CaMKII Nitrosylation in Cardiac Myocytes at Cys273 suppresses activation, but at Cys 290 mediates intrinsic mechanical afterload-induced enhancement of Calcium transients

By

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Molecular, Cellular, Integrative Physiology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2021

ACKNOWLEDGEMENT

My people say it takes a village to raise a child. My journey to this PhD is a testament of this fact. I wholeheartedly thank everyone who has been instrumental in my path so far, my friends and mentors, my advisors and committee members, my colleagues in the lab and other MCIP cohort, all my students past and present and finally my nuclear and extended family. Thank you for the outstanding love and support while I strived to contribute a spec in the big circle of known scientific facts – you are my village.

"Not to know is bad; not to wish to know is worse."

- **Nigerian Elders**

ABSTRACT

Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) is a key regulator of cardiac function and dysfunction in pathological states via its influence on ion channels, Ca^{2+} balance, Ca^{2+} handling proteins involved in cell death, transcriptional activation of hypertrophy, inflammation, and arrhythmias. CaMKII signaling regulates diverse cardiac cellular processes including excitation-contraction coupling, excitation-transcription coupling, mechanics and energetics. Chronic activation of CaMKII results in significant cellular remodeling and alterations in Ca^{2+} handling, ion channel function, cell-to-cell coupling and metabolism leading to increased risk of atrial and ventricular arrhythmias. The prominent role of CaMKII is also well-established in the pathophysiology of several common heart diseases, including cardiac hypertrophy, heart failure, ischemia-reperfusion injury and post-myocardial infarction (MI) remodeling, atrial fibrillation and ventricular arrhythmias. Pharmacological or genetic inhibition of CaMKII limits arrhythmias and progression of HF, so CaMKII is widely considered a bona fide drug target for heart disease. Post-translational modifications of CaMKII include *S*-nitrosylation of Cys273 and Cys290. Notably, while *S*-Nitrosylation of Cys290 on CaMKII promotes autonomous kinase activity, *S*-Nitrosylation of Cys273 suppresses activation by Ca-CaM.

Mechanical stress can affect Ca^{2+} dynamics in cardiomyocytes and lead to cardiac remodeling, hypertrophy, arrhythmias, and heart failure. Mechanical loading can also evoke physiological adaptations that help the heart cope with increased preload or end-diastolic volume (via the Frank-Starling effect) and with afterload or aortic pressure (via the Anrep effect). Both nitric oxide (NO) produced by NOS1 and CaMKII signaling are required mediators of mechano-chemo-transduction (MCT) whereby mechanical afterload promotes enhanced Ca^{2+} transients

and stronger contraction. To test whether Cys290 in CaMKIIδ mediates the acute *S*-nitrosylation of CaMKIIδ that promotes increased sarcoplasmic reticulum (SR) Ca release, we developed a novel CaMKIIδ knock-in mouse (C290A substitution). This knock-in mouse exhibited normal cardiac size and function, myocyte Ca transients and β-adrenergic responses. However, compared to wildtype (WT) littermates the C290A myocytes failed to exhibit an increase in spontaneous SR Ca^{2+} release events (Ca^{2+} sparks) in response to *S*-nitrosylating agent, GSNO. Next, we tested whether this single amino acid on CaMKIIδ is necessary for afterload-induced increase in Ca^{2+} sparks and Ca^{2+} transients, using our cell-in-gel system to exert multiaxial 3D mechanical stress during contraction. In WT myocytes, mechanical afterload increased Ca^{2+} spark frequency, Ca^{2+} transient amplitude and SR Ca uptake vs. load-free WT myocytes, which enhances contractility to better meet mechanical afterload against which the myocyte or heart has to work. All of these MCT effects were abolished in either cardiac-specific CaMKIIδ knockout mice, or the point mutant C290A CaMKIIδ knock-in myocytes. Thus, data here shows that CaMKIIδ activation by nitrosylation at the Cys290 site is essential to acute mechanical-stress induced Ca^{2+} upregulation in cardiomyocytes. They also suggest that NOS1 activation is upstream of *S*-nitrosylation at Cys290 of CaMKII and enhanced SR Ca uptake and release in this intrinsic MCT autoregulatory pathway in the heart. Since NO and CaMKII signaling are altered in disease, this may raise novel therapeutic strategies for treating cardiac disease.

I also investigate the effect of the post translational modification - nitrosylation - on CaMKII Cys273 via Fluorescence Lifetime Imaging Microscopy (FLIM) and Fluorescence Recovery After Photobleaching (FRAP). Results show that nitric oxide (NO) alters CaMKIIδ activation state and mobility in adult cardiomyocytes. I then investigate the relevance of the Cys273 site on CaMKIIδ for NO-induced suppression of CaMKIIδ activation and mobility in adult ventricular

myocytes. The data derived shows for the first time in ventricular cardiomyocytes, that nitrosylation of CaMKII at Cys273 prior to electrical field stimulation and activation by Ca^{2+} or β-adrenergic agonists, suppresses CaMKIIδ activity and mobility – whereas mutation of the Cysteine residue at 273 to Serine, eliminates this effect.

The multifunctional signaling molecule CaMKII has received considerable attention over recent years for its central role in maladaptive remodeling and arrhythmias in the setting of chronic disease. Hopefully, with continued research, these basic science discoveries will contribute to a greater understanding of cardiac function and heart disease and ultimately translate into new therapies for human patients.

Chapter 1:

Understanding the *S*-Nitrosylation of CaMKII

The *overall goal of this dissertation is to understand how nitrosylation regulates CaMKIIδ signaling in the heart*. This introduction chapter is in two parts: first an overview of Nitrosylation and then an overview of CaMKII signaling in cardiomyocytes.

1. Nitrosylation Overview

1.1. **Background and Introduction**

Nitric Oxide (NO) was initially discovered in the vascular endothelium as an endothelium-derived relaxing factor (EDRF) in 1980 (Furchgott & Zawadzki, 1980). It has since emerged as an integral signaling molecule with numerous molecular targets. As a free radical with a single, unpaired electron, it is highly labile (a half-life of only a few seconds or less) and chemically reactive (Phaniendra et al., 2015). Thus, the bioactivity of NO can be partially compromised because it rapidly reacts with numerous inactivating species present in the bloodstream and cellular milieu (Subelzu et al., 2015). Reduced protein thiol groups were reported decades ago to serve as rich NO carriers that could not only stabilize NO and extend its half-life but also protect its biological activity from oxidative inactivation (Cooke et al., 1990). Via this route, NO controls several regulatory functions. In mammals, it acts as a signaling molecule in the nervous and cardiovascular systems and has cytoprotective and cytotoxic effects (under disease conditions) in the immune system's response to infection. Disruptions in NO signaling are linked to

hypertension, erectile dysfunction, neurodegeneration, stroke, and heart disease (Condorelli et al., 2013; Serafim et al., 2012). Here I review the production, spatiotemporal regulation and post translational modifications of NO, specifically as relates to the cardiovascular system.

1.2. **NO Synthesis**

NO, originally discovered as endothelium-derived relaxation factor is an endogenous, gaseous signaling molecule that is produced by: 1) nitric oxide synthase and 2) nitrate reduction.

1.2.1. Nitric Oxide Synthase

Physiological NO is synthesized by L-arginine through three isoforms of the enzyme nitric oxide synthase (NOS). Each NOS isoform is encoded by a distinct gene and all consist of an N-terminal oxidase domain and a C-terminal reductase domain (Campbell et al., 2014). Shown in Figure 1.1, the isozymes are referred to as neuronal NOS (nNOS; encoded by NOS1) (Knowles et al., 1989), inducible NOS (iNOS; encoded by NOS2) (Lowenstein et al., 1992; Stuehr & Marletta, 1987), and endothelial NOS (eNOS; encoded by NOS3) (Palmer & Moncada, 1989). They all utilize l-arginine and molecular oxygen as substrates and require the cofactors reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6*R*-)5,6,7,8-tetrahydrobiopterin (BH4). All NOS bind calmodulin and contain a ferric heme cluster (Stuehr & Haque, 2019). NOS functions as a dimer and must incorporate heme to dimerize (Baek et al., 1993; Klatt et al., 1996; Rodríguez-Crespo et al., 1996). Upon NOS activation, the flavins in the reductase domain transfer NADPH-derived electrons to the heme in the oxygenase domain of the other monomer, allowing oxygen (O_2) binding on the reduced heme iron (Fe^{2+}) and the conversion of L-arginine to hydroxy-L-arginine, then NO and L-citrulline (Kwon et al., 1990). The recruitment of the Ca^{2+}/calmodulin complex is essential for this transfer of electrons to occur, however, the CaM-binding site of NOS2 can bind Ca^{2+}-free CaM

(ApoCaM) with high affinity (Busse & Mülsch, 1990; Cho et al., 1992). In NOS1 and NOS3, calmodulin binding occurs with an acute increase in intracellular Ca^{2+} concentrations (between 200-400 nM) (Bredt & Snyder, 1990; Knowles et al., 1989; Pollock et al., 1991). Ca^{2+}-Calmodulin

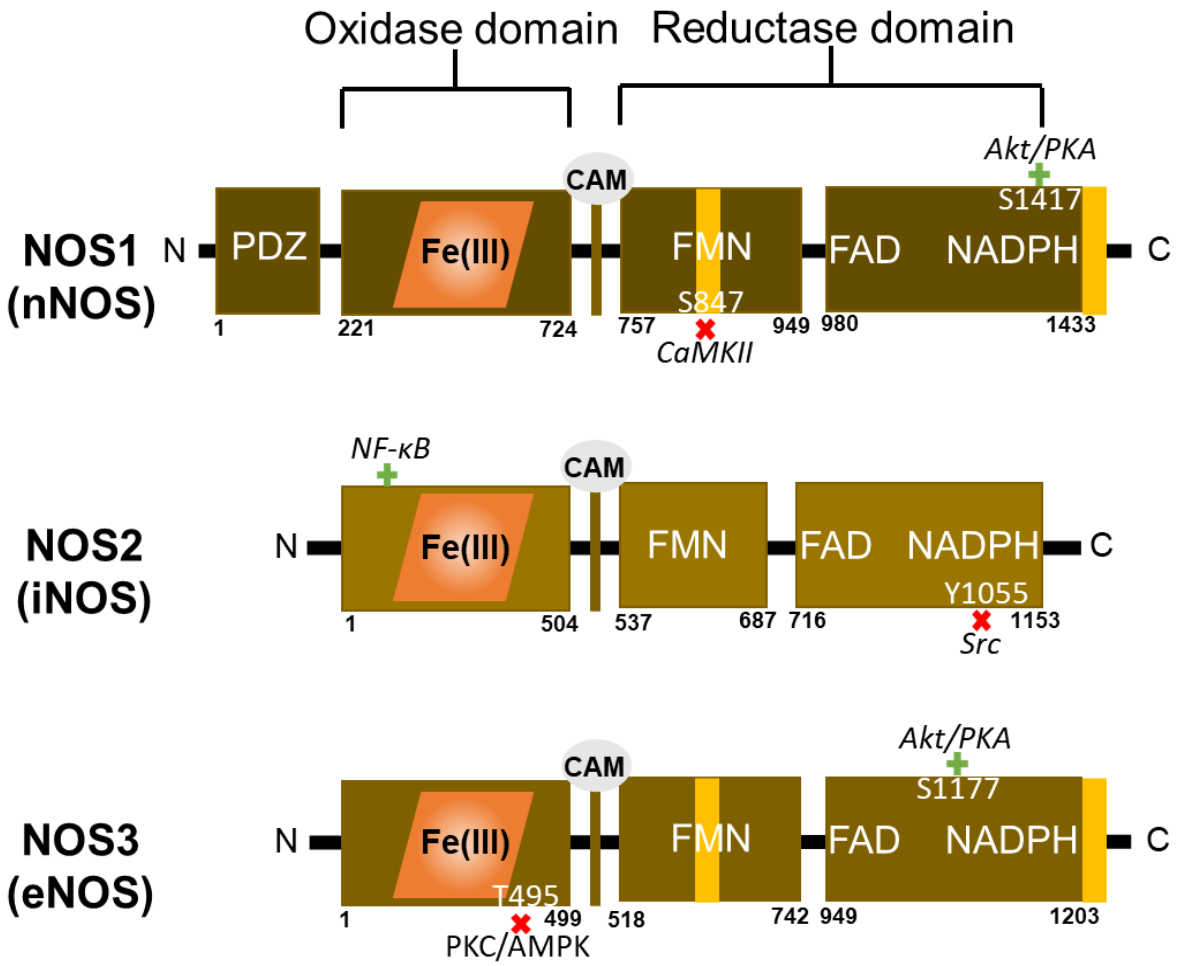

Figure 1.1: Mammalian nitric oxide synthases (NOS) domain organization. Each NOS monomer is composed of two domains: the oxidase domain and the reductase domain. The heme cofactor (orange) binds within the oxidase domain. The reductase domain is divided further into the FAD/NADPH-binding and the FMN-binding subdomains. CaM binds to a small helical segment that connects the oxidase and reductase domain. Although largely similar, each NOS isoform possesses a few unique features. iNOS is the smallest (260 kDa for the homodimer without CaM bound) and lacks the autoinhibitory helix within the FMN subdomain and the C-terminal extension (both shown in yellow) present in eNOS and nNOS. eNOS has a molecular weight of 266 kDa for the homodimer, and nNOS is the largest at 321 kDa for the homodimer as a result of an N-terminal extension of ~250 aa containing a PDZ domain. Transcriptional and post-translational regulation sites are shown for all isoforms, Green +'s and red x's indicate positive and negative regulation, respectively. Modified from (Campbell et al., 2014).

binds NOS1 with a K_D of 9.43±1.78 nM and NOS3 K_D = 4.0 nM (Song et al., 2001). When Ca^{2+}-CaM binds to NOS, it facilitates the flow of electrons from NADPH in the reductase domain to the heme in the oxygenase domain. In NOS2, calmodulin can bind strongly even at basal/low cytosolic Ca^{2+} concentrations (below 40 nM) due to a different amino acid structure of the calmodulin-binding site (Cho et al., 1992; Hemmens & Mayer, 1998), so it remains constitutively active even in resting cells whenever it is expressed.

1.2.2. Nitrate-Nitrite Reduction

The nitrite/nitrate (NO_3) axis can also be used to produce NO in a NOS-independent manner. Nitrate can enter the body via diet and the oxidation of NOS-derived NO. Once ingested, nitrate originating from a nitrate-rich diet is reduced to nitrite by nitrate reductase and salivary commensal bacteria in the oral microbiome of the oral cavity (Duncan et al., 1995). After swallowing, nitrite reaches the stomach and is, in part, reduced by nitrite reductase from enteric bacteria (Benjamin et al., 1994; Lundberg et al., 1994). Nitrate and nitrite circulate to various tissues, and NO is produced either by enzyme-mediated reactions or under acidic conditions, such as hypoxia (Omar et al., 2016). Notably, nitrite reduction does not require O_2 and is greatly enhanced under low O_2 conditions as well as acidosis (Modin et al., 2001). In the presence of oxygen and water, NO can undergo a reversible reaction to produce dinitrogen trioxide and nitrite, which under anaerobic or non-aqueous conditions can become NO donors (Fukuto et al., 2012; Hughes, 2008). These varying chemistries help to dictate the biological interactions between NO and macromolecules, and along with concentration, determine its cytoprotective or cytotoxic properties (Wink & Mitchell, 1998). By acting as a new source of NO, nitrite crucially affects multiple NO-dependent parameters and processes such as blood pressure (Kapil et al., 2015; Kreusser et al., 2014), platelet

aggregation (Velmurugan et al., 2013; Webb et al., 2008) and cardio-protection in both heart failure (Bhushan et al., 2014) and ischemia (Duranski et al., 2005).

1.3. NO based Signaling: cGMP and S-Nitrosylation

One of the earliest described intracellular receptors for NO is the soluble guanylyl cyclase (sGC) (Kots et al., 2009; Murad, 1986). Physiologically, NO binding to the heme group of sGC induces vasorelaxation (Kukreja & Xi, 2007; Montfort et al., 2017; Rainer & Kass, 2016). Upon binding sGC, NO induces the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). cGMP acts as a second messenger to activate protein kinase G (PKG), which reduces vascular tone, vascular smooth muscle (VSMC) proliferation and platelet aggregation (Münzel et al., 2003) and induces cardiac positive lusitropic effects (Massion & Balligand, 2003).

Most of the cellular influence exerted by NO is cGMP independent, however. An alternative molecular mechanism for NO regulation of cellular function is the NO-mediated modification of protein cysteine residues (to generate an S-nitrosothiol), referred to as *S*-nitrosylation (Foster et al., 2003; Hess et al., 2005) – Figure 1.2.

1.4. Molecular Basis of SNO formation

The term *S*-nitrosylation was first coined by Jonathan Stamler in 1992 to describe the reversible formation of the *S*-nitrosothiols (SNOs) of reduced thiols from protein sulfhydryl groups that were exposed to NO (Stamler, Simon, et al., 1992). Simply put, *S*-nitrosylation is the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine (Figure 1.2).

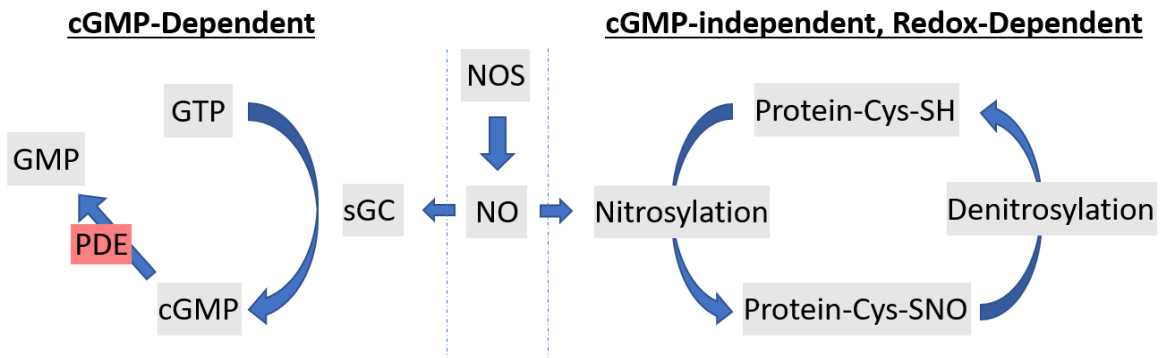

Figure 1.2: Roles of cGMP and S-nitrosylation in NO-based signaling. NOS synthesizes NO, which may activate sGC and thereby enhance production of cGMP (left) or protein S-nitrosylation (right). The cGMP-dependent pathway is deactivated by cGMP-phosphodiesterase, which hydrolyzes cGMP to GMP (phosphodiesterase may also be activated allosterically by cGMP). The SNO-based mechanisms are dynamically regulated via S-nitrosylation and de-nitrosylation of a multitude of cysteine-containing proteins. In contrast to the multiple elements regulated by S-nitrosylation, the cGMP-based signaling system relies primarily on the cGMP-dependent protein kinase, PKG. Modified from (Lima et al., 2010)

1.4.1. Protein S-Nitrosothiols

Most proteins in the human body possess such thiol groups and can function as substrates for *S*-nitrosylation, making it a ubiquitous post-translational modification (PTM) in biology (Hess et al., 2005). Cysteine is unique among the coded amino acids because of its thiol side chain. Thiol is an organic compound containing a sulfhydryl (-SH) functional group with a sulfur atom bonded to a hydrogen atom (Miseta & Csutora, 2000). This functional group is nucleophilic, acidic (pKa≈8) and redox active because of its hybridized p- and d-orbitals, which, together, underlie the large range of reactivities for cysteine residues within proteins (Lima et al., 2010). Numerous redox reactions occur on cysteine's thiol side chain that affect protein structure, reactivity, stability and function (Kovacs & Lindermayr, 2013). The most relevant example being the formation of a protein SNO from the redox reaction of NO and a cysteine thiol. Since NO functions as an integrative element in electron transfer reactions, *S*-nitrosylation is now considered a key mechanism that mediates extensive redox-based cellular signal transduction. Additionally, *S*-

nitrosylation can promote or inhibit the formation of disulphide linkages within or between proteins, depending on thiol proximity and orientation (Arnelle & Stamler, 1995; Stamler & Toone, 2002). Many cysteines can be either *S*-nitrosylated or oxidized by ROS. Oxidative stress (increased levels of ROS) can lead to NOS uncoupling which generates the superoxide anion (O_2^-) and promotes cytotoxic pathways (Roe & Ren, 2012; Vásquez-Vivar et al., 1998; Xia et al., 1998).

1.4.2. Low molecular weight S-Nitrosothiols

In addition to the SNO in proteins, SNO also occurs in low-molecular-weight (LMW) thiols that produce SNO-LMW, such as S-nitrosocysteine (CysNO), S-nitrosoglutathione (GSNO), and S-nitroso-CoA (SNO-CoA) (Stomberski et al., 2019). Glutathione is the most abundant low molecular-weight thiol found to date and has been most extensively studied as a key element in regulating cellular redox homeostasis (Ferguson & Bridge, 2019). It is composed of three amino acids, which are cysteine, glutamate, and glycine. The S-nitrosylation of glutathione (GSH) produces GSNO, which serves as a stable and mobile NO reservoir especially in cardiomyocytes. Because GSNO exists in steady-state with protein SNO, it is the main endogenous NO donor to other proteins via transnitrosylation (i.e., the NO-group transfer) (Belcastro et al., 2017; Hogg, 2002). The equilibrium between GSNO and protein SNO is determined in part by the enzyme GSNO-reductase (GSNOR), which metabolizes GSNO.

In comparison with other PTMs of proteins - like phosphorylation - *S*-nitrosylation has emerged as a key regulatory mechanism in various cellular functions and has been studied in many physiological and disease conditions (Barouch et al., 2002; Boehning & Snyder, 2003; Foster et al., 2003; Gow et al., 2002; Stamler et al., 2001). It has been reported that *S*-nitrosylation plays an important role in vascular homeostasis, cellular functions (in aspects of gene regulation and

immune modulation), respiratory and neuronal signaling (Selemidis et al., 2007). Conversely, its impairment has been implicated in many diseases such as cardiac hypertrophy, neurodegenerative diseases, diabetes, and endotoxic shock (Irie et al., 2015; Liu et al., 2004; Qian et al., 2018; Shahani & Sawa, 2012; Yasukawa et al., 2005).

The stability of *S*-nitrosothiols is also affected by denitrosylation, trans-*S*-nitrosylation and *S*-thiolation (Hogg, 2002). Protein denitrosylation, is the removal of NO groups primarily from Cys thiol side chains in proteins. In trans-*S*-nitrosylation, the nucleophilic thiolate of R′SH attacks the nitrogen of *S*-nitrosothiols to form R′SNO and RSH, whereas in *S*-thiolation, the nucleophilic thiolate interacts with the sulfur of an *S*-nitrosothiol to form a mixed disulfide and NO^- (Maron et al., 2013). Additionally, despite the covalent nature of the S-NO bond, in the presence of transition metals (*i.e.*, Fe^{2+} and Cu^+) and reducing agents (*i.e.*, ascorbate), S-NO may decompose by homolytic or heterolytic cleavage resulting in the release of NO, NO^+, or NO^- (Maron et al., 2013).

Various assays have been developed to measure/detect *S*-nitrosylated proteins in biological systems. "NO-based" assays are a standard method for probing *S*-nitrosylation in vivo (Hausladen et al., 2007; Marzinzig et al., 1997; Palmer & Gaston, 2008; Park & Kostka, 1997). These assays detect NO or nitrite that's liberated from SNO homolysis and are sensitive to nM SNO concentrations. With complex biological samples like cell lysates, NO-based assays can determine the absolute amount of SNO per sample but cannot readily detect an individual protein–SNO (Forrester et al., 2009). The Biotin Switch Technique (BST) is a "Sulphur-based" assay that covalently targets the sulphur atom of each SNO, thus facilitating relative quantitation and protein–SNO identification (Forrester et al., 2009). First free cysteine thiols are blocked, then SNOs are converted to thiols and biotinylated. The extent of biotinylation – and therefore S-nitrosylation – is ascertained by either anti-biotin immunoblotting or streptavidin pulldown followed by

immunoblotting. Because the biotin tag is added to the protein of interest and independent of liberated NO, the BST can detect individual protein–SNOs in a complex mixture. This approach can be and has been adapted to suit specific goals (Camerini et al., 2007; Friedman & Lilley, 2008; Han et al., 2008; Hao et al., 2006; Kettenhofen et al., 2008).

1.5. NO signaling in the heart

In the heart, NO was primarily recognized for being a vasodilator, but its biological functions have proven to extend far beyond that, as it is produced by multiple synthases and present in both physiological and pathological conditions. Both NOS3 and NOS1 are physiologically expressed in the heart whereas NOS2 is typically expressed in disease states (Carnicer et al., 2013; Seddon et al., 2007; Takimoto et al., 2002).

1.5.1. NOS Isoforms and Localization

Cellular localization dictates compartmentalized NO production and downstream signaling to specific effectors. To enhance specificity for responses to calcium, NOS enzymes concentrate in specific subcellular domains allowing NOS activity to respond selectively to calcium mobilization from localized calcium pools. The reactive and diffusive nature of the NO signal also lends to the need for proximal localization/compartmentalization to the vicinity of target proteins as is essential to aid specificity and decrease cytotoxicity. This is understandable because NO has a short half-life (10s) and limited diffusion area (due to abundant NO scavenging myoglobin and NO-interacting biochemicals and proteins (Y. H. Zhang et al., 2014). NO changes ion currents through membranes, inhibits the cellular respiration and various SH-dependent enzyme activities, mediates DNA damage, and affects the cellular transcription machinery. In susceptible cells these multiple effects may lead to cellular necrosis or apoptosis depending on the cell type or on local NO concentrations (Kröncke et al., 1997). Localization therefore enhances signaling specificity for

the reactive NO mediator and minimizes toxicity that arises from the free radical nature of NO (Barouch et al., 2002; Ziolo et al., 2008). Each isoform modulates cardiac function differently.

1.5.1.1. Neuronal Nitric oxide synthase – NOS1

Although first identified in the brain hence the name neuronal NOS, expression of this isozyme has been found in numerous cell types and is alternatively referred to as NOS1. NOS1 is expressed in human coronary artery smooth muscle cells (Han et al., 2007) and maintains the basal blood flow (Seddon et al., 2009). NOS1 is also the only NOS isoform that is expressed in intrinsic cardiac neurons from autonomic nerves and ganglions (Choate et al., 2001; Danson et al., 2005; Mohan et al., 2000) and controls parasympathetic and sympathetic regulation of cardiac rhythm and contractility.

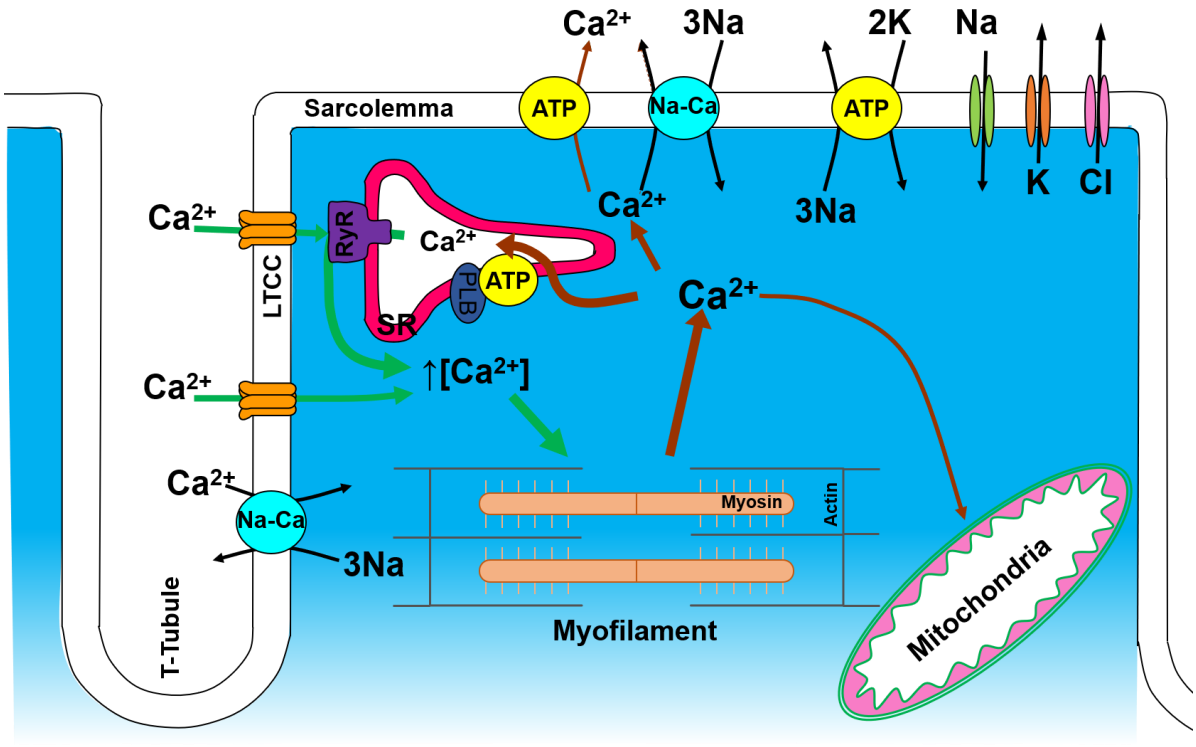

Figure 1.3: Ca^{2+} transport in ventricular myocytes. Na-Ca: Na^{+}/Ca^{2+} exchanger; RyR: Ryanodine receptor; ATP: ATPase; PLB: phospholamban; SR: sarcoplasmic reticulum. Modified from Bers, 2002.

