attenuated AngII-mediated PKD activation in mouse hearts (Fig. 5E and F). Similarly, HDAC phosphorylation was blunted in Sal treated group (Fig. 5E and G). PKD phosphorylation of HDAC is partially responsible mediating MEF-dependent hypertrophic gene transcription (179). Salmeterol also significantly inhibited AngII-driven cardiac hypertrophy as measured as a reduction in heart size (HW/BW) compared to animals treated with AngII alone (Fig. 5H). These

observations together suggest β2ARs inhibit development of hypertrophy in mice by preventing detrimental AngII-mediated PLCε signaling in cardiomyocytes. However, we cannot exclude the possibility that Sal mediated protection in mice involves inhibition of AngII-mediated hypertension.

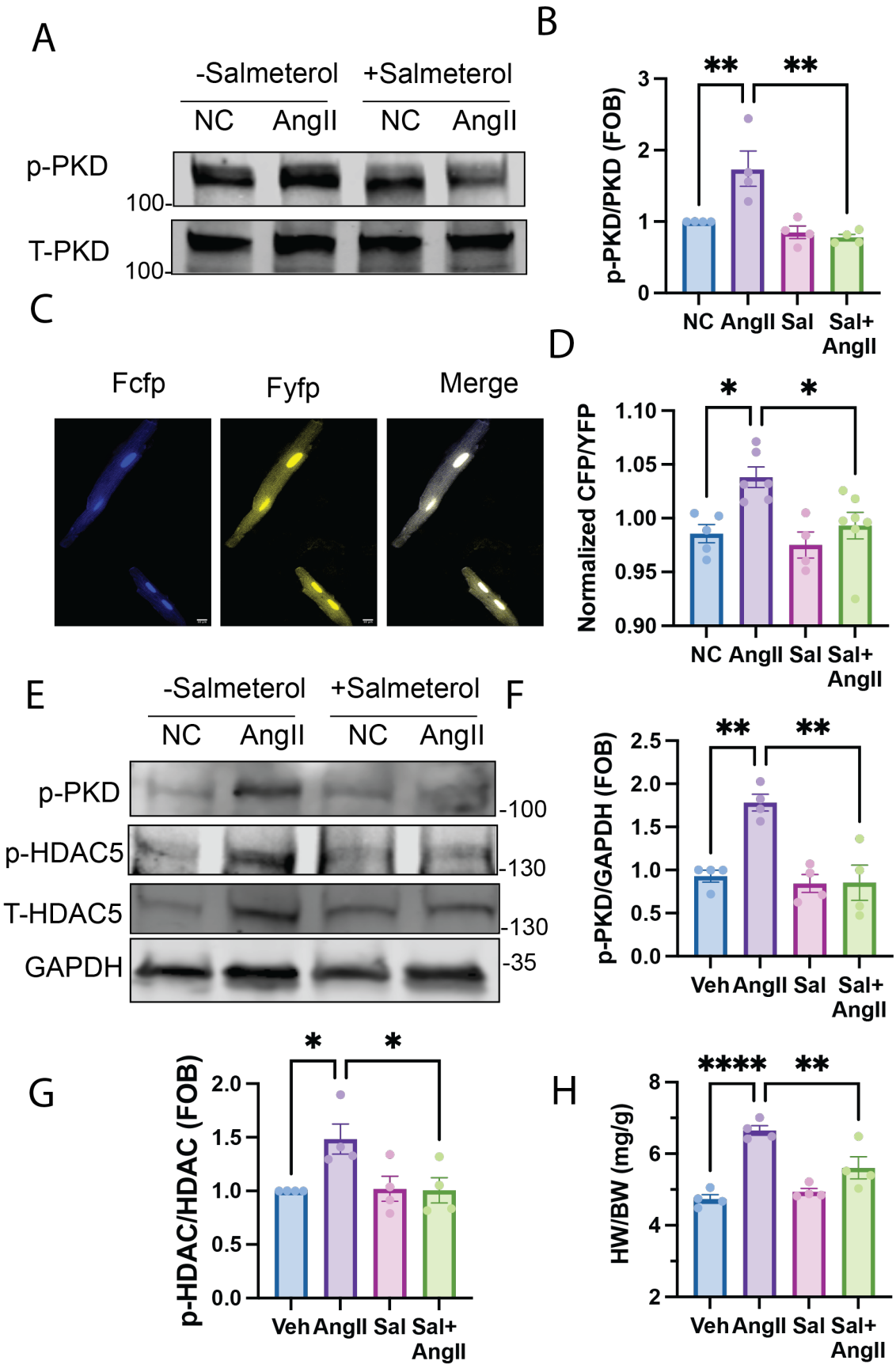

A
-Salmeterol
+Salmeterol
NC
AngII
NC
AngII
p-PKD
100-
T-PKD
100-
B
p-PKD/PKD (FOB)
NC AngII Sal Sal+ AngII
C
Fcfp
Fyfp
Merge
D
Normalized CFP/YFP
NC AngII Sal Sal+ AngII
E
-Salmeterol
+Salmeterol
NC
AngII
NC
AngII
p-PKD
p-HDAC5
T-HDAC5
GAPDH
-100
-130
-130
-35
F
p-PKD/GAPDH (FOB)
Veh AngII Sal Sal+ AngII
G
p-HDAC/HDAC (FOB)
Veh AngII Sal Sal+ AngII
H
HW/BW (mg/g)
Veh AngII Sal Sal+ AngII

Figure 3-5 Nuclear PKD activation downstream of PLCε is suppressed by β2AR activation.
A-B) NRVMs were pretreated with either Sal or vehicle before addition of AngII (1 μM) for 30 min and followed by western blotting to for p-PKD and total PKD. Shown is a representative western blot from four separate preparations of NRVMs. Quantitation is shown in B. C) Representative images of AVMs expressing nuclear-DKAR. D) The nuclear region of AVMs expressing nDKAR was selected and the CFP/YFP ratio was measured before and after addition of AngII (1μM) for 30 min addition in the presence with or without pretreatment with Sal for 30 min. E) Heart lysates from WT mice infused with either saline (Veh) or salmeterol (Sal) (25 μg/kg/day) together with or without AngII (1.5 mg/kg/day) for 14 days. Western blotting was performed to determine the level of p-PKD, p-HDAC, total HDAC and GAPDH. Shown is a representative western blot from 4 mice each group. F) Quantitation of PKD phosphorylation from E. G) Quantitation of HDAC phosphorylation from E. H) Heart weight/body weight (HW/BW) ratios from AngII and AngII+Sal treated mice. N=4 animals each condition. Data was analyzed by a one-way ANOVA with Sidak's pots-hoc test. *$p<0.05$; **$p<0.001$; **** $p<0.00001$ using GraphPad Prism 9.

3.4.7 Dob-induced NRVM hypertrophy is effectively inhibited by β2ARs activation.

In our previous studies, we showed that Golgi resident β1ARs signaling to PLCε is required for catecholamine-mediated cardiomyocyte hypertrophy in NRVMs (42). NRVMs were treated with Dob (100nM) with or without Sal for 48 hours and cell area and expression of the hypertrophic marker, atrial natriuretic factor (ANF), were measured. Dob stimulated a significant increase in cell area (Fig 6A, B) and ANF expression (Fig 6C, D). Co-treatment with Sal blunted Dob-induced hypertrophy assessed by these two measurements (Fig 6A, B, C, D), demonstrating that β2AR signaling is protective against cardiomyocyte hypertrophy consistent with the results from mice.

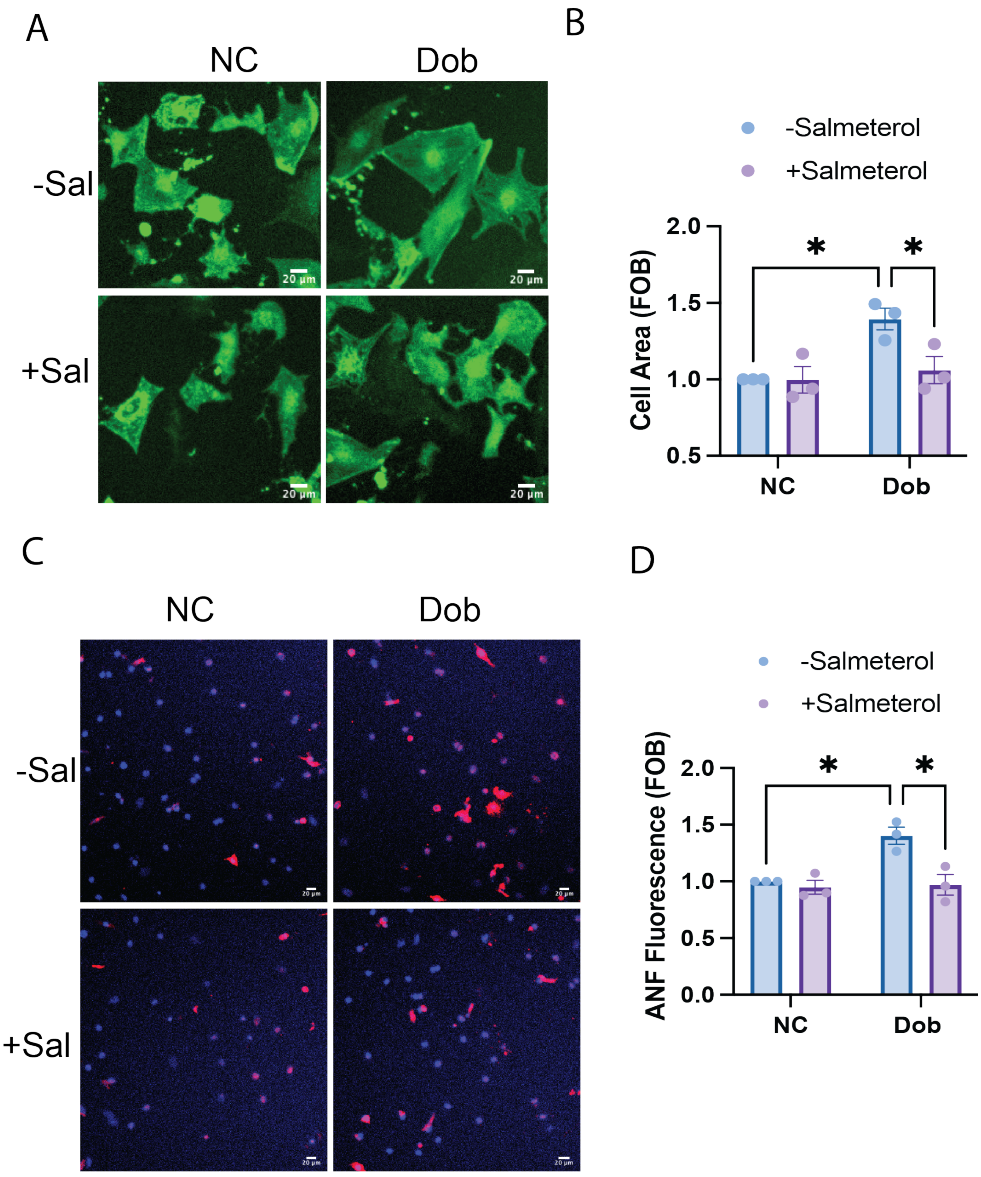


Figure 3-6 Activation of β2ARs inhibits dobutamine induced cardiomyocyte hypertrophic growth.
A-B) NRVMs were stimulated with dobutamine for 48 hours in the presence of Sal or vehicle control and followed by fixation and staining for a-actinin to identify cardiomyocytes and quantitated for cell area by image J. C-D) NRVMs were stimulated with dobutamine for 48 hours in the presence of Sal or vehicle control and followed by fixation, cells were stained for ANF expression and with DAPI to identify nuclei. The fluorescence intensity of ANF rings was quantified by image J. Data was quantified from at least n=350 cells from 3 separate preparations of NRVMs. Data was analyzed by a one-way ANOVA with Tukey's pots-hoc test. *p<0.05; *p<0.001 using GraphPad Prism 9.