In 1999, Xu et al. revealed NOS1 as constitutively expressed on the sarcoplasmic reticulum (SR) of cardiac myocytes, and super resolution microscopy later showed NOS1 was closely colocalized with ryanodine receptor (RyR) (Jian et al., 2014; Xu et al., 1999). The SR functions as an intracellular store for free calcium, enabling calcium release (for myocyte contraction) via the RyR and calcium re-uptake (for myocyte relaxation) via the SR Ca^{2+} pump (SERCA) – see Figure 1.3. More details on cardiomyocyte excitation-contraction coupling (ECC) can be found in **section 2.1** below. In myocytes, NOS1 is also found at the mitochondria (Burkard et al., 2010) and sarcolemmal membrane (Williams et al., 2006). It acts as a major regulator of Ca^{2+} handling and contractile studies in myocytes with genetic NOS1 deletion (*NOS1*$^{-/-}$) show depressed basal contraction as well as prolonged relaxation (H. Wang, M. J. Kohr, C. J. Traynham, et al., 2008). These knockout mice and mice with pharmacological inhibition of NOS1 also exhibited a blunted force-frequency response (FFR) and reduced contractile response to β-adrenergic stimulation (Barouch et al., 2002; Khan et al., 2003; H. Wang, M. J. Kohr, C. J. Traynham, et al., 2008). NOS1 also targets the RyR via *S*-nitrosylation (Wang et al., 2010). Several studies observed a decrease in RyR protein levels in *NOS1*$^{-/-}$ (Chen et al., 2010; Gonzalez et al., 2007; Sears et al., 2003) which is believed to be compensatory for the decreased calcium handling in these mice.

Five NOS1 variants have been identified - NOS1α, NOS1β, NOS1μ, NOS1γ, and NOS11-2. NOS1α and NOS1μ contain a PDZ domain (small modular protein—protein interaction interfaces that mediate assembly of protein complexes at cell junctions), which plays a major role in targeting/constraining the NOS1 to specific cellular membranes (Brenman & Bredt, 1997). NOS1β and NOS1γ do not contain a PDZ domain and may be localized in the cytosol. However, little is conventionally known about the variants' specificity, functional relevance and mechanisms in cardiac myocytes. To date, two main NOS1 phosphorylation residues have been described in

cardiac cells: the Ca^{2+}/calmodulin kinase II (CaMKII) dependent phosphorylation of Ser852 (corresponding to Ser847 in the mouse sequence), which reduces NOS1 activity by inhibiting Ca^{2+}/calmodulin binding (Lekontseva et al., 2011); and the Akt-dependent phosphorylation of Ser1417 (corresponding to Ser1412 in the mouse sequence), which increases NOS1 activity in cardiac myocytes (Adak et al., 2001; Trappanese et al., 2015). More details on CaMKII below.

Activated by elevated local $[Ca]_i$, NOS1 signaling occurs at the subcellular SR domain to directly (S-nitrosylation) and indirectly (phosphorylation) regulate protein function and contraction. The spatial confinement and compartmentalization of NOS1 signaling ensure its ability to regulate diverse intracellular signaling pathways and myocardial functions in normal and diseased hearts. Syntrophin, a member of the dystrophin-associated proteins, acts as scaffold protein for NOS1 and the plasma membrane Ca^{2+}-ATPase (PMCA) where it inhibits NO production (Williams et al., 2006). Mutations in syntrophin disrupts this inhibition and favors interaction of NOS1 with the Na^+ channel SCN5A. The Ala390→Val mutation in syntrophin leads to an increase in late Na^+ currents, a pro-arrhythmic event recapitulating long-QT syndrome (Ueda et al., 2008).

1.5.1.2. Endothelial Nitric oxide synthase – NOS3

eNOS was first identified in endothelial cells (vascular endothelium and endocardium) (Palmer et al., 1988) and then in myocardial cells (Balligand et al., 1995; Petroff et al., 2001). It has since been renamed "NOS 3" as it is also expressed in numerous cell types. NOS3 keeps blood vessels dilated, controls blood pressure, and has numerous other vasoprotective and anti-atherosclerotic effects. In cardiomyocytes, NOS3 is localized to the caveolae where it closely associates with L-type Ca^{2+} channels (LTCC) and β-adrenergic receptors (β-AR) (H. Wang, M. J. Kohr, D. G. Wheeler, et al., 2008). In contrast to *NOS1*$^{-/-}$ myocytes, *NOS3*$^{-/-}$ myocytes showed an increase in

functional response to β-AR stimuli (Barouch et al., 2002; Champion et al., 2004; Gyurko et al., 2000; H. Wang, M. J. Kohr, D. G. Wheeler, et al., 2008) whereas mice with NOS3 overexpression showed decreased functional response to β-AR stimulated contraction (Brunner et al., 2001; Massion et al., 2004). In $NOS3^{-/-}$ mice, CaT amplitudes are increased due to an enhanced β-adrenergic stimulated L-type Ca^{2+} current (Wang et al., 2012). NOS3 therefore, limits the action potential duration with β-adrenergic stimulation indicating its protective nature against adrenergic toxicity. Recently, the Allen group showed that NOS3 is localized to the nucleus where it can regulate transcription via nuclear β-AR (Vaniotis et al., 2013). It also mediates preload mechanical stress stimulation of intracellular Ca^{2+} release from ryanodine receptors (Petroff et al., 2001) (in contrast to afterload effects mediated by NOS1 (Jian et al., 2014)). NOS3 expression levels have been shown to decrease in heart failure (Piech et al., 2002), which could be detrimental to the failing heart.

Shear stress and stretch are well-known activators of NOS3 mRNA stabilization and protein expression through cooperative regulation by the transcription factors nuclear factor-κB (NF-κB) and Krüppel-like factor 2 (KLF2) (Balligand et al., 2009). Reactive oxygen species (ROS) also mediate NOS3 transcription through oxidant-responsive kinases (for example, p38 mitogen-activated protein kinase [MAPK]) in response to receptor-mediated or physical stimuli (Farah et al., 2018). Activity of NOS3 is fine-tuned post-translationally by phosphorylation (Dimmeler et al., 1999; Fisslthaler et al., 2008; Mattagajasingh et al., 2007), protein–protein interactions (Feron et al., 1998), *S*-nitrosylation (Erwin et al., 2005), and *S*-glutathionylation (Chen et al., 2010).

1.5.1.3. Inducible Nitric oxide synthase - NOS2

iNOS expression can be “induced” in various cells including cardiomyocytes during an inflammatory response (Schulz et al., 1992). It is alternatively referred to as NOS2 for consistency

with the modern nomenclature. Transcription of NOS2 occurs in pro-inflammatory or oxidative conditions in a large range of cells, including leukocytes, endothelial cells, VSMCs, nerve cells, and cardiac myocytes (Balligand et al., 1994; Wilcox et al., 1997). It contributes to the pathophysiology of inflammatory diseases and septic shock (Liu et al., 2004). In contrast to NOS3 and NOS1, NOS2 is calcium-independent as it is maximally active at Ca^{2+} concentrations as low as 0.1 nM (Stuehr et al., 1991). When assembled, the dimeric enzyme's catalytic activity is 100–1,000 times higher than that of NOS3 and NOS1 under basal intracellular Ca^{2+} concentrations (and fivefold to tenfold higher than that of Ca^{2+}/calmodulin activated NOS3 or NOS1) and maintains a high NO output until exhaustion of substrate and cofactors or enzyme degradation (MacMicking et al., 1997). In 1996, Haywood showed that NOS2 expression in the cardiovascular system is mostly associated with pathological remodeling (Haywood et al., 1996). NOS2 is now widely considered to be involved in various pathophysiological conditions of the myocardium, such as ischemia-reperfusion injury (Wildhirt et al., 1999), septicemia (Ichinose et al., 2003; Ullrich et al., 2000; Ziolo et al., 2001a), aging (Yang et al., 2004), infarction (Sam et al., 2001), and heart failure (Ziolo et al., 2004) where its expression contributes to cardiac dysfunction (among others) by blunting contractility. Given all this, specific NOS2 knockdown or NOS2 inhibition could be a therapeutic approach for heart failure.

1.5.2. Post-translational Modifications of NOS isoforms

NO signaling can be controlled in part by regulating the source, and each of the NOS isoforms has its own form of regulation. Phosphorylation of the NOS isoforms is an important mechanism for enhancing or suppressing NO production as these modifications influence electron flow through the oxidase and reductase domains, altering l-arginine reduction, and thus altering NO output (McCabe et al., 2000). Activation of NOS1 is regulated by phosphorylation and can be inhibited

when phosphorylated by CaMKII at Ser847 (Komeima et al., 2000). Phosphorylation of NOS3 at serines 615, 633, and 1177 by phosphoinositide 3-kinase (PI3K), Akt/PKB, PKG, PKA, adenosine monophosphate-activated protein kinase (AMPK), and Ca^{2+}/calmodulin kinase II enhance NO synthesis (Boo et al., 2003; Fulton et al., 1999; Morell et al., 2018; Nakane et al., 1991; Partovian et al., 2005). Phosphorylation of NOS3 at threonine 495 by PKC in the absence of calmodulin is known to be inhibitory (Fleming et al., 2001).

1.5.3. NO in the heart

NO regulates cardiovascular function via both *S*-nitrosylation and the activation of sGC and its downstream stimulation of cGMP production and PKG-1 activation (Maron et al., 2013). PKG-1 is expressed in two isoforms, PKG-1α and PKG-1β, with the PKG-1α form being predominant in cardiac myocytes. By inhibiting the LTCC (Klein et al., 2000), and phosphorylating phospholamban at Ser16 (Wollert et al., 2003), PKG-1α reduces intracellular Ca^{2+} content enhances SR Ca reuptake and thus accelerates cardiac relaxation. PKG-Iα can also induce cardiac relaxation by modulating Ca^{2+} sensitivity in the myofilaments - through the phosphorylation of troponin I (Lee et al., 2010), myosin-binding protein C (MyBP-C) (Hamdani, Bishu, et al., 2013), and titin (Krüger et al., 2009). The abundance of cGMP is regulated by the phosphodiesterase enzymes (PDEs), which hydrolyze the phosphodiester bond of cGMP (and/or cAMP). Notably, by activating PDE2 and inhibiting PDE3, cGMP can modulate cAMP–PKA signaling in the cardiac myocytes, thereby extending its influence on ECC (Weber et al., 2017; Zaccolo & Movsesian, 2007). Additionally, sGC is sensitive to oxidation, which can interrupt NO signaling.

Dysfunctional NOSs could lead to oxidative stress in heart failure, and consequently contractile dysfunction, hypertrophy, and adverse remodeling. NOS2 inhibition would be beneficial for heart

failure. Trials however, were terminated early due to negative effects in patients most likely due to inhibition of the other beneficial NOS isoforms (Bailey et al., 2007).

1.5.4. SNO in the heart

Protein *S*-nitrosylation appears to play a role in virtually every aspect of cardiac function, including: the regulation of inflammation, apoptosis, and angiogenesis that contribute to atherogenesis (Lee et al., 2005; Suhara et al., 2004), Ca^{2+} flux that regulates myocardial contractility and electrophysiologic signaling (Burger et al., 2009), cardiovascular responses to hypoxia (Stamler, Singel, et al., 1992), mitochondrial bioenergetics in vascular tissue (Clementi et al., 1998), and platelet function, among others (Chen et al., 2008; Fauconnier et al., 2010; Stamler et al., 1997; Thibeault et al., 2010). In contrast to the cGMP axis that uses a single principal molecular effector (*i.e.,* PKG) to carry out the downstream functions of NO, *S*-nitrosylation allows for a wide range of NO-mediated functions, as a plethora of proteins can undergo this modification (Foster et al., 2003; Hess et al., 2005). *S*-Nitrosylation therefore, helps to explain the wide range of cellular effects of NO in the cardiovascular system. *S*-nitrosylation might influence levels of cGMP via the inhibition of sGC (Sayed et al., 2008) and cGMP phosphodiesterase (Murray et al., 2008), as well as NOS3 itself (Ravi et al., 2004) and NOS3-regulating proteins including heat shock protein 90 (Martínez-Ruiz et al., 2005) and Akt/PKB (Yasukawa et al., 2005). PKG has regulatory thiols as well, which may be susceptible to S-nitrosylation.

SNO modulates several ion channels involved in ECC and Ca^{2+} handling to affect contractility and arrhythmogenesis. SR Ca^{2+} release via the RyR2 can be regulated by poly-*S*-nitrosylation and activated by SNO compounds in vitro, increasing RyR2 open probability (Stoyanovsky et al., 1997; Xu et al., 1998). NO is also able to modulate β-adrenergic stimulated RyR2 activity (measured as decrease in Ca^{2+} spark frequency) in intact cardiac myocytes (Ziolo et al., 2001b).

Following myocardial infarction, a proarrhythmic state is associated with diminished *S*-nitrosylation of RyR2, SERCA and the LTCC (Burger et al., 2009). Several other ion channels involved in regulating the action potential are also subject to *S*-nitrosylation. *S*-nitrosylation of the α1C subunit of the LTCC inhibits the L-type Ca^{2+} current (Sun et al., 2006) while *S*-nitrosylation of SCN5A channels enhances the Na^{+} current (Ahern et al., 2000). S-nitrosylation of the KCNQ1 subunit facilitates the slowly activating component of the delayed rectifier K^{+} current (I_{Ks}), whereas *S*-nitrosylation exerts an inhibitory influence on Kv4.3 and thus the transient outward potassium current (Asada et al., 2009; Gómez et al., 2008). Cysteine *S*-nitrosylation in Kir2.1 of the inward-rectifying K channel that mediates I_{K1} and shortens the action potential duration (Gómez et al., 2009).

1.6. Sex Differences

Baseline heart physiology and response to disease in male versus female hearts are known to differ (Czubryt et al., 2006). Understanding CVD in women is a research priority for the NIH and AHA, and sex as a variable is now required as a factor for inclusion in studies.

1.6.1. Sex differences in cardiovascular disease

Major cardiovascular conditions where sex differences are present include myocardial infarctions, hypertension, cardiac hypertrophy, and ischemia–reperfusion (I/R) injury. Female hearts exhibit endogenous sex-dependent cardio-protection and reduced susceptibility to ischemic heart injury. This cardio-protection is evident both in female pre-clinical animal models (Murphy & Steenbergen, 2007; Shao et al., 2016; Sun et al., 2006) and in premenopausal women compared to age-matched men (Chandrasekhar et al., 2018; Mosca et al., 2011). Estrogen could have cardio-protective properties, thus explaining why pre-menopausal women are less likely than men to

suffer from cardiovascular diseases (Randolph et al., 2003; Salerni et al., 2015). Hormone replacement therapy, as shown in previous studies, had no cardiovascular benefits and might in fact cause an increased risk of cardiovascular diseases (Hulley et al., 1998; Rossouw et al., 2002). However, more recent studies in both animal and humans, show estrogen supplementation and hormone therapy to improve diastolic function (Michalson et al., 2018; Sabbatini & Kararigas, 2020). Estrogen also plays a putative role in the prevention and treatment of heart failure with preserved ejection fraction (HFpEF) (Tadic et al., 2019; Zhao et al., 2018).

Gender variation also can be seen in patients with heart failure as the incidence of heart failure is higher in men than in women (Czubryt et al., 2006). The prevalence of heart failure, however, is about the same for both males and females (Czubryt et al., 2006). This is because even though more males are diagnosed with heart failure, the number of males and females living with heart failure is similar, as women with heart failure usually have a lower mortality rate than men. There is also a difference in roughly half of the heart failure population. That is, diastolic heart failure (now called heart failure with preserved ejection fraction or HFpEF) occurs more often in women, whereas systolic heart failure (with reduced ejection fraction or HFrEF) occurs more often in men (Czubryt et al., 2006).

1.6.2. Sex differences in NO signaling

Sex differences in NO signaling have been extensively studied over time. In humans, the female sex is associated with greater nitric oxide content in heart tissues sampled before surgery (Schuh et al., 2018). Sex differences with regard to NOS isoforms expression and NOS activation have been noted in the heart (Ruiz-Holst et al., 2010; Shao et al., 2017; Shao et al., 2016; Wang & Abdel-Rahman, 2002). In 2006, the Murphy group demonstrated that endogenous female cardio-protection was derived in part from SNO of the LTCC, thereby reducing Ca^{2+} entry and the

potential for I/R-induced Ca^{2+} overload (Sun et al., 2006). Interestingly, this protection was dependent upon both NOS3 and NOS1, as knockout of either isoform ablated sex-dependent cardio-protection. They also showed that under hyper-contractile conditions, female hearts have more protein SNO than males. The Kohr group reported that hearts from intact female mice had more SNO proteins at baseline, yet more GSNO-R activity compared to males with no difference in GSNO-R expression (Shao et al., 2016). Casin et al. recently demonstrated that GSNO-R inhibition or genetic deletion impaired endogenous cardio-protection in female hearts, in part by increasing post-ischemic protein SNO levels (Casin et al., 2018). These studies support SNO, GSNO, and GSNO-R as essential mediators of cardio-protection, especially sex-dependent protection.

These data, along with epidemiologic and preclinical data indicating that female hearts have lower risk and injury from cardiovascular disease, suggest that baseline NO signaling may be important for preventing cardiovascular disease development.

2. CaMKII Overview

2.1 Introduction

At the center of cardiovascular physiology is the heart, which drives blood flow to all organ systems, delivering oxygen and collecting waste, to maintain homeostasis. The basis for this blood flow results from contraction of the heart, and each heartbeat is as a result of combined effort of several ions and proteins. Calcium is a ubiquitous second messenger that regulates several physiological processes in general, and the cardiovascular contractile system specifically. During the cardiac action potential, membrane depolarization opens the L-type calcium channel (LTCC)

allowing the influx of calcium which induces the release of more intracellular calcium (a process termed Ca^{2+} induced Ca^{2+} release – CICR) from the sarcoplasmic reticulum (SR) via the type-2 ryanodine receptors (RyR2). This transient rise in free intracellular calcium ($[Ca^{2+}]_i$) leads to cardiac contraction. Calcium is then transported out of the cytosol via SR Calcium-ATPase (SERCA), sarcolemmal Na/Ca exchanger (NCX), sarcolemmal calcium-ATPase or mitochondrial calcium uniporter for relaxation to occur. Given the significance of calcium, perturbations or irregularities in intracellular calcium handling machinery are considered pathognomic.

Ca^{2+}/calmodulin-dependent kinases (CaMK) are a family of serine/threonine kinases that respond to intracellular Ca^{2+} changes [$Ca^{2+}{}_i$] and consist of three members: CaMKI, CaMKII, and CaMKIV (Racioppi & Means, 2012). Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) is a key regulator of cardiac function and dysfunction in pathological states via its influence on ion channels, Ca^{2+} balance, Ca^{2+} handling proteins involved in cell death, transcriptional activation of hypertrophy, inflammation, and arrhythmias. CaMKII delicately senses and translates the periodic intracellular Ca^{2+} concentration rises in cardiomyocytes during excitation-contraction coupling to kinase activity (Bers, 2002). CaMKII signaling regulates diverse cardiac cellular processes including excitation-contraction coupling, excitation-transcription coupling, mechanics and energetics (Backs et al., 2009; Bers, 2008; Bossuyt et al., 2008). Chronic activation of CaMKII results in a significant cellular remodeling and alterations in Ca^{2+} handling, ion channels, cell-to-cell coupling and metabolism leading to increased risk of atrial and ventricular arrhythmias (Ai et al., 2005; Anderson et al., 2011; Chelu et al., 2009; van Oort et al., 2010). The prominent role of CaMKII is also well-established in the pathophysiology of several common heart diseases, including cardiac hypertrophy (Anderson et al., 2011; Backs et al., 2009; Bers, 2008), heart failure (Ai et al., 2005; Bossuyt et al., 2008), I/R injury and post-myocardial infarction (MI) remodeling

(Ling et al., 2013; Weinreuter et al., 2014), atrial fibrillation (Chelu et al., 2009; Voigt et al., 2012) and ventricular arrhythmias (van Oort et al., 2010; Wagner et al., 2015). Pharmacological or genetic inhibition of CaMKII limits arrhythmias and progression of HF, so CaMKII is widely considered a bona fide drug target for heart disease (He et al., 2019; Hoeker et al., 2016; Kreusser et al., 2016; Neef et al., 2018; Pellicena & Schulman, 2014).

2.2 Overview of CaMKII structure

CaMKII is a multifunctional serine/threonine protein kinase with wide tissue distribution (Maier & Bers, 2007; Rellos et al., 2010; Solà et al., 1999) and a broad range of protein substrates. There

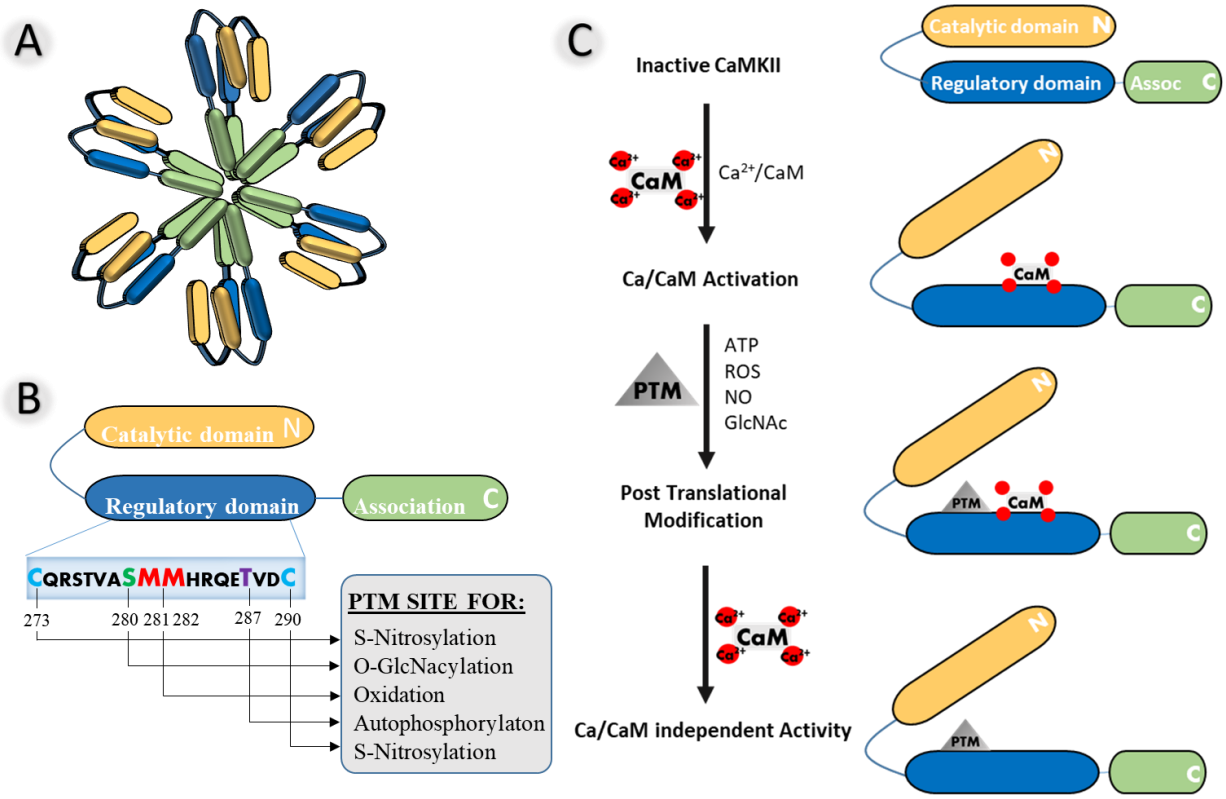

Figure 1.4: CaMKII structural domains and activation mechanisms. Each CaMKII monomer consists of an N-terminal catalytic domain, a regulatory domain, and a C-terminal association domain. General structure of the dodecameric holoenzyme, CaMKII (**a**) and potential regulatory sites (**b**). Under resting conditions, the regulatory domain inhibits the catalytic domain (autoinhibition). This inhibition is relieved by the binding of Ca/CaM, activating the kinase. Post-translational modification including autophosphorylation, allows CaMKII activity persist autonomously independent of Ca/CaM (**c**). Modified from (Zhang,

are 4 isoforms of CaMKII (α, β, δ and γ) expressed in various tissues including neurons (mostly α and β) and cardiomyocytes (mostly δ and some γ) (Gaertner et al., 2004; Kreusser et al., 2014). In cardiomyocytes, CaMKIIδ accounts for 85–90% of CaMKII activity, with the remainder being mostly CaMKIIγ (Kreusser et al., 2014). **Studies reported in this dissertation focus on CaMKIIδ.** Two CaMKIIδ isoforms have been actively described, CaMKIIδb and CaMKIIδc, which differ only in the presence of a nuclear localization sequence in CaMKIIδb (Mishra et al., 2011). CaMKII exists mainly as a dodecamer in which each CaMKII monomer has 3 domains: An N-terminal catalytic domain, an autoinhibitory regulatory domain, and a C-terminal association domain (Figure 1.4). The C-terminal association domains direct multimeric assembly, binding together to form a central hub in the holoenzyme that is connected by a linker to the regulatory domain. The regulatory domain controls enzyme activation or inhibition and binds either the catalytic domain (in its inactive state), or calmodulin (CaM, in its active state) with a K_D of 10–50 nM (Gaertner et al., 2004; Hoelz et al., 2003). The catalytic domain performs the kinase function of CaMKII, containing ATP- and substrate binding-sites. The C-terminal has an oligomerization domain that organizes the enzyme into two ring-shaped hexamers stacked on each other, with the catalytic domains of each subunit near the outer edge, in close proximity to facilitate rapid autoactivation (Rellos et al., 2010). Although the stoichiometry and geometry of the oligomeric assembly in CaMKII holoenzyme varies slightly, in humans the hub domain majorly forms a dodecameric or tetradecameric ring (Bhattacharyya et al., 2020). Notably, when activated the dodecameric and tetradecameric forms of CaMKII may interconvert by the reversible addition and release of vertical dimeric subunits (Bhattacharyya et al., 2016).

2.3 CaMKII Activation

Under resting conditions, CaMKII is inhibited by its autoregulatory domain, which forms a tight association with the catalytic domain, preventing substrate binding (Rellos et al., 2010). Binding of Ca^{2+}/CaM triggers unfolding of the inhibitory helix, relieving the autoinhibitory effect, releasing the kinase domain and activating the enzyme (Hudmon & Schulman, 2002; Rosenberg et al., 2005). In prolonged conditions of elevated Ca^{2+}, inter-subunit autophosphorylation occurs at the Thr287 residue, preventing the reassociation of the regulatory and catalytic domains (Rellos et al., 2010; Schworer et al., 1988). This auto-phosphorylation raises Ca^{2+}/CaM affinity and slows deactivation or re-association with the catalytic domain – also allowing the kinase to remain partially active independently of Ca^{2+}/CaM.

2.4 Post-translational Modifications

Besides the Ca^{2+}/calmodulin-dependent activation of CaMKII that may lead to autophosphorylation (Thr287), additional post-translational modifications (PTMs) of CaMKII via oxidation, *O*-GlcNAcylation and *S*-nitrosylation have been identified.

In 2004, Howe et al. showed CaMKII can be activated by oxidation in Ca^{2+} free conditions and Erickson et al., later identified M281/M282 as the sites for oxidation (Erickson et al., 2008; Howe et al., 2004). This activation blocks autoinhibition in a similar manner to Thr287 autophosphorylation. CaMKII can also be modified via *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) at S280, leading to persistent activity with increased glucose concentrations (Erickson et al., 2013). In 2013, Gutierrez et al. showed that CaMKII can be activated via formation of endogenous NO during β-adrenergic stimulation, leading to an increased spark rate (Gutierrez et al., 2013). In 2014, Coultrap and Bayer then showed that *S*-nitrosylation of CaMKIIα at Cys280/289 leads to autonomous activity and pathological functions in neurons (Coultrap & Bayer, 2014). In 2015, the Bers group confirmed this with CaMKIIδ, extending it to show that *S*-

nitrosylation at the analogous Cys290 after Ca^{2+}-CaM binding to CaMKII led to persistent activity (Erickson et al., 2015). However, they also showed that *S*-nitrosylation before Ca^{2+}-CaM binding prevented kinase activation at Cys273 (a site present in CaMKIIβ, γ and δ but not CaMKIIα). Thus, while *S*-Nitrosylation of Cys290 on CaMKIIδ activates the kinase, *S*-Nitrosylation of Cys273 suppresses activation by Ca^{2+}-CaM. Notably there is no inhibitory nitrosylation in the neuronal CaMKIIα, compared to cardiac CaMKIIδ where it could be inhibitory and stimulatory.

A key part of cardiac pathology is due to sustained activation of CaMKII via these PTMs, however, little is known on how these PTMs contribute individually to pathology and how they interact. M281/282 oxidation has been shown to reset the Ca^{2+}-sensitivity favoring CaMKII activation at low $[Ca^{2+}]_i$ (Palomeque et al., 2009) and S280 *O*-GlcNAcylation of CaMKII promotes T287 autophosphorylation (Erickson et al., 2013). Studies in this dissertation use P TM mutants to investigate specific role in chapters 3, 4 and 5.

2.5 Localization and Function

CaMKII subcellular localization is dependent on the nature, location and presence of interacting domains on its target. As discussed earlier, CaMKIIδ, the predominant cardiac isoform, is alternatively spliced to generate multiple subtypes (Edman & Schulman, 1994). CaMKIIδb and CaMKIIδc the predominant subtypes are identical except for the insertion of an 11-amino acid sequence in the variable domain of CaMKIIδb which is responsible for nuclear localization (Schworer et al., 1993; Srinivasan et al., 1994). CaMKIIδc is distributed in high density near the t-tubules of cardiomyocytes, close to the LTCC (Cav1.2) and to RyR2 channels of the SR. CaMKII is also found near the intercalated disc (Swaminathan et al., 2012). Additionally, the Bossuyt group found CaMKII to be highly mobile in resting myocytes as at low $[Ca^{2+}]$, CaMKII was concentrated at Z-lines near the dyad but spread throughout the sarcomere upon activation via

pacing (Wood et al., 2018). α- and β-subunits of LTCC, which are phosphorylated by CaMKII, bind with CaMKII, because of the homology between the phosphorylation sites and the auto-inhibitory region of CaMKII (Anderson, 2009; Bers & Morotti, 2014). A homology domain, like the LTCC β-subunit, is also found in the actin-associated protein, βIV-spectrin, to which CaMKII is known to bind. This interaction is a prerequisite for the CaMKII-mediated phosphorylation of the voltage-gated Na^+ channels at the intercalated disc in cardiomyocytes (Makara et al., 2014).

Chronic elevations in CaMKIIδ expression and activity are observed in humans with heart failure (Hoch et al., 1999). CaMKIIδb regulates gene expression as a result of its actions in the nucleus. In 1997, Ramirez et al., showed expressing CaMKIIδb in neonatal rat ventricular myocytes induces atrial natriuretic factor (ANF) expression and leads to increased myofilament organization; both hallmarks of cardiac hypertrophy, while expressing CaMKIIδc did not (Ramirez et al., 1997). CaMKIIδb signaling activates several transcription factors including myocyte enhancer factor 2 (MEF2), GATA4, and heat shock factor 1 (HSF1) (Little et al., 2009; Lu et al., 2010; Peng et al., 2010; Zhang et al., 2002). These nuclear transcription effects are largely driven by CaMKII-dependent phosphorylation of histone deacetylase (HDAC 4 and 5) which drives nuclear export of these HDACS and de-repression of hypertrophy-associated transcription (e.g. by MEF2) (Wu et al., 2006; Zhang et al., 2007). It induces the expression of hypertrophic genes in myocytes and transgenic mice, consistent with its primarily nuclear localization (Ramirez et al., 1997; Zhang et al., 2002). The overexpression of CaMKIIδb in transgenic mice led to hypertrophy and moderate cardiac dysfunction within 4 months (Zhang et al., 2002), demonstrating the enhanced expression of hypertrophic markers and that CaMKIIδb expression appears to be sufficient to induce cardiac hypertrophy. In comparison, CaMKIIδc transgenic mice rapidly progress to heart failure and premature death (Zhang et al., 2003). By 6 weeks, they display marked changes in cardiac

morphology and by 12 weeks, severe cardiac dysfunction and upregulation of hypertrophic genes. The dysregulation of ECC by CaMKII is supported by studies showing both increased incidence of arrhythmogenic events in transgenic mice and enhanced susceptibility of mice to arrhythmogenic challenge by β-adrenergic stimulation (Anderson et al., 1998; Wagner et al., 2006; Wu et al., 2002). Treatment with CaMKII inhibitor, KN-93 or genetic ablation of CaMKIIδ, significantly suppressed the proarrhythmogenic effects of β-adrenergic stimulation on Ca^{2+} handling (Sag et al., 2009).

CaMKII expression and activity are increased during heart failure (Bossuyt et al., 2008; Zhang et al., 2003), while genetic inhibition or ablation of cardiac CaMKII protects against the transition to structural heart disease (Ling et al., 2009; Zhang et al., 2005).

2.5.1. Regulation of ion channels and calcium handling proteins

CaMKII has been shown to regulate several ion channels and transporters in the heart by phosphorylation to alter ionic current properties, or gene transcription to affect expression levels. This regulation can result in alterations to cell electrophysiology, action potential properties, and contribute to arrhythmogenesis. Chronic and acute CaMKII activity may differentially regulate ion channel function. With acute CaMKII activation under physiological conditions, CaMKII upregulates K^+ current via direct phosphorylation of the channel protein, and tends to increase inward Ca^{2+} and late Na^+ currents which tends to prolong action potential duration, but also accelerates I_{to} recovery from inactivation – increasing K^+ currents that tend to shorten action potential duration (Hegyi et al., 2019; Wagner et al., 2009). Notably, pathological CaMKII activation can downregulate K^+ current via reduced gene and protein expression (Hegyi et al., 2019). CaMKII significantly enhances the late Na^+ current ($I_{Na,L}$), which contributes to AP prolongation and intracellular Na^+ loading under pathological conditions (Glynn et al., 2015).

CaMKII increases L-type Ca^{2+} current ($I_{Ca,L}$) amplitude and slows $I_{Ca,L}$ inactivation upon repetitive stimulation, and several studies have implicated increases in CaMKII activity as a direct mechanism governing HF-related changes in I_{CaL} (Y. Wang et al., 2008; Wu et al., 2002). T-type Ca^{2+} current amplitude can also be enhanced by CaMKII-dependent phosphorylation (Welsby et al., 2003). Conversely, chronic CaMKII activation may decrease $Ca_v1.2$ expression (Xu et al., 2010).

Phospholamban (PLB) is an SR transmembrane protein that binds to and inhibits SERCA2 in cardiac myocytes and PLB phosphorylation by CaMKII relieves its inhibition and enhances SERCA2 activity. The RyR is directly phosphorylated by CaMKII which activates RyR gating during both diastole and ECC (Wehrens et al., 2004; Yang et al., 2007). In HF, where CaMKII expression and activation are increased, RyR phosphorylation and diastolic SR Ca^{2+} leak are increased (Ai et al., 2005); and this Ca^{2+} leak can initiate delayed afterdepolarizations (DADs) where the depolarizing current is inward Na/Ca exchange current. In general, CaMKII can phosphorylate numerous ion channels and Ca^{2+} transport proteins to result in altered cellular electrophysiology and ineffective ECC which are major causes for arrhythmogenesis and contractile dysfunction seen in HF (Bers & Grandi, 2009).

2.5.2. Regulation of contractile proteins

CaMKII regulates several proteins that are important in the force and kinetics of muscle contraction. It can phosphorylate MyBP-C, which contributes to the frequency-dependent increase in the force and kinetics of normal cardiac muscle contractions (Tong et al., 2015). CaMKII phosphorylation of the cardiac bovine troponin T and rat troponin I via an Epac-dependent pathway in ventricular myocytes have also been reported which may increase myofilament Ca^{2+}-

sensitivity (Cazorla et al., 2009; Jaquet et al., 1995). CaMKII phosphorylation of myosin light chain-2 has also been implicated, based on *in situ* experiments with the CaMKII inhibitor KN-93 in cardiomyocytes, and this effect may contribute to positive inotropy by facilitating the crossbridge formation of the myofilaments (Eikemo et al., 2016). CaMKII also phosphorylates titin on the N2B and PEVK elements (Hidalgo et al., 2013), which likely increases passive tension. It is responsible for the passive elasticity of the muscle, and mutations or altered phosphorylation are associated with diastolic dysfunction of the heart (Hamdani, Krysiak, et al., 2013).

2.5.3. Regulation of inflammatory response in cardiac diseases

Inflammation is a physiological process by which the body responds to external insults and stress conditions, and it is characterized by the production of pro-inflammatory mediators such as cytokines. Heart-specific cytokines, neurohormones and pro-inflammatory molecules, which can be referred to as cardiokines, actively drive the progression of cardiac dysfunction in heart failure (Doroudgar & Glembotski, 2011; Wu et al., 2018). CaMKII has been shown to enhance the expression of proinflammatory genes (e.g. complement factor B) through modulation of NFκB signaling following MI (Singh et al., 2009). CaMKII is activated in several diseases with an inflammatory component, such as myocardial infarction, ischemia/reperfusion injury, pressure overload/hypertrophy, and arrhythmic syndromes, where it actively regulates pro-inflammatory transcriptional signaling, thus contributing to pathological cardiac remodeling (Rusciano et al., 2019). CaMKII inhibition also attenuates the inflammatory response (reduced expression of TNFα, IL-1β, and ICAM-1) and fibrosis (reduced expression of collagen 1 and TGFβ) by influencing NFκB and MAPK signaling following I/R injury (Gray et al., 2017; Ling et al., 2013; Suetomi et al., 2019; Suetomi et al., 2018) and free fatty acid/hyperlipidemia-induced cellular remodeling (Zhong et al., 2017). Furthermore, CaMKII mediates cardiomyocyte apoptosis during

sustained β-adrenergic stimulation (Yang et al., 2006) by promoting mitochondrial Ca^{2+} overload and/or affecting p53 and BAX protein expression (Toko et al., 2010; Zhang et al., 2010). Notably, the inhibition of CaMKII has been suggested as a novel therapeutic target to treat cardiac arrhythmias, heart failure, and hypertrophy (Cipolletta et al., 2015; Rokita & Anderson, 2012; Zhang et al., 2005).

2.5.4. CaMKII and GPCR

G protein-coupled receptor (GPCR) agonists can activate CaMKII indirectly by phosphorylation at Thr287, oxidation of Met281/282 and via PKA, PKC, Epac and β-arrestin among others (Erickson et al., 2008; Mangmool et al., 2010; Pereira et al., 2017; Wu et al., 2006; Xu et al., 2010). CaMKII is activated by GPCR signaling both for acute regulation of ECC and also for long term regulation of gene expression, especially in hypertrophy and heart failure (Maier & Bers, 2007; Wu et al., 2002). GPCRs can be regulated by various protein kinases which control phosphorylation-dephosphorylation balance, and actively modify GPCR expression and function. CaMKII is one of such kinases, identified to maintain homeostasis in the brain via GPCR phosphorylation (Guo et al., 2010). Not much is known on how CaMKII regulates GPCR signaling in the heart.

Our group recently showed that pacing-induced activation of CaMKIIδ in cardiomyocytes enhances its mobility and rapid translocation to its extra-dyadic targets, providing a mechanism for broad myocyte target phosphorylation (Wood et al., 2018). However, there are gaps in knowledge relating to CaMKIIδ subcellular targeting, and how activation at its many known targets is regulated. It is not known whether GPCR-induced CaMKIIδ activation likewise stimulates its mobility. It is also unknown to what extent each PTM contributes to CaMKIIδ activation by specific GPCR stimulation – although initial experiments suggest isoproterenol is

more auto-phosphorylation dependent while angiotensin II, more oxidation dependent (Erickson et al., 2011). We investigate these in chapters 4 and 5.

2.6 CaMKII Nitrosylation

Previous studies have reported a modulatory role for NO in cardiac calcium handling (Cutler et al., 2012; Gonzalez et al., 2007; Wang et al., 2010), and interestingly tight interplays between NO and Ca^{2+} signaling was confirmed in plants as well (Trapet et al., 2015). CaMKIIδ has been well established as a key mediator of altered calcium fluxes, ion channels, and pathological transcriptional regulation in the heart (Anderson et al., 2011; Bers & Grandi, 2009; Hegyi et al., 2019). Zhang et al., showed that CaMKIIδ activity is enhanced by NO donors and Curran et al., showed CaMKII in cardiomyocytes can be nitrosylated (Curran et al., 2014; D. M. Zhang et al., 2014). In 2015, underlying the connection between NO signaling and cardiac function, Erickson et al. (Erickson et al., 2015) established the mechanism by which NO activates CaMKIIδ. *S*-nitrosylation can either activate or inhibit CaMKIIδ activity depending on the timing of NO exposure relative to Ca^{2+}/CaM binding. Exposure to NO after Ca^{2+}/CaM binding to CaMKIIδ results in autonomous kinase activation, which is abolished by mutation of the Cys290 site. However, exposure of CaMKIIδ to GSNO prior to Ca^{2+}/CaM exposure strongly suppresses kinase activation and conformational change by Ca^{2+}/CaM, and this inhibition was ablated by mutation of the Cys273 site (Erickson et al., 2015). A mechanism by which nitrosylation-dependent CaMKII activation may influence cellular physiology is in modulating Ca^{2+} release events from the SR in myocytes. In 2017, Pereira et al. suggested a pathway for the *S*-nitrosylation of CaMKII (β-AR-cAMP-Epac-PI3K-Akt-NOS1-CaMKII), showing that without the Epac-induced *S*-nitrosylation of CaMKII, autophosphorylation does not occur (Pereira et al., 2017).

The emergence of SNOs as second messengers and of *S*-nitrosylation as the preeminent NO-based signal has led to a great number of discoveries in the mechanisms underlying the pathogenesis of heart diseases. Emerging evidence indicates that *S*-nitrosylation is a potential target of cardiac protection ergo, unraveling the molecular underpinnings of SNO-based cardiovascular function and pathophysiology will undoubtedly yield novel therapeutic targets with great potential to improve clinical outcomes.

The key functional role that CaMKII plays in numerous cardiac pathologies, makes it essential to understand how CaMKII is regulated in adult cardiac myocytes. Given that localized nitric oxide signaling and the CaMKII signaling pathway have been identified as mediators of mechanochemotransduction (MCT), in this dissertation, I test the hypothesis that CaMKII nitrosylation at Cys290 plays a key role in mediating MCT. Identifying key mediators in MCT can provide molecular therapeutic targets for treating mechanical stress induced Ca^{2+} dysregulation, arrythmias and cardiomyopathy. I also investigate the effect of CaMKII nitrosylation at Cys273 and its effect on limiting CaMKII activation. Generally, the studies in this dissertation will have a major impact on understanding how CaMKII activity is regulated in the heart. Studies will also contribute to understanding mechanical stress effects on the heart, and why excessive afterload in disease conditions worsens cardiac function.

References

Adak, S., Santolini, J., Tikunova, S., Wang, Q., Johnson, J. D., & Stuehr, D. J. (2001). Neuronal nitric-oxide synthase mutant (Ser-1412 --> Asp) demonstrates surprising connections

between heme reduction, NO complex formation, and catalysis. *J Biol Chem*, *276*(2), 1244-1252. https://doi.org/10.1074/jbc.M006857200

Ahern, G. P., Hsu, S. F., Klyachko, V. A., & Jackson, M. B. (2000). Induction of persistent sodium current by exogenous and endogenous nitric oxide. *J Biol Chem*, *275*(37), 28810-28815. https://doi.org/10.1074/jbc.M003090200

Ai, X., Curran, J. W., Shannon, T. R., Bers, D. M., & Pogwizd, S. M. (2005). Ca2+/calmodulin-dependent protein kinase modulates cardiac ryanodine receptor phosphorylation and sarcoplasmic reticulum Ca2+ leak in heart failure. *Circ Res*, *97*(12), 1314-1322. https://doi.org/10.1161/01.RES.0000194329.41863.89

Anderson, M. E. (2009). Sticky fingers: CaMKII finds a home on another ion channel. *Circ Res*, *104*(6), 712-714. https://doi.org/10.1161/CIRCRESAHA.109.195503

Anderson, M. E., Braun, A. P., Wu, Y., Lu, T., Schulman, H., & Sung, R. J. (1998). KN-93, an inhibitor of multifunctional Ca++/calmodulin-dependent protein kinase, decreases early afterdepolarizations in rabbit heart. *J Pharmacol Exp Ther*, *287*(3), 996-1006. https://www.ncbi.nlm.nih.gov/pubmed/9864285

Anderson, M. E., Brown, J. H., & Bers, D. M. (2011). CaMKII in myocardial hypertrophy and heart failure. *J Mol Cell Cardiol*, *51*(4), 468-473. https://doi.org/10.1016/j.yjmcc.2011.01.012

Arnelle, D. R., & Stamler, J. S. (1995). NO+, NO, and NO- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation. *Arch Biochem Biophys*, *318*(2), 279-285. https://doi.org/10.1006/abbi.1995.1231

Asada, K., Kurokawa, J., & Furukawa, T. (2009). Redox- and calmodulin-dependent S-nitrosylation of the KCNQ1 channel. *J Biol Chem*, *284*(9), 6014-6020. https://doi.org/10.1074/jbc.M807158200

Backs, J., Backs, T., Neef, S., Kreusser, M. M., Lehmann, L. H., Patrick, D. M., Grueter, C. E., Qi, X., Richardson, J. A., Hill, J. A., Katus, H. A., Bassel-Duby, R., Maier, L. S., & Olson, E. N. (2009). The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. *Proc Natl Acad Sci U S A*, *106*(7), 2342-2347. https://doi.org/10.1073/pnas.0813013106

Baek, K. J., Thiel, B. A., Lucas, S., & Stuehr, D. J. (1993). Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. *J Biol Chem*, *268*(28), 21120-21129. https://www.ncbi.nlm.nih.gov/pubmed/7691806

Bailey, A., Pope, T. W., Moore, S. A., & Campbell, C. L. (2007). The tragedy of TRIUMPH for nitric oxide synthesis inhibition in cardiogenic shock: where do we go from here? *Am J Cardiovasc Drugs*, *7*(5), 337-345. https://doi.org/10.2165/00129784-200707050-00003

Balligand, J. L., Feron, O., & Dessy, C. (2009). eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues. *Physiol Rev*, *89*(2), 481-534. https://doi.org/10.1152/physrev.00042.2007

Balligand, J. L., Kobzik, L., Han, X., Kaye, D. M., Belhassen, L., O'Hara, D. S., Kelly, R. A., Smith, T. W., & Michel, T. (1995). Nitric oxide-dependent parasympathetic signaling is due to activation of constitutive endothelial (type III) nitric oxide synthase in cardiac myocytes. *J Biol Chem*, *270*(24), 14582-14586. https://doi.org/10.1074/jbc.270.24.14582

Balligand, J. L., Ungureanu-Longrois, D., Simmons, W. W., Pimental, D., Malinski, T. A., Kapturczak, M., Taha, Z., Lowenstein, C. J., Davidoff, A. J., & Kelly, R. A. (1994). Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. Characterization and regulation of iNOS expression and detection of iNOS activity in single cardiac myocytes in vitro. *J Biol Chem*, *269*(44), 27580-27588. https://www.ncbi.nlm.nih.gov/pubmed/7525557

Barouch, L. A., Harrison, R. W., Skaf, M. W., Rosas, G. O., Cappola, T. P., Kobeissi, Z. A., Hobai, I. A., Lemmon, C. A., Burnett, A. L., O'Rourke, B., Rodriguez, E. R., Huang, P. L., Lima, J. A., Berkowitz, D. E., & Hare, J. M. (2002). Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature*, *416*(6878), 337-339. https://doi.org/10.1038/416337a

Belcastro, E., Gaucher, C., Corti, A., Leroy, P., Lartaud, I., & Pompella, A. (2017). Regulation of protein function by S-nitrosation and S-glutathionylation: processes and targets in cardiovascular pathophysiology. *Biol Chem*, *398*(12), 1267-1293. https://doi.org/10.1515/hsz-2017-0150

Benjamin, N., O'Driscoll, F., Dougall, H., Duncan, C., Smith, L., Golden, M., & McKenzie, H. (1994). Stomach NO synthesis. *Nature*, *368*(6471), 502. https://doi.org/10.1038/368502a0

Bers, D. M. (2002). Cardiac excitation-contraction coupling. *Nature*, *415*(6868), 198-205. https://doi.org/10.1038/415198a

Bers, D. M. (2008). Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol*, *70*, 23-49. https://doi.org/10.1146/annurev.physiol.70.113006.100455

Bers, D. M., & Grandi, E. (2009). Calcium/calmodulin-dependent kinase II regulation of cardiac ion channels. *J Cardiovasc Pharmacol*, *54*(3), 180-187. https://doi.org/10.1097/FJC.0b013e3181a25078

Bers, D. M., & Morotti, S. (2014). Ca(2+) current facilitation is CaMKII-dependent and has arrhythmogenic consequences. *Front Pharmacol*, *5*, 144. https://doi.org/10.3389/fphar.2014.00144

Bhattacharyya, M., Karandur, D., & Kuriyan, J. (2020). Structural Insights into the Regulation of Ca. *Cold Spring Harb Perspect Biol*, *12*(6). https://doi.org/10.1101/cshperspect.a035147

Bhattacharyya, M., Stratton, M. M., Going, C. C., McSpadden, E. D., Huang, Y., Susa, A. C., Elleman, A., Cao, Y. M., Pappireddi, N., Burkhardt, P., Gee, C. L., Barros, T., Schulman, H., Williams, E. R., & Kuriyan, J. (2016). Molecular mechanism of activation-triggered subunit exchange in Ca(2+)/calmodulin-dependent protein kinase II. *Elife*, *5*. https://doi.org/10.7554/eLife.13405

Bhushan, S., Kondo, K., Polhemus, D. J., Otsuka, H., Nicholson, C. K., Tao, Y. X., Huang, H., Georgiopoulou, V. V., Murohara, T., Calvert, J. W., Butler, J., & Lefer, D. J. (2014). Nitrite therapy improves left ventricular function during heart failure via restoration of nitric oxide-mediated cytoprotective signaling. *Circ Res*, *114*(8), 1281-1291. https://doi.org/10.1161/CIRCRESAHA.114.301475

Boehning, D., & Snyder, S. H. (2003). Novel neural modulators. *Annu Rev Neurosci*, *26*, 105-131. https://doi.org/10.1146/annurev.neuro.26.041002.131047

Boo, Y. C., Sorescu, G. P., Bauer, P. M., Fulton, D., Kemp, B. E., Harrison, D. G., Sessa, W. C., & Jo, H. (2003). Endothelial NO synthase phosphorylated at SER635 produces NO without

requiring intracellular calcium increase. *Free Radic Biol Med*, *35*(7), 729-741. https://doi.org/10.1016/s0891-5849(03)00397-6

Bossuyt, J., Helmstadter, K., Wu, X., Clements-Jewery, H., Haworth, R. S., Avkiran, M., Martin, J. L., Pogwizd, S. M., & Bers, D. M. (2008). Ca2+/calmodulin-dependent protein kinase IIdelta and protein kinase D overexpression reinforce the histone deacetylase 5 redistribution in heart failure. *Circ Res*, *102*(6), 695-702. https://doi.org/10.1161/CIRCRESAHA.107.169755

Bredt, D. S., & Snyder, S. H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci U S A*, *87*(2), 682-685. https://doi.org/10.1073/pnas.87.2.682

Brenman, J. E., & Bredt, D. S. (1997). Synaptic signaling by nitric oxide. *Curr Opin Neurobiol*, *7*(3), 374-378. https://doi.org/10.1016/s0959-4388(97)80065-7

Brunner, F., Andrew, P., Wölkart, G., Zechner, R., & Mayer, B. (2001). Myocardial contractile function and heart rate in mice with myocyte-specific overexpression of endothelial nitric oxide synthase. *Circulation*, *104*(25), 3097-3102. https://doi.org/10.1161/hc5001.101966

Burger, D. E., Lu, X., Lei, M., Xiang, F. L., Hammoud, L., Jiang, M., Wang, H., Jones, D. L., Sims, S. M., & Feng, Q. (2009). Neuronal nitric oxide synthase protects against myocardial infarction-induced ventricular arrhythmia and mortality in mice. *Circulation*, *120*(14), 1345-1354. https://doi.org/10.1161/CIRCULATIONAHA.108.846402

Burkard, N., Williams, T., Czolbe, M., Blömer, N., Panther, F., Link, M., Fraccarollo, D., Widder, J. D., Hu, K., Han, H., Hofmann, U., Frantz, S., Nordbeck, P., Bulla, J., Schuh, K., & Ritter, O. (2010). Conditional overexpression of neuronal nitric oxide synthase is cardioprotective in ischemia/reperfusion. *Circulation*, *122*(16), 1588-1603. https://doi.org/10.1161/CIRCULATIONAHA.109.933630

Busse, R., & Mülsch, A. (1990). Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. *FEBS Lett*, *265*(1-2), 133-136. https://doi.org/10.1016/0014-5793(90)80902-u

Camerini, S., Polci, M. L., Restuccia, U., Usuelli, V., Malgaroli, A., & Bachi, A. (2007). A novel approach to identify proteins modified by nitric oxide: the HIS-TAG switch method. *J Proteome Res*, *6*(8), 3224-3231. https://doi.org/10.1021/pr0701456

Campbell, M. G., Smith, B. C., Potter, C. S., Carragher, B., & Marletta, M. A. (2014). Molecular architecture of mammalian nitric oxide synthases. *Proc Natl Acad Sci U S A*, *111*(35), E3614-3623. https://doi.org/10.1073/pnas.1413763111

Carnicer, R., Crabtree, M. J., Sivakumaran, V., Casadei, B., & Kass, D. A. (2013). Nitric oxide synthases in heart failure. *Antioxid Redox Signal*, *18*(9), 1078-1099. https://doi.org/10.1089/ars.2012.4824

Casin, K. M., Fallica, J., Mackowski, N., Veenema, R. J., Chan, A., St Paul, A., Zhu, G., Bedja, D., Biswal, S., & Kohr, M. J. (2018). S-Nitrosoglutathione Reductase Is Essential for Protecting the Female Heart From Ischemia-Reperfusion Injury. *Circ Res*, *123*(11), 1232-1243. https://doi.org/10.1161/CIRCRESAHA.118.313956

Cazorla, O., Lucas, A., Poirier, F., Lacampagne, A., & Lezoualc'h, F. (2009). The cAMP binding protein Epac regulates cardiac myofilament function. *Proc Natl Acad Sci U S A*, *106*(33), 14144-14149. https://doi.org/10.1073/pnas.0812536106

Champion, H. C., Georgakopoulos, D., Takimoto, E., Isoda, T., Wang, Y., & Kass, D. A. (2004). Modulation of in vivo cardiac function by myocyte-specific nitric oxide synthase-3. *Circ Res*, *94*(5), 657-663. https://doi.org/10.1161/01.RES.0000119323.79644.20

Chandrasekhar, J., Gill, A., & Mehran, R. (2018). Acute myocardial infarction in young women: current perspectives. *Int J Womens Health*, *10*, 267-284. https://doi.org/10.2147/IJWH.S107371

Chelu, M. G., Sarma, S., Sood, S., Wang, S., van Oort, R. J., Skapura, D. G., Li, N., Santonastasi, M., Müller, F. U., Schmitz, W., Schotten, U., Anderson, M. E., Valderrábano, M., Dobrev, D., & Wehrens, X. H. (2009). Calmodulin kinase II-mediated sarcoplasmic reticulum Ca2+ leak promotes atrial fibrillation in mice. *J Clin Invest*, *119*(7), 1940-1951. https://doi.org/10.1172/jci37059

Chen, C. A., Wang, T. Y., Varadharaj, S., Reyes, L. A., Hemann, C., Talukder, M. A., Chen, Y. R., Druhan, L. J., & Zweier, J. L. (2010). S-glutathionylation uncouples eNOS and regulates its cellular and vascular function. *Nature*, *468*(7327), 1115-1118. https://doi.org/10.1038/nature09599

Chen, S. C., Huang, B., Liu, Y. C., Shyu, K. G., Lin, P. Y., & Wang, D. L. (2008). Acute hypoxia enhances proteins' S-nitrosylation in endothelial cells. *Biochem Biophys Res Commun*, *377*(4), 1274-1278. https://doi.org/10.1016/j.bbrc.2008.10.144

Cho, H. J., Xie, Q. W., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., & Nathan, C. (1992). Calmodulin is a subunit of nitric oxide synthase from macrophages. *J Exp Med*, *176*(2), 599-604. https://doi.org/10.1084/jem.176.2.599

Choate, J. K., Danson, E. J., Morris, J. F., & Paterson, D. J. (2001). Peripheral vagal control of heart rate is impaired in neuronal NOS knockout mice. *Am J Physiol Heart Circ Physiol*, *281*(6), H2310-2317. https://doi.org/10.1152/ajpheart.2001.281.6.H2310

Cipolletta, E., Rusciano, M. R., Maione, A. S., Santulli, G., Sorriento, D., Del Giudice, C., Ciccarelli, M., Franco, A., Crola, C., Campiglia, P., Sala, M., Gomez-Monterrey, I., De

Luca, N., Trimarco, B., Iaccarino, G., & Illario, M. (2015). Targeting the CaMKII/ERK Interaction in the Heart Prevents Cardiac Hypertrophy. *PLoS One*, *10*(6), e0130477. https://doi.org/10.1371/journal.pone.0130477

Clementi, E., Brown, G. C., Feelisch, M., & Moncada, S. (1998). Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc Natl Acad Sci U S A*, *95*(13), 7631-7636. https://doi.org/10.1073/pnas.95.13.7631

Condorelli, R. A., Calogero, A. E., Vicari, E., Favilla, V., Morgia, G., Cimino, S., & La Vignera, S. (2013). Vascular regenerative therapies for the treatment of erectile dysfunction: current approaches. *Andrology*, *1*(4), 533-540. https://doi.org/10.1111/j.2047-2927.2013.00087.x

Cooke, J. P., Stamler, J., Andon, N., Davies, P. F., McKinley, G., & Loscalzo, J. (1990). Flow stimulates endothelial cells to release a nitrovasodilator that is potentiated by reduced thiol. *Am J Physiol*, *259*(3 Pt 2), H804-812. https://doi.org/10.1152/ajpheart.1990.259.3.H804

Coultrap, S. J., & Bayer, K. U. (2014). Nitric oxide induces Ca2+-independent activity of the Ca2+/calmodulin-dependent protein kinase II (CaMKII). *J Biol Chem*, *289*(28), 19458-19465. https://doi.org/10.1074/jbc.M114.558254

Curran, J., Tang, L., Roof, S. R., Velmurugan, S., Millard, A., Shonts, S., Wang, H., Santiago, D., Ahmad, U., Perryman, M., Bers, D. M., Mohler, P. J., Ziolo, M. T., & Shannon, T. R. (2014). Nitric oxide-dependent activation of CaMKII increases diastolic sarcoplasmic reticulum calcium release in cardiac myocytes in response to adrenergic stimulation. *PLoS One*, *9*(2), e87495. https://doi.org/10.1371/journal.pone.0087495

Cutler, M. J., Plummer, B. N., Wan, X., Sun, Q. A., Hess, D., Liu, H., Deschenes, I., Rosenbaum, D. S., Stamler, J. S., & Laurita, K. R. (2012). Aberrant S-nitrosylation mediates calcium-

triggered ventricular arrhythmia in the intact heart. *Proc Natl Acad Sci U S A*, *109*(44), 18186-18191. https://doi.org/10.1073/pnas.1210565109

Czubryt, M. P., Espira, L., Lamoureux, L., & Abrenica, B. (2006). The role of sex in cardiac function and disease. *Can J Physiol Pharmacol*, *84*(1), 93-109. https://doi.org/10.1139/y05-151

Danson, E. J., Choate, J. K., & Paterson, D. J. (2005). Cardiac nitric oxide: emerging role for nNOS in regulating physiological function. *Pharmacol Ther*, *106*(1), 57-74. https://doi.org/10.1016/j.pharmthera.2004.11.003

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., & Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*, *399*(6736), 601-605. https://doi.org/10.1038/21224

Doroudgar, S., & Glembotski, C. C. (2011). The cardiokine story unfolds: ischemic stress-induced protein secretion in the heart. *Trends Mol Med*, *17*(4), 207-214. https://doi.org/10.1016/j.molmed.2010.12.003

Duncan, C., Dougall, H., Johnston, P., Green, S., Brogan, R., Leifert, C., Smith, L., Golden, M., & Benjamin, N. (1995). Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med*, *1*(6), 546-551. https://doi.org/10.1038/nm0695-546