3.5 Discussion

Our previous work has led to development of a model where stimulation of Golgi β1ARs by a membrane permeant drug Dob produces a local pool of cAMP with privileged access to the EPAC/PLCε/mAKAP complex, generating DAG derived from PI4P depletion, activating PKD and mediating cardiac hypertrophy (38, 42). Unexpectedly, while low concentrations of Dob induced robust PI4P hydrolysis, saturating concentrations of Dob did not. Here we demonstrate that β2AR activation opposes activation of Golgi PLCε-PKD pathway by two clinically relevant hypertrophic stimuli which could play an essential role in the ability of β2ARs to limit cardiac hypertrophy. In cardiac myocytes we show that the mechanism involves agonist-driven internalization of β2ARs where they couple to ERK activation via Gi-Gβγ at the endosome which in turn inhibits prohypertrophic PLCε signaling at the Golgi. Heart failure is often associated with up-regulation of Gi and enhanced β2AR-Gi signaling (180, 181) which may be a compensatory mechanism to overcome the detrimental effects induced by circulating mediators and sympathetic neurotransmitters, many of which are likely to signal through PLCε.

3.5.1 Compartment specific ERK signaling in the heart.

Extensive studies suggest ERK is a key player in regulating cardiomyopathy. ERK signaling can produce both beneficial and deleterious effects depending on the context. Transgenic mice overexpressing activated ERK are reported to show maladaptive(182) or adaptive(183) hypertrophic responses, while other studies utilizing mice with ERK deletion mice suggest ERK1/2 signaling may be dispensable in pathologic cardiac hypertrophy(184, 185). In addition, an in vitro study indicates that ERK1/2 activation is required for the ANF mediated antihypertrophic response(186). It is now becoming clear that the signaling cascades and outcomes

downstream of ERK largely depend on the subcellular locations of phosphorylated ERK1/2 (187). Therefore, a more sophisticated dissection of ERK signaling mechanisms is required.

ERK is activated downstream of multiple GPCRs which are themselves spatially compartmentalized(188). GPCRs inside the cell activate G proteins and produce local cAMP accumulation to initiate distinct subcellular signaling events (189, 190). G proteins including Gs, Gq, Gi and Gβγ subunit or β-arrestin-mediated signaling pathways activate ERK cascades. Different ligands cause different subcellular destinations of activated ERK via their preference to shift GPCR coupling to G-proteins or β-arrestin (191, 192). Recently, it has been shown that after ligand-stimulated β2ARs endocytosis, endosomal ERK signaling is activated by an endosome-localized active Gαs to subsequently stimulate nuclear ERK activity to control gene expression in HEK cells(131). Our studies support the idea that in AVMs, β2AR-induced accumulated ERK signaling requires receptor endocytosis and endosomal Gβγ released from Gi. This endosome-receptor initiated ERK signaling serves as a repressor for PLCε which has been implicated in mediating cardiac hypertrophy. In our model, we utilized Sal, a highly selective partial β2AR agonist (3000 food selectivity of β2AR over β1AR), which preferentially interacts G protein relative to arrestin, thereby resulting in lower receptor internalization than the full agonist Iso (176, 177). This could explain why we observed delayed Sal-mediated endosomal ERK activation. Nevertheless, our study provides a functional effect of this endosomal β2AR-ERK signaling axis in preventing the development of cardiac hypertrophy.

3.5.2 PKD signaling in cardiac remodeling

Studies have reported that cardiac-specific deletion of PKD improves cardiac function in response to pressure overload or angiotensin II signaling (193), making it a promising therapeutic target to treat heart failure resulting from maladaptive cardiac hypertrophic signaling. We

previously demonstrated that DAG generated from PI hydrolysis by the perinuclear scaffolded PLCε activates PKD in close proximity to the nucleus, where it phosphorylates HDAC to regulate hypertrophic gene expression (38). Our data suggest that acute activation of β2ARs can protect against pathological cardiac hypertrophy through inhibition of phosphorylation of nuclear PKD activation and perhaps other kinases such as CamKII. Hence, currently clinically effective β2AR agonists can serve as potent PKD repressors. In addition to the role of PKD in cardiac hypertrophy and fibrosis, it is also a critical signaling molecule in cancer-associated functions such as migration, cell proliferation, or survival (194). Thus, our observations could extend to other pathophysiological systems.

Molecular mechanisms underlying the cardioprotective effects of β2AR signaling have been investigated previously. Gi-dependent-PI3K-AKT signaling downstream of β2-ARs contributes to its cardioprotective effects through prevention of apoptosis (195). In addition, β2-ARs can mediate EGFR and PDGFR transactivation, promoting cardiomyocyte survival (196). These studies focused on cardiomyocyte apoptosis. We propose that inhibition of hypertrophic PLCε signaling is an additional mechanism for β2AR dependent cardiac protection against detrimental cardiac remodeling.

3.5.3 Combined therapy with a β2AR agonist and a selective β1AR blocker.

β-blockers (carvedilol, bisoprolol, and metoprolol) are clinically used to treat patients with heart failure by blocking detrimental β1AR-G protein signaling, although their complete mechanism of action is not fully understood. More recently, it has been suggested that effective β-blockers tend to be hydrophobic which allows them to access the internal pools of β1ARs (42, 81). As a non-selective β-blocker, carvedilol has been shown to produce superior clinical outcomes relative to other β-blockers (197), though the mechanism is unclear. Interestingly, carvedilol has

been suggested to activate β2ARs and ERK signaling (198). It has been proposed that carvedilol might have dual therapeutic benefits in heart failure by preventing the deleterious signaling from β1ARs and promoting the beneficial effects of β2ARs (160). This therapeutic concept combining β1AR blockade with a β2AR agonist has been previously proposed and investigated in a coronary ligation model mice and rats where the combination treatment was more effective at preserving cardiac function that β1 blockade alone (195, 199, 200). Our data indicate that in addition to effects on vascular tone selective β2ARs ligands can act directly through cardiac myocytes to improve cardiac function by preventing the deleterious PLCε stimulated by circulating catecholamines and angiotensin.

3.5.4 Isoproterenol paradox

Isoproterenol (Iso), a non-selective membrane impermeant β1AR and β2AR agonist, does not induce acute PLCε activation(43), but with chronic exposure, it causes cardiac hypertrophy in vitro and vivo. One possible explanation for this discrepancy could be with acute treatment of Iso, β2ARs signal to counteract the deleterious effects from other mediators. However, with chronic exposure to a strong stimulation by Iso, β2ARs, while not downregulated may undergo desensitization leading to the loss of their protective signaling. Alternatively, Iso may depend on a different plasma membrane mediated hypertrophic pathway. These speculations need to be experimentally verified.

Chapter 4 Subcellular Control of Heart Failure Pathways by Golgi β1-Adrenergic Receptors in Cardiac Myocytes

4.1 Abstract

Chronic stimulation of Gs-coupled β1-adrenergic receptor (β1-AR) signaling by catecholamines induces cardiac hypertrophy and ultimately heart failure. However, the mechanism remains incompletely understood. GPCRs at different intracellular compartments are recognized as novel mediators of distinct localized signaling outputs. We have demonstrated Golgi-β1-ARs are involved in the regulation of the activity of subcellular PLCε by generating a localized pool of cAMP, leading to cardiac hypertrophy in cardiomyocytes. However, its role in an animal model of heart failure has not been determined. We developed a Golgi-targeted construct, eNOS (1-33)-mApple-NB80 to deliver the β1AR inhibitor, nanobody 80 (NB80), specifically to the Golgi apparatus. We confirmed the selective localization to the Golgi apparatus of cardiac myocytes and equal expression of eNOS-mApple-NB80 and eNOS-mApple (negative control without NB80) in mouse hearts. Additionally, the activation of the prohypertrophic Golgi PLCε pathway by an agonist was inhibited in isolated adult cardiac myocytes by expressing eNOS-mApple-NB80. We performed transaortic constriction on eNOS-mApple-NB80, eNOS-mApple, or GFP expressing animals. Our analysis of the animal hearts did not show a significant restoration of cardiac function, heart weight, and key signaling pathways, though the data trended towards possible significance. More animals, an appropriate Golgi targeting sequence, or catecholamine-perfused heart failure animal models may be required to definitely determine the role of Golgi-β1-ARs in heart failure.

4.2 Introduction

In pathological cardiac hypertrophy, elevated and chronic neurohumoral stimulation induced by conditions such as hypertension, myocardial infarction, or valve diseases are responsible for cardiac remodeling. These conditions activate maladaptive cellular process including fetal gene re-expression and protein synthesis, inducing progressive loss of ventricular function and, in the long term, evolves to the symptoms of heart failure.(201). Treatments that target chronic adrenergic stimulation, such as β-adrenergic receptor blockers and calcium channel blockers, ameliorate the symptoms of heart failure and slow the deterioration of cardiac function, but they have significant side effects(202). Hence, understanding the mechanisms of cardiac hypertrophy will provide insights into novel therapeutic targets or improve current therapeutic strategies for the treatment of heart failure.

One key factor that contributes to cardiac hypertrophy and subsequent heart failure is chronic activation of β1-AR signaling(203). β1-ARs are Gs coupled receptors that stimulate adenylyl cyclase (AC), to produce cyclic adenosine monophosphate (cAMP)(4). cAMP then phosphorylates protein kinase A (PKA), resulting in PKA dependent phosphorylation of a variety of intracellular proteins such as the type 2 ryanodine receptor (RyR2), cardiac myosin binding protein-C (cMyBP-C), and phospholamban, etc. EPAC (exchange protein directly activated by cAMP) is also one of the downstream effectors of cAMP(11). Our laboratory identified a Golgi/nuclear envelope localized signaling pathway where cAMP-dependent Epac activation stimulates PLCε-dependent Golgi PI4P hydrolysis, generating local diacylglycerol (DAG) and subsequent local protein kinase D (PKD) activation(38, 40, 42, 43, 129). PKD phosphorylates histone deacetylases (HDACs) resulting in their nuclear export leading to MEF2-dependent hypertrophic gene expression(204, 205).

Several studies have implicated cAMP signals generated by GPCRs at multiple subcellular locations. GPCRs including β2ARs, the parathyroid hormone (PTH) receptors, and opioid receptors are internalized into endosomes upon stimulation via clathrin-mediated endocytosis(81, 146, 148). While β1-ARs are poorly internalized, recent studies demonstrate some β1ARs reside at the Golgi apparatus and studies from our laboratory demonstrated that norepinephrine (NE) activates β1-ARs at the Golgi apparatus through an organic cation transporter 3 in cardiac myocytes(42). Activation of these intracellular β1-ARs triggers hypertrophic signaling from the Golgi apparatus in cardiac myocytes through the Epac/PLCε pathway described above, but activation of cell surface β1-ARs does not. A recent study suggests β1-ARs are present in sarcoplasmic reticulum (SR) produce a local pool of cAMP leading to phosphorylation of PLB to regulate cardiomyocyte contraction(97).

Activatable internal pools of GPCRs are rapidly emerging as novel mediators of cellular processes(206). Most of the previous studies were performed in tool cell lines that are easier to handle but with no real physiological significance. Our studies in cardiomyocytes were the first to report a physiological role for internal β1ARs and we have identified its downstream signaling pathways(42). However, no studies have been conducted on animals to show the role of Golgi-β1ARs in heart failure. Interestingly, β-AR blockers such as bisoprolol, carvedilol and metoprolol, which show beneficial effects in heart failure, tend to be hydrophobic and capable of crossing cell membranes to access intracellular compartments. Therefore, characterizing the roles of β1ARs at different intracellular compartments in intact animals under pathological conditions will have significant implications for developing therapeutic strategies for the treatment of heart failure. This chapter aims to explore the functional consequences of inhibition of Golgi resident β1ARs in a mouse model of pressure overload induced cardiac hypertrophy: transaortic constriction (TAC).