Duranski, M. R., Greer, J. J., Dejam, A., Jaganmohan, S., Hogg, N., Langston, W., Patel, R. P., Yet, S. F., Wang, X., Kevil, C. G., Gladwin, M. T., & Lefer, D. J. (2005). Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest*, *115*(5), 1232-1240. https://doi.org/10.1172/JCI22493

Edman, C. F., & Schulman, H. (1994). Identification and characterization of delta B-CaM kinase and delta C-CaM kinase from rat heart, two new multifunctional Ca2+/calmodulin-dependent protein kinase isoforms. *Biochim Biophys Acta*, *1221*(1), 89-101. https://doi.org/10.1016/0167-4889(94)90221-6

Eikemo, H., Moltzau, L. R., Hussain, R. I., Nguyen, C. H., Qvigstad, E., Levy, F. O., Skomedal, T., & Osnes, J. B. (2016). CaMKII in addition to MLCK contributes to phosphorylation of regulatory light chain in cardiomyocytes. *Biochem Biophys Res Commun*, *471*(1), 219-225. https://doi.org/10.1016/j.bbrc.2016.01.132

Erickson, J. R. (2014). Mechanisms of CaMKII Activation in the Heart. *Front Pharmacol*, *5*, 59. https://doi.org/10.3389/fphar.2014.00059

Erickson, J. R., Joiner, M. L., Guan, X., Kutschke, W., Yang, J., Oddis, C. V., Bartlett, R. K., Lowe, J. S., O'Donnell, S. E., Aykin-Burns, N., Zimmerman, M. C., Zimmerman, K., Ham, A. J., Weiss, R. M., Spitz, D. R., Shea, M. A., Colbran, R. J., Mohler, P. J., & Anderson, M. E. (2008). A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell*, *133*(3), 462-474. https://doi.org/10.1016/j.cell.2008.02.048

Erickson, J. R., Nichols, C. B., Uchinoumi, H., Stein, M. L., Bossuyt, J., & Bers, D. M. (2015). S-Nitrosylation Induces Both Autonomous Activation and Inhibition of Calcium/Calmodulin-dependent Protein Kinase II δ. *J Biol Chem*, *290*(42), 25646-25656. https://doi.org/10.1074/jbc.M115.650234

Erickson, J. R., Patel, R., Ferguson, A., Bossuyt, J., & Bers, D. M. (2011). Fluorescence resonance energy transfer-based sensor Camui provides new insight into mechanisms of calcium/calmodulin-dependent protein kinase II activation in intact cardiomyocytes. *Circ Res*, *109*(7), 729-738. https://doi.org/10.1161/CIRCRESAHA.111.247148

Erickson, J. R., Pereira, L., Wang, L., Han, G., Ferguson, A., Dao, K., Copeland, R. J., Despa, F., Hart, G. W., Ripplinger, C. M., & Bers, D. M. (2013). Diabetic hyperglycaemia activates CaMKII and arrhythmias by O-linked glycosylation. *Nature*, *502*(7471), 372-376. https://doi.org/10.1038/nature12537

Erwin, P. A., Lin, A. J., Golan, D. E., & Michel, T. (2005). Receptor-regulated dynamic S-nitrosylation of endothelial nitric-oxide synthase in vascular endothelial cells. *J Biol Chem*, *280*(20), 19888-19894. https://doi.org/10.1074/jbc.M413058200

Farah, C., Michel, L. Y. M., & Balligand, J. L. (2018). Nitric oxide signalling in cardiovascular health and disease. *Nat Rev Cardiol*, *15*(5), 292-316. https://doi.org/10.1038/nrcardio.2017.224

Fauconnier, J., Thireau, J., Reiken, S., Cassan, C., Richard, S., Matecki, S., Marks, A. R., & Lacampagne, A. (2010). Leaky RyR2 trigger ventricular arrhythmias in Duchenne muscular dystrophy. *Proc Natl Acad Sci U S A*, *107*(4), 1559-1564. https://doi.org/10.1073/pnas.0908540107

Ferguson, G. D., & Bridge, W. J. (2019). The glutathione system and the related thiol network in Caenorhabditis elegans. *Redox Biol*, *24*, 101171. https://doi.org/10.1016/j.redox.2019.101171

Feron, O., Michel, J. B., Sase, K., & Michel, T. (1998). Dynamic regulation of endothelial nitric oxide synthase: complementary roles of dual acylation and caveolin interactions. *Biochemistry*, *37*(1), 193-200. https://doi.org/10.1021/bi972307p

Fisslthaler, B., Loot, A. E., Mohamed, A., Busse, R., & Fleming, I. (2008). Inhibition of endothelial nitric oxide synthase activity by proline-rich tyrosine kinase 2 in response to

fluid shear stress and insulin. *Circ Res*, *102*(12), 1520-1528. https://doi.org/10.1161/CIRCRESAHA.108.172072

Fleming, I., Fisslthaler, B., Dimmeler, S., Kemp, B. E., & Busse, R. (2001). Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res*, *88*(11), E68-75. https://doi.org/10.1161/hh1101.092677

Forrester, M. T., Foster, M. W., Benhar, M., & Stamler, J. S. (2009). Detection of protein S-nitrosylation with the biotin-switch technique. *Free Radic Biol Med*, *46*(2), 119-126. https://doi.org/10.1016/j.freeradbiomed.2008.09.034

Foster, M. W., McMahon, T. J., & Stamler, J. S. (2003). S-nitrosylation in health and disease. *Trends Mol Med*, *9*(4), 160-168. https://doi.org/10.1016/s1471-4914(03)00028-5

Friedman, D. B., & Lilley, K. S. (2008). Optimizing the difference gel electrophoresis (DIGE) technology. *Methods Mol Biol*, *428*, 93-124. https://doi.org/10.1007/978-1-59745-117-8_6

Fukuto, J. M., Carrington, S. J., Tantillo, D. J., Harrison, J. G., Ignarro, L. J., Freeman, B. A., Chen, A., & Wink, D. A. (2012). Small molecule signaling agents: the integrated chemistry and biochemistry of nitrogen oxides, oxides of carbon, dioxygen, hydrogen sulfide, and their derived species. *Chem Res Toxicol*, *25*(4), 769-793. https://doi.org/10.1021/tx2005234

Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., & Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*, *399*(6736), 597-601. https://doi.org/10.1038/21218

Furchgott, R. F., & Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, *288*(5789), 373-376. https://doi.org/10.1038/288373a0

Gaertner, T. R., Kolodziej, S. J., Wang, D., Kobayashi, R., Koomen, J. M., Stoops, J. K., & Waxham, M. N. (2004). Comparative analyses of the three-dimensional structures and enzymatic properties of alpha, beta, gamma and delta isoforms of Ca2+-calmodulin-dependent protein kinase II. *J Biol Chem*, *279*(13), 12484-12494. https://doi.org/10.1074/jbc.M313597200

Glynn, P., Musa, H., Wu, X., Unudurthi, S. D., Little, S., Qian, L., Wright, P. J., Radwanski, P. B., Gyorke, S., Mohler, P. J., & Hund, T. J. (2015). Voltage-Gated Sodium Channel Phosphorylation at Ser571 Regulates Late Current, Arrhythmia, and Cardiac Function In Vivo. *Circulation*, *132*(7), 567-577. https://doi.org/10.1161/CIRCULATIONAHA.114.015218

Gómez, R., Caballero, R., Barana, A., Amorós, I., Calvo, E., López, J. A., Klein, H., Vaquero, M., Osuna, L., Atienza, F., Almendral, J., Pinto, A., Tamargo, J., & Delpón, E. (2009). Nitric oxide increases cardiac IK1 by nitrosylation of cysteine 76 of Kir2.1 channels. *Circ Res*, *105*(4), 383-392. https://doi.org/10.1161/CIRCRESAHA.109.197558

Gómez, R., Núñez, L., Vaquero, M., Amorós, I., Barana, A., de Prada, T., Macaya, C., Maroto, L., Rodríguez, E., Caballero, R., López-Farré, A., Tamargo, J., & Delpón, E. (2008). Nitric oxide inhibits Kv4.3 and human cardiac transient outward potassium current (Ito1). *Cardiovasc Res*, *80*(3), 375-384. https://doi.org/10.1093/cvr/cvn205

Gonzalez, D. R., Beigi, F., Treuer, A. V., & Hare, J. M. (2007). Deficient ryanodine receptor S-nitrosylation increases sarcoplasmic reticulum calcium leak and arrhythmogenesis in

cardiomyocytes. *Proc Natl Acad Sci U S A*, *104*(51), 20612-20617. https://doi.org/10.1073/pnas.0706796104

Gow, A. J., Chen, Q., Hess, D. T., Day, B. J., Ischiropoulos, H., & Stamler, J. S. (2002). Basal and stimulated protein S-nitrosylation in multiple cell types and tissues. *J Biol Chem*, *277*(12), 9637-9640. https://doi.org/10.1074/jbc.C100746200

Gray, C. B., Suetomi, T., Xiang, S., Mishra, S., Blackwood, E. A., Glembotski, C. C., Miyamoto, S., Westenbrink, B. D., & Brown, J. H. (2017). CaMKIIδ subtypes differentially regulate infarct formation following ex vivo myocardial ischemia/reperfusion through NF-κB and TNF-α. *J Mol Cell Cardiol*, *103*, 48-55. https://doi.org/10.1016/j.yjmcc.2017.01.002

Guo, M. L., Liu, Z., Chu, X. P., Mao, L. M., & Wang, J. Q. (2010). CaMKIIα, a modulator of M4 muscarinic acetylcholine receptors. *Commun Integr Biol*, *3*(5), 465-467. https://doi.org/10.4161/cib.3.5.12476

Gutierrez, D. A., Fernandez-Tenorio, M., Ogrodnik, J., & Niggli, E. (2013). NO-dependent CaMKII activation during β-adrenergic stimulation of cardiac muscle. *Cardiovasc Res*, *100*(3), 392-401. https://doi.org/10.1093/cvr/cvt201

Gyurko, R., Kuhlencordt, P., Fishman, M. C., & Huang, P. L. (2000). Modulation of mouse cardiac function in vivo by eNOS and ANP. *Am J Physiol Heart Circ Physiol*, *278*(3), H971-981. https://doi.org/10.1152/ajpheart.2000.278.3.H971

Hamdani, N., Bishu, K. G., von Frieling-Salewsky, M., Redfield, M. M., & Linke, W. A. (2013). Deranged myofilament phosphorylation and function in experimental heart failure with preserved ejection fraction. *Cardiovasc Res*, *97*(3), 464-471. https://doi.org/10.1093/cvr/cvs353

Hamdani, N., Krysiak, J., Kreusser, M. M., Neef, S., Dos Remedios, C. G., Maier, L. S., Krüger, M., Backs, J., & Linke, W. A. (2013). Crucial role for Ca2(+)/calmodulin-dependent protein kinase-II in regulating diastolic stress of normal and failing hearts via titin phosphorylation. *Circ Res*, *112*(4), 664-674. https://doi.org/10.1161/CIRCRESAHA.111.300105

Han, G., Ma, H., Chintala, R., Miyake, K., Fulton, D. J., Barman, S. A., & White, R. E. (2007). Nongenomic, endothelium-independent effects of estrogen on human coronary smooth muscle are mediated by type I (neuronal) NOS and PI3-kinase-Akt signaling. *Am J Physiol Heart Circ Physiol*, *293*(1), H314-321. https://doi.org/10.1152/ajpheart.01342.2006

Han, P., Zhou, X., Huang, B., Zhang, X., & Chen, C. (2008). On-gel fluorescent visualization and the site identification of S-nitrosylated proteins. *Anal Biochem*, *377*(2), 150-155. https://doi.org/10.1016/j.ab.2008.03.023

Hao, G., Derakhshan, B., Shi, L., Campagne, F., & Gross, S. S. (2006). SNOSID, a proteomic method for identification of cysteine S-nitrosylation sites in complex protein mixtures. *Proc Natl Acad Sci U S A*, *103*(4), 1012-1017. https://doi.org/10.1073/pnas.0508412103

Hausladen, A., Rafikov, R., Angelo, M., Singel, D. J., Nudler, E., & Stamler, J. S. (2007). Assessment of nitric oxide signals by triiodide chemiluminescence. *Proc Natl Acad Sci U S A*, *104*(7), 2157-2162. https://doi.org/10.1073/pnas.0611191104

Haywood, G. A., Tsao, P. S., von der Leyen, H. E., Mann, M. J., Keeling, P. J., Trindade, P. T., Lewis, N. P., Byrne, C. D., Rickenbacher, P. R., Bishopric, N. H., Cooke, J. P., McKenna, W. J., & Fowler, M. B. (1996). Expression of inducible nitric oxide synthase in human heart failure. *Circulation*, *93*(6), 1087-1094. https://doi.org/10.1161/01.cir.93.6.1087

He, Q., Cheng, J., & Wang, Y. (2019). Chronic CaMKII inhibition reverses cardiac function and cardiac reserve in HF mice. *Life Sci*, *219*, 122-128. https://doi.org/10.1016/j.lfs.2019.01.010

Hegyi, B., Bers, D. M., & Bossuyt, J. (2019). CaMKII signaling in heart diseases: Emerging role in diabetic cardiomyopathy. *J Mol Cell Cardiol*, *127*, 246-259. https://doi.org/10.1016/j.yjmcc.2019.01.001

Hemmens, B., & Mayer, B. (1998). Enzymology of nitric oxide synthases. *Methods Mol Biol*, *100*, 1-32. https://doi.org/10.1385/1-59259-749-1:1

Hess, D. T., Matsumoto, A., Kim, S. O., Marshall, H. E., & Stamler, J. S. (2005). Protein S-nitrosylation: purview and parameters. *Nat Rev Mol Cell Biol*, *6*(2), 150-166. https://doi.org/10.1038/nrm1569

Hidalgo, C. G., Chung, C. S., Saripalli, C., Methawasin, M., Hutchinson, K. R., Tsaprailis, G., Labeit, S., Mattiazzi, A., & Granzier, H. L. (2013). The multifunctional Ca(2+)/calmodulin-dependent protein kinase II delta (CaMKIIδ) phosphorylates cardiac titin's spring elements. *J Mol Cell Cardiol*, *54*, 90-97. https://doi.org/10.1016/j.yjmcc.2012.11.012

Hoch, B., Meyer, R., Hetzer, R., Krause, E. G., & Karczewski, P. (1999). Identification and expression of delta-isoforms of the multifunctional Ca2+/calmodulin-dependent protein kinase in failing and nonfailing human myocardium. *Circ Res*, *84*(6), 713-721. https://doi.org/10.1161/01.res.84.6.713

Hoeker, G. S., Hanafy, M. A., Oster, R. A., Bers, D. M., & Pogwizd, S. M. (2016). Reduced Arrhythmia Inducibility With Calcium/Calmodulin-dependent Protein Kinase II Inhibition

in Heart Failure Rabbits. *J Cardiovasc Pharmacol*, *67*(3), 260-265. https://doi.org/10.1097/FJC.0000000000000343

Hoelz, A., Nairn, A. C., & Kuriyan, J. (2003). Crystal structure of a tetradecameric assembly of the association domain of Ca2+/calmodulin-dependent kinase II. *Mol Cell*, *11*(5), 1241-1251. https://doi.org/10.1016/s1097-2765(03)00171-0

Hogg, N. (2002). The biochemistry and physiology of S-nitrosothiols. *Annu Rev Pharmacol Toxicol*, *42*, 585-600. https://doi.org/10.1146/annurev.pharmtox.42.092501.104328

Howe, C. J., Lahair, M. M., McCubrey, J. A., & Franklin, R. A. (2004). Redox regulation of the calcium/calmodulin-dependent protein kinases. *J Biol Chem*, *279*(43), 44573-44581. https://doi.org/10.1074/jbc.M404175200

Hudmon, A., & Schulman, H. (2002). Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J*, *364*(Pt 3), 593-611. https://doi.org/10.1042/BJ20020228

Hughes, M. N. (2008). Chemistry of nitric oxide and related species. *Methods Enzymol*, *436*, 3-19. https://doi.org/10.1016/S0076-6879(08)36001-7

Hulley, S., Grady, D., Bush, T., Furberg, C., Herrington, D., Riggs, B., & Vittinghoff, E. (1998). Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA*, *280*(7), 605-613. https://doi.org/10.1001/jama.280.7.605

Ichinose, F., Hataishi, R., Wu, J. C., Kawai, N., Rodrigues, A. C., Mallari, C., Post, J. M., Parkinson, J. F., Picard, M. H., Bloch, K. D., & Zapol, W. M. (2003). A selective inducible NOS dimerization inhibitor prevents systemic, cardiac, and pulmonary hemodynamic

dysfunction in endotoxemic mice. *Am J Physiol Heart Circ Physiol*, *285*(6), H2524-2530. https://doi.org/10.1152/ajpheart.00530.2003

Irie, T., Sips, P. Y., Kai, S., Kida, K., Ikeda, K., Hirai, S., Moazzami, K., Jiramongkolchai, P., Bloch, D. B., Doulias, P. T., Armoundas, A. A., Kaneki, M., Ischiropoulos, H., Kranias, E., Bloch, K. D., Stamler, J. S., & Ichinose, F. (2015). S-Nitrosylation of Calcium-Handling Proteins in Cardiac Adrenergic Signaling and Hypertrophy. *Circ Res*, *117*(9), 793-803. https://doi.org/10.1161/CIRCRESAHA.115.307157

Jaquet, K., Fukunaga, K., Miyamoto, E., & Meyer, H. E. (1995). A site phosphorylated in bovine cardiac troponin T by cardiac CaM kinase II. *Biochim Biophys Acta*, *1248*(2), 193-195. https://doi.org/10.1016/0167-4838(95)00028-s

Jian, Z., Han, H., Zhang, T., Puglisi, J., Izu, L. T., Shaw, J. A., Onofiok, E., Erickson, J. R., Chen, Y. J., Horvath, B., Shimkunas, R., Xiao, W., Li, Y., Pan, T., Chan, J., Banyasz, T., Tardiff, J. C., Chiamvimonvat, N., Bers, D. M., Lam, K. S., & Chen-Izu, Y. (2014). Mechanochemotransduction during cardiomyocyte contraction is mediated by localized nitric oxide signaling. *Sci Signal*, *7*(317), ra27. https://doi.org/10.1126/scisignal.2005046

Kapil, V., Khambata, R. S., Robertson, A., Caulfield, M. J., & Ahluwalia, A. (2015). Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study. *Hypertension*, *65*(2), 320-327. https://doi.org/10.1161/HYPERTENSIONAHA.114.04675

Kettenhofen, N. J., Wang, X., Gladwin, M. T., & Hogg, N. (2008). In-gel detection of S-nitrosated proteins using fluorescence methods. *Methods Enzymol*, *441*, 53-71. https://doi.org/10.1016/S0076-6879(08)01204-4

Khan, S. A., Skaf, M. W., Harrison, R. W., Lee, K., Minhas, K. M., Kumar, A., Fradley, M., Shoukas, A. A., Berkowitz, D. E., & Hare, J. M. (2003). Nitric oxide regulation of myocardial contractility and calcium cycling: independent impact of neuronal and endothelial nitric oxide synthases. *Circ Res*, *92*(12), 1322-1329. https://doi.org/10.1161/01.RES.0000078171.52542.9E

Klatt, P., Pfeiffer, S., List, B. M., Lehner, D., Glatter, O., Bächinger, H. P., Werner, E. R., Schmidt, K., & Mayer, B. (1996). Characterization of heme-deficient neuronal nitric-oxide synthase reveals a role for heme in subunit dimerization and binding of the amino acid substrate and tetrahydrobiopterin. *J Biol Chem*, *271*(13), 7336-7342. https://doi.org/10.1074/jbc.271.13.7336

Klein, G., Drexler, H., & Schröder, F. (2000). Protein kinase G reverses all isoproterenol induced changes of cardiac single L-type calcium channel gating. *Cardiovasc Res*, *48*(3), 367-374. https://doi.org/10.1016/s0008-6363(00)00194-2

Knowles, R. G., Palacios, M., Palmer, R. M., & Moncada, S. (1989). Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc Natl Acad Sci U S A*, *86*(13), 5159-5162. https://doi.org/10.1073/pnas.86.13.5159

Komeima, K., Hayashi, Y., Naito, Y., & Watanabe, Y. (2000). Inhibition of neuronal nitric-oxide synthase by calcium/ calmodulin-dependent protein kinase IIalpha through Ser847 phosphorylation in NG108-15 neuronal cells. *J Biol Chem*, *275*(36), 28139-28143. https://doi.org/10.1074/jbc.M003198200

Kots, A. Y., Martin, E., Sharina, I. G., & Murad, F. (2009). A short history of cGMP, guanylyl cyclases, and cGMP-dependent protein kinases. *Handb Exp Pharmacol*(191), 1-14. https://doi.org/10.1007/978-3-540-68964-5_1

Kovacs, I., & Lindermayr, C. (2013). Nitric oxide-based protein modification: formation and site-specificity of protein S-nitrosylation. *Front Plant Sci*, *4*, 137. https://doi.org/10.3389/fpls.2013.00137

Kreusser, M. M., Lehmann, L. H., Keranov, S., Hoting, M. O., Oehl, U., Kohlhaas, M., Reil, J. C., Neumann, K., Schneider, M. D., Hill, J. A., Dobrev, D., Maack, C., Maier, L. S., Gröne, H. J., Katus, H. A., Olson, E. N., & Backs, J. (2014). Cardiac CaM Kinase II genes δ and γ contribute to adverse remodeling but redundantly inhibit calcineurin-induced myocardial hypertrophy. *Circulation*, *130*(15), 1262-1273. https://doi.org/10.1161/CIRCULATIONAHA.114.006185

Kreusser, M. M., Lehmann, L. H., Wolf, N., Keranov, S., Jungmann, A., Gröne, H. J., Müller, O. J., Katus, H. A., & Backs, J. (2016). Inducible cardiomyocyte-specific deletion of CaM kinase II protects from pressure overload-induced heart failure. *Basic Res Cardiol*, *111*(6), 65. https://doi.org/10.1007/s00395-016-0581-2

Kröncke, K. D., Fehsel, K., & Kolb-Bachofen, V. (1997). Nitric oxide: cytotoxicity versus cytoprotection--how, why, when, and where? *Nitric Oxide*, *1*(2), 107-120. https://doi.org/10.1006/niox.1997.0118

Krüger, M., Kötter, S., Grützner, A., Lang, P., Andresen, C., Redfield, M. M., Butt, E., dos Remedios, C. G., & Linke, W. A. (2009). Protein kinase G modulates human myocardial passive stiffness by phosphorylation of the titin springs. *Circ Res*, *104*(1), 87-94. https://doi.org/10.1161/CIRCRESAHA.108.184408

Kukreja, R. C., & Xi, L. (2007). eNOS phosphorylation: a pivotal molecular switch in vasodilation and cardioprotection? *J Mol Cell Cardiol*, *42*(2), 280-282. https://doi.org/10.1016/j.yjmcc.2006.10.011

Kwon, N. S., Nathan, C. F., Gilker, C., Griffith, O. W., Matthews, D. E., & Stuehr, D. J. (1990). L-citrulline production from L-arginine by macrophage nitric oxide synthase. The ureido oxygen derives from dioxygen. *J Biol Chem*, *265*(23), 13442-13445. https://www.ncbi.nlm.nih.gov/pubmed/1696255

Lee, D. I., Vahebi, S., Tocchetti, C. G., Barouch, L. A., Solaro, R. J., Takimoto, E., & Kass, D. A. (2010). PDE5A suppression of acute beta-adrenergic activation requires modulation of myocyte beta-3 signaling coupled to PKG-mediated troponin I phosphorylation. *Basic Res Cardiol*, *105*(3), 337-347. https://doi.org/10.1007/s00395-010-0084-5

Lee, S. J., Kim, K. M., Namkoong, S., Kim, C. K., Kang, Y. C., Lee, H., Ha, K. S., Han, J. A., Chung, H. T., Kwon, Y. G., & Kim, Y. M. (2005). Nitric oxide inhibition of homocysteine-induced human endothelial cell apoptosis by down-regulation of p53-dependent Noxa expression through the formation of S-nitrosohomocysteine. *J Biol Chem*, *280*(7), 5781-5788. https://doi.org/10.1074/jbc.M411224200

Lekontseva, O., Chakrabarti, S., Jiang, Y., Cheung, C. C., & Davidge, S. T. (2011). Role of neuronal nitric-oxide synthase in estrogen-induced relaxation in rat resistance arteries. *J Pharmacol Exp Ther*, *339*(2), 367-375. https://doi.org/10.1124/jpet.111.183798

Lima, B., Forrester, M. T., Hess, D. T., & Stamler, J. S. (2010). S-nitrosylation in cardiovascular signaling. *Circ Res*, *106*(4), 633-646. https://doi.org/10.1161/CIRCRESAHA.109.207381

Ling, H., Gray, C. B., Zambon, A. C., Grimm, M., Gu, Y., Dalton, N., Purcell, N. H., Peterson, K., & Brown, J. H. (2013). Ca2+/Calmodulin-dependent protein kinase II δ mediates

myocardial ischemia/reperfusion injury through nuclear factor-κB. *Circ Res*, *112*(6), 935-944. https://doi.org/10.1161/CIRCRESAHA.112.276915

Ling, H., Zhang, T., Pereira, L., Means, C. K., Cheng, H., Gu, Y., Dalton, N. D., Peterson, K. L., Chen, J., Bers, D., Brown, J. H., & Heller Brown, J. (2009). Requirement for Ca2+/calmodulin-dependent kinase II in the transition from pressure overload-induced cardiac hypertrophy to heart failure in mice. *J Clin Invest*, *119*(5), 1230-1240. https://doi.org/10.1172/JCI38022

Little, G. H., Saw, A., Bai, Y., Dow, J., Marjoram, P., Simkhovich, B., Leeka, J., Kedes, L., Kloner, R. A., & Poizat, C. (2009). Critical role of nuclear calcium/calmodulin-dependent protein kinase IIdeltaB in cardiomyocyte survival in cardiomyopathy. *J Biol Chem*, *284*(37), 24857-24868. https://doi.org/10.1074/jbc.M109.003186

Liu, L., Yan, Y., Zeng, M., Zhang, J., Hanes, M. A., Ahearn, G., McMahon, T. J., Dickfeld, T., Marshall, H. E., Que, L. G., & Stamler, J. S. (2004). Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell*, *116*(4), 617-628. https://doi.org/10.1016/s0092-8674(04)00131-x

Lowenstein, C. J., Glatt, C. S., Bredt, D. S., & Snyder, S. H. (1992). Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc Natl Acad Sci U S A*, *89*(15), 6711-6715. https://doi.org/10.1073/pnas.89.15.6711

Lu, Y. M., Shioda, N., Yamamoto, Y., Han, F., & Fukunaga, K. (2010). Transcriptional upregulation of calcineurin Abeta by endothelin-1 is partially mediated by calcium/calmodulin-dependent protein kinase IIdelta3 in rat cardiomyocytes. *Biochim Biophys Acta*, *1799*(5-6), 429-441. https://doi.org/10.1016/j.bbagrm.2010.02.004

Lundberg, J. O., Weitzberg, E., Lundberg, J. M., & Alving, K. (1994). Intragastric nitric oxide production in humans: measurements in expelled air. *Gut*, *35*(11), 1543-1546. https://doi.org/10.1136/gut.35.11.1543

MacMicking, J., Xie, Q. W., & Nathan, C. (1997). Nitric oxide and macrophage function. *Annu Rev Immunol*, *15*, 323-350. https://doi.org/10.1146/annurev.immunol.15.1.323

Maier, L. S., & Bers, D. M. (2007). Role of Ca2+/calmodulin-dependent protein kinase (CaMK) in excitation-contraction coupling in the heart. *Cardiovasc Res*, *73*(4), 631-640. https://doi.org/10.1016/j.cardiores.2006.11.005

Makara, M. A., Curran, J., Little, S. C., Musa, H., Polina, I., Smith, S. A., Wright, P. J., Unudurthi, S. D., Snyder, J., Bennett, V., Hund, T. J., & Mohler, P. J. (2014). Ankyrin-G coordinates intercalated disc signaling platform to regulate cardiac excitability in vivo. *Circ Res*, *115*(11), 929-938. https://doi.org/10.1161/CIRCRESAHA.115.305154

Mangmool, S., Shukla, A. K., & Rockman, H. A. (2010). beta-Arrestin-dependent activation of Ca(2+)/calmodulin kinase II after beta(1)-adrenergic receptor stimulation. *J Cell Biol*, *189*(3), 573-587. https://doi.org/10.1083/jcb.200911047

Maron, B. A., Tang, S. S., & Loscalzo, J. (2013). S-nitrosothiols and the S-nitrosoproteome of the cardiovascular system. *Antioxid Redox Signal*, *18*(3), 270-287. https://doi.org/10.1089/ars.2012.4744

Martínez-Ruiz, A., Villanueva, L., González de Orduña, C., López-Ferrer, D., Higueras, M. A., Tarín, C., Rodríguez-Crespo, I., Vázquez, J., & Lamas, S. (2005). S-nitrosylation of Hsp90 promotes the inhibition of its ATPase and endothelial nitric oxide synthase regulatory activities. *Proc Natl Acad Sci U S A*, *102*(24), 8525-8530. https://doi.org/10.1073/pnas.0407294102

Marzinzig, M., Nussler, A. K., Stadler, J., Marzinzig, E., Barthlen, W., Nussler, N. C., Beger, H. G., Morris, S. M., & Brückner, U. B. (1997). Improved methods to measure end products of nitric oxide in biological fluids: nitrite, nitrate, and S-nitrosothiols. *Nitric Oxide*, *1*(2), 177-189. https://doi.org/10.1006/niox.1997.0116

Massion, P. B., & Balligand, J. L. (2003). Modulation of cardiac contraction, relaxation and rate by the endothelial nitric oxide synthase (eNOS): lessons from genetically modified mice. *J Physiol*, *546*(Pt 1), 63-75. https://doi.org/10.1113/jphysiol.2002.025973

Massion, P. B., Dessy, C., Desjardins, F., Pelat, M., Havaux, X., Belge, C., Moulin, P., Guiot, Y., Feron, O., Janssens, S., & Balligand, J. L. (2004). Cardiomyocyte-restricted overexpression of endothelial nitric oxide synthase (NOS3) attenuates beta-adrenergic stimulation and reinforces vagal inhibition of cardiac contraction. *Circulation*, *110*(17), 2666-2672. https://doi.org/10.1161/01.CIR.0000145608.80855.BC

Mattagajasingh, I., Kim, C. S., Naqvi, A., Yamamori, T., Hoffman, T. A., Jung, S. B., DeRicco, J., Kasuno, K., & Irani, K. (2007). SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A*, *104*(37), 14855-14860. https://doi.org/10.1073/pnas.0704329104

McCabe, T. J., Fulton, D., Roman, L. J., & Sessa, W. C. (2000). Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. *J Biol Chem*, *275*(9), 6123-6128. https://doi.org/10.1074/jbc.275.9.6123

Michalson, K. T., Groban, L., Howard, T. D., Shively, C. A., Sophonsritsuk, A., Appt, S. E., Cline, J. M., Clarkson, T. B., Carr, J. J., Kitzman, D. W., & Register, T. C. (2018). Estradiol Treatment Initiated Early After Ovariectomy Regulates Myocardial Gene Expression and Inhibits Diastolic Dysfunction in Female Cynomolgus Monkeys: Potential Roles for

Calcium Homeostasis and Extracellular Matrix Remodeling. *J Am Heart Assoc*, *7*(21), e009769. https://doi.org/10.1161/JAHA.118.009769

Miseta, A., & Csutora, P. (2000). Relationship between the occurrence of cysteine in proteins and the complexity of organisms. *Mol Biol Evol*, *17*(8), 1232-1239. https://doi.org/10.1093/oxfordjournals.molbev.a026406

Mishra, S., Gray, C. B., Miyamoto, S., Bers, D. M., & Brown, J. H. (2011). Location matters: clarifying the concept of nuclear and cytosolic CaMKII subtypes. *Circ Res*, *109*(12), 1354-1362. https://doi.org/10.1161/CIRCRESAHA.111.248401

Modin, A., Björne, H., Herulf, M., Alving, K., Weitzberg, E., & Lundberg, J. O. (2001). Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand*, *171*(1), 9-16. https://doi.org/10.1046/j.1365-201X.2001.00771.x

Mohan, R. M., Choate, J. K., Golding, S., Herring, N., Casadei, B., & Paterson, D. J. (2000). Peripheral pre-synaptic pathway reduces the heart rate response to sympathetic activation following exercise training: role of NO. *Cardiovasc Res*, *47*(1), 90-98. https://doi.org/10.1016/s0008-6363(00)00066-3

Montfort, W. R., Wales, J. A., & Weichsel, A. (2017). Structure and Activation of Soluble Guanylyl Cyclase, the Nitric Oxide Sensor. *Antioxid Redox Signal*, *26*(3), 107-121. https://doi.org/10.1089/ars.2016.6693

Morell, M., Burgos, J. I., Gonano, L. A., & Vila Petroff, M. (2018). AMPK-dependent nitric oxide release provides contractile support during hyperosmotic stress. *Basic Res Cardiol*, *113*(1), 7. https://doi.org/10.1007/s00395-017-0665-7

Mosca, L., Barrett-Connor, E., & Wenger, N. K. (2011). Sex/gender differences in cardiovascular disease prevention: what a difference a decade makes. *Circulation*, *124*(19), 2145-2154. https://doi.org/10.1161/CIRCULATIONAHA.110.968792

Münzel, T., Feil, R., Mülsch, A., Lohmann, S. M., Hofmann, F., & Walter, U. (2003). Physiology and pathophysiology of vascular signaling controlled by guanosine 3',5'-cyclic monophosphate-dependent protein kinase [corrected]. *Circulation*, *108*(18), 2172-2183. https://doi.org/10.1161/01.CIR.0000094403.78467.C3

Murad, F. (1986). Cyclic guanosine monophosphate as a mediator of vasodilation. *J Clin Invest*, *78*(1), 1-5. https://doi.org/10.1172/JCI112536

Murphy, E., & Steenbergen, C. (2007). Gender-based differences in mechanisms of protection in myocardial ischemia-reperfusion injury. *Cardiovasc Res*, *75*(3), 478-486. https://doi.org/10.1016/j.cardiores.2007.03.025

Murray, C. I., Gebska, M. A., Haile, A., Zhang, M., Kass, D. A., Champion, H. C., & Eyk, J. E. V. (2008). cGMP Specific Phosphodiesterase Type 5A Activity is Regulated by S-nitrosylation at Cys 181. In (Vol. 118). Circulation: https://doi.org/10.1161/circ.118.suppl_18.S_415-b.