We developed and characterized a Golgi-targeted Nanobody 80 (NB80)(81) to specifically inhibit Golgi-β1-ARs. We delivered this genetically encodable subcellular targeted β-blocker into mouse hearts using a recombinant adeno-associated virus 9 (AAV9) based system, followed by TAC surgery to understand the role of Golgi-localized β1-ARs in a heart failure animal model.

4.3 Methods

Isolation and transduction of neonatal rat ventricular myocytes (NRVMs) and mouse adult cardiac myocytes (ACMs)

Neonatal rat ventricular myocytes were isolated from 2 to 4 day-old Sprague-Dawley rats as described previously(40). Briefly, ventricles were separated from the hearts and minced well before adding digestion buffer (Collagenase type II in Hanks buffered saline solution without calcium). Following digestion, supernatant was collected, and cells were centrifuged at 1200 rpm for 2 min. Fibroblasts were removed by pre-plating cells onto tissue culture plastic for one hour at 37C. Purified NRVMs in the supernatant were transferred onto gelatin-coated glass-bottom dishes or 6 well plates. NRVMs were cultured in DMEM supplemented with 10% FBS, 100U/mL penicillin, 100 ug/mL streptomycin, 2 mg/mL vitamin B12, and 10 mM cytosine arabinoside. 24 hours later, media was changed to 1%FBS or serum free media. For adenovirus transduction, 50 MOI of adenovirus was incubated overnight.

Isolation of adult ventricular myocytes (from 2-5 month-old wild type C57BL/6J mice) was performed following the protocol from Auerbach et al (150). Mice were anesthetized with ketamine (100mg/kg body weight) and xylazine (5mg/kg body weight). Hearts were then removed, cannulated, and perfused with perfusion buffer (10 mM HEPES, 0.6 mM Na2PO4, 113 mM NaCl, 4.7 mM KCl, 12 mM NaCO3, 0.6 mM KH2PO4, 1.2 mM MgSO4, 10 mM KHCO3, 30 mM

Taurine, 10 mM BDM, 5.5 mM Glucose) via the aorta. Subsequently, digestion buffer (collagenase type II (773.48 U/ml), trypsin (0.14 mg/ml), and calcium chloride (12.5μM) in perfusion buffer) was perfused via aorta. Following digestion, hearts were minced in stopping buffer (10% FBS and 12.5 μM calcium chloride in perfusion buffer), debris was allowed to settle, the supernatant was collected, and calcium was added back to a final 1 mM concentration. Cells were then centrifuged at 18g for 3min before plating onto laminin-coated 20mm glass bottom dishes for confocal microscopy imaging. AVMs were maintained in minimum essential medium (MEM) supplemented with 0.35 g/L sodium bicarbonate, 100 U/mL penicillin, 100 U/mL streptomycin, and 10mM BDM.

Transduction of AVMs with adenovirus and imaging.

To transduce AVMs with adenovirus, 100 MOI of indicated adenoviruses were added to cells in BDM-free media for 4 hours. Following this, the virus was removed, and replaced with the culture media supplemented with BDM.18-24 hours later, cells were imaged by confocal microscopy with 40X oil-immersion lens for measurement of FAPP-PH-GFP fluorescence. EGFP was excited at 488 nm and images were acquired with 25 ms exposure at 2.5 min intervals.

NES-Venus-mini-Gs imaging

NRVMs were plated into gelatin-coated 20 mm glass bottom cell culture dishes. Cells were transfected the following day with plasmids (500-800 ng of β1-ARs and 250-400 ng of NES-Venus-mini-Gs per dish) using lipofectamine 3000. Media was changed to 1% FBS the next day and transduced with adenovirus-expressing CFP-Giantin overnight. Cells were imaged in confocal mode with a Leica DMi8 equipped with a Crest-optics X-light V2 confocal unit and a 100x 1.4

NA oil-immersion lens. Venus was excited at 515 with an X-Cite Xled1 light source, and emission monitored imaged on a backlit CMOS Photometrics Prime 95B camera.

AAV9 mediated gene delivery into neonatal mice

AAV9-eNos-mApple or AAV9-eNos-mApple-Nb80 were generated by University of Michigan Viral Vector Core. AAV9-eNos-mApple or AAV9-eNos-mApple-Nb80 were injected into mediastinum of 7 days-old C57BL/6J wild-type mice at a dose of 10^12 viral genomes per mouse. Adult myocytes were isolated at 8 weeks of age and expression and localization were confirmed by confocal microscopy.

Western blotting

Hearts were minced and homogenized in RIPA buffer supplemented with protease inhibitor cocktail. Tissues were lysed at 4C° for 45min with gentle rotation and followed by centrifuged at 13,000rmp for 15min. Tissue lysates were then diluted in 1X Laemmli sample buffer, boiled, and loaded onto 4-20% gradient mini-PROTEAN TGX gels (4561094, Bio-Rad). Proteins were then transferred to nitrocellulose membranes overnight at 25mA or 2 hours at 300mA. Membranes were blocked with 4% bovine serum albumin (Fisher BP1600) in TBST (0.1% Tween-20 in Tris-buffered saline) for 2 hours. Primary antibodies were incubated overnight at 4℃ followed by 3X washes with TBST. Secondary antibodies were incubated for 1 hour at room temperature followed by 3X washes with TBST. Western blots were imaged and quantified with a LI-COR Odyssey imaging system.

RNA extraction and RT-qPCR

Heart tissue was homogenized in TRIzol reagent (Fisher, 15596026) and total RNA was extracted using RNeasy miniKit (QIAGEN, 74104) according to the manufacturer's instructions. iScript cDNA synthesis kit (Bio-rad, 1708890) was used to reverse transcribe RNA to cDNA. Gene expression was quantified by RT PCR detection system (Bio-rad) using iQ SYBR green supermix (Biorad, 1708882). Gene expression was normalized to 18S.

Statistics

Data were presented as mean +/- SEM from independent preparations of cells. Data were analyzed by two way anova with sidak's post-hoc test or one way anova with Tukey's multiple comparison test

4.4 Results

4.4.1 A Golgi-targeted nanobody specifically inhibits Golgi-β1-AR activation in NRVMs.

Nanobody 80 (NB80) is a nanobody directed to the intracellular surface of β-ARs that competes for Gs, providing a genetically encodable β-AR inhibitor that can be directed to intracellular compartments(81). It was shown that rapamycin-induced translocation of NB80 to the Golgi apparatus inhibits β1AR signaling in HELA cells(81) and in NRVMs and that this prevents NE-dependent cardiac hypertrophy(42). To circumvent the requirement of rapamycin induction in vivo, we fused a Golgi targeting sequence from eNos(167) to target NB80 to the Golgi constitutively and mApple for visualization (eNOS-mApple-NB80). To determine if eNOS-mApple-NB80 localizes to the Golgi apparatus NRVMs were co-transfected with this construct and a Golgi marker, CFP-giantin. eNOS-mApple-NB80 strongly colocalized with the Golgi marker (Fig. 4.1A). To further confirm the functional role of eNOS-mApple-NB80, a miniGs-

Venus fusion protein sensor (mGs-Venus)(89) was utilized to reported the activity of Golgi-localized β1ARs. It can be recruited to Gs-coupled receptors upon their activation. In NRVMs, β1-ARs, mGs-Venus, and eNOS-mApple-NB80 or negative control eNOS-mApple were co-expressed, and followed by stimulation with dobutamine (Dob), a membrane permeant β1AR agonist at 100nM. In the NRVMs expressing eNOS-mApple-NB80, Dob treatment induced rapid recruitment of mGs-Venus to the plasma membrane but we did not observe mGs-Venus accumulation at the Golgi (Fig. 4.1B). Whereas in NRVMs expressing eNOS-mApple, dob induced robust NB80 recruitment to the Golgi apparatus (Fig. 4.1C). Together, these data showed that eNOS-mApple-NB80 can specifically inhibit β1-AR activation at the Golgi apparatus without interrupting those at the plasma membrane.

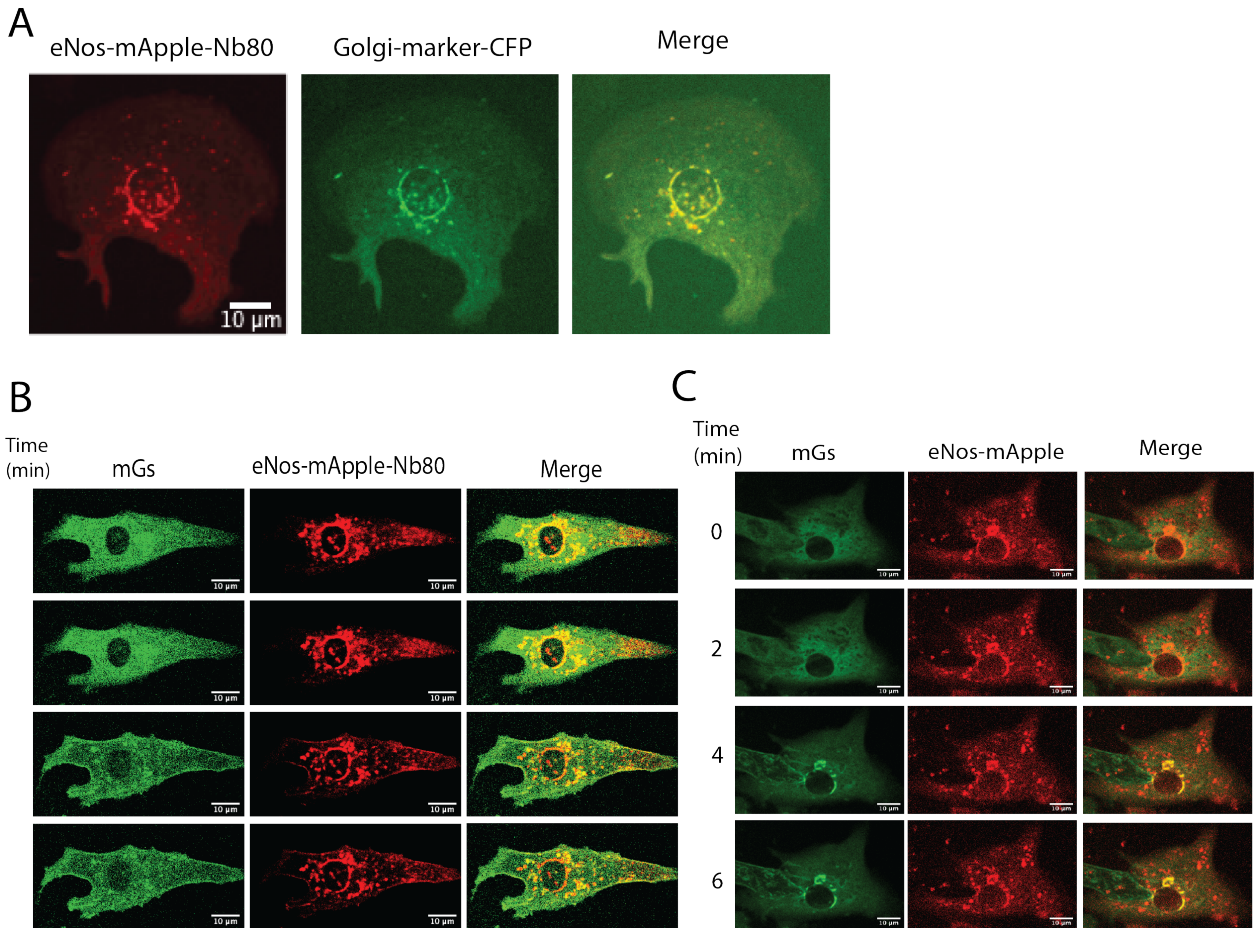


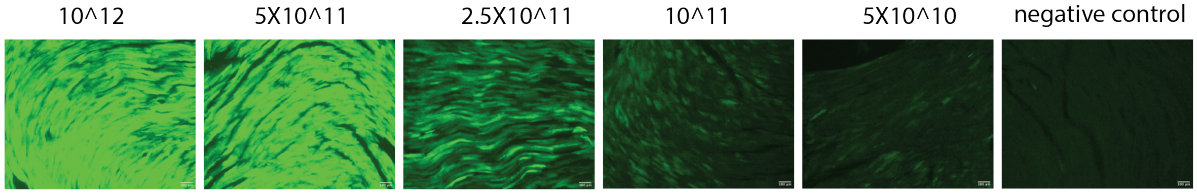
Figure 4-1 eNos-mApple-NB80 are targeted to Golgi in NRVMs.
(A) Co-localization of eNOS-mApple-NB80 with CFP-Giantin marker in NRVMs. (B) NRVMs were co-transfected with NES-Venus-miniGs and eNOS-mApple-NB80. Representative confocal images of NES-Venus-mini-Gs recruitment by dobutamine (100nM). (C) NRVMs were co-transfected with NES-Venus-miniGs and eNOS-mApple. Representative confocal images of NES-Venus-mini-Gs recruitment by dobutamine (100nM). Scale bar=10 µm.