Nakane, M., Mitchell, J., Förstermann, U., & Murad, F. (1991). Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase. *Biochem Biophys Res Commun*, *180*(3), 1396-1402. https://doi.org/10.1016/s0006-291x(05)81351-8

Neef, S., Steffens, A., Pellicena, P., Mustroph, J., Lebek, S., Ort, K. R., Schulman, H., & Maier, L. S. (2018). Improvement of cardiomyocyte function by a novel pyrimidine-based

CaMKII-inhibitor. *J Mol Cell Cardiol, 115*, 73-81. https://doi.org/10.1016/j.yjmcc.2017.12.015

Omar, S. A., Webb, A. J., Lundberg, J. O., & Weitzberg, E. (2016). Therapeutic effects of inorganic nitrate and nitrite in cardiovascular and metabolic diseases. *J Intern Med*, *279*(4), 315-336. https://doi.org/10.1111/joim.12441

Palmer, L. A., & Gaston, B. (2008). S-nitrosothiol assays that avoid the use of iodine. *Methods Enzymol*, *440*, 157-176. https://doi.org/10.1016/S0076-6879(07)00809-9

Palmer, R. M., Ashton, D. S., & Moncada, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, *333*(6174), 664-666. https://doi.org/10.1038/333664a0

Palmer, R. M., & Moncada, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem Biophys Res Commun*, *158*(1), 348-352. https://doi.org/10.1016/s0006-291x(89)80219-0

Palomeque, J., Rueda, O. V., Sapia, L., Valverde, C. A., Salas, M., Petroff, M. V., & Mattiazzi, A. (2009). Angiotensin II-induced oxidative stress resets the Ca2+ dependence of Ca2+-calmodulin protein kinase II and promotes a death pathway conserved across different species. *Circ Res, 105*(12), 1204-1212. https://doi.org/10.1161/CIRCRESAHA.109.204172

Park, J. K., & Kostka, P. (1997). Fluorometric detection of biological S-nitrosothiols. *Anal Biochem*, *249*(1), 61-66. https://doi.org/10.1006/abio.1997.2159

Partovian, C., Zhuang, Z., Moodie, K., Lin, M., Ouchi, N., Sessa, W. C., Walsh, K., & Simons, M. (2005). PKCalpha activates eNOS and increases arterial blood flow in vivo. *Circ Res, 97*(5), 482-487. https://doi.org/10.1161/01.RES.0000179775.04114.45

Pellicena, P., & Schulman, H. (2014). CaMKII inhibitors: from research tools to therapeutic agents. *Front Pharmacol*, *5*, 21. https://doi.org/10.3389/fphar.2014.00021

Peng, W., Zhang, Y., Zheng, M., Cheng, H., Zhu, W., Cao, C. M., & Xiao, R. P. (2010). Cardioprotection by CaMKII-deltaB is mediated by phosphorylation of heat shock factor 1 and subsequent expression of inducible heat shock protein 70. *Circ Res*, *106*(1), 102-110. https://doi.org/10.1161/CIRCRESAHA.109.210914

Pereira, L., Bare, D. J., Galice, S., Shannon, T. R., & Bers, D. M. (2017). β-Adrenergic induced SR Ca. *J Mol Cell Cardiol*, *108*, 8-16. https://doi.org/10.1016/j.yjmcc.2017.04.005

Petroff, M. G., Kim, S. H., Pepe, S., Dessy, C., Marbán, E., Balligand, J. L., & Sollott, S. J. (2001). Endogenous nitric oxide mechanisms mediate the stretch dependence of Ca2+ release in cardiomyocytes. *Nat Cell Biol*, *3*(10), 867-873. https://doi.org/10.1038/ncb1001-867

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free radicals: properties, sources, targets, and their implication in various diseases. *Indian J Clin Biochem*, *30*(1), 11-26. https://doi.org/10.1007/s12291-014-0446-0

Piech, A., Massart, P. E., Dessy, C., Feron, O., Havaux, X., Morel, N., Vanoverschelde, J. L., Donckier, J., & Balligand, J. L. (2002). Decreased expression of myocardial eNOS and caveolin in dogs with hypertrophic cardiomyopathy. *Am J Physiol Heart Circ Physiol*, *282*(1), H219-231. https://doi.org/10.1152/ajpheart.2002.282.1.H219

Pollock, J. S., Förstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H. H., Nakane, M., & Murad, F. (1991). Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc Natl Acad Sci U S A*, *88*(23), 10480-10484. https://doi.org/10.1073/pnas.88.23.10480

Qian, Q., Zhang, Z., Orwig, A., Chen, S., Ding, W. X., Xu, Y., Kunz, R. C., Lind, N. R. L., Stamler, J. S., & Yang, L. (2018). -Nitrosoglutathione Reductase Dysfunction Contributes to Obesity-Associated Hepatic Insulin Resistance via Regulating Autophagy. *Diabetes*, *67*(2), 193-207. https://doi.org/10.2337/db17-0223

Racioppi, L., & Means, A. R. (2012). Calcium/calmodulin-dependent protein kinase kinase 2: roles in signaling and pathophysiology. *J Biol Chem*, *287*(38), 31658-31665. https://doi.org/10.1074/jbc.R112.356485

Rainer, P. P., & Kass, D. A. (2016). Old dog, new tricks: novel cardiac targets and stress regulation by protein kinase G. *Cardiovasc Res*, *111*(2), 154-162. https://doi.org/10.1093/cvr/cvw107

Ramirez, M. T., Zhao, X. L., Schulman, H., & Brown, J. H. (1997). The nuclear deltaB isoform of Ca2+/calmodulin-dependent protein kinase II regulates atrial natriuretic factor gene expression in ventricular myocytes. *J Biol Chem*, *272*(49), 31203-31208. https://doi.org/10.1074/jbc.272.49.31203

Randolph, J. F., Sowers, M., Gold, E. B., Mohr, B. A., Luborsky, J., Santoro, N., McConnell, D. S., Finkelstein, J. S., Korenman, S. G., Matthews, K. A., Sternfeld, B., & Lasley, B. L. (2003). Reproductive hormones in the early menopausal transition: relationship to ethnicity, body size, and menopausal status. *J Clin Endocrinol Metab*, *88*(4), 1516-1522. https://doi.org/10.1210/jc.2002-020777

Ravi, K., Brennan, L. A., Levic, S., Ross, P. A., & Black, S. M. (2004). S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc Natl Acad Sci U S A*, *101*(8), 2619-2624. https://doi.org/10.1073/pnas.0300464101

Rellos, P., Pike, A. C., Niesen, F. H., Salah, E., Lee, W. H., von Delft, F., & Knapp, S. (2010). Structure of the CaMKIIdelta/calmodulin complex reveals the molecular mechanism of CaMKII kinase activation. *PLoS Biol*, *8*(7), e1000426. https://doi.org/10.1371/journal.pbio.1000426

Rodríguez-Crespo, I., Gerber, N. C., & Ortiz de Montellano, P. R. (1996). Endothelial nitric-oxide synthase. Expression in Escherichia coli, spectroscopic characterization, and role of tetrahydrobiopterin in dimer formation. *J Biol Chem*, *271*(19), 11462-11467. https://doi.org/10.1074/jbc.271.19.11462

Roe, N. D., & Ren, J. (2012). Nitric oxide synthase uncoupling: a therapeutic target in cardiovascular diseases. *Vascul Pharmacol*, *57*(5-6), 168-172. https://doi.org/10.1016/j.vph.2012.02.004

Rokita, A. G., & Anderson, M. E. (2012). New therapeutic targets in cardiology: arrhythmias and Ca2+/calmodulin-dependent kinase II (CaMKII). *Circulation*, *126*(17), 2125-2139. https://doi.org/10.1161/CIRCULATIONAHA.112.124990

Rosenberg, O. S., Deindl, S., Sung, R. J., Nairn, A. C., & Kuriyan, J. (2005). Structure of the autoinhibited kinase domain of CaMKII and SAXS analysis of the holoenzyme. *Cell*, *123*(5), 849-860. https://doi.org/10.1016/j.cell.2005.10.029

Rossouw, J. E., Anderson, G. L., Prentice, R. L., LaCroix, A. Z., Kooperberg, C., Stefanick, M. L., Jackson, R. D., Beresford, S. A., Howard, B. V., Johnson, K. C., Kotchen, J. M., Ockene, J., & Investigators, W. G. f. t. W. s. H. I. (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA*, *288*(3), 321-333. https://doi.org/10.1001/jama.288.3.321

Ruiz-Holst, C., Bölck, B., Ghanem, A., Tiemann, K., Brokat, S., Regitz-Zagrosek, V., Bloch, W., Schwinger, R. H., & Brixius, K. (2010). eNOS phosphorylation and translocation are altered in male but not female mice by increased activation of the Gαq protein. *Can J Physiol Pharmacol*, *88*(2), 121-129. https://doi.org/10.1139/Y09-115

Rusciano, M. R., Sommariva, E., Douin-Echinard, V., Ciccarelli, M., Poggio, P., & Maione, A. S. (2019). CaMKII Activity in the Inflammatory Response of Cardiac Diseases. *Int J Mol Sci*, *20*(18). https://doi.org/10.3390/ijms20184374

Sabbatini, A. R., & Kararigas, G. (2020). Menopause-Related Estrogen Decrease and the Pathogenesis of HFpEF: JACC Review Topic of the Week. *J Am Coll Cardiol*, *75*(9), 1074-1082. https://doi.org/10.1016/j.jacc.2019.12.049

Sag, C. M., Wadsack, D. P., Khabbazzadeh, S., Abesser, M., Grefe, C., Neumann, K., Opiela, M. K., Backs, J., Olson, E. N., Brown, J. H., Neef, S., Maier, S. K., & Maier, L. S. (2009). Calcium/calmodulin-dependent protein kinase II contributes to cardiac arrhythmogenesis in heart failure. *Circ Heart Fail*, *2*(6), 664-675. https://doi.org/10.1161/CIRCHEARTFAILURE.109.865279

Salerni, S., Di Francescomarino, S., Cadeddu, C., Acquistapace, F., Maffei, S., & Gallina, S. (2015). The different role of sex hormones on female cardiovascular physiology and function: not only oestrogens. *Eur J Clin Invest*, *45*(6), 634-645. https://doi.org/10.1111/eci.12447

Sam, F., Sawyer, D. B., Xie, Z., Chang, D. L., Ngoy, S., Brenner, D. A., Siwik, D. A., Singh, K., Apstein, C. S., & Colucci, W. S. (2001). Mice lacking inducible nitric oxide synthase have improved left ventricular contractile function and reduced apoptotic cell death late after myocardial infarction. *Circ Res*, *89*(4), 351-356. https://doi.org/10.1161/hh1601.094993

Sayed, N., Kim, D. D., Fioramonti, X., Iwahashi, T., Durán, W. N., & Beuve, A. (2008). Nitroglycerin-induced S-nitrosylation and desensitization of soluble guanylyl cyclase contribute to nitrate tolerance. *Circ Res*, *103*(6), 606-614. https://doi.org/10.1161/CIRCRESAHA.108.175133

Schuh, A. K., Sheybani, B., Jortzik, E., Niemann, B., Wilhelm, J., Boening, A., & Becker, K. (2018). Redox status of patients before cardiac surgery. *Redox Rep*, *23*(1), 83-93. https://doi.org/10.1080/13510002.2017.1418620

Schulz, R., Nava, E., & Moncada, S. (1992). Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. *Br J Pharmacol*, *105*(3), 575-580. https://doi.org/10.1111/j.1476-5381.1992.tb09021.x

Schworer, C. M., Colbran, R. J., Keefer, J. R., & Soderling, T. R. (1988). Ca2+/calmodulin-dependent protein kinase II. Identification of a regulatory autophosphorylation site adjacent to the inhibitory and calmodulin-binding domains. *J Biol Chem*, *263*(27), 13486-13489. https://www.ncbi.nlm.nih.gov/pubmed/3417668

Schworer, C. M., Rothblum, L. I., Thekkumkara, T. J., & Singer, H. A. (1993). Identification of novel isoforms of the delta subunit of Ca2+/calmodulin-dependent protein kinase II. Differential expression in rat brain and aorta. *J Biol Chem*, *268*(19), 14443-14449. https://www.ncbi.nlm.nih.gov/pubmed/8390994

Sears, C. E., Bryant, S. M., Ashley, E. A., Lygate, C. A., Rakovic, S., Wallis, H. L., Neubauer, S., Terrar, D. A., & Casadei, B. (2003). Cardiac neuronal nitric oxide synthase isoform regulates myocardial contraction and calcium handling. *Circ Res*, *92*(5), e52-59. https://doi.org/10.1161/01.RES.0000064585.95749.6D

Seddon, M., Melikian, N., Dworakowski, R., Shabeeh, H., Jiang, B., Byrne, J., Casadei, B., Chowienczyk, P., & Shah, A. M. (2009). Effects of neuronal nitric oxide synthase on human coronary artery diameter and blood flow in vivo. *Circulation*, *119*(20), 2656-2662. https://doi.org/10.1161/CIRCULATIONAHA.108.822205

Seddon, M., Shah, A. M., & Casadei, B. (2007). Cardiomyocytes as effectors of nitric oxide signalling. *Cardiovasc Res*, *75*(2), 315-326. https://doi.org/10.1016/j.cardiores.2007.04.031

Selemidis, S., Dusting, G. J., Peshavariya, H., Kemp-Harper, B. K., & Drummond, G. R. (2007). Nitric oxide suppresses NADPH oxidase-dependent superoxide production by S-nitrosylation in human endothelial cells. *Cardiovasc Res*, *75*(2), 349-358. https://doi.org/10.1016/j.cardiores.2007.03.030

Serafim, R. A., Primi, M. C., Trossini, G. H., & Ferreira, E. I. (2012). Nitric oxide: state of the art in drug design. *Curr Med Chem*, *19*(3), 386-405. https://doi.org/10.2174/092986712803414321

Shahani, N., & Sawa, A. (2012). Protein S-nitrosylation: role for nitric oxide signaling in neuronal death. *Biochim Biophys Acta*, *1820*(6), 736-742. https://doi.org/10.1016/j.bbagen.2011.07.010

Shao, Q., Casin, K. M., Mackowski, N., Murphy, E., Steenbergen, C., & Kohr, M. J. (2017). Adenosine A1 receptor activation increases myocardial protein S-nitrosothiols and elicits protection from ischemia-reperfusion injury in male and female hearts. *PLoS One*, *12*(5), e0177315. https://doi.org/10.1371/journal.pone.0177315

Shao, Q., Fallica, J., Casin, K. M., Murphy, E., Steenbergen, C., & Kohr, M. J. (2016). Characterization of the sex-dependent myocardial S-nitrosothiol proteome. *Am J Physiol Heart Circ Physiol*, *310*(4), H505-515. https://doi.org/10.1152/ajpheart.00681.2015

Singh, M. V., Kapoun, A., Higgins, L., Kutschke, W., Thurman, J. M., Zhang, R., Singh, M., Yang, J., Guan, X., Lowe, J. S., Weiss, R. M., Zimmermann, K., Yull, F. E., Blackwell, T. S., Mohler, P. J., & Anderson, M. E. (2009). Ca2+/calmodulin-dependent kinase II triggers cell membrane injury by inducing complement factor B gene expression in the mouse heart. *J Clin Invest*, *119*(4), 986-996. https://doi.org/10.1172/JCI35814

Solà, C., Tusell, J. M., & Serratosa, J. (1999). Comparative study of the distribution of calmodulin kinase II and calcineurin in the mouse brain. *J Neurosci Res*, *57*(5), 651-662. https://www.ncbi.nlm.nih.gov/pubmed/10462689

Song, Y., Zweier, J. L., & Xia, Y. (2001). Determination of the enhancing action of HSP90 on neuronal nitric oxide synthase by EPR spectroscopy. *Am J Physiol Cell Physiol*, *281*(6), C1819-1824. https://doi.org/10.1152/ajpcell.2001.281.6.C1819

Srinivasan, M., Edman, C. F., & Schulman, H. (1994). Alternative splicing introduces a nuclear localization signal that targets multifunctional CaM kinase to the nucleus. *J Cell Biol*, *126*(4), 839-852. https://doi.org/10.1083/jcb.126.4.839

Stamler, J. S., Jia, L., Eu, J. P., McMahon, T. J., Demchenko, I. T., Bonaventura, J., Gernert, K., & Piantadosi, C. A. (1997). Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science*, *276*(5321), 2034-2037. https://doi.org/10.1126/science.276.5321.2034

Stamler, J. S., Lamas, S., & Fang, F. C. (2001). Nitrosylation. the prototypic redox-based signaling mechanism. *Cell*, *106*(6), 675-683. https://doi.org/10.1016/s0092-8674(01)00495-0

Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., & Loscalzo, J. (1992). S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. *Proc Natl Acad Sci U S A*, *89*(1), 444-448. https://doi.org/10.1073/pnas.89.1.444

Stamler, J. S., Singel, D. J., & Loscalzo, J. (1992). Biochemistry of nitric oxide and its redox-activated forms. *Science*, *258*(5090), 1898-1902. https://doi.org/10.1126/science.1281928

Stamler, J. S., & Toone, E. J. (2002). The decomposition of thionitrites. *Curr Opin Chem Biol*, *6*(6), 779-785. https://doi.org/10.1016/s1367-5931(02)00383-6

Stomberski, C. T., Hess, D. T., & Stamler, J. S. (2019). Protein S-Nitrosylation: Determinants of Specificity and Enzymatic Regulation of S-Nitrosothiol-Based Signaling. *Antioxid Redox Signal*, *30*(10), 1331-1351. https://doi.org/10.1089/ars.2017.7403

Stoyanovsky, D., Murphy, T., Anno, P. R., Kim, Y. M., & Salama, G. (1997). Nitric oxide activates skeletal and cardiac ryanodine receptors. *Cell Calcium*, *21*(1), 19-29. https://doi.org/10.1016/s0143-4160(97)90093-2

Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F., & Nathan, C. F. (1991). Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A*, *88*(17), 7773-7777. https://doi.org/10.1073/pnas.88.17.7773

Stuehr, D. J., & Haque, M. M. (2019). Nitric oxide synthase enzymology in the 20 years after the Nobel Prize. *Br J Pharmacol*, *176*(2), 177-188. https://doi.org/10.1111/bph.14533

Stuehr, D. J., & Marletta, M. A. (1987). Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. *J Immunol*, *139*(2), 518-525. https://www.ncbi.nlm.nih.gov/pubmed/3110273

Subelzu, N., Bartesaghi, S., De Bem, A., & Radi, R. (2015). Oxidative inactivation of nitric oxide and peroxynitrite formation in the vasculature. In (Vol. 2, pp. 91-145). ACS Symposium Series: ACS Publications.

Suetomi, T., Miyamoto, S., & Brown, J. H. (2019). Inflammation in nonischemic heart disease: initiation by cardiomyocyte CaMKII and NLRP3 inflammasome signaling. *Am J Physiol Heart Circ Physiol*, *317*(5), H877-H890. https://doi.org/10.1152/ajpheart.00223.2019

Suetomi, T., Willeford, A., Brand, C. S., Cho, Y., Ross, R. S., Miyamoto, S., & Brown, J. H. (2018). Inflammation and NLRP3 Inflammasome Activation Initiated in Response to Pressure Overload by Ca. *Circulation*, *138*(22), 2530-2544. https://doi.org/10.1161/CIRCULATIONAHA.118.034621

Suhara, T., Fukuo, K., Yasuda, O., Tsubakimoto, M., Takemura, Y., Kawamoto, H., Yokoi, T., Mogi, M., Kaimoto, T., & Ogihara, T. (2004). Homocysteine enhances endothelial apoptosis via upregulation of Fas-mediated pathways. *Hypertension*, *43*(6), 1208-1213. https://doi.org/10.1161/01.HYP.0000127914.94292.76

Sun, J., Picht, E., Ginsburg, K. S., Bers, D. M., Steenbergen, C., & Murphy, E. (2006). Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca2+ channel alpha1 subunit and reduced ischemia/reperfusion injury. *Circ Res*, *98*(3), 403-411. https://doi.org/10.1161/01.RES.0000202707.79018.0a

Swaminathan, P. D., Purohit, A., Hund, T. J., & Anderson, M. E. (2012). Calmodulin-dependent protein kinase II: linking heart failure and arrhythmias. *Circ Res*, *110*(12), 1661-1677. https://doi.org/10.1161/CIRCRESAHA.111.243956

Tadic, M., Cuspidi, C., Plein, S., Belyavskiy, E., Heinzel, F., & Galderisi, M. (2019). Sex and Heart Failure with Preserved Ejection Fraction: From Pathophysiology to Clinical Studies. *J Clin Med*, *8*(6). https://doi.org/10.3390/jcm8060792

Takimoto, Y., Aoyama, T., Tanaka, K., Keyamura, R., Yui, Y., & Sasayama, S. (2002). Augmented expression of neuronal nitric oxide synthase in the atria parasympathetically decreases heart rate during acute myocardial infarction in rats. *Circulation*, *105*(4), 490-496. https://doi.org/10.1161/hc0402.102662

Thibeault, S., Rautureau, Y., Oubaha, M., Faubert, D., Wilkes, B. C., Delisle, C., & Gratton, J. P. (2010). S-nitrosylation of beta-catenin by eNOS-derived NO promotes VEGF-induced endothelial cell permeability. *Mol Cell*, *39*(3), 468-476. https://doi.org/10.1016/j.molcel.2010.07.013

Toko, H., Takahashi, H., Kayama, Y., Oka, T., Minamino, T., Okada, S., Morimoto, S., Zhan, D. Y., Terasaki, F., Anderson, M. E., Inoue, M., Yao, A., Nagai, R., Kitaura, Y., Sasaguri, T., & Komuro, I. (2010). Ca2+/calmodulin-dependent kinase IIdelta causes heart failure by accumulation of p53 in dilated cardiomyopathy. *Circulation*, *122*(9), 891-899. https://doi.org/10.1161/CIRCULATIONAHA.109.935296

Tong, C. W., Wu, X., Liu, Y., Rosas, P. C., Sadayappan, S., Hudmon, A., Muthuchamy, M., Powers, P. A., Valdivia, H. H., & Moss, R. L. (2015). Phosphoregulation of Cardiac Inotropy via Myosin Binding Protein-C During Increased Pacing Frequency or β1-Adrenergic Stimulation. *Circ Heart Fail*, *8*(3), 595-604. https://doi.org/10.1161/CIRCHEARTFAILURE.114.001585

Trapet, P., Kulik, A., Lamotte, O., Jeandroz, S., Bourque, S., Nicolas-Frances, V., Rosnoblet, C., Besson-Bard, A., & Wendehenne, D. (2015). NO signaling in plant immunity: a tale of

messengers. *Phytochemistry*, *112*, 72-79. https://doi.org/10.1016/j.phytochem.2014.03.015

Trappanese, D. M., Liu, Y., McCormick, R. C., Cannavo, A., Nanayakkara, G., Baskharoun, M. M., Jarrett, H., Woitek, F. J., Tillson, D. M., Dillon, A. R., Recchia, F. A., Balligand, J. L., Houser, S. R., Koch, W. J., Dell'Italia, L. J., & Tsai, E. J. (2015). Chronic β1-adrenergic blockade enhances myocardial β3-adrenergic coupling with nitric oxide-cGMP signaling in a canine model of chronic volume overload: new insight into mechanisms of cardiac benefit with selective β1-blocker therapy. *Basic Res Cardiol*, *110*(1), 456. https://doi.org/10.1007/s00395-014-0456-3

Ueda, K., Valdivia, C., Medeiros-Domingo, A., Tester, D. J., Vatta, M., Farrugia, G., Ackerman, M. J., & Makielski, J. C. (2008). Syntrophin mutation associated with long QT syndrome through activation of the nNOS-SCN5A macromolecular complex. *Proc Natl Acad Sci U S A*, *105*(27), 9355-9360. https://doi.org/10.1073/pnas.0801294105

Ullrich, R., Scherrer-Crosbie, M., Bloch, K. D., Ichinose, F., Nakajima, H., Picard, M. H., Zapol, W. M., & Quezado, Z. M. (2000). Congenital deficiency of nitric oxide synthase 2 protects against endotoxin-induced myocardial dysfunction in mice. *Circulation*, *102*(12), 1440-1446. https://doi.org/10.1161/01.cir.102.12.1440

van Oort, R. J., McCauley, M. D., Dixit, S. S., Pereira, L., Yang, Y., Respress, J. L., Wang, Q., De Almeida, A. C., Skapura, D. G., Anderson, M. E., Bers, D. M., & Wehrens, X. H. (2010). Ryanodine receptor phosphorylation by calcium/calmodulin-dependent protein kinase II promotes life-threatening ventricular arrhythmias in mice with heart failure. *Circulation*, *122*(25), 2669-2679. https://doi.org/10.1161/CIRCULATIONAHA.110.982298

Vaniotis, G., Glazkova, I., Merlen, C., Smith, C., Villeneuve, L. R., Chatenet, D., Therien, M., Fournier, A., Tadevosyan, A., Trieu, P., Nattel, S., Hébert, T. E., & Allen, B. G. (2013). Regulation of cardiac nitric oxide signaling by nuclear β-adrenergic and endothelin receptors. *J Mol Cell Cardiol*, *62*, 58-68. https://doi.org/10.1016/j.yjmcc.2013.05.003

Vásquez-Vivar, J., Kalyanaraman, B., Martásek, P., Hogg, N., Masters, B. S., Karoui, H., Tordo, P., & Pritchard, K. A. (1998). Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A*, *95*(16), 9220-9225. https://doi.org/10.1073/pnas.95.16.9220

Velmurugan, S., Kapil, V., Ghosh, S. M., Davies, S., McKnight, A., Aboud, Z., Khambata, R. S., Webb, A. J., Poole, A., & Ahluwalia, A. (2013). Antiplatelet effects of dietary nitrate in healthy volunteers: involvement of cGMP and influence of sex. *Free Radic Biol Med*, *65*, 1521-1532. https://doi.org/10.1016/j.freeradbiomed.2013.06.031