4.4.2 The pro-hypertrophic Golgi β1AR mediated PLCε/PI4P pathway is inhibited by eNOS-mApple-NB80 in adult myocytes isolated from mice expressing eNOS-mApple-NB80.

To deliver the targeted genes into mouse heart tissue, we adopted a recombinant adeno-associated virus 9 (rAAV9) gene delivery system. rAAV9 has a tropism for the heart and the vector contains the cardiac troponin T (cTNT) promoter to direct cardiomyocyte-specific expression. To examine the rAAV9 transduction efficiency and specificity in mice hearts, we injected rAAV9-GFP into the thoracic cavity of one-week old CD-1 mice at the doses of 10^12, 5X10^11, 2.5X10^11, 10^11, and 5X10^10 viral particles/mouse. Two weeks after viral administration, cryosections were performed and GFP expression were monitored. As shown in Figure 4.2A, rAAV9-GFP transduced cardiomyocytes in a dose-dependent manner, and it is capable of transducing most of cardiomyocytes at a minimum dose of 2.5X10^11. rAAV9-eNOS-mApple-NB80, the negative control (rAAV9-eNOS-mApple), or rAAV9-GFP were administered into one-week old CD-1 male/female mice at a dose of 10^12 viral genomes per mouse, respectively. After two weeks, the expression was confirmed by western blotting (Fig. 4.2B). Furthermore, around 80% adult ventricular myocytes (AVMs) isolated from mice expressing eNOS-mApple-NB80 or eNOS-mApple showed a positive expression of a Golgi associated fluorescence confirming the Golgi specific localization (Fig. 4.2C). No transgene expression was observed in adult cardiac fibroblasts (Fig. 4.2D). To further determine if eNOS-mApple-NB80 inhibits βARs-stimulated PI4P hydrolysis, AVMs were isolated from mice expressing eNOS-mApple-NB80 or eNOS-mApple and followed by adenoviral transduction of Golgi specific PI4P biosensor, FAPP-PH-GFP (Fig. 4.2E). Dob was added and PLCε dependent PI4P depletion was assessed by monitoring Golgi PI4P associated fluorescence over time using live cell microscopy. As expected, Dob (100nM) induced a robust PLCε activation and PI4P hydrolysis in AVMs expressing the control eNOS-

mApple (Fig. 4.2E). However, Dob-induced PI4P hydrolysis was blocked by eNOS-mApple-NB80 (Fig. 4.2E) strongly support that this approach will likely block this pathway in vivo in cardiomyocytes.

A



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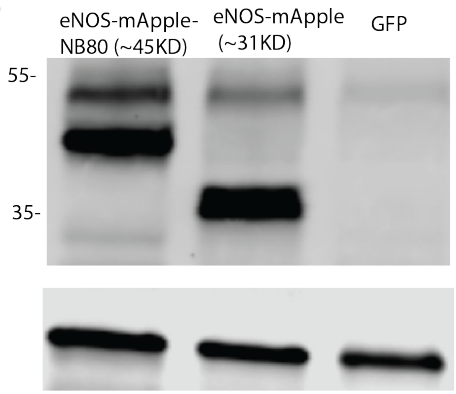

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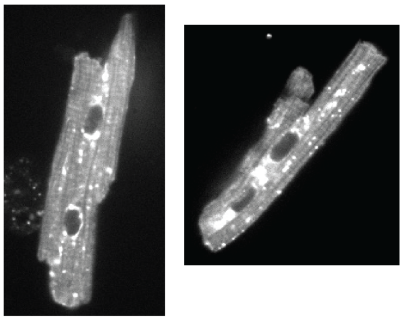
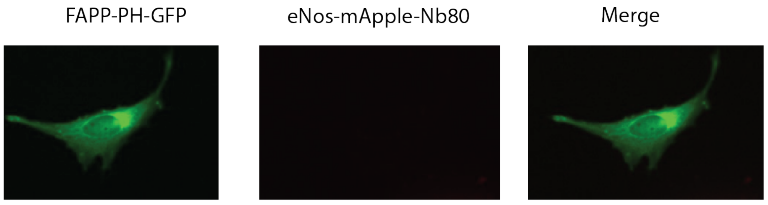


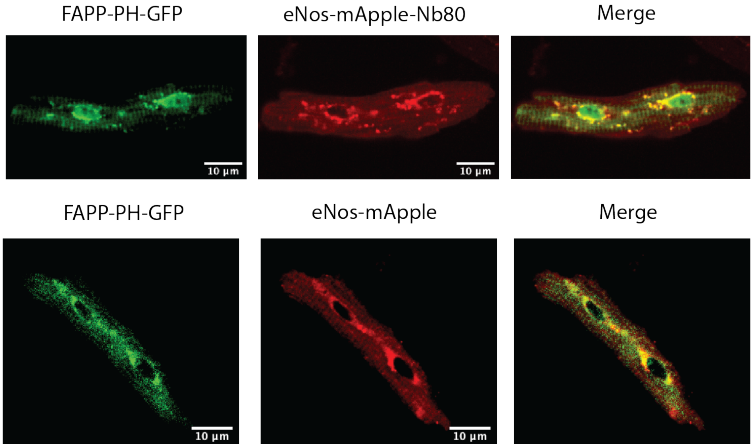
D

Adult cardiac fibroblasts



E

Adult cardiac myocytes



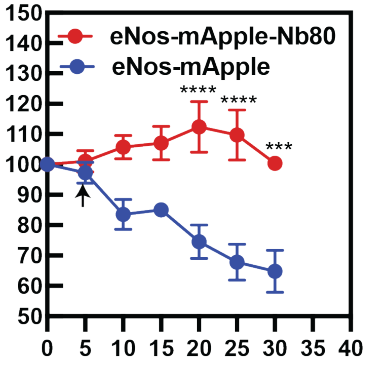


Figure 4-2 AAV9 mediated gene delivery of eNOS-mApple-NB80 in CD-1 mice.
(A) rAAV9 delivers GFP into cardiomyocytes in vivo. Representative images of heart cryosections from mice injected with increasing doses of rAAV9 viral particles. (B) Mice were administered with 10^12 eNOS-mApple-NB80, eNOS-mApple or GFP viral genomes per mouse respectively. After 2 weeks, heart lysates were analyzed by western blotting. (C) Adult ventricular myocytes were isolated from the mice overexpressing eNOS-mApple-NB80 or eNOS-mApple. Representative pictures showing Golgi specific locations of eNOS1-33. (D) Adult fibroblasts were isolated from the mice overexpressing eNOS-mApple-NB80 and followed by adenoviral transduction of FAPP-PH-GFP. (E) Adult myocytes were isolated from the mice overexpressing eNOS-mApple-NB80 and followed by adenoviral transduction of FAPP-PH-GFP. Representative images showing adult myocytes co-expressing eNOS-mApple-NB80 and FAPP-PH-GFP (left). AVMs were treated with Dob (100nM) at the arrow (right). Scale bar=10μm. Images for PI4P hydrolysis were taken from n=3-4 cells from 3 independent preparations of AVMs. Data were presented as mean +/- SEM and was analyzed by a two-way unpaired ANOVA with Sidak's pots-hoc test. *** $p<0.0001$, ****$p<0.00001$ using GraphPad Prism 9.

4.4.3 A pilot study in CD-1 mice showed potential inhibition of the Golgi βARs could prevent the development of heart failure in vivo.

To definitively define the role for Golgi-β1-ARs in the progression to heart failure, we transduced 8 CD-1 mice (4-7 days old, male and female mice) each with rAAV9 expressing either with eNOS-mApple-NB80 or GFP. After 2 months, TAC surgeries were performed. Animals were assessed by echocardiography to measure cardiac structure and function and heart tissues were analyzed by western blotting to assess the key signal transduction pathways after 6 weeks of TAC. No significant differences in ejection fraction, a measurement of cardiac function, were seen at baseline level suggesting blockade of Golgi-β1-ARs does not affect heart development or baseline cardiovascular parameters (Fig. 4.3A). However, due to technical issues, 4 animals in eNOS-mApple-NB80 group were lost. We didn't observe TAC-induced decrease in ejection fraction in GFP group in CD-1 mice (Fig. 4.3A) possibly due to this species of mice not being very responsive to TAC surgery and might require a longer time to develop heart failure in our hands. We didn't observe a decrease in heart weight to body weight (HW/BW) in eNOS-NB80 group compared to GFP group, possibly due to a lack of animals (Fig. 4.3A). We further confirmed the expression of eNOS-NB80 after TAC surgery and analyzed the key hypertrophic signaling pathways. Though the expression of eNOS-mApple-NB80 after surgery is not as strong as that prior to surgery, we still detected a significant amount of eNOS-mApple-NB80 (Fig. 4.3B). We found that mice expressing eNOS-mApple-NB80 trended toward having lower levels of ANF expression, protein kinase D (PKD) phosphorylation and ERK phosphorylation but not troponin I phosphorylation (Fig. 4.3 B, C). While these data are promising, more animals are needed to solidify the data.