Voigt, N., Li, N., Wang, Q., Wang, W., Trafford, A. W., Abu-Taha, I., Sun, Q., Wieland, T., Ravens, U., Nattel, S., Wehrens, X. H., & Dobrev, D. (2012). Enhanced sarcoplasmic reticulum Ca2+ leak and increased Na+-Ca2+ exchanger function underlie delayed afterdepolarizations in patients with chronic atrial fibrillation. *Circulation*, *125*(17), 2059-2070. https://doi.org/10.1161/CIRCULATIONAHA.111.067306

Wagner, S., Dybkova, N., Rasenack, E. C., Jacobshagen, C., Fabritz, L., Kirchhof, P., Maier, S. K., Zhang, T., Hasenfuss, G., Brown, J. H., Bers, D. M., & Maier, L. S. (2006). Ca2+/calmodulin-dependent protein kinase II regulates cardiac Na+ channels. *J Clin Invest*, *116*(12), 3127-3138. https://doi.org/10.1172/JCI26620

Wagner, S., Hacker, E., Grandi, E., Weber, S. L., Dybkova, N., Sossalla, S., Sowa, T., Fabritz, L., Kirchhof, P., Bers, D. M., & Maier, L. S. (2009). Ca/calmodulin kinase II differentially

modulates potassium currents. *Circ Arrhythm Electrophysiol*, *2*(3), 285-294. https://doi.org/10.1161/CIRCEP.108.842799

Wagner, S., Maier, L. S., & Bers, D. M. (2015). Role of sodium and calcium dysregulation in tachyarrhythmias in sudden cardiac death. *Circ Res*, *116*(12), 1956-1970. https://doi.org/10.1161/CIRCRESAHA.116.304678

Wang, H., Bonilla, I. M., Huang, X., He, Q., Kohr, M. J., Carnes, C. A., & Ziolo, M. T. (2012). Prolonged Action Potential and After depolarizations Are Not due to Changes in Potassium Currents in NOS3 Knockout Ventricular Myocytes. *J Signal Transduct*, *2012*, 645721. https://doi.org/10.1155/2012/645721

Wang, H., Kohr, M. J., Traynham, C. J., Wheeler, D. G., Janssen, P. M., & Ziolo, M. T. (2008). Neuronal nitric oxide synthase signaling within cardiac myocytes targets phospholamban. *Am J Physiol Cell Physiol*, *294*(6), C1566-1575. https://doi.org/10.1152/ajpcell.00367.2007

Wang, H., Kohr, M. J., Wheeler, D. G., & Ziolo, M. T. (2008). Endothelial nitric oxide synthase decreases beta-adrenergic responsiveness via inhibition of the L-type Ca2+ current. *Am J Physiol Heart Circ Physiol*, *294*(3), H1473-1480. https://doi.org/10.1152/ajpheart.01249.2007

Wang, H., Viatchenko-Karpinski, S., Sun, J., Györke, I., Benkusky, N. A., Kohr, M. J., Valdivia, H. H., Murphy, E., Györke, S., & Ziolo, M. T. (2010). Regulation of myocyte contraction via neuronal nitric oxide synthase: role of ryanodine receptor S-nitrosylation. *J Physiol*, *588*(Pt 15), 2905-2917. https://doi.org/10.1113/jphysiol.2010.192617

Wang, X., & Abdel-Rahman, A. A. (2002). Estrogen modulation of eNOS activity and its association with caveolin-3 and calmodulin in rat hearts. *Am J Physiol Heart Circ Physiol*, *282*(6), H2309-2315. https://doi.org/10.1152/ajpheart.00772.2001

Wang, Y., Tandan, S., Cheng, J., Yang, C., Nguyen, L., Sugianto, J., Johnstone, J. L., Sun, Y., & Hill, J. A. (2008). Ca2+/calmodulin-dependent protein kinase II-dependent remodeling of Ca2+ current in pressure overload heart failure. *J Biol Chem*, *283*(37), 25524-25532. https://doi.org/10.1074/jbc.M803043200

Webb, A. J., Patel, N., Loukogeorgakis, S., Okorie, M., Aboud, Z., Misra, S., Rashid, R., Miall, P., Deanfield, J., Benjamin, N., MacAllister, R., Hobbs, A. J., & Ahluwalia, A. (2008). Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension*, *51*(3), 784-790. https://doi.org/10.1161/HYPERTENSIONAHA.107.103523

Weber, S., Zeller, M., Guan, K., Wunder, F., Wagner, M., & El-Armouche, A. (2017). PDE2 at the crossway between cAMP and cGMP signalling in the heart. *Cell Signal*, *38*, 76-84. https://doi.org/10.1016/j.cellsig.2017.06.020

Wehrens, X. H., Lehnart, S. E., Reiken, S. R., & Marks, A. R. (2004). Ca2+/calmodulin-dependent protein kinase II phosphorylation regulates the cardiac ryanodine receptor. *Circ Res*, *94*(6), e61-70. https://doi.org/10.1161/01.RES.0000125626.33738.E2

Weinreuter, M., Kreusser, M. M., Beckendorf, J., Schreiter, F. C., Leuschner, F., Lehmann, L. H., Hofmann, K. P., Rostosky, J. S., Diemert, N., Xu, C., Volz, H. C., Jungmann, A., Nickel, A., Sticht, C., Gretz, N., Maack, C., Schneider, M. D., Gröne, H. J., Müller, O. J., Katus, H. A., & Backs, J. (2014). CaM Kinase II mediates maladaptive post-infarct remodeling and pro-inflammatory chemoattractant signaling but not acute myocardial

ischemia/reperfusion injury. *EMBO Mol Med*, *6*(10), 1231-1245. https://doi.org/10.15252/emmm.201403848

Welsby, P. J., Wang, H., Wolfe, J. T., Colbran, R. J., Johnson, M. L., & Barrett, P. Q. (2003). A mechanism for the direct regulation of T-type calcium channels by Ca2+/calmodulin-dependent kinase II. *J Neurosci*, *23*(31), 10116-10121. https://www.ncbi.nlm.nih.gov/pubmed/14602827

Wilcox, J. N., Subramanian, R. R., Sundell, C. L., Tracey, W. R., Pollock, J. S., Harrison, D. G., & Marsden, P. A. (1997). Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol*, *17*(11), 2479-2488. https://doi.org/10.1161/01.atv.17.11.2479

Wildhirt, S. M., Weismueller, S., Schulze, C., Conrad, N., Kornberg, A., & Reichart, B. (1999). Inducible nitric oxide synthase activation after ischemia/reperfusion contributes to myocardial dysfunction and extent of infarct size in rabbits: evidence for a late phase of nitric oxide-mediated reperfusion injury. *Cardiovasc Res*, *43*(3), 698-711. https://doi.org/10.1016/s0008-6363(99)00080-2

Williams, J. C., Armesilla, A. L., Mohamed, T. M., Hagarty, C. L., McIntyre, F. H., Schomburg, S., Zaki, A. O., Oceandy, D., Cartwright, E. J., Buch, M. H., Emerson, M., & Neyses, L. (2006). The sarcolemmal calcium pump, alpha-1 syntrophin, and neuronal nitric-oxide synthase are parts of a macromolecular protein complex. *J Biol Chem*, *281*(33), 23341-23348. https://doi.org/10.1074/jbc.M513341200

Wink, D. A., & Mitchell, J. B. (1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic Biol Med*, *25*(4-5), 434-456. https://doi.org/10.1016/s0891-5849(98)00092-6

Wollert, K. C., Yurukova, S., Kilic, A., Begrow, F., Fiedler, B., Gambaryan, S., Walter, U., Lohmann, S. M., & Kuhn, M. (2003). Increased effects of C-type natriuretic peptide on contractility and calcium regulation in murine hearts overexpressing cyclic GMP-dependent protein kinase I. *Br J Pharmacol*, *140*(7), 1227-1236. https://doi.org/10.1038/sj.bjp.0705567

Wood, B. M., Simon, M., Galice, S., Alim, C. C., Ferrero, M., Pinna, N. N., Bers, D. M., & Bossuyt, J. (2018). Cardiac CaMKII activation promotes rapid translocation to its extra-dyadic targets. *J Mol Cell Cardiol*, *125*, 18-28. https://doi.org/10.1016/j.yjmcc.2018.10.010

Wu, X., Zhang, T., Bossuyt, J., Li, X., McKinsey, T. A., Dedman, J. R., Olson, E. N., Chen, J., Brown, J. H., & Bers, D. M. (2006). Local InsP3-dependent perinuclear Ca2+ signaling in cardiac myocyte excitation-transcription coupling. *J Clin Invest*, *116*(3), 675-682. https://doi.org/10.1172/JCI27374

Wu, Y., Temple, J., Zhang, R., Dzhura, I., Zhang, W., Trimble, R., Roden, D. M., Passier, R., Olson, E. N., Colbran, R. J., & Anderson, M. E. (2002). Calmodulin kinase II and arrhythmias in a mouse model of cardiac hypertrophy. *Circulation*, *106*(10), 1288-1293. https://doi.org/10.1161/01.cir.0000027583.73268.e7

Wu, Y. S., Zhu, B., Luo, A. L., Yang, L., & Yang, C. (2018). The Role of Cardiokines in Heart Diseases: Beneficial or Detrimental? *Biomed Res Int*, *2018*, 8207058. https://doi.org/10.1155/2018/8207058

Xia, Y., Tsai, A. L., Berka, V., & Zweier, J. L. (1998). Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem*, *273*(40), 25804-25808. https://doi.org/10.1074/jbc.273.40.25804

Xu, K. Y., Huso, D. L., Dawson, T. M., Bredt, D. S., & Becker, L. C. (1999). Nitric oxide synthase in cardiac sarcoplasmic reticulum. *Proc Natl Acad Sci U S A*, *96*(2), 657-662. https://doi.org/10.1073/pnas.96.2.657

Xu, L., Eu, J. P., Meissner, G., & Stamler, J. S. (1998). Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science*, *279*(5348), 234-237. https://doi.org/10.1126/science.279.5348.234

Xu, L., Lai, D., Cheng, J., Lim, H. J., Keskanokwong, T., Backs, J., Olson, E. N., & Wang, Y. (2010). Alterations of L-type calcium current and cardiac function in CaMKII{delta} knockout mice. *Circ Res*, *107*(3), 398-407. https://doi.org/10.1161/CIRCRESAHA.110.222562

Yang, B., Larson, D. F., & Watson, R. R. (2004). Modulation of iNOS activity in age-related cardiac dysfunction. *Life Sci*, *75*(6), 655-667. https://doi.org/10.1016/j.lfs.2003.09.076

Yang, D., Zhu, W. Z., Xiao, B., Brochet, D. X., Chen, S. R., Lakatta, E. G., Xiao, R. P., & Cheng, H. (2007). Ca2+/calmodulin kinase II-dependent phosphorylation of ryanodine receptors suppresses Ca2+ sparks and Ca2+ waves in cardiac myocytes. *Circ Res*, *100*(3), 399-407. https://doi.org/10.1161/01.RES.0000258022.13090.55

Yang, Y., Zhu, W. Z., Joiner, M. L., Zhang, R., Oddis, C. V., Hou, Y., Yang, J., Price, E. E., Gleaves, L., Eren, M., Ni, G., Vaughan, D. E., Xiao, R. P., & Anderson, M. E. (2006). Calmodulin kinase II inhibition protects against myocardial cell apoptosis in vivo. *Am J Physiol Heart Circ Physiol*, *291*(6), H3065-3075. https://doi.org/10.1152/ajpheart.00353.2006

Yasukawa, T., Tokunaga, E., Ota, H., Sugita, H., Martyn, J. A., & Kaneki, M. (2005). S-nitrosylation-dependent inactivation of Akt/protein kinase B in insulin resistance. *J Biol Chem*, *280*(9), 7511-7518. https://doi.org/10.1074/jbc.M411871200

Zaccolo, M., & Movsesian, M. A. (2007). cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res*, *100*(11), 1569-1578. https://doi.org/10.1161/CIRCRESAHA.106.144501

Zhang, D. M., Chai, Y., Erickson, J. R., Brown, J. H., Bers, D. M., & Lin, Y. F. (2014). Intracellular signalling mechanism responsible for modulation of sarcolemmal ATP-sensitive potassium channels by nitric oxide in ventricular cardiomyocytes. *J Physiol*, *592*(5), 971-990. https://doi.org/10.1113/jphysiol.2013.264697

Zhang, P. (2017). CaMKII: The molecular villain that aggravates cardiovascular disease. *Exp Ther Med*, *13*(3), 815-820. https://doi.org/10.3892/etm.2017.4034

Zhang, R., Khoo, M. S., Wu, Y., Yang, Y., Grueter, C. E., Ni, G., Price, E. E., Thiel, W., Guatimosim, S., Song, L. S., Madu, E. C., Shah, A. N., Vishnivetskaya, T. A., Atkinson, J. B., Gurevich, V. V., Salama, G., Lederer, W. J., Colbran, R. J., & Anderson, M. E. (2005). Calmodulin kinase II inhibition protects against structural heart disease. *Nat Med*, *11*(4), 409-417. https://doi.org/10.1038/nm1215

Zhang, T., Guo, T., Mishra, S., Dalton, N. D., Kranias, E. G., Peterson, K. L., Bers, D. M., & Brown, J. H. (2010). Phospholamban ablation rescues sarcoplasmic reticulum Ca(2+) handling but exacerbates cardiac dysfunction in CaMKIIdelta(C) transgenic mice. *Circ Res*, *106*(2), 354-362. https://doi.org/10.1161/CIRCRESAHA.109.207423

Zhang, T., Johnson, E. N., Gu, Y., Morissette, M. R., Sah, V. P., Gigena, M. S., Belke, D. D., Dillmann, W. H., Rogers, T. B., Schulman, H., Ross, J., & Brown, J. H. (2002). The

cardiac-specific nuclear delta(B) isoform of Ca2+/calmodulin-dependent protein kinase II induces hypertrophy and dilated cardiomyopathy associated with increased protein phosphatase 2A activity. *J Biol Chem*, *277*(2), 1261-1267. https://doi.org/10.1074/jbc.M108525200

Zhang, T., Kohlhaas, M., Backs, J., Mishra, S., Phillips, W., Dybkova, N., Chang, S., Ling, H., Bers, D. M., Maier, L. S., Olson, E. N., & Brown, J. H. (2007). CaMKIIdelta isoforms differentially affect calcium handling but similarly regulate HDAC/MEF2 transcriptional responses. *J Biol Chem*, *282*(48), 35078-35087. https://doi.org/10.1074/jbc.M707083200

Zhang, T., Maier, L. S., Dalton, N. D., Miyamoto, S., Ross, J., Bers, D. M., & Brown, J. H. (2003). The deltaC isoform of CaMKII is activated in cardiac hypertrophy and induces dilated cardiomyopathy and heart failure. *Circ Res*, *92*(8), 912-919. https://doi.org/10.1161/01.RES.0000069686.31472.C5

Zhang, Y. H., Jin, C. Z., Jang, J. H., & Wang, Y. (2014). Molecular mechanisms of neuronal nitric oxide synthase in cardiac function and pathophysiology. *J Physiol*, *592*(15), 3189-3200. https://doi.org/10.1113/jphysiol.2013.270306

Zhao, D., Guallar, E., Ouyang, P., Subramanya, V., Vaidya, D., Ndumele, C. E., Lima, J. A., Allison, M. A., Shah, S. J., Bertoni, A. G., Budoff, M. J., Post, W. S., & Michos, E. D. (2018). Endogenous Sex Hormones and Incident Cardiovascular Disease in Post-Menopausal Women. *J Am Coll Cardiol*, *71*(22), 2555-2566. https://doi.org/10.1016/j.jacc.2018.01.083

Zhong, P., Quan, D., Peng, J., Xiong, X., Liu, Y., Kong, B., & Huang, H. (2017). Role of CaMKII in free fatty acid/hyperlipidemia-induced cardiac remodeling both in vitro and in vivo. *J Mol Cell Cardiol*, *109*, 1-16. https://doi.org/10.1016/j.yjmcc.2017.06.010

Ziolo, M. T., Katoh, H., & Bers, D. M. (2001a). Expression of inducible nitric oxide synthase depresses beta-adrenergic-stimulated calcium release from the sarcoplasmic reticulum in intact ventricular myocytes. *Circulation*, *104*(24), 2961-2966. https://doi.org/10.1161/hc4901.100379

Ziolo, M. T., Katoh, H., & Bers, D. M. (2001b). Positive and negative effects of nitric oxide on Ca(2+) sparks: influence of beta-adrenergic stimulation. *Am J Physiol Heart Circ Physiol*, *281*(6), H2295-2303. https://doi.org/10.1152/ajpheart.2001.281.6.H2295

Ziolo, M. T., Kohr, M. J., & Wang, H. (2008). Nitric oxide signaling and the regulation of myocardial function. *J Mol Cell Cardiol*, *45*(5), 625-632. https://doi.org/10.1016/j.yjmcc.2008.07.015

Ziolo, M. T., Maier, L. S., Piacentino, V., Bossuyt, J., Houser, S. R., & Bers, D. M. (2004). Myocyte nitric oxide synthase 2 contributes to blunted beta-adrenergic response in failing human hearts by decreasing Ca2+ transients. *Circulation*, *109*(15), 1886-1891. https://doi.org/10.1161/01.CIR.0000124231.98250.A8

Chapter 2:

2. Overview of Fluorescent Techniques and Cell-in-gel system

This section briefly explains the specific fluorescent techniques used in this dissertation studies. A basic knowledge of fluorescence is assumed.

2.1 Fluorescence recovery after photobleaching

2.1.1 Background

Proteins involved in phosphorylation activities, nuclear modeling and several other biological processes are able to translocate and interact with binding partners/targets to exert their function. Being able to measure and quantify these properties can be indispensable in elucidating the molecular mechanisms driving these events. Fluorescence recovery after photobleaching (FRAP) is a very useful approach for quantitative measurements and studies involving the mobility and also fractional immobilization of proteins at normal physiological conditions in living organisms. Developed by Axelrod and coworkers in the 1970s, early FRAP investigations focused on the mobility of fluorescently labelled constituents of the cell membrane[1]. With the development of confocal microscopy and increase in availability of fluorescent proteins, FRAP has grown in its usage. It can reveal information on the rate of diffusion, molecular binding kinetics, compartmentalization, and active transport.

2.1.2 Photobleach and recovery profile

The technique involves rapidly and irreversibly photobleaching a population of fluorescently labeled molecules in a small region of interest (ROI) within the cell, followed by observing and characterizing the recovery of fluorescence in that region over time (Figure 2.1). The underlying principle is that prior to bleaching the system under study is already at a steady state. In this steady state the labeled molecules may be stationary with very little or no movement (tightly bound to a structurally restricted target, freely diffusing within their environment, undergoing active transport, transiently binding to other molecules, or a complex mixture of all. Due to high molecular concentrations within living cells, and the diffraction limit of light, these conditions are typically indistinguishable from one another under a light microscope, appearing as a static unvarying signal. However, following a spatially restricted bleach, properties about the mobility of the labeled molecules are revealed in the kinetics of any fluorescent recovery. Immediately after a bleach-pulse that causes a majority of the GFP-tagged proteins (GFP-proteins) within the region to irreversibly lose their fluorescent properties. In a situation where all GFP-proteins are mobile, uniformly distributed and not bound to fixed targets, fluorescent proteins outside the region will diffuse into the bleached region resulting in fluorescence recovery after photobleach (FRAP) in that ROI. This will continue until the signal inside the bleached ROI reaches the signal outside the ROI. Note that the final fluorescence level outside the ROI will be slightly lower than the initial value, to an extent determined by the fraction of total cell GFP that is bleached. In this case the FRAP is mainly limited by the apparent diffusion coefficient of the fluorescent protein in its cellular environment). In the other extreme, if all of the GFP-tagged protein is permanently immobile (bound permanently to immobile targets), neither the bleached proteins in the ROI nor the fluorescent protein outside the ROI will diffuse, resulting in zero

FRAP. More often, there is a mixture of fluorescent protein that is mobile and bound *reversibly* to relatively fixed sites. In this usual case, FRAP at those fixed sites will be determined by both the off-rate of the bleached protein from its target and diffusion of bleached protein out and unbleached protein into the bleached region. So, there may be a fast FRAP component that is diffusion limited (related to unbound GFP-protein) and a slower component that may reflect, in large part, dissociation of the bleached GFP-protein from fixed targets within the bleached ROI. Very strongly bound GFP-protein may have negligible dissociation during the time course of a FRAP experiments, and that would appear as residual fluorescence difference between the bleached ROI and the remainder of the cell. Thus, FRAP characteristic can be informative about diffusion and binding of GFP-proteins.

2.1.3 Overview of FRAP Variants

Several variants of FRAP have been developed (Figure 2.1), including spot-FRAP, strip-FRAP, fluorescence loss in photobleaching (FLIP), combined FLIP-FRAP and inverted FRAP (iFRAP).

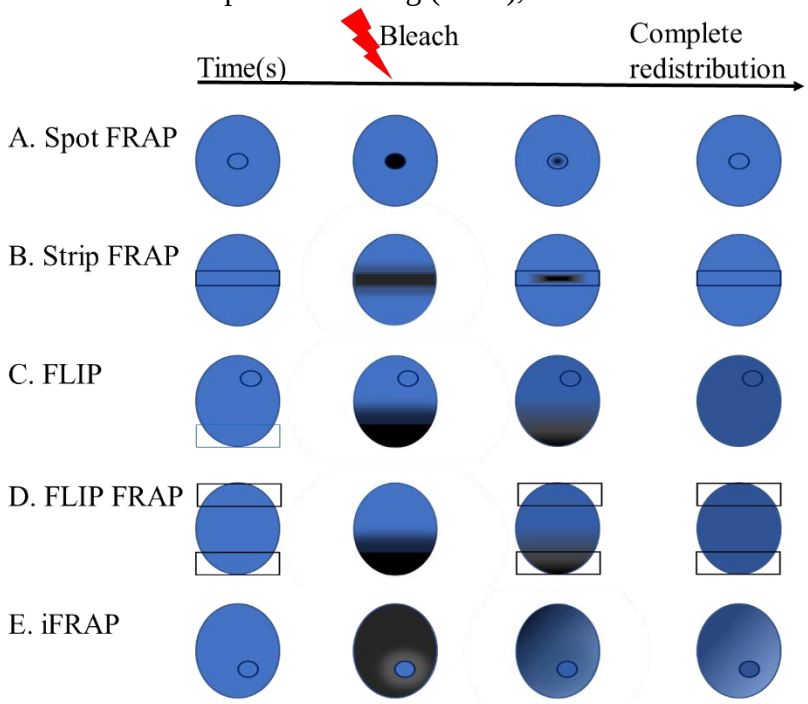

Figure 2.1: Schematic of FRAP variants

Spot-FRAP is based on photobleaching of a small spot whereas in strip-FRAP, a larger region. Strip FRAP is favorable when expression of the GFP-tagged protein is low. In FLIP, the loss of fluorescence in a region or structure distant from the bleached region is monitored. Combining FLIP and FRAP is useful to determine residence time of protein within structures - the recovery of the fluorescence in a region at one end of the cell after bleaching for a relative long period (FRAP) is measured in parallel with the decrease of the signal in a similar region at the other end of the cell (FLIP). iFRAP on the other hand involves bleaching the entire cell with the exception of the protein of interest. For CaMKII studies in cardiomyocytes, spot-FRAP is most often used, as in this study. However, Strip FRAP (or confocal line FRAP) can also be useful in distinguishing longitudinal vs. transverse diffusion in structurally oriented cells like cardiac myocytes[2].

2.2 Fluorescence Resonance Energy Transfer

Fluorescence Resonance Energy Transfer (FRET) is simply the transfer of fluorescent energy between two closely juxtaposed molecules (within 10 nm of each other, see Figure 2.2) – a donor and an acceptor. The energy transfer is dependent on the distance between the interacting molecules and can thus be used as a spectroscopic ruler and reporter, great for detecting protein–protein interactions in live cells (Figure 2.2). With the aid of fluorescent probes, we can visually detect, measure and quantify these spatial changes.

FRET involves two fluorescent species — a donor (e.g. GFP tag on the N-terminal of CaMKII) and an acceptor (RFP tag on the C-terminal). When CaMKII is in its inactive conformation, the fluorescent probes are proximal enough to interact and FRET occurs[3]. When activated, FRET

decreases as the distance between probes is increased. Fluorescence read out here can thus be an indicator of CaMKII activity state. Other than fluorescence intensity, FRET can also be quantified

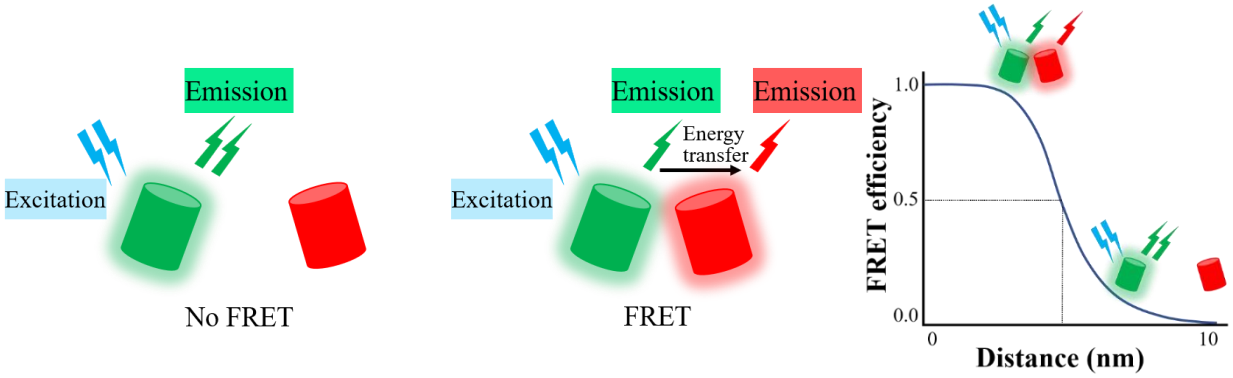

Figure 2.2: Schematic of the FRET principle. Interacting partners must be within 1-10nM for maximum FRET efficiency.

by fluorescence lifetime, quantum efficiency, and anisotropy[4].

2.3 Fluorescence Lifetime Imaging Microscopy (FLIM)

The fluorescence lifetime of a fluorophore is a measure of the average time a fluorophore remains in the excited state[5]. Time-correlated single photon counting (TCSPC) is used for high speed FLIM data acquisition[6]. In this approach, the timing between the laser pulse and detection of single photons from fluorescence is recorded. However, a large number of photons is necessary to accurately fit an exponential curve. For a homogenous population of molecules, the resulting fluorescence decay is a single exponential function. FLIM is used to discriminate different fractions of the same fluorophore in different states of interaction with its environment as it is dependent on environmental conditions such as ions, pH, oxygen content, and interactions with other proteins (including FRET acceptors). Fluorescence lifetime measurements are insensitive to

instrument settings like laser intensity or detector gain and are largely independent of fluorophore concentration, photobleaching and light scattering. Therefore, fluorescence lifetime imaging allows us to perform accurate measurements that circumvent some limitations of typical Fluorescence Resonance Energy Transfer (FRET) analysis.

2.4 FLIM-FRET measurements

The FLIM-FRET technique used in this study is based on the measurement of the fluorescence lifetime of the donor. The time constant of this function, the fluorescence lifetime (τ), is the reciprocal sum of the rate constants of all possible return paths: emitting a photon (EP), non-radiative relaxation (NR), and FRET.

No FRET: $\tau_{donor} = \frac{1}{K_{EP}+K_{NR}}$ …Equation 1

With FRET: $\tau_{donor} = \frac{1}{K_{EP}+K_{NR}+K_{FRET}}$ …Equation 2

FRET efficiency is obtained by comparing the fluorescence lifetimes of the donor in presence and absence of FRET (Figure 2.3). The average lifetime is shorter with FRET given the additional rate of transfer K_{FRET}. The rate constants, and thus the fluorescence lifetime, depend on the type of the molecule, its conformation and on the way the molecule interacts with its environment. In its inactive state where FRET occurs, CaMKII has shorter lifetimes compared to its active state with reduced FRET.

An advantage of FLIM-based FRET measurement is that FLIM can distinguish between interacting and noninteracting donor fractions. Also, only donor lifetimes need to be recorded, avoiding problems associated with bleed-through or direct excitation of acceptor. Lifetime measurements are insensitive to laser intensity, detector gain and other instrument settings.

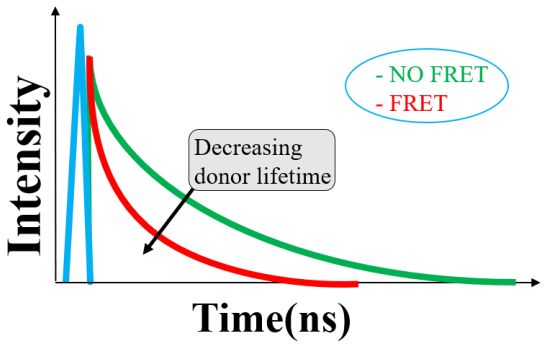

Figure 2.3: Time correlated single photon counting. FRET effect on donor fluorescence decay. FRET is a quenching process, i.e. offers an additional non-radiative decay pathway in Eq. (2) and thus shortens the donor fluorescence

2.3 Cell-in-gel System

The cardiomyocyte contracts as calcium in the cytosol binds contractile proteins to activate cross-bridge cycling and sarcomere shortening. Mechano-transduction mechanisms autoregulate cardiac inotropy and cell contractility, allowing the heart to adapt efficiently to changing mechanical loads. Early research on cardiac function brought about the Frank starling law, which states that the force of contraction increases with increased venous return and preload. Another mechanical load phenomenon observed is the Anrep effect where increased cardiac afterload causes a positive inotropic response. The investigate the molecular mechanisms and signaling pathways behind these, an experimental platform that mimics the myocardium's mechanical properties was developed. This platform is referred to as the Cell-in-gel system. The system enables cells to be embedded into a viscoelastic hydrogel and simulates mechanical loading in vivo. The gel consists of a polyvinyl alcohol (PVA) polymer cross-linked with polyethylene glycol (PEG)-boronate

cross-linker to produce a 3-D viscoelastic matrix. To embed cardiomyocytes, the PEG boronate links the PVA polymer matrix to the cis diols of glycans on the sarcolemma surface, effectively fastening the entire cell surface to the gel and transducing mechanical forces via constraint. The viscoelastic hydrogel was characterized by rheological methods and its material properties tuned to closely match those of a cell contracting in the native environment. Tuning hydrogels can change their material properties to match those of different tissues and disease conditions, with stiffer hydrogels mimicking the diseased myocardium for instance.

References

1 Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. & Webb, W. W. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys J* **16**, 1055-1069, doi:10.1016/S0006-3495(76)85755-4 (1976).

2 Picht, E. *et al.* Dynamic calcium movement inside cardiac sarcoplasmic reticulum during release. *Circ Res* **108**, 847-856, doi:10.1161/CIRCRESAHA.111.240234 (2011).

3 Bunt, G. & Wouters, F. S. FRET from single to multiplexed signaling events. *Biophys Rev* **9**, 119-129, doi:10.1007/s12551-017-0252-z (2017).

4 Lidke, D. S. *et al.* Imaging molecular interactions in cells by dynamic and static fluorescence anisotropy (rFLIM and emFRET). *Biochem Soc Trans* **31**, 1020-1027, doi:10.1042/bst0311020 (2003).

5 Meyer-Almes, F. J. Fluorescence lifetime based bioassays. *Methods Appl Fluoresc* **5**, 042002, doi:10.1088/2050-6120/aa7c7a (2017).

6 Becker, W. *et al. High-speed FLIM data acquisition by time-correlated single-photon counting*. Vol. 5323 PWB (SPIE, 2004).

Chapter 3:

Nitrosylation of Cardiac CaMKII at Cys290 Mediates Mechano-chemo-transduction

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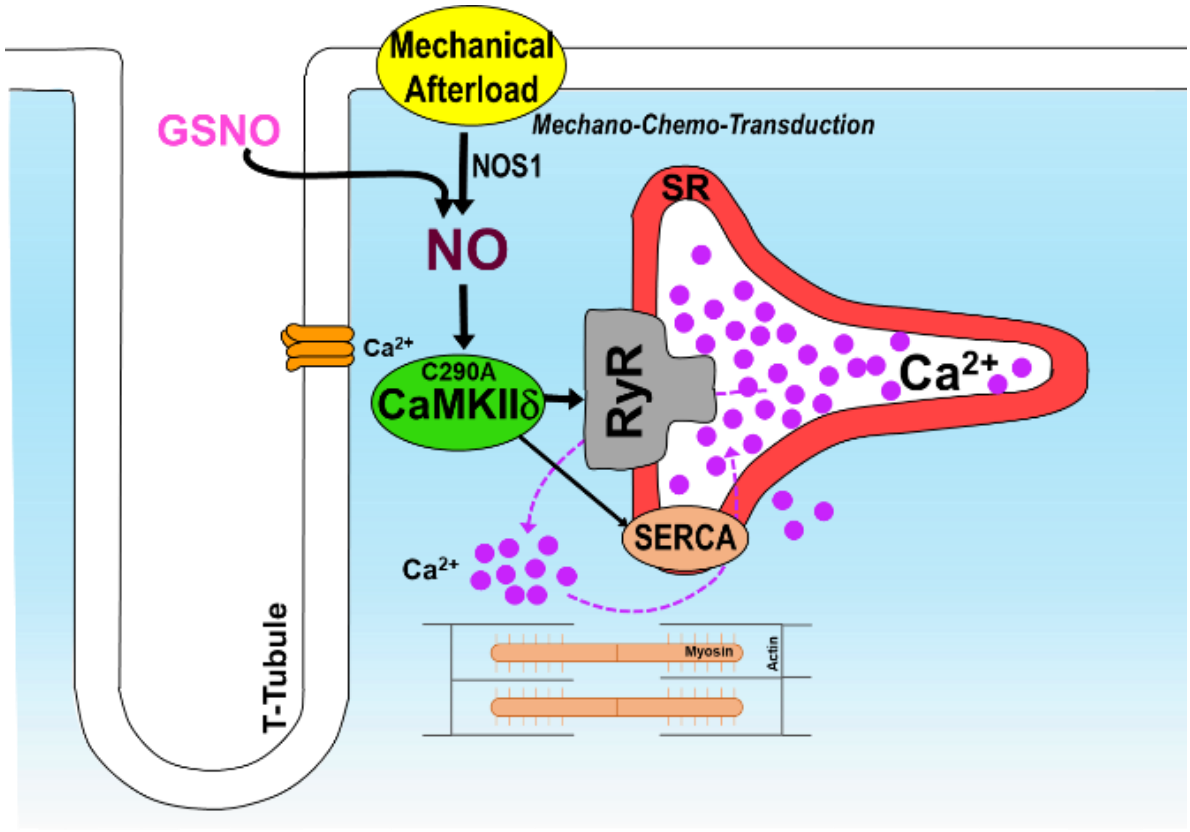
Abstract

Mechanical stress can affect Ca^{2+} (Ca) dynamics in cardiomyocytes and lead to cardiac remodeling, hypertrophy, arrhythmias, and heart failure. Both nitric oxide (NO) produced by NOS1 and Ca-calmodulin kinase II (CaMKII) signaling are required mediators of mechano-chemo-transduction (MCT) whereby mechanical afterload promotes enhanced Ca^{2+} transients and stronger contraction. To test whether Cys290 in CaMKIIδ mediates the acute *S*-nitrosylation of CaMKIIδ that promotes sarcoplasmic reticulum (SR) Ca release, we developed a novel CaMKIIδ knock-in mouse (C290A substitution This knock-in mouse exhibited normal heart size, cardiac function, Ca transients and β-adrenergic responses. However, compared to wildtype (WT) littermates the C290A myocytes failed to exhibit an increase in Ca spark frequency in response to *S*-nitrosylating agent GSNO. Next, we tested whether this single amino acid on CaMKIIδ is necessary for afterload-induced increase in Ca sparks and Ca transients, using our cell-in-gel system to exert multiaxial 3D mechanical stress on the cardiomyocyte during contraction. In WT myocytes, mechanical afterload increased Ca spark frequency, Ca transient amplitude and SR Ca uptake vs. load-free WT myocytes. All of these MCT effects were abolished in either cardiac-specific CaMKIIδ knockout mice, or the point mutant C290A CaMKIIδ knock-in myocytes. Thus, our data show that CaMKIIδ activation by nitrosylation at the C290 site is essential to mediate mechanical stress induced acute Ca^{2+} upregulation in cardiomyocytes. The data also suggest that NOS1 activation is upstream of *S*-nitrosylation at C290 of CaMKII, and increased CaMKII activity leads to enhanced SR Ca uptake and release and increased Ca^{2+} transient. Understanding the molecular players in the MCT pathway in the heart may provide novel therapeutic strategies for treating cardiac diseases associated with mechanical stress.

Keywords: Cardiac myocyte, Calcium, Mechano-chemo-transduction, *S*-Nitrosylation, CaMKII, Post-translational modifications



Introduction

The heart is a mechanical pump, undergoing contraction under mechanical loads[1] that vary with changes in position, emotional state, physical exertion, and chemical stimulants. How the heart senses mechanical load and alters Ca^{2+} dynamics to compensate – termed mechano-chemo-transduction (MCT) – has been studied for decades. In normal cardiac physiology, MCT pathways provide autoregulation of Ca^{2+} signaling and contractility but in pathological conditions, mechanical load may cause Ca^{2+} dysregulation and cardiac dysfuncti, adding medical relevance to these studies. Cardiac remodeling, hypertrophy, arrhythmias, and heart failure among others, are often accompanied by altered cardiac mechanics[2-6]. Understanding the MCT signaling pathways and key molecular determinants may hold the key to developing drug therapies that would effectively combat arrhythmias and cardiomyopathy in patients with mechanical stress related issues such as hypertension, muscular dystrophy, and dilated cardiomyopathy.

Several methods have been used to study MCT, and the role of nitric oxide (NO) has been increasingly recognized. Imposing mechanical afterload (via a hydrogel) on contracting myocytes increases the systolic Ca^{2+} transient and causes spontaneous Ca^{2+} sparks during diastole in a NO dependent manner involving NO synthase 1 (NOS1)[7]. Physiologic stress from stretching cardiomyocytes with glass rods (increased preload) activates endothelial nitric oxide synthase (NOS3)[8] and nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2)[9] to enhance Ca^{2+} transients and induce spontaneous Ca^{2+} sparks. The stretch induces X-ROS signaling, which sensitizes the ryanodine receptor (RyR); and in Duchenne muscular dystrophy models, it contributes to cardiomyopathy via abnormal Ca^{2+} release from the sarcoplasmic reticulum (SR)[9]. Other studies have shown excessive afterload can alter Ca^{2+} cycling or cause maladaptive hypertrophy with increased fibrosis, inflammation, cardiomyocyte apoptosis, heart failure and

increased mortality[10-12]. The CaMKII signaling pathway has also been implicated in acute afterload induced changes on Ca^{2+} handling[7] as well as the long-term maladaptive processes mentioned above[13,14]. CaMKII has four isoforms and is found in most cell types, but the predominant isoform in cardiac myocytes is CaMKIIδ. It delicately senses and translates the periodic rises in intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) in cardiomyocytes during excitation-contraction coupling to kinase activity[15]. Besides the Ca^{2+}/calmodulin-dependent activation of CaMKIIδ that may lead to autophosphorylation (pThr287)[16], additional post-translational modifications (PTMs) within the regulatory hub (Fig 3.1A) such as oxidation (M281/M282)[17], *O*-GlcNAcylation (S280)[18], and *S*-nitrosylation (C290)[19,20] have likewise been shown to trigger autonomous activity. Notably, nitrosylation of Cys290 promotes autonomous, pathologically sustained kinase activity (molecular memory) leading to spontaneous calcium mishandling and arrhythmogenic activities[21].

The cellular and molecular mechanisms that transduce mechanical load to influence Ca^{2+} handling and electrical activities in the cardiomyocyte are far from fully understood. In this study, we show a connection between two pathways associated with MCT. Both NOS1 and CaMKIIδ signaling pathways have been identified as mediators of mechano-chemo-transduction that respond to mechanical load during cardiomyocyte contraction and regulate Ca^{2+} handling[7]. Given the PTM sites for nitrosylation on CaMKIIδ and its influence on kinase activity, we hypothesize that the S-nitrosylation of Cys290 on CaMKIIδ positively mediates mechano-chemo-transduction. Using the cell-in-gel system[7] to impose afterload, we monitor Ca^{2+} handling from the SR Ca^{2+} release unit (co-localized L-type calcium channels and RyR proteins which are downstream targets of CaMKII) as summated Ca^{2+} transients and spontaneous Ca^{2+} release at rest, using confocal linescan imaging.

Methods

CaMKIIδ C290A knock-in mouse model

CaMKIIδ C290A knock-in mice were generated via the UC Davis Mouse Biology Program, to ablate one of the nitrosylation sites on CaMKIIδ. To convert the wildtype Cys residue 'TGC' into an Ala residue 'GCC' at position 290, we use CRISPR/Cas9 homology directed repair to knock-in the Ala codon into the CaMKIIδ mouse locus in place of that for Cys (Fig 3.1B). We took advantage of an offset oligo (5'-atgacttctgctttcagtagctttcacctcaccagatcaaccctccagccctcccacaaca-atgtcaaatttcttaccttcagtttccgtctagcattaaatttcttcaag**gc**gtctacagtctcctgcctgtgcatcatggaggcaacagtagagcg ttgctaa-3') that is complementary to the target strand and distal to the protospacer adjacent motif (PAM). Additionally, we used the following sgRNA 5'-tgcctccatgatgcacaggc**agg**-3' and 5'-tgaagaaatttaatgctaga**cgg**-3', which cleaves the genome upstream and downstream from the engineered mutation, knocking in via a semi long ssODN method to ensure highest possible success.

The mice were genotyped and CaMKIIδ mRNA was sequenced to confirm the amino acid substitution in the C290A knock-in. To determine mouse genotype, polymerase chain reaction (PCR) was performed on genomic DNA from 0.4 cm lengths of mouse tails obtained at birth. All PCR reagents were from Quantabio (Beverly, MA). Thirty-five cycles were performed on the samples using ProFlex PCR System (ThermoFisher Scientific) as follows: denaturation at 95°C for 0.5 min, annealing at 60°C for 0.5 min, extension at 72°C for 0.5 min, final extension cycle at 72°C for 5 min, an a soak cycle at 4°C. Reaction products were analyzed on 2% agarose gels in which the following bands were expected: wildtype (+/+) allele: 137 bp; knockout (−/−) allele: 137 bp; heterotypic (+/−) alleles: both bands.

Conventional echocardiography and Doppler imaging

Transthoracic echocardiography was performed using a VisualSonics Vevo 2100 system equipped with MS550 40 MHz transducer (FUJIFILM VisualSonics, Toronto, ON, Canada). Systolic function (cardiac contractile function) indices were obtained from short-axis M-mode scans at the midventricular level. Apical four-chamber views were assessed for diastolic function measurements using pulsed-wave and tissue Doppler imaging at the level of the mitral valve. Body temperature was carefully monitored, and anesthesia was adjusted to achieve 400-450 beats/min heart rate in each animal. Measurements were collected at baseline conditions for both C290A and WT mice. All parameters were measured at least three times, and means are presented.

Cardiomyocyte Isolation

All animal handling and laboratory procedures were conducted in compliance with the NIH guidelines for animal research and with approval of the Institutional Animal Care and Use Committee at the University of California, Davis. Adult mice between 10-12 weeks old were used including wild-type (Jackson Laboratory, Stock No. 000664), CaMKIIδ cardiac-specific KOs, and the novel CaMKIIδ C290A mice with a cysteine to alanine substitution at position 290. For the enzymatic isolation of left ventricular cardiomyocytes, the perfusion system was filled with MEM solution and cleared of any bubbles (bubbles are cleared by fast forwarding the motor). The perfusion system water bath set to 37℃ and a rate of ~5 mL/minute. The top section of the perfusion system was cleared of MEM, then filled with MEM + enzyme (50 mL MEM + 60 mg collagenase type 2 and 1.3 mg protease). The mice were injected subcutaneously with diluted heparin (~0.3 mL diluted heparin) and anesthetized with 5% isoflurane. After sedation mice were moved onto an absorbent pad with the nose/mouth in a nose cone which continuously delivers 2% isoflurane. The mice were taped down to the absorbent pad and reactions to noxious stimuli

checked. When areflexia was achieved, hearts were excised and cleaned in cold wash solution (100 mL MEM + 0.1 mL heparin), with any extra tissue or fat cut off. The entire heart was then cannulated in cold wash solution on ice. Hearts were retrograde perfused on constant flow ~5mL/min, 37ºC. When adequately digested, a cut was made below the atria to drop the ventricles into a small dish with stopping solution (mouse MEM + FBS). Ventricular myocytes were dispersed mechanically and filtered through a nylon mesh and allowed to sediment for ~10 minutes. The sedimentation was repeated three times using increasing [Ca^{2+}] from 0.125 to 0.25 then 0.5 mmol/L. Finally, ventricular myocytes were kept in Tyrode's solution (0.5 mmol/L [Ca^{2+}]) at room temperature until use.

Fluorescent measurement of [Ca^{2+}] using Fluo-4 AM dye loading

[Ca^{2+}]$_i$ was measured using a single wavelength Calcium indicator, Fluo-4 AM (10 μM/L, Thermo Fisher scientific, Catalog number: F14201, Lot number: 2146860). After isolation, freshly isolated cardiomyocytes were loaded with Fluo-4 AM and Pluronic acid (0.02%, Thermo Fisher scientific, Catalog number: P3000MP, Lot number: 1990297). The dye was loaded for 30 minutes at room temperature followed by wash and de-esterification for 30 minutes. Supernatant was removed and replaced with Normal Tyrode (135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4, 23 °C) three times - increasing [Ca^{2+}] with each wash to a final of 1.8mM. All loading was done at room temperature.

Cell-in-gel system

Elastic gel matrix was made of a PVA hydrogel system composed of underivatized PVA (98 kD) and a tetravalent boronate-PEG cross-linker, all custom made[22]. Freshly isolated cardiomyocytes were loaded with Fluo-4 AM as described above, suspended in a 7% PVA solution; then, a 7.5% cross-linker solution was added in equal volume. Optionally, submicron fluorescent beads can be

embedded in the gel to track displacement. Upon incubation for 15 minutes at room temperature, the boronate group cross-links PVA hydrogel, embedding the cell in the 3D gel matrix. The boronate group also cross-links the cis diols of the cell surface glycans to PVA, thereby tethering the cell surface to the gel. The optically transparent gel allows for Ca^{2+} signals to be observed while the cell-in-gel system is perfused. This gel system helps investigate MCT signaling pathways by activating comparable mechanical mechanisms in contracting myocytes.

Confocal imaging of Ca^{2+} signals

Ca^{2+} transients and diastolic Ca^{2+} events (sparks and waves) were detected via linescan imaging with a laser scanning confocal microscope - Bio-Rad Radiance 2100, equipped with a 40× oil immersion objective lens, at 6 ms/line. Fluo-4 was excited with a 488 nm line of the argon laser and the emitted fluorescence was collected through a 500-530 nm bandpass emission filter. To measure systolic calcium transient, intact cardiomyocytes were plated on laminin-coated coverslips or loaded in-gel as described above and paced at 0.5 Hz in a field stimulation chamber, until Ca^{2+} cycling reached steady state. Fluo-4 fluorescence was recorded for at least 5 beats during steady state. To assess SR calcium load, 20 mM caffeine in normal Tyrode was applied to rapidly deplete the SR content. To evaluate the SR calcium load, the exponential rate constant of the calcium transient amplitude induced by rapid caffeine application was used as an indicator of the SR Ca^{2+} release through RyRs and the Ca^{2+} extrusion through sodium-calcium exchanger (NCX)[23]. ImageJ was used for image processing and analysis; Ca sparks were detected using SparkMaster plugin[24] first, and then verified by human inspection. Ca sparks were measured from ~10 secs after stimulation stopped. To analyze Ca sparks with SparkMaster, the Scanning Speed (in lines per second) and Pixelsize (in μm) are adjusted according to the microscope setting during image acquisition, and Criteria Values were set to 3.8 as recommended[24]. Based on non-cellular

fluorescent regions in image, the background is determined and regions where the fluorescence in the data image is below the determined noncellular background level are excluded from the analysis. Images with Ca waves were also excluded from analysis. Fluorescence intensities were background-subtracted and normalized to the corrected baseline.

Results

To allow us to test the role of CaMKIIδ-C290 in mediating MCT, we generated a C290A knock in mouse. Figure 3.1A illustrates 5 known PTM sites in the highly conserved regulatory region of CaMKII, immediately adjacent to the CaM (calmodulin) binding site. These include O-GlcNAcylation (S280), oxidation (MM281/282), autophosphorylation (pT287), and S-nitrosylation (C290), each of which promotes autonomous kinase activity after Ca^{2+}-CaM dissociation (memory)[17,18,20,25]. *S*-nitrosylation of C273 suppresses activation by Ca^{2+}-CaM[19]. Figure 3.1B shows the design of the CRISPR strategy and PAM sites used to replace the endogenous cysteine in CaMKIIδ with a serine. The mice were genotyped and CaMKIIδ mRNA was sequenced (see Methods) to confirm the amino acid substitution in the C290A knock-in. Figure 3.1C shows blots for WT and C290A mutant, so, allowing identification of homozygous WT and C290A mice, as well as heterozygous mice expressing both WT and mutant alleles.

The new CaMKII C290A knock-in mice were born at expected Mendelian genotype ratios and exhibited normal growth and maturation. CaMKIIδ C290A mice, vs WT littermates at the age we studied (10-12 weeks) exhibited unaltered baseline heart rate and cardiac contractile function on echocardiography (Figure 3.1C), and normal heart and body weights (Figure 3.1D). Thus, the new C290 Nitrosylation resistant mutant mice exhibit no obvious baseline phenotype differences from the WT mice.

CaMKIIδ C290A knock-in mice prevents CaMKIIδ S-nitrosylation at C290

First, we investigated the effects of CaMKII C290A mutation on myocyte Ca^{2+} handling. Figure 3.2A illustrates the experimental protocol with a confocal line-scan Ca^{2+} imaging series. Mouse

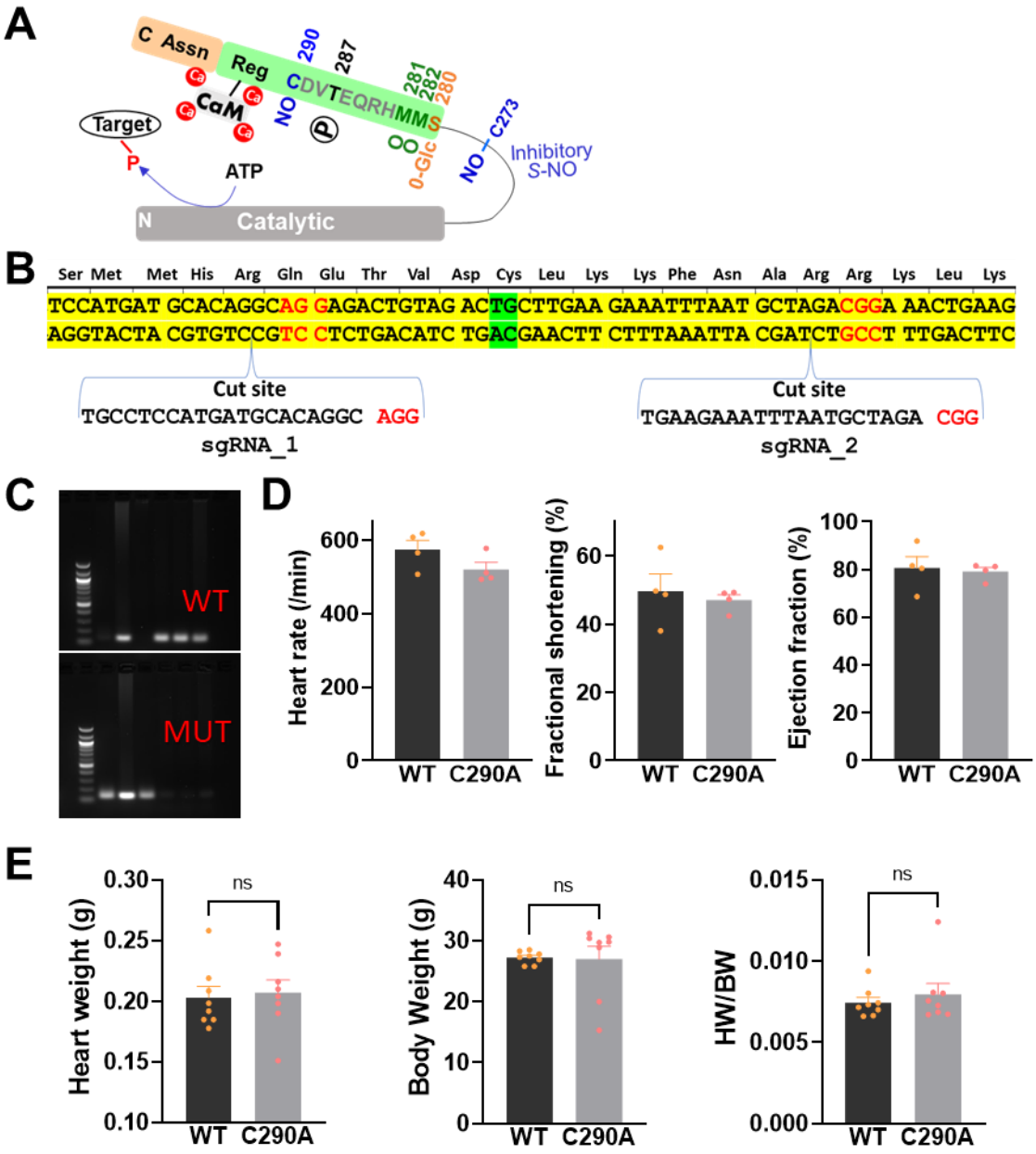


Figure 3.1: C290A mice model. A) Schematic of a CaMKII monomer and sequence of the regulatory hub domain. B) Homology directed repair Knock-in schematic for Cys290→Ala mouse generation with exon 11 in yellow and PAM sequence in red (left) and C) western blot showing genotype results for WT, MUT and HET mice. D) Unaltered heart rate and echocardiographic fractional shortening and ejection fraction in C290A mice. E) Normal heart weight, body weight and their ratio in CaMKIIδ C290A mice at baseline.

cardiomyocytes were paced at 0.5 Hz for 30 s and the last five (5) stabilized Ca^{2+} transients (CaTs; Fig 3.2A) were used for quantitative analysis. After pacing, myocytes were rested for one minute during which spontaneous Ca^{2+} sparks were recorded, and then 20 mM caffeine was rapidly applied to release all SR Ca^{2+} and assess the SR load. CaMKIIδ C290A knock-in mice exhibited unaltered Ca^{2+} amplitude and time to peak $[Ca^{2+}]_i$ in comparison to their WT littermates (Figures 3.2B). There was an acceleration of twitch $[Ca^{2+}]_i$ decline (faster time constant τ in Figure 3.2B). However, some unusually slow individual τ values, especially in WT myocytes, alerted us to the possible slowing of $[Ca^{2+}]_i$ decline because of high Ca^{2+} buffering that occurs at higher indicator loading. To assess this aspect, we plotted the τ of twitch $[Ca^{2+}]_i$ decline as a function of baseline fluorescence (F_0, as a crude index of indicator loading; Fig 3.2C, top). As expected, higher indicator loading was associated with slowed $[Ca^{2+}]_i$ decline, but there was no significant difference in linear regressions of the C290A vs. WT myocytes (neither slopes nor Y-intercepts). Moreover, only in the WT was there a cluster of myocytes with the highest levels of loading (boxed in Fig 3.2C) that was not seen in any C290A mice. If those data had been excluded the WT vs. C290A P value for τ would drop (from 0.231 to 0.009). This suggests that SR Ca^{2+} uptake was not significantly altered between the groups. The higher Fluo4 loading in some WT myocytes may also have damped the peak $[Ca^{2+}]_i$ values in those cells, and indeed that same cluster of highly Fluo4 loaded WT myocytes had relatively low peak $[Ca^{2+}]_i$ values. Exclusion of those cells increased the mean WT CaT amplitude to 5.4, very close to that seen in the C290A; P=0.500, still non-significant). Thus, in agreement with unaltered echocardiographic differences in C290A vs. WT myocytes (Figure 3.1C) we conclude that baseline myocyte Ca transients were also unaltered.

Furthermore, both C290A knock-in and WT mice showed a classical response towards the β-adrenergic agonist, isoproterenol (ISO; at 100 nM), which was added 5 min before pacing began. ISO significantly increased both the CaT amplitude (Figure 3.2D-F) and rate of Ca^{2+} transient decline. In contrast, acute exposure to 150 µM *S*-Nitrosoglutathione (GSNO; an NO donor) for 5

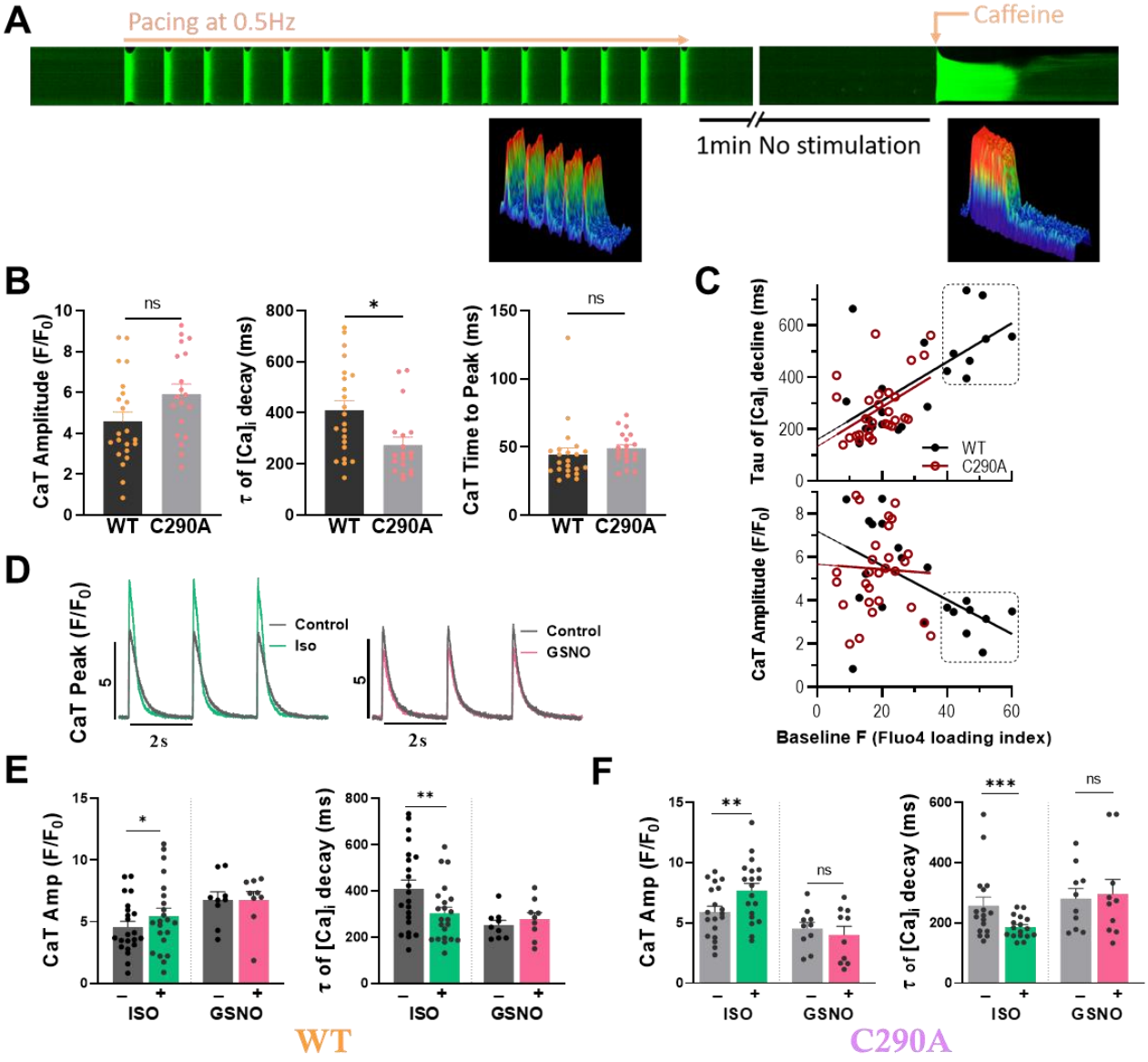


Figure 3.2: Characterization of novel C290A mice. A) Experimental protocol representation. B) Mean ± SEM values of baseline Ca^{2+} transient (CaT) data for C290A mice in comparison with their wild-type littermates (WT) under load-free conditions C) Influence of Fluo4 loading level on tau of $[Ca^{2+}]_i$ decline and CaT amplitude. D) CaT peaks of C290A cardiomyocytes under control conditions and with 100nM isoproterenol (ISO; left), or with 150µM *S*-nitrosoglutathione (GSNO; right) all stimulated at 0.5Hz. E) CaT amplitude and tau

min) did not alter Ca^{2+} transient amplitude or kinetics of $[Ca^{2+}]_i$ decline in either WT or C290A mouse myocytes (Figure 3.2D-F).