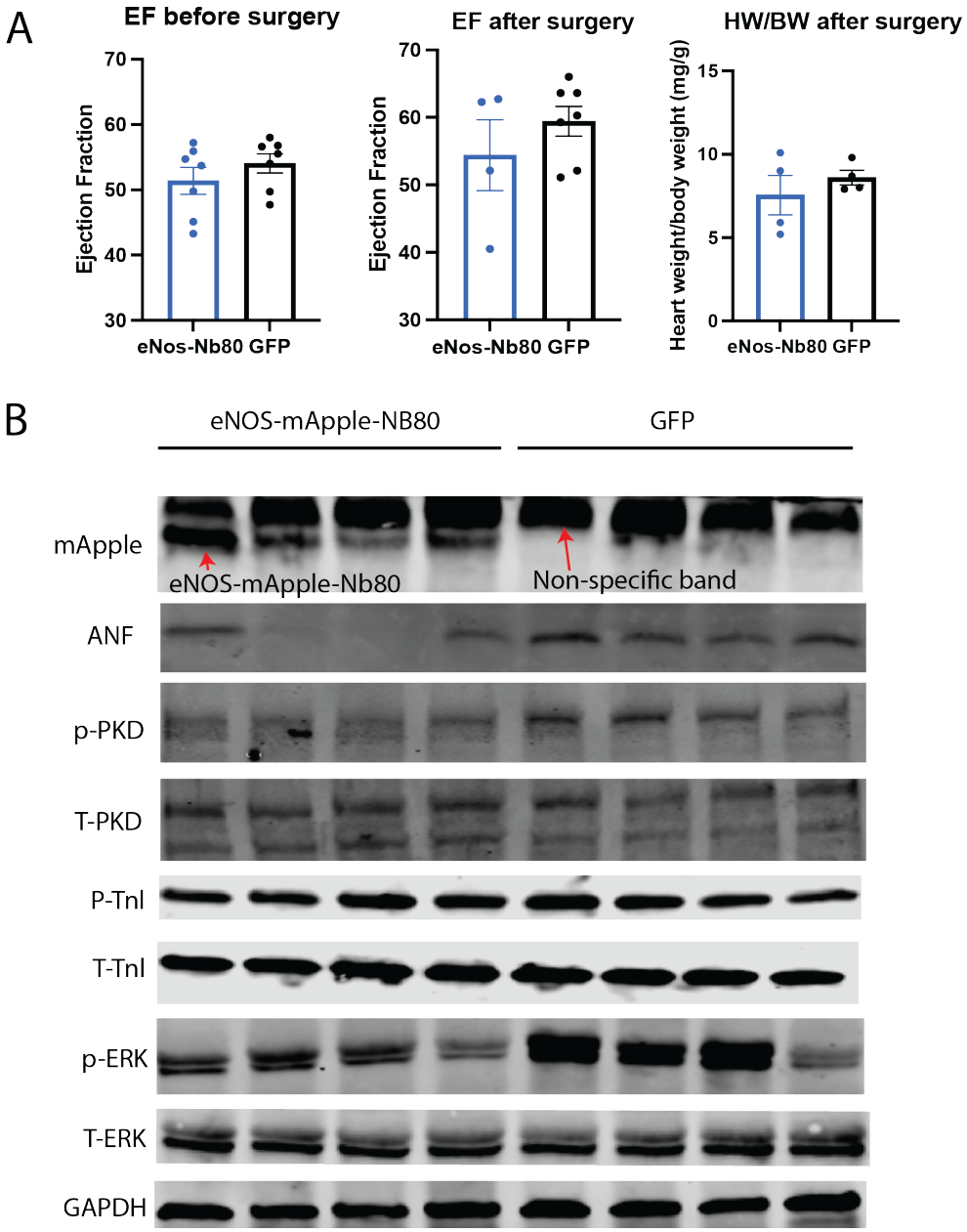

A
EF before surgery
EF after surgery
HW/BW after surgery
Ejection Fraction
Heart weight/body weight (mg/g)
eNos-Nb80 GFP
B
eNOS-mApple-NB80
GFP
mApple
eNOS-mApple-Nb80
Non-specific band
ANF
p-PKD
T-PKD
P-TnI
T-TnI
p-ERK
T-ERK
GAPDH
C
ANF
p-PKD
p-ERK
0.1014
0.1313
0.1111
ANF/GAPDH
p-PKD/T-PKD
p-ERK/T-ERK
eNos-Nb80 GFP

Figure 4-3 Analysis of cardiac hypertrophic signaling from CD-1 mouse hearts overexpressing eNOS-mApple-NB80 or GFP.
TAC was performed for 6 weeks on CD-1 mice overexpressing control GFP or eNOS-mApple-NB80 at 2-month-old. (A) Cardiac function measured by echocardiography before TAC (left), cardiac function measured by echocardiography after 6-weeks of TAC (middle), heart weight to body weight ratio after 6-weeks of TAC (right). (B) Western blots of the remaining eNOS-mApple-NB80 expression level and hypertrophic signaling pathway analysis after 6-weeks of TAC. (C) Quantified protein expression level of ANF, p-PKD, and p-ERK after 6-weeks of TAC. Data were presented as mean +/- SEM and was analyzed by an unpaired t-test. P values were presented as numeric values in the figure.

4.4.4 Golgi-targeted βAR inhibitor did not preserve cardiac function but could potentially prevent cardiac hypertrophy.

Since we didn't observe a TAC-induced decrease in ejection fraction in a cohort of CD-1 mice in the pilot study, we switched to C57BL/6J mice. Similar to CD-1 mice, C57BL/6J mice were received AAV9-eNOS-mApple-NB80, eNOS-mApple, or GFP at a dose of 10^12 viral genome/ mouse respectively. When mice were 8 weeks old, they were analyzed by echocardiography to establish baseline function, followed by TAC or sham surgery for 6 weeks and analysis of the mice by echocardiography, qRT-PCR or western blotting to determine cardiac hypertrophy and cardiac function. 6-weeks of TAC induced a significant decrease in ejection fraction in control C57BL/6J mice, however, expression of eNOS-mApple-NB80 did not rescue it (Fig. 4.4A). The increase in heart weight to tibia length induced by TAC was significantly inhibited by eNOS-mApple-NB80 relative to GFP and not to eNOS-mApple (targeting sequence control) consistent with the result of hypertrophic marker gene ANF mRNA level (Fig. 4.4 B, C). In addition, we didn't observe a significant difference of the fibrotic gene (periostin) and the inflammatory gene (MCP-1) between eNOS-mApple-NB80 and GFP (Fig. 4.4 D, E). eNOS-mApple-NB80 mice are trending toward lower ANF protein level after TAC, they did not reach the statistical difference (Fig. 4.4 F). In addition, we did not see a difference in PKD phosphorylation (Fig. 4.4 F). We further analyze the expression level of eNOS-mApple-NB80 after TAC surgery, we found a dramatic decrease in the protein expression level (Fig. 4.4 G), this could partially explain why we did not see the expected effects of inhibition of Golgi-β1ARs on TAC-induced cardiac hypertrophy and dysfunction.

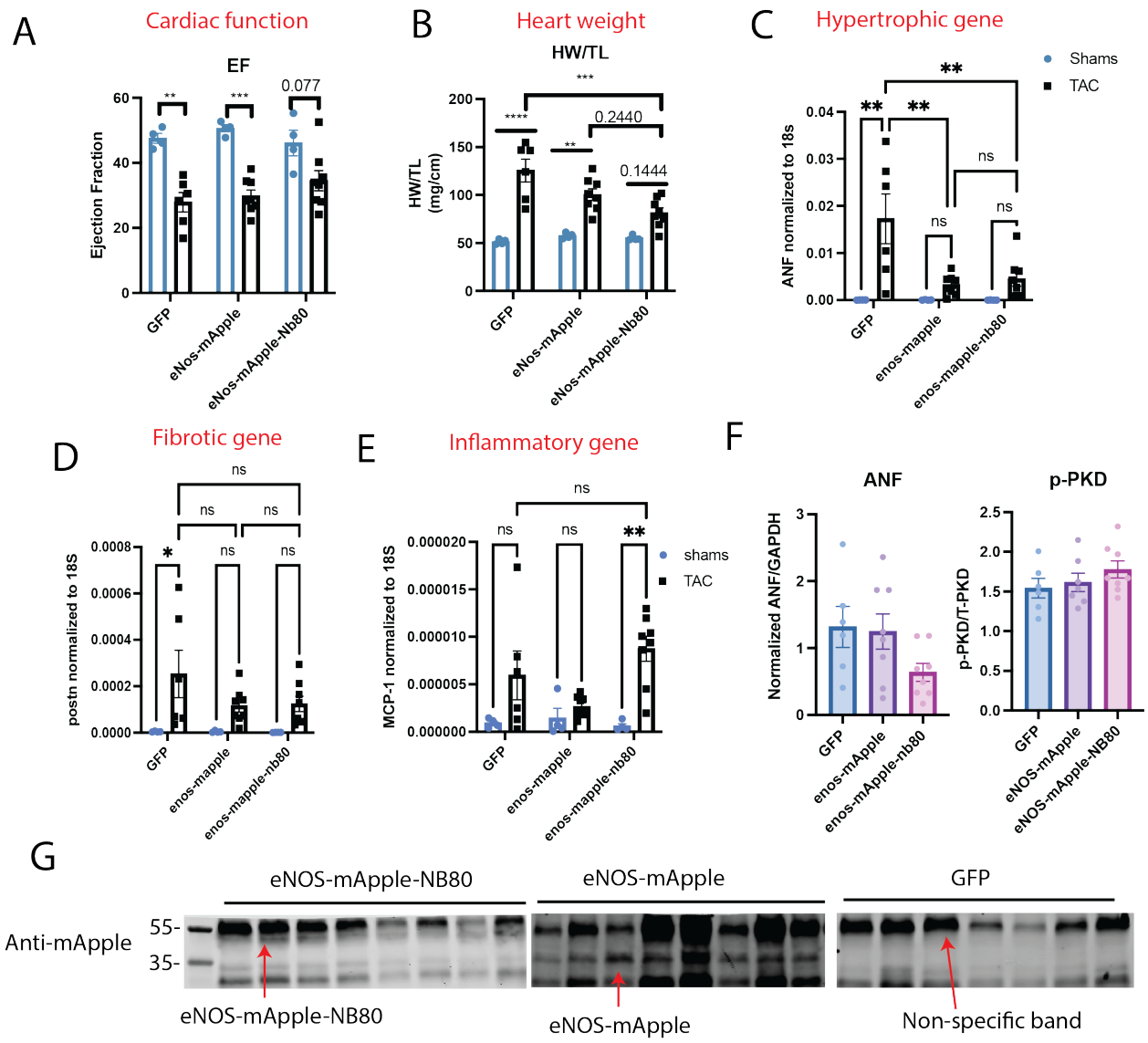


Figure 4-4 Analysis of cardiac hypertrophic signaling from C57BL/6J mouse hearts overexpressing eNOS-mApple-NB80, eNOS-mApple or GFP, respectively.
TAC was performed for 6 weeks on C57BL/6J mice overexpressing control GFP or eNOS-mApple-NB80 at 2-month-old. (A) Cardiac function measured by echocardiography after 6-weeks of TAC. (B) Heart weight to tibial length ratio after 6-weeks of TAC. RT-qPCR quantification of hypertrophic gene (ANF, C), fibrotic gene (periostin, D), and inflammatory gene (MCP-1, E) after 6-weeks of TAC. (F) Quantified protein expression level of ANF and p-PKD after 6-weeks of TAC (G) Western blots of the remaining eNOS-mApple-NB80, eNOS-mApple, and GFP expression level after 6-weeks of TAC. Data was analyzed by a two-way unpaired ANOVA with Sidak's pots-hoc test. *p<0.05; **P<0.001; ***P<0.0001; ****p<0.00001; ns, not significant using GraphPad Prism 9.

4.5 Discussion

In isolated cardiac myocytes, we have shown that Golgi localized β1ARs are the key mediators of catecholamine-dependent cardiac hypertrophy and may be key targets of β-blockers treatment in heart failure patients.(42) Here, in this chapter, we have characterized a genetically encodable β-blocker that is directed to the Golgi apparatus (eNOS-mApple-NB80) can inhibit Golgi-β1AR activation in isolated NRVMs and prevent PLCε-dependent hypertrophic signaling in AVMs. By utilizing AAV9 based approach, we delivered the Golgi targeted β-AR inhibitor specifically to cardiomyocytes in vivo to study the role of Golgi β1-ARs in cardiac hypertrophy. AAV9 mediated gene delivery is safe and efficient and can serve as a useful tool for studying cardiomyopathy in vivo and renders it a potential tool of gene therapy for humans.

Here, in our study, we observed a strain difference in ejection fraction (EF) after transverse aortic constriction (TAC). 6-weeks post TAC, CD-1 mice did not show a decrease in EF compared to that prior to surgery. In contrast, C57BL/6J mice showed a significant and consistent decrease in EF regardless of gender. In the TAC model, a constriction is placed around the transverse aorta thereby inducing a pressure-overload in the left ventricle and mimic the high blood pressure. This is often related to Angiotensin II receptor type I (AT1R) activation. It might be more involved in the release of hormones that regulate Gq or G12/13 coupled receptor. Though it has been shown β-blocker is effective in preventing TAC-induced heart failure(207), β1-AR knockout animals showed comparable responses to wild type mice(72, 73). Our hypothesis is not supported at this stage possibly due to the reason that TAC induced cardiac hypertrophy may not be driven by adrenergic tone. Also, we observed a strong reduction in the expression of Golgi-β-blocker after 6-weeks TAC surgery which could partially explain why we do not observe an expected restore in cardiac function by blockade of Golgi-β1ARs in C57BL/6J mice. We could potentially use a

catecholamine infusion induced cardiac hypertrophy and fibrosis mouse model for a duration of 2 weeks in our study. Different heart failure animals were discussed in the following section.

4.5.1 Heart failure animal models.

Based on left ventricular (LV) ejection fraction (LVEF), heart failure (HF) can be divided into heart failure with preserved ejection fraction (HFpEF) with ejection fraction >50% or heart failure with reduced ejection fraction (HFrEF) with ejection fraction <40%(208). About 50% of HF patients are afflicted with HFpEF(209). Small animal models have been generated as a useful system to decipher the mechanism of the pathogenesis of HF. The first surgical animal model for HFrEF is TAC resulting from pressure-overload by impeding blood flow across the aortic arch described by Rockman et al(210). It has been reported that the development of HF post-TAC is different between mouse strain background(211). Ischemia/reperfusion (I/R) is one of the most commonly used surgical induced myocardial infarction (MI) model to mimic HFrEF by introducing coronary artery ligation(212). Several factors including strain, gender, location and degree of occlusion can impact the development of I/R model and mortality rate(213). Chronically infusion with Angiotensin II (AngII) causes hypertension and followed by cardiac hypertrophy in mice(214). Chronically stimulation of β-adrenergic receptors with isoproterenol induces cardiomyocyte hypertrophy and fibrosis in mice possibly due to cardiomyocyte apoptosis(215, 216). There are fewer animal models that recapitulate HFpEF. Recently, it was reported that combination of high-fat diet and inhibition of constitutive nitric oxide synthase can mimic the cardiovascular features of HFpEF patients(217). This model will be useful for identification of signaling pathways and development of therapies for HFpEF.

4.5.2 Confounding issues.

One issue that confounded this study is the lack of animal numbers due to the loss of animals by AAV injection caused death and surgical complications. Also, it is hard to separate male and female animals prior to the AAV injections when the animals are neonatal pups at P7. Therefore, more animals are required to separate the analysis of male and female mice to get conclusive data.

AAV9 mediated gene expression should be sustained and stable for up to 9 months(218). However, we saw a significant decrease in the expression of eNOS-mApple-NB80 post-TAC. Somehow, the expression of the Golgi-β-blocker is downregulated in post-TAC heart tissues. To solve this problem, transgenic mice might be required in this study.

We observed that the targeting sequence (eNOS-mApple) alone could mediate a decrease in heart weight and hypertrophic marker (ANF) expression. Though it is unlikely that the first 33 amino acids of eNOS can play a role in improving cardiovascular function, eNOS is critical in maintenance of blood flow, vasodilation, autonomic reflexes and so on. The first 33 amino acids of eNOS might have other unknown effects other than Golgi targeting. The data is analyzed from a mixture of males and females. More animals are required to conclude that eNOS 1-33 itself is cardioprotective.

Overexpression of βAR inhibitors at the Golgi apparatus may affect the receptor export to the cell surface. To show the overall surface β1AR numbers in these mice are unaltered, we could isolate sarcolemmal membrane fractions and quantify βAR numbers using radioligand binding with ^{125}I-cyanopindolol. We could also isolate adult cardiomyocytes and measure Iso-dependent cAMP production.

4.5.3 Highlights.

Here, in vitro, we successfully utilized NB80 to block the activation of β1ARs confirming its effect as an inhibitor at high concentration other than a biosensor. Also, we added further evidence that PLCε activation at nuclear envelope/Golgi by Dob requires Golgi β1ARs in AVMs. Also, blockade of Golgi-β1ARs does not affect baseline cardiac function. Though it does not reach statistically significant differences for various measurements including gene expression, heart weight, and key signaling pathways involved in the regulation of cardiac hypertrophy, it shows promising statistically trends supporting our overall hypothesis. In the future, more animal numbers are required to separate males and females, possibly utilize a different Golgi targeting sequence, and catecholamine-prefusion induced cardiac hypertrophy model will be needed to definitively define a role for Golgi-β1ARs in the progression of heart failure.

Chapter 5 Discussion

5.1 Summary and significance

Here, we uncover a role for Golgi β1ARs for activation of the pro-hypertrophic cAMP-Epac-PLCε-PI4P hydrolysis pathway at nuclear envelope/Golgi interface. Importantly, blockade of intracellular β1ARs prevents cardiomyocyte hypertrophy. This work arose from the previous studies from our laboratory of investigating compartmentalized phospholipase C signaling in cardiac myocytes. Our initial observation was that in cardiac myocyte specific PLCε knockout mice produced by using cardiomyocyte-specific, tamoxifen inducible Cre (aMHC-Mer-Cre-Mer) mice crossed with PLCε floxed mice, were protected from cardiac hypertrophy in a transaortic constriction (TAC) animal model(38). By further dissecting the signaling pathways, our laboratory reported that one role of nuclear envelope localized PLCε is to produce local DAG to mediate nuclear PKD activation(38). Further studies from our laboratory found that adenylyl cyclase activator, forskolin and cpTOME, a cAMP analog that specifically activates Epac, strongly stimulated nuclear envelope PLCε activation. Surprisingly, isoproterenol, a membrane impermeant full βAR agonist, did not despite strongly elevating cAMP level in cardiac myocytes(43). To solve the puzzle, in my study, we used a membrane permeant βAR agonist, Dob, with or without membrane permeant and impermeant antagonists show that intracellular β1ARs are required to generate a compartmentalized cAMP pool that has privileged access to the Epac/PLCε/mAKAP complex. Unexpectedly, when we were analyzing dose dependent effects of Dob, we found that Dob at a concentration of 100nM induces optimal PLCε activation 10μM does

not. Dob can activate β2ARs at a higher concentration so we started to investigate the role of β2ARs in this scenario. Interestingly, we found that endosomal Gβγ released from activated internalized β2ARs can mediate ERK activation, this inhibits PLCε activity downstream of Golgi-β1ARs, Epac, and AT2R. Remarkably, activation of β2ARs inhibits AngII mediated PKD activation, one of the signaling consequences of nuclear envelope-PLCε activation, in NRVMs, AVMs and animals (Fig. 5.1). We also developed a Golgi-targeted "genetically encoded β-blocker". We characterized it in vitro and successfully delivered it into cardiomyocytes in animals by AAV9 based approach to test the role of Golgi-β1ARs in the regulation of TAC model of cardiac hypertrophy in animals.

Emerging data in the GPCR field supports the idea that GPCRs have unique compartmentalized signaling functions inside the cells(188, 206, 219). The majority of these studies have been conducted in cell lines with low relevancy to real physiology. The studies described in my dissertation are the first to demonstrate a physiological role for the Golgi-β1ARs in an architecturally complicated and physiologically relevant cell system, and we have also uncovered an associated signaling transduction pathway. In addition, β2ARs are known to exert cardioprotective effects but the mechanism is poorly understood. We are the first to report the role of endosomal β2ARs in the regulation of PLCε associated cardiac hypertrophic signaling. Consistent with recently published data in HEK cells(131), we found that endosomal β2AR signaling significantly contributes to overall ERK signaling in adult cardiac myocytes. Together, such information would lead to new more effective and specific pharmacological approaches to heart failure therapy with ligands efficiently targeted internal β1ARs and meanwhile combining a β2AR agonist.

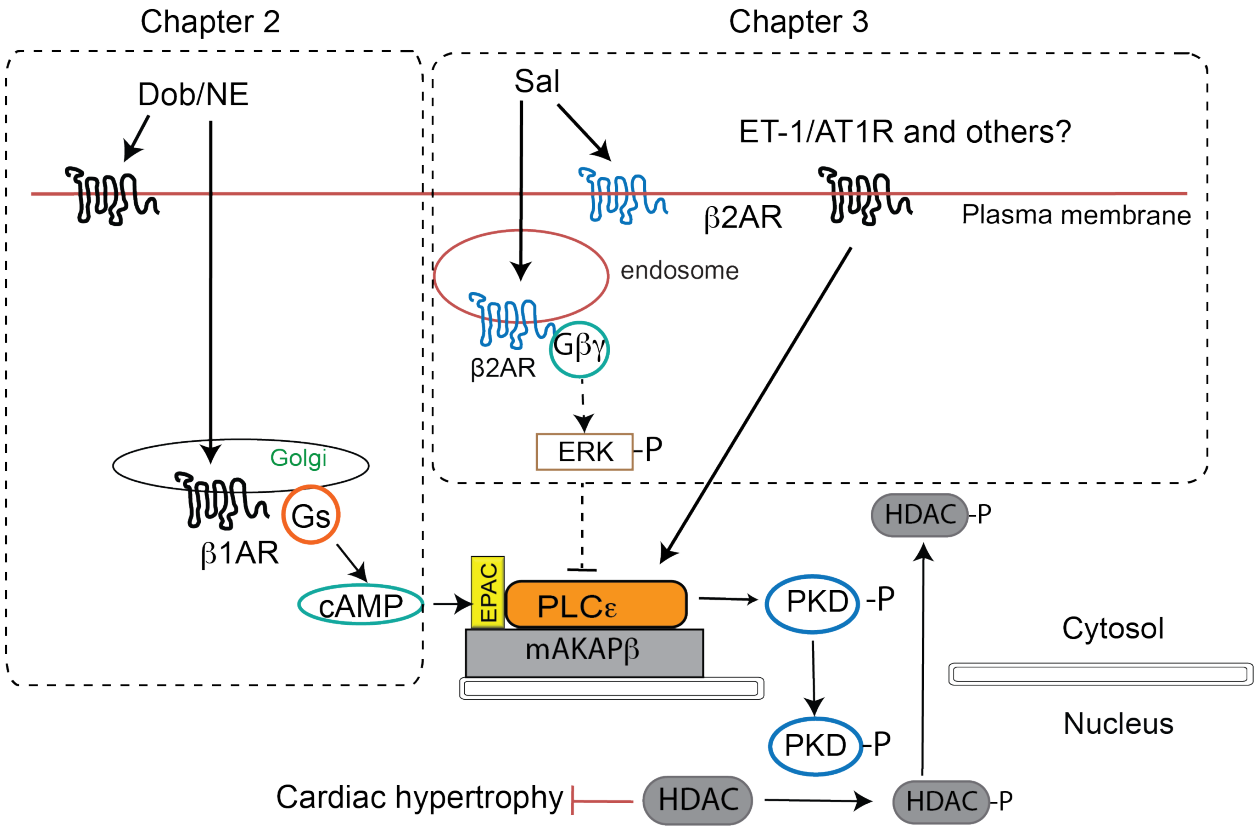


Figure 5-1 Signal transduction by Golgi β1ARs and endosomal β2ARs.
β1AR stimulates Gs and subsequent production of cAMP locally once activated in the Golgi. Local cAMP has privileged access to the Epac/PLCε/mAKAPβ complex which leads to PLCε-dependent production of local DAG from PI4P. This drives nuclear PKD activation which in turn phosphorylates HDAC resulting in sequestration of HDAC in the cytoplasm, allowing hypertrophic gene transcription. Activation of β2ARs leads to receptor internalization. Endosomal localized β2ARs are continued to signal and mediated ERK activation by releasing Gβγ and further suppress PLCε's activity which can be activated by many circulating mediators including ET-1 or angiotensin II. This could be the mechanism of the cardioprotective effects of β2ARs.