Figure 3.3 shows Ca^{2+} spark analysis for this group of myocytes during the post-stimulation period indicated in Figure 3.2A. In WT myocytes, both ISO and GSNO treatment significantly increased Ca^{2+} spark frequency, and to similar extents (Figure 3.3D-E). However, in C290A myocytes, GSNO failed to increase Ca^{2+} spark frequency, in comparison to a similar ISO-induced increase

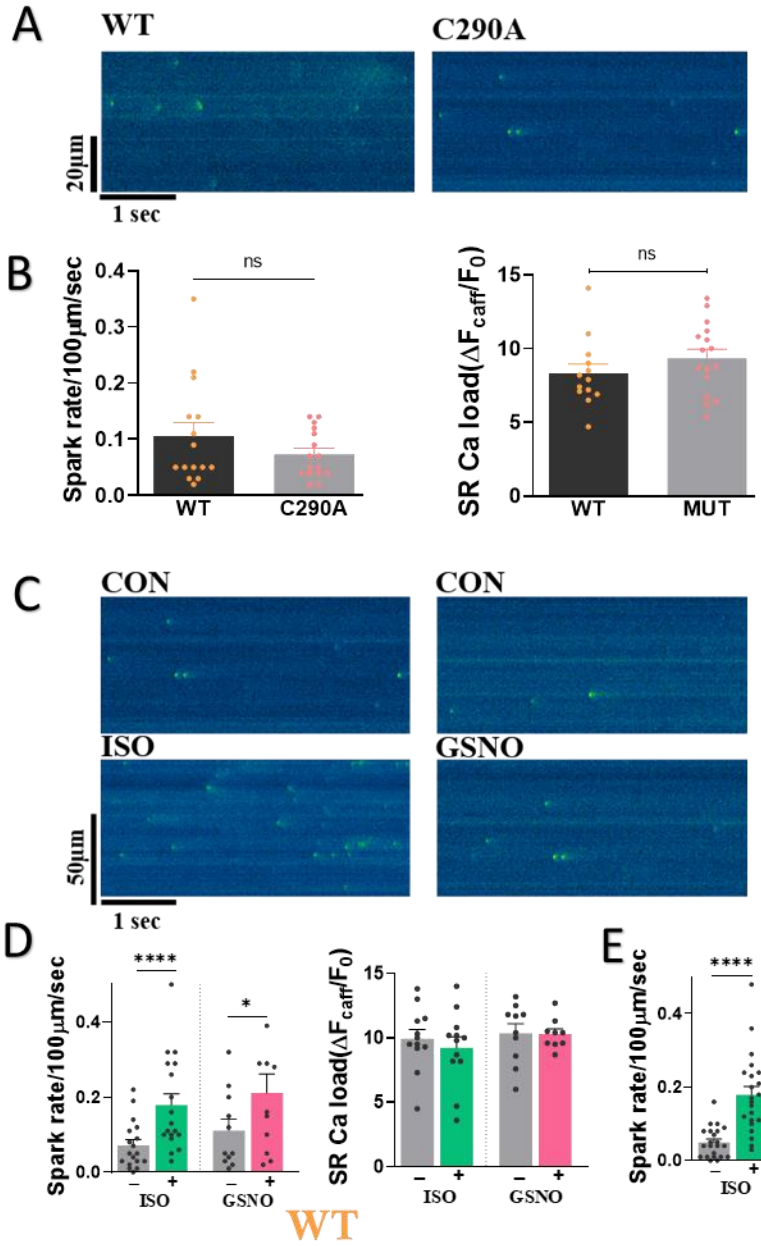


Figure 3.3: Characterization of novel C290A mice. A) Representative spontaneous Ca^{2+} sparks seen in the pseudo-colored confocal linescan images of C290A cardiomyocytes and their wild-type (WT) littermates. B) Mean ± SEM values of Ca^{2+} spark rate under load-free conditions in C290A (n = 16 cells) vs WT cardiomyocytes (n = 15 cells) C) Representative pseudo-colored confocal line-scans from quiescent C290A cardiomyocytes under control conditions and with 100nM isoproterenol (ISO, left), or with 150μM *S*-nitrosoglutathione (GSNO, right). D) Mean ± SEM Ca^{2+} spark rate data are shown for WT (n = 18) and E) C290A (n = 22) cardiomyocytes in response to ISO (left) and GSNO (right).

in Ca^{2+} spark frequency in C290A as in WT myocytes (Fig 3.3C-D). These data from intact myocytes are consistent with GSNO-induced CaMKII nitrosylation and activation with consequent RyR sensitization, and that this effect is abolished by the single point mutation of C290A in CaMKIIδ.

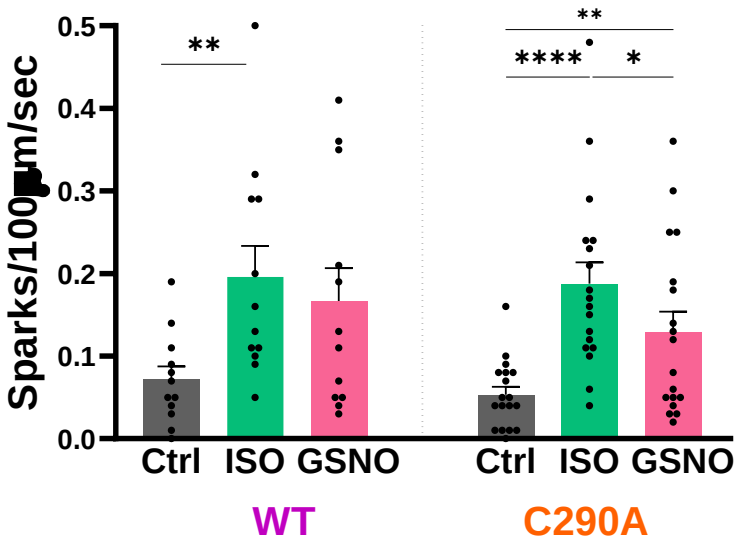


Figure 3.4: GSNO induced a persistent SR Ca^{2+} leak (consistent with autonomous activation state in the WT) which was suppressed in the C290A nitrosylation mutant. NB: Ctrl solution here had the same components as NT used elsewhere, but with varying concentrations (NaCl 125 mM, KCl 74.55 mM, Hepes 238.3 mM, MgCl 2.6 H2O 203.3 mM, Glucose 180.16 mM).

Figure 3.4 shows a slightly different protocol, where we applied ISO and GSNO sequentially. While at rest, myocytes were perfused with ISO for five minutes before pacing (0.5 HZ for 30 s), followed by resting Ca^{2+} spark measurement. Subsequently, myocytes were perfused with GSNO (ISO washout) for 2 minutes and then paced at 0.5 Hz for 30 s before Ca^{2+} spark measurements. In WT myocytes applying ISO first induces the usual increase in Ca^{2+} spark frequency, and if we then wash out ISO while applying GSNO, the elevated Ca^{2+} spark frequency is maintained. In contrast, in the absence of C290 (C290A knock-in myocytes) the ISO-induced increase in Ca^{2+} spark frequency is not maintained by GSNO addition as ISO is washed out. This shows that while GSNO induced a persistent SR Ca^{2+} leak, consistent with autonomous activation state in the WT, this autonomous activation state was suppressed in the C290A nitrosylation mutant. Collectively, our data demonstrate that the CaMKIIδ C290A knock-in displayed normal physiological myocyte

function, similar to their WT littermates. However, the GSNO-induced increase in arrhythmogenic SR Ca^{2+} leak seen in WT myocytes is suppressed in C290A myocytes.

Afterload-induced diastolic SR calcium leak requires CaMKIIδ S-nitrosylation

To extend our above controlled induction of *S*-nitrosylation by GSNO in myocytes, we next tested whether a myocyte-intrinsic *S*-nitrosylation effect induced by increased mechanical afterload would also be altered in the C290A myocytes. We used a cell-in-gel system, in which myocytes encounter a mechanical afterload as they contract against a viscoelastic hydrogel. Others in our group previously showed that NOS1 and CaMKII were both involved in mediating afterload-induced increase in intracellular CaTs and Ca^{2+} sparks in intact ventricular cardiomyocytes from WT mice[7]. Figure 3.5A shows that WT cardiomyocytes contracting in the gel (vs. load-free) displayed enhanced systolic CaT amplitudes (Figure 3.5D), faster $[Ca^{2+}]_i$ decline (Figure 3.5E), faster time to peak $[Ca^{2+}]_i$ (Figure 3.5F), reduced fractional shortening (Figure 3.5G) and a robust increase in diastolic Ca^{2+} sparks (Figure 3.6). The increase in CaTs and Ca^{2+} sparks cardiomyocyte under afterload in-gel are consistent with an increase in RyR sensitivity, while the faster CaT decay would be consistent with enhanced SR Ca^{2+} uptake. As a control, we tested whether individual gel forming components might alter cardiomyocyte function. We preincubated cardiomyocytes with PVA or cross-linker alone, followed by perfusion with normal Tyrode solution and contraction measurements. There was no difference in contraction measured as sarcomere length (SL) shortening for both either PVA or cross-linker treatment – without mixing to form hydrogel (Figure 3.5H). Previous in-gel studies used the non-specific CaMKII inhibitor KN-93 to test the role of CaMKII in the cell-in-gel MCT effect on Ca^{2+} handling, and used NOS1 knockout to confirm the role of that NOS isoform as the NO source in the MCT pathway. Here, we used cardiac specific CaMKIIδ knockout mice (cKO) to more explicitly test whether CaMKIIδ, in particular,

is required for mediating the afterload-induced effects in Ca^{2+} handling. Figure 3.4B, D-E shows that compared to WT myocytes in the same conditions, the afterload-induced increase in CaT amplitude and rate of $[Ca]_i$ decline were completely prevented in the CaMKIIδ-cKO myocytes. Moreover, in the absence of CaMKIIδ, CaT amplitude became smaller and slower under afterload (Figure 3.5D-E). Thus, the CaMKIIδ isoform is required for this intrinsic MCT-mediated increase in Ca^{2+} transients.

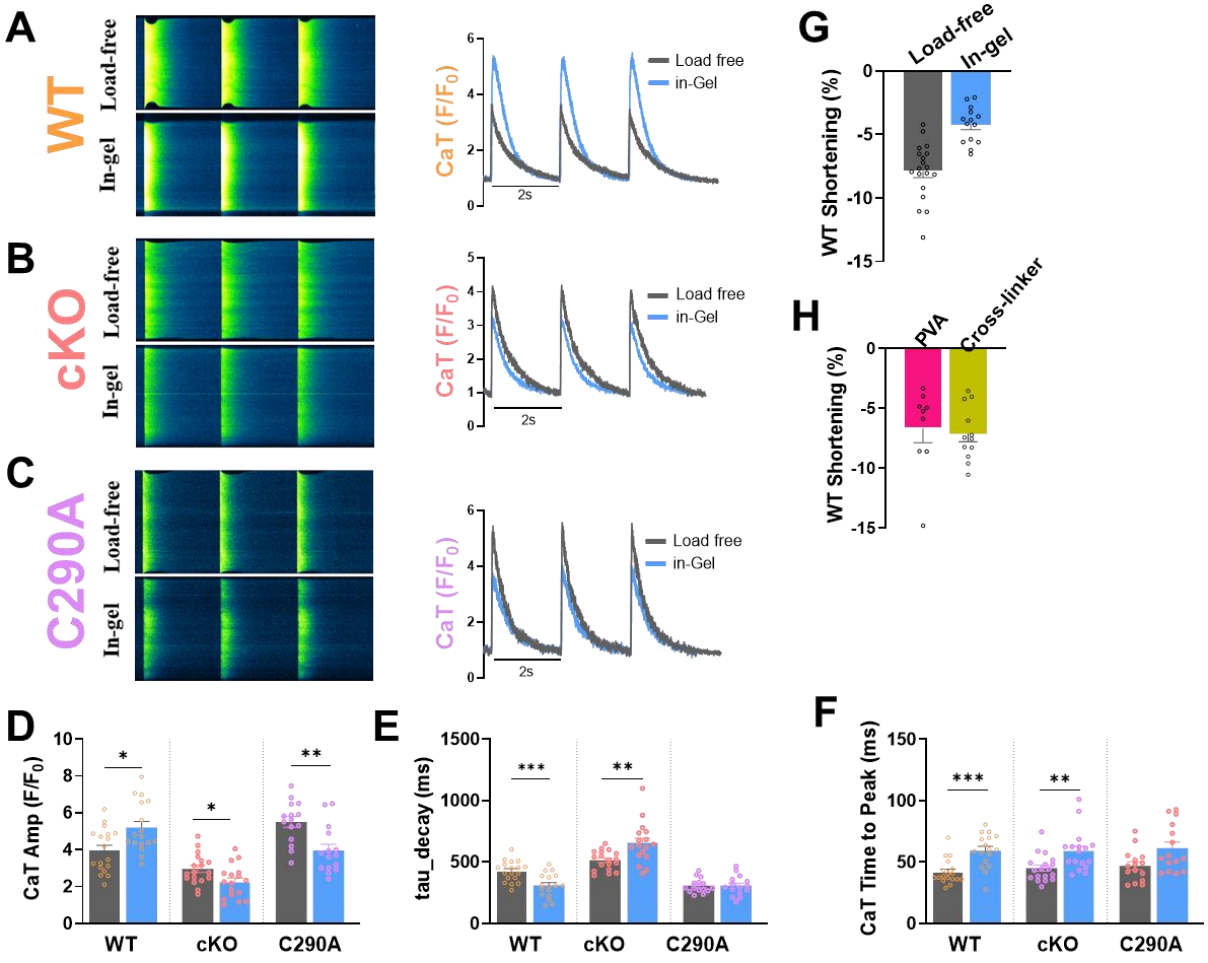


Figure 3.5: Representative pseudo-colored line scan images of systolic Ca^{2+} transients (left) obtained using Fluo-4 confocal imaging AND resultant Ca^{2+} transient (CaT) peak (right) in load-free control and cell-in-gel for A) WT B) cKO C) C290A mice. D) CaT Amp E) tau decay and F) Time to peak for WT, C290A, KO. G) Fractional shortening in WT and H) Fractional shortening with PVA and Crosslinker.

Furthermore, the new CaMKIIδ C290A knock-in myocytes provides a more explicit test of whether this single cysteine (C290) is critically important for the MCT effects. Figure 3.5C-F shows that contrary to their WT littermates, C290A myocytes contracting in the gel (vs. load-free contractions), displayed a decrease in CaT amplitude (Fig 3.5D) and no change in the τ of twitch $[Ca]_i$ decline (Fig 3.5E). Thus, this specific *S*-nitrosylation target on CaMKIIδ-C290 is essential to the afterload-induced increase in CaTs and accelerated SR Ca^{2+} uptake.

Figure 3.6 shows that CaMKIIδ-cKO myocytes (vs. WT) exhibited significantly lower baseline Ca^{2+} spark frequency, which also failed to increase upon afterloaded contractions (contrasting to WT). In the C290A knock-in myocytes the baseline Ca^{2+} spark frequency was higher than in the

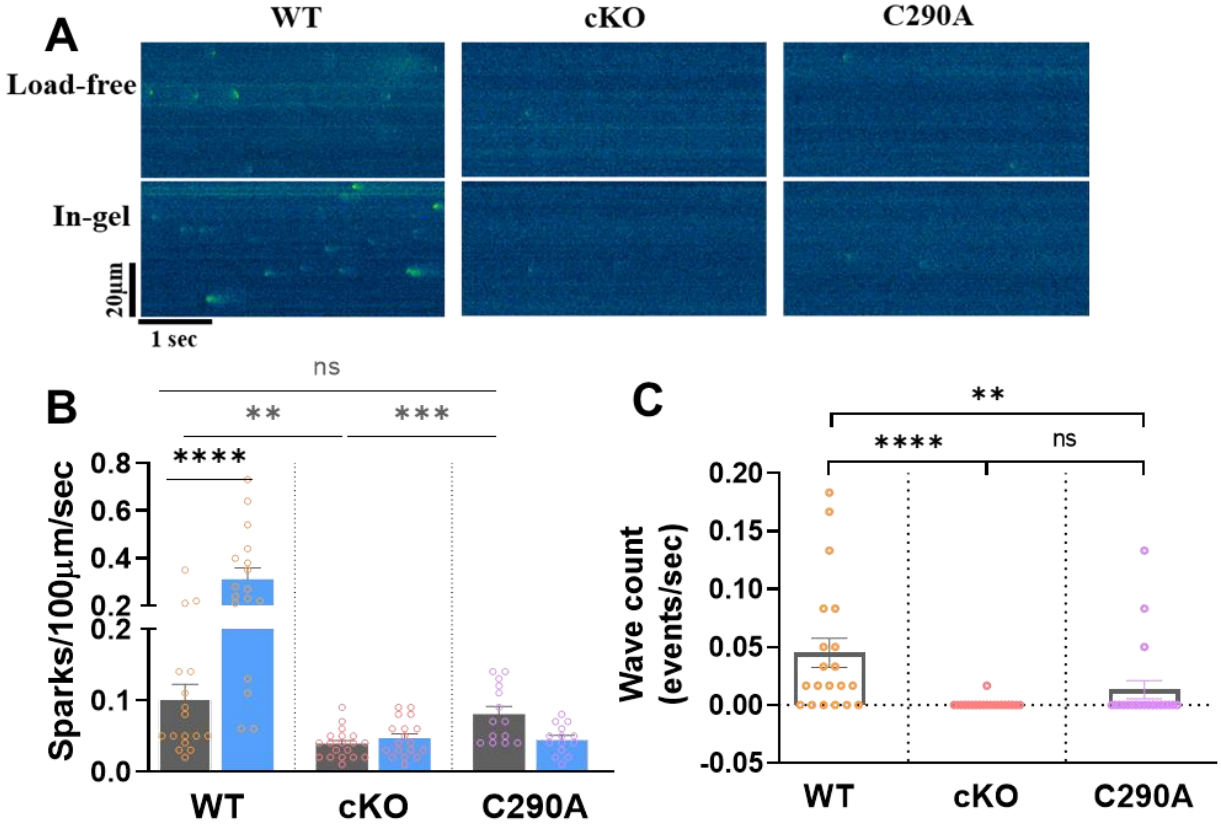


Figure 3.6: A) Representative spontaneous Ca^{2+} sparks seen in the pseudo-colored confocal linescan images of cardiomyocytes with and without load in wild-type (WT, left), cardiac specific CaMKII KO (cKO, middle) and CaMKII nitrosylation resistant (C290A, right) mice. B) Mean ± SEM values of Ca^{2+} spark rate in WT (n = 17), cKO (n = 18) and C290A (n = 14) cardiomyocytes under load-free conditions (grey) and in-gel (blue).

complete absence of CaMKIIδ (in cKO), but not significantly different than in WT myocytes. However, as in the cKO, there was no increase in SR Ca^{2+} sparks in response to afterloaded contractions. In fact, there was a slight decrease in Ca^{2+} spark frequency in C290A myocytes, which could conceivably be due to *S*-nitrosylation of the CaMKIIδ inhibitory *S*-nitrosylation site (C273). In the above Ca^{2+} spark frequency analysis, sweeps were excluded from spark analysis if propagating Ca^{2+} waves occurred, because such waves would tend to reduce SR Ca content and hence the probability of Ca^{2+} sparks. The WT myocytes under afterload conditions exhibited many more arrhythmogenic Ca^{2+} waves than were seen in either CaMKIIδ-cKO or C290A knock-in myocytes (Fig 3.6C).

Taken together, these MCT studies point to CaMKIIδ C290 as the critical mediator of the intrinsic afterload-induced increase in cardiac Ca^{2+} transient enhancement that allows the heart to contract more strongly in response to greater circulatory load. Indeed, C290 was required for not only the afterload-induced increase in Ca^{2+} transient amplitude and faster SR Ca^{2+} uptake, but also for the afterload-induced increase in arrhythmogenic Ca^{2+} waves. Ergo, S-nitrosylation of CaMKIIδ C290 is necessary for the mediation of MCT, but can also be pro-arrhythmic.

Discussion

The influence of mechanical stress on contraction and Ca^{2+} cycling is a new area of study that is gaining more attention in the cardiovascular field. In a model of familial hypertrophic cardiomyopathy, selective inhibition of NOS1 and CaMKII suppressed afterload-induced spontaneous Ca^{2+} activities[7], highlighting the involvement of both nitrosylation and CaMKII in MCT. We know from previous studies that CaMKII can be nitrosylated, so the big question was

whether CaMKII gets nitrosylated in the MCT pathway and how does it contribute to contraction. To investigate this, we used CaMKII C290A nitrosylation-resistant mice to test the role of CaMKII nitrosylation in MCT, with the cell-in-gel system to exert mechanical load. The cell-in-gel system mimics in-vivo mechanical conditions in two ways, first it imposes multiaxial 3D mechanical stress during contraction, and next it tethers the cell surface to the gel and imposes both normal and shear stress to the cell surface. With this system, we have established that afterload-induced increase in CaT amplitude, enhanced contractility, and increase in afterload induced spontaneous spark frequency are mediated by the nitrosylation of CaMKIIδ at Cys290. We found that in CaMKII C290A mice, inhibiting this nitrosylation site decreased contraction and Ca^{2+} kinetics with mechanical load. SR Ca^{2+} release, quantified by CaT amplitude, was decreased and the rate of SR Ca^{2+} reuptake was also decreased. On this basis, we establish that CaMKII nitrosylation at C290 is a signaling pathway for MCT in cardiomyocytes.

The cell shortening in the C290A mice was reduced with and without mechanical load (compared to WT). A relevant follow-up experiment would be to determine if cardiac output decreases more in C290A mice than in WT when under pressure-overload. NOS1 was identified as a mediator of mechano-chemo-transduction given its role in that transducing mechanical afterload to Ca^{2+} handling[7]. Mechanical preload, has also been shown to promote increases in Ca spark frequency, involving NADPH oxidase (NOX) that produces reactive oxygen species (ROS)[9], so it is possible that there are somewhat separate, but overlapping signaling pathways in mediating intrinsic afterload- and preload-induced changes in myocytes Ca^{2+} handling. NOX2 can be activated by NO signaling[26], suggesting a complex interplay between these two pathways. It might be worthwhile to investigate the specific role of CaMKII oxidation in mechano-chemo-transduction.

CaMKIIδ has two known sites for nitrosylation, C290 and C273. While *S*-Nitrosylation of C290 on CaMKIIδ promotes autonomous kinase activity, *S*-Nitrosylation of C273 suppresses activation by Ca-CaM[19]. Given the role of Nitrosylation in MCT, the differential effect of nitrosylation at these two sites suggests an added layer of complexity, as activating the inhibitory C273 site could complicate the physiological MCT process.

Mechano-chemo-transduction through S-nitrosylation of CaMKIIδ suggests a novel point of therapeutic intervention for treating mechanical stress-induced Ca^{2+} dysregulation, arrhythmias, and cardiomyopathy. As demonstrated with the C290A mice, mitigating or blocking the CaMKII C290 site could be a therapeutic strategy.

References

1 Waldman, L. K., Fung, Y. C. & Covell, J. W. Transmural myocardial deformation in the canine left ventricle. Normal in vivo three-dimensional finite strains. *Circ Res* **57**, 152-163, doi:10.1161/01.res.57.1.152 (1985).

2 Ruwhof, C. & van der Laarse, A. Mechanical stress-induced cardiac hypertrophy: mechanisms and signal transduction pathways. *Cardiovasc Res* **47**, 23-37, doi:10.1016/s0008-6363(00)00076-6 (2000).

3 Wernig, F., Mayr, M. & Xu, Q. Mechanical stretch-induced apoptosis in smooth muscle cells is mediated by beta1-integrin signaling pathways. *Hypertension* **41**, 903-911, doi:10.1161/01.HYP.0000062882.42265.88 (2003).

4 Abrahams, C., Janicki, J. S. & Weber, K. T. Myocardial hypertrophy in Macaca fascicularis. Structural remodeling of the collagen matrix. *Lab Invest* **56**, 676-683 (1987).

5 Weber, K. T. *et al.* Collagen remodeling of the pressure-overloaded, hypertrophied nonhuman primate myocardium. *Circ Res* **62**, 757-765, doi:10.1161/01.res.62.4.757 (1988).

6 Conrad, C. H. *et al.* Myocardial fibrosis and stiffness with hypertrophy and heart failure in the spontaneously hypertensive rat. *Circulation* **91**, 161-170, doi:10.1161/01.cir.91.1.161 (1995).

7 Jian, Z. *et al.* Mechanochemotransduction during cardiomyocyte contraction is mediated by localized nitric oxide signaling. *Sci Signal* **7**, ra27, doi:10.1126/scisignal.2005046 (2014).

8 Petroff, M. G. *et al.* Endogenous nitric oxide mechanisms mediate the stretch dependence of Ca2+ release in cardiomyocytes. *Nat Cell Biol* **3**, 867-873, doi:10.1038/ncb1001-867 (2001).

9 Prosser, B. L., Ward, C. W. & Lederer, W. J. X-ROS signaling: rapid mechano-chemo transduction in heart. *Science* **333**, 1440-1445, doi:10.1126/science.1202768 (2011).

10 Toischer, K. *et al.* Elevated afterload, neuroendocrine stimulation, and human heart failure increase BNP levels and inhibit preload-dependent SERCA upregulation. *Circ Heart Fail* **1**, 265-271, doi:10.1161/CIRCHEARTFAILURE.108.785279 (2008).

11 Toischer, K. *et al.* Differential cardiac remodeling in preload versus afterload. *Circulation* **122**, 993-1003, doi:10.1161/CIRCULATIONAHA.110.943431 (2010).

12 Shin, S. H. *et al.* Mechanical dyssynchrony after myocardial infarction in patients with left ventricular dysfunction, heart failure, or both. *Circulation* **121**, 1096-1103, doi:10.1161/CIRCULATIONAHA.109.863795 (2010).

13 Anderson, M. E., Brown, J. H. & Bers, D. M. CaMKII in myocardial hypertrophy and heart failure. *J Mol Cell Cardiol* **51**, 468-473, doi:10.1016/j.yjmcc.2011.01.012 (2011).

14 Hegyi, B., Bers, D. M. & Bossuyt, J. CaMKII signaling in heart diseases: Emerging role in diabetic cardiomyopathy. *J Mol Cell Cardiol* **127**, 246-259, doi:10.1016/j.yjmcc.2019.01.001 (2019).

15 Bers, D. M. Cardiac excitation-contraction coupling. *Nature* **415**, 198-205, doi:10.1038/415198a (2002).

16 Hudmon, A. & Schulman, H. Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J* **364**, 593-611, doi:10.1042/BJ20020228 (2002).

17 Erickson, J. R. *et al.* A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell* **133**, 462-474, doi:10.1016/j.cell.2008.02.048 (2008).

18 Erickson, J. R. *et al.* Diabetic hyperglycaemia activates CaMKII and arrhythmias by O-linked glycosylation. *Nature* **502**, 372-376, doi:10.1038/nature12537 (2013).

19 Erickson, J. R. *et al.* S-Nitrosylation Induces Both Autonomous Activation and Inhibition of Calcium/Calmodulin-dependent Protein Kinase II δ. *J Biol Chem* **290**, 25646-25656, doi:10.1074/jbc.M115.650234 (2015).

20 Coultrap, S. J. & Bayer, K. U. Nitric oxide induces Ca2+-independent activity of the Ca2+/calmodulin-dependent protein kinase II (CaMKII). *J Biol Chem* **289**, 19458-19465, doi:10.1074/jbc.M114.558254 (2014).

21 Curran, J. *et al.* Nitric oxide-dependent activation of CaMKII increases diastolic sarcoplasmic reticulum calcium release in cardiac myocytes in response to adrenergic stimulation. *PLoS One* **9**, e87495, doi:10.1371/journal.pone.0087495 (2014).

22 Onofiok, E., Lam, K. S. & Luo, J. Three-Dimensional Cell Adhesion Matrix. Patent WO/2010/148346 (2010).

23 Guo, T., Ai, X., Shannon, T. R., Pogwizd, S. M. & Bers, D. M. Intra-sarcoplasmic reticulum free [Ca2+] and buffering in arrhythmogenic failing rabbit heart. *Circ Res* **101**, 802-810, doi:10.1161/CIRCRESAHA.107.152140 (2007).

24 Picht, E., Zima, A. V., Blatter, L. A. & Bers, D. M. SparkMaster: automated calcium spark analysis with ImageJ. *Am J Physiol Cell Physiol* **293**, C1073-1081, doi:10.1152/ajpcell.00586.2006 (2007).

25 Howe, C. J., Lahair, M. M., McCubrey, J. A. & Franklin, R. A. Redox regulation of the calcium/calmodulin-dependent protein kinases. *J Biol Chem* **279**, 44573-44581, doi:10.1074/jbc.M404175200 (2004).

26 Girouard, H. *et al.* NMDA receptor activation increases free radical production through nitric oxide and NOX2. *J Neurosci* **29**, 2545-2552, doi:10.1523/JNEUROSCI.0133-09.2009 (2009).

Chapter