5.2 Physiological Roles for β1AR Compartmentation

What is the physiological significance of having separate pools of β1AR at the Golgi apparatus or nuclear envelope, and the sarcolemma? A possible function might be to separate short-term sympathetic stimulation from changes in gene expression. In this scenario, during fight or flight responses, acute catecholamine release would access receptors at the cell surface but would be insufficient in duration and magnitude to access intracellular pathways through OCT3. The kinetics of Golgi β1AR activation in cardiac myocytes and HeLa cells are significantly slower than the activation of these receptors at the cell surface(42, 81) perhaps because of the kinetic properties of the transporter. The efficiency and rate of uptake of catecholamines depend on the Km, Kcat, and expression level of OCT3. The km's of OCT3 for Epi and NE are in the high mM range (~500–1000 μM)(159), whereas affinities for the βARs are in the low μM range (1–15 μM)(60). One might expect that during acute stimulation, surface β1ARs would be rapidly stimulated, but NE or Epi may not reach sufficient concentrations long enough for significant uptake by OCT3. Under chronic cardiac stress and sustained catecholamine elevation, NE and Epi could achieve sufficient concentrations long enough to accumulate intracellularly and access the Golgi pool of β1ARs to regulate gene expression through the mAKAP/EPAC/PLCε pathway. This mechanism would prevent inappropriate activation of PLCε-dependent cardiac hypertrophic responses to acute catecholamine exposure. In addition, with sustained sympathetic stimulation and development of heart failure, β1AR signaling from the plasma membrane is significantly blunted. Under these conditions, Golgi-β1AR–mediated signaling may become more prevalent. Having separate internal β1AR pools also likely allows cells to generate precise signals that regulate specific cellular responses instead of activating uncontrolled global signal events. These speculations remain to be experimentally verified.

5.3 β2AR agonists as a potential therapeutic strategy in the treatment of HF

It is now well-established that the excessive β1-AR stimulation leads to pathogenesis of HF. B-blocker combined with other medications including angiotensin system blocker and diuretics is the standard of care for patients with HFrEF. One of the key mechanisms for the effectiveness of β-blockers is to restore the responsiveness to catecholamines(220, 221). Considering that the expression level of β1ARs drops by 50% and the expression level of β2ARs remains unchanged in HF, the mechanism can only apply to the blockade of β1ARs. It is controversial whether to use β2-agonists in the treatment of HF. Long acting β2AR agonists (LABAs) have been suggested to associated with the increased risk of adverse cardiovascular events(222, 223). But a recent meta-analysis showed that LABAs are not associated with overall cardiovascular adverse events(224). It is also possible that the adverse effects associated with β2AR agonists is off-target stimulation of β1ARs.

The cardioprotective roles of β2ARs have been suggested in a large body of studies. As we have discussed in chapter 1, several studies confirmed the observations that a low to mild level of β2AR overexpression confers enhanced atrial contractility and left ventricular functions in mice(62-65). It has been shown that mice with β2AR deletion led to development of exaggerated hypertrophy in TAC-induced cardiac hypertrophy(73). In CHW-1102 cells, Thr164Ile mutation in the ADRB2 (β2AR) gene displayed a lower binding affinity for catecholamines including epi, NE, Iso and agonists-induced internalization(225). Strikingly, HF patients harboring this loss of function Thr164Ile mutation displayed a higher risk of death compared to wild type Thr164(226). An in vitro study showed enhanced agonist-mediated downregulation of β2AR expression when substitute Arg with Gly at amino acid 16(227). A recent study reported that HF patients harboring

Arg16Gly allele also have an increased risk of cardiovascular death and importantly, β2AR Arg16Gly variant is associated with the defective β2AR-Gi signaling(228). β2AR-Gi signaling has been implicated in the antiapoptotic effects in cardiac myocytes(195). It has been suggested that β2AR-Gs signaling is also cardioprotective. This idea is supported by several studies showing a Gs-baised β2AR agonist fenoterol is beneficial in different animal models of heart failure(199, 229-232). Both β1ARs and β2ARs have been shown to mediate tyrosine kinase receptor transactivation to promote cardiomyocyte survival(175, 196, 233). The mechanism of action has been associated with a β-arrestin dependent but G protein-independent transactivation of EGFR(234).

Consistent with these findings, our data suggest a protective role for a β2AR agonist (salmeterol) in the prevention of cardiac hypertrophy. The mechanism involves in the inhibition of nuclear envelope PLCε activation through endosomal Gβγ. Most β2AR agonists activate both Gs and Gi, however, it has been shown that fenoterol selectively activates Gs (232). It will be worth measuring the effects of fenoterol in the regulation of the activity of PLCε. Fenoterol is possible to inhibit the activation of nuclear envelope PLCε activation through Gs-cAMP-PKA pathway. Overall, the evidence from both basic and translational work favors the hypothesis that β2AR agonists can be used in the treatment of HF.

5.4 Future directions

5.4.1 On and off states of endosomal Gβγ signaling.

Though we have shown that ERK could be downstream targets of endosomal Gβγ, the molecular mechanism by which endosomal Gβγ activate ERK is unclear. To gain the mechanistic understanding of this cellular process, the spatial interactome mapping of endosomal Gβγ is needed to find some endosomal specific targets. In order to identify its proximal neighbors, we

could employee split proximity labeling methods. Split-TurboID is consist of two fragments, the Tb (N), an N terminal fragment, and the Tb (C), a C terminal fragment. These two fragments can be brought together to reconstitute an active enzyme when protein-protein interaction happens(235). Thus, we could fuse one fragment of split-TurboID with endosomal targeting sequence (FYVE domain) and the other fragment with Gβγ. We could compare treatment of vehicle, salmeterol (activates both Gs and Gi), and fenoterol (activates Gs) in split-TurboID expressing cells and focus on the targets that is significantly enriched in the salmeterol group. Also, we could target two fragments of split-TurboID (the N and C terminal fragments) to endosome to determine the background endosomal targets.

The question of how to turn off the endosomal Gβγ signaling downstream of β2ARs has been raised. It has been reported that the deactivation of ER-localized Gβγ downstream of D2R requires reassociation with Gαo at the plasma membrane(121). They showed that Gαo remains entirely at the plasma membrane after activation. To test if the deactivation mechanism could apply to our system, we could utilize a similar location-specific BRET strategy. By monitoring the BRET signaling produced by the interaction of Venus-Gβγ and GRK2CT-Nluc fused to an endosomal targeting sequence, we could monitor the association and dissociation of Gβγ from the endosomal membrane. To determine if endosomal Gβγ signaling termination is driven by Gαi deactivation at the plasma membrane, we could overexpress plasma membrane targeted RGS proteins and measure the deactivation rate of Gβγ at endosomes.

5.4.2 Monitor endogenous Golgi-localized β1-adrenergic receptor activation by proximity-dependent transcription factor release.

Though in chapter 2, we have shown that in isolated NRVMs Golgi-β1-AR activation by NE, can be detected by overexpressing β1ARs and a fluorescent tagged miniG protein,

successfully monitoring of the activation of endogenous Golgi-β1ARs in vivo under pathological conditions would add further evidence for a functional role of Golg-β1ARs. Thus, designing a tool to sensitively detect the endogenous Golgi-localized β-ARs activation is valuable.

To enable the selective detection of Golgi-β-AR activation, we could employee a genetically encodable tool that utilizes proximity-dependent transcription factor release, previously designed to monitor GPCRs at the plasma membrane, modified to monitor GPCR activation at the Golgi. Specifically, we could modify the approach called Tango assay(236). For our design a transcription factor with a protease cleavage site would be targeted to the Golgi apparatus by fusing to a Golgi-targeting sequence (eNOS-Flag-TEVcs-tTA). NB80 is a nanobody that can bind to the activated βARs. By fusing a protease to NB80 (NB80-GFP-TEV), the protease will be recruited to the sites where β-ARs are activated. Upon the activation of Golgi-β1ARs, NB80 with the protease can recognize the protease cleavage site at the Golgi apparatus resulting in transcription factor release. Then the released transcription factor will translocate to nucleus and drive the expression of mCherry from a TRE-mCherry reporter plasmid (Fig. 5.2). Because this signal is durable we can isolate the hearts and monitor mCherry expression in tissue sections. We could apply this system in animal models of TAC-induced cardiac hypertrophy, or infusion of dobutamine or catecholamines with different membrane permeabilities via osmotic pump implantation into animals. However, challenges would be to successfully infect cells with correct molar ratios of different plasmids of this system to actively monitor the activation of receptors in vitro and to transition from in vitro to in vivo by AAV9 gene-delivery system. This system could also be employed in other intracellular compartments such as SR. This will characterize the activity of Golgi-β1ARs under disease condition VS healthy condition in vivo and inform new strategies for the development of β-blockers for heart failure.

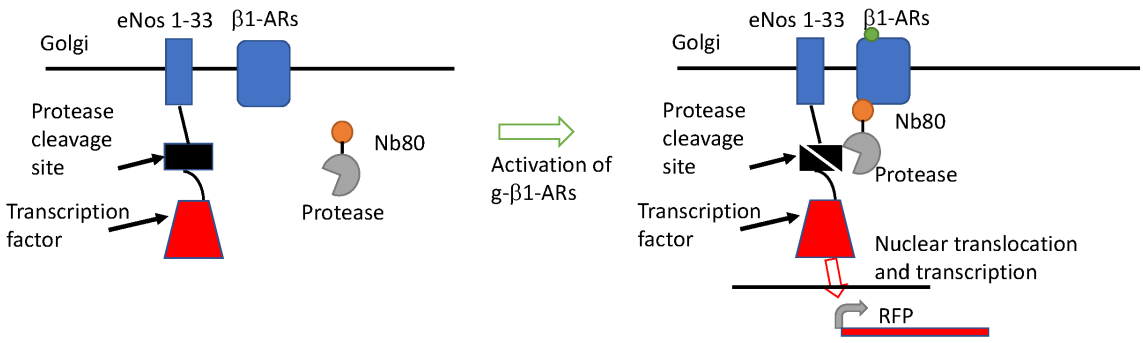


Figure 5-2 Detect endogenous Golgi-β1-AR activation in vivo.
A transcription factor with a protease cleavage site is targeted to the Golgi apparatus. Upon the activation of Golgi β1-ARs, NB80 (β-AR biosensor) fused with a protease will be recruited to the Golgi where the receptor is activated. Then the protease will interact with the protease cleavage site and the transcription factor (tTA) will be released, further driving the expression of mCherry (TRE-mCherry).

5.4.3 Blockade of Golgi-β1ARs in mouse models of norepinephrine infusion induced hypertrophy and fibrosis.

In chapter 2, we defined a prohypertrophic signaling pathway associated with Golgi-β1ARs in isolated cardiomyocyte system in vitro. Based on our data, Golgi-β1ARs are necessary for the development of hypertrophy in response to elevated catecholamines in vitro. β-blocker used treatment for HF includes bisoprolol, metoprolol, and carvedilol. These all have relatively high lipid solubility. Bisoprolol and metoprolol are β1-selective, but carvedilol is non-selective. Among these β-blockers, carvedilol has been shown to activate ERK by a process dependent on β2AR activation and β-arrestin recruitment(198) but others showed that carvedilol activates ERK dependent on β2AR-Gαs signaling and does not require β-arrestin(237). Nevertheless, the positive effects on β2AR activation could contribute to its superior effects in the treatment of HF. Overall, the higher lipid solubility and selectivity of β1ARs are often associated with higher clinical efficacy. Thus, these observations lead to the hypothesis that the site of action of the clinically effective β-blocker is the Golgi-β1ARs.

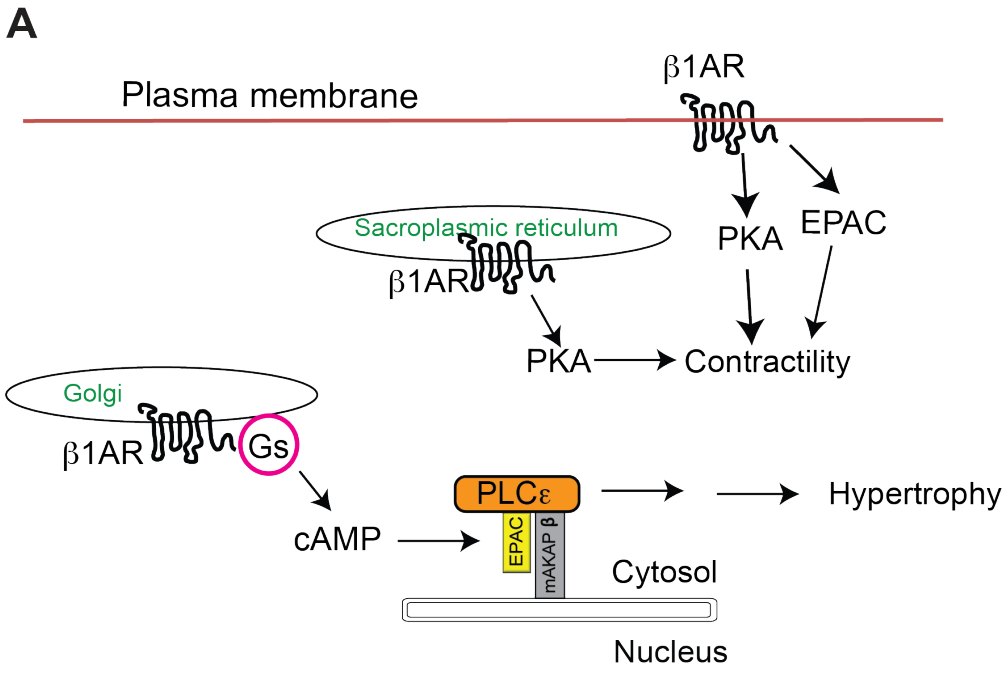
To test role of Golgi-β1ARs in a physiological in vivo model of cardiac hypertrophy, we could utilize osmotic pumps to chronically infusion animal with a series of catecholamines that have different membrane permeabilities. C57BL/6J mice overexpressing Golgi-targeted β-blocker or the targeting sequence that we used in chapter 4 would be implanted with osmotic minipumps filled with vehicle, Iso (a membrane impermeant agonist), Dob (a membrane permeant agonist), or NE (transported into cells via OCT3) respectively. In addition, NE requires active transportation through OCT3 to pass the plasma membrane but dobutamine enters cells through passive diffusion. To further validate the requirement of intracellular β1ARs, we could co-infuse an OCT3 transporter inhibitor with NE or dob in wild-type animals. Furthermore, we could identify key

signal transduction pathways and downstream genes associated with Golgi-β1ARs by analyzing these heart tissues.

5.4.4 Determine the role of PM and SR localized β1ARs function.

A recent study demonstrated that functional β1ARs localize at SR and are critical in regulating contractility dependent on PKA mediated phospholamban phosphorylation(97). In this study, they utilized OCT3 knockout animals and membrane permeant/impermeant antagonists/agonist and followed by measurement of contractility. This work adds further evidence of intracellular β1ARs in the regulations of contractility, however, these strategies cannot separate SR from other intracellular compartments like Golgi. Thus, the role of SR-β1ARs in contractility needs to be more carefully and explicitly examined. To determine the role of SR-β1ARs, we can create an SR targeted NB80 to inhibit β1AR specifically at SR but not other intracellular compartments, a similar strategy used to create Golgi-NB80. In animals expressing SR targeted β-blocker, cardiac contractility can be measured by echocardiography or pressure/volume loop (PV loop) analysis after acute IP injection of varying agonists with different membrane permeabilities.

Current evidence suggests roles for Golgi β1ARs in mediating cardiac hypertrophy, SR β1ARs in mediating cardiac contractility, and PM β1ARs regulating contractility through PKA and Epac-CamKII signaling(37)(Fig 5.3A). However, the effects of specific blockade of PM β1ARs has not been examined. To definitely determine the function of PM β1ARs relative to other locations, we created plasma membrane targeted NB80 (FYN-NB80) as shown in Figure 5.3B. We could use similar strategy for SR-NB80 to determine the role of PM β1ARs in the regulation of cardiac contractility.



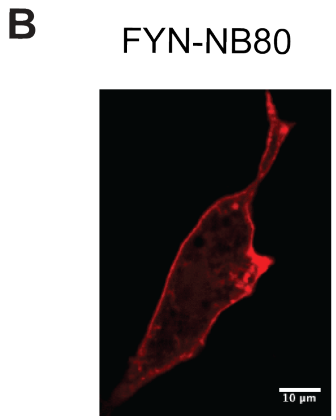


Figure 5-3 The roles of β1ARs in the regulation of cardiac function.
(A) β1ARs are resident in the plasma membrane, Golgi apparatus and sarcoplasmic reticulum. Activation of Golgi-β1ARs generates a local cAMP pool that induces cardiomyocyte hypertrophy through mAKAP/EPAC/PLCε pathway. Golgi β1 ARs likely also regulate PKA and may contribute to contractile or other processes. β1ARs at sarcoplasmic reticulum modulate contractility through PKA-dependent phospholamban phosphorylation. Plasma membrane resident β1ARs can aksi regulate contractility via PKA, or CamKII downstream of EPAC. (B) Representative images of NRVMs expressing FYN-NB80. Scale bar, 10μm.

5.4.5 Measure localized cAMP generation at different intracellular compartments.

There is substantial evidence showing localized degradation of cAMP by PDEs to create cAMP microdomains(238). However, with emerging ideas of intracellular β1ARs at Golgi or SR, additional mechanism that contributes to cAMP in distinct microdomains could be the residence of Golgi or SR β1ARs. To monitor the spatially restricted cAMP generated by Golgi or SR β1ARs, we could utilize the Golgi/SR targeting sequences fused to a cAMP sensor. A fluorescence resonance energy transfer (fret)-based cAMP sensor, CUTie has previously been used for spatial measurement of cAMP in adult cardiac myocytes(104). AVMs could be transduced with the Golgi or SR targeted CUTie. Cells can then be stimulated with NE or Dob under various conditions: 1) in cells with and without pretreatment with an OCT inhibitor (corticosterone), 2) with or without membrane permeant (metoprolol) or impermeant (sotalol) β1-ARs antagonists. Targeted CUTie FRET changes in specific locations can be monitored to indicate the cAMP pools can be actively generated by β1ARs at Golgi or SR.

The demonstration of the presence of adenylyl cyclase (AC) at the Golgi or other intracellular compartments is difficult due to the inadequacy of AC antibodies and the likely low levels of this enzyme. Nevertheless, binding of AC5 to mAKAPβ in cardiac myocytes has been previously demonstrated. Successful detection of cAMP pools downstream of intracellular β1ARs can be indirect evidence of the presence of subcellularly localized AC.

5.4.6 Determine the role of endosomal Gβγ released by β2ARs in cardiac signaling.

We proposed that endosomal β2-Gβγ-ERK signaling axis is cardioprotective via inhibition of nuclear envelope localized PLCε. However, this is only experimentally verified in vitro. To test this in vivo, we could overexpress FYVE-GRK2CT, the endosomal targeted Gβγ inhibitor, or FYVE targeting sequence as a negative control, using rAAV9 in cardiomyocytes. Animals would

be implanted with osmotic minipumps containing AngII, or AngII and salmeterol. Heart tissue analysis of hypertrophic responses and RNAseq can be performed to determine the role of endosomal Gβγ in cardiac signaling.

Also, AVMs/NRVMs expressing FYVE-GRK2CT or FYVE could be treated with salmeterol, a highly selective β2 agonist, to definitely determine if endosomal Gβγ downstream of β2ARs contributes to the ERK activation. Endosomal ERK activity can be monitored by a ERK fret reporter (EKA4) fused with endosomal targeting sequence. By utilizing FYVE-GRK2CT and FYVE-EKA4, we can definitely determine the role of endosomal Gβγ in the regulation of endosomal ERK activation. In addition, by targeting ERK fret reporter (EKAR4) to endosomes, we could be able to monitor endosomal ERK activity in response to a series of β2-agonists with different kinetics in mediating receptor internalization or preferences in activating Gs or Gi.

5.4.7 Define the molecular mechanisms of β1AR retention at the Golgi apparatus during biosynthesis.

Transport of newly synthesized GPCRs from the Golgi apparatus to the plasma membrane is highly regulated(239). GPCR retention at the Golgi apparatus is controlled by the structural features of the receptors as well as the C-terminal interacting proteins. β1ARs but not β2ARs are localized at the Golgi/perinuclear regions in cardiac myocytes(92). This is likely due to the differential potential of the two receptors to bind scaffolding or transport proteins. The greatest difference between β1ARs and β2ARs is the C-terminal region. It has been shown that overexpression of PIST (protein interacting specifically with Tc10) enhances the retention of β1-ARs at the Golgi apparatus via the interaction with C-terminal PDZ ligand motifs(240, 241). Also, proteins such as golgin-160 interact with β1ARs to regulate receptor expression at the plasma membrane(242).

To determine the domain within β1ARs involved in the regulation of its Golgi retention, we can convert C-terminus of PDZ binding domain, the last 50 amino acids in the C-terminus, or the whole C-tail (100 amino acids) of β1-ARs into the corresponding C-terminus of β2-ARs. The seven transmembrane helices of GPCRs are connected by three extracellular loops (ECL) and three intracellular loops (ICL). ECL2 is the most diverse loop among the three loops and is essential in ligand selectivity, receptors stability, and activity(243). It has been demonstrated that ECL2 regulates the cell surface expression and degradation of adhesion G protein-coupled receptor 2(244). Therefore, we could also convert the ECL2 of β1-ARs into the corresponding ECL2 of β2-ARs.

Furthermore, we could characterize proteins that specifically interact with the domain responsible for the Golgi-β1-AR retention and evaluate its functional role in NRVMs. We could purify the identified domains of β1ARs and the corresponding domains of β2ARs and scramble peptide. We could incubate the three peptides respectively with the NRVMs/AVMs cell lysates and submit for mass spectrometry and we will focus on the novel targets known to localize at the Golgi apparatus.

5.5 Closing remarks

The GPCR pharmacology has entered a new era of studying spatially biased signaling. The signaling profiles change in response to discrete spatially distributed receptors, bringing new insights in designing the "location bias" therapies. The work presented here have uncovered an opposing role of Golgi-β1ARs and endosomal-β2ARs in regulating the activity of PLCε and therefore cardiac hypertrophy. This informs a novel therapeutic approach of combining a β2 agonist with hydrophobic β1 selective blocker. Potentially, in the future, we could use AAV based approach to target Golgi β1ARs as a new therapeutical strategy to combat cardiac diseases.

Moreover, the comprehensive mapping of the connections between GPCR locations and functions holds the tremendous implication in achieving functional selectivity and strategically design therapies to avoid undesired effects.

5.6 Contributions

Alan V Smrcka provided Figure 2.8 and contributed to conceptualization, editing and preparation of the figures and text.

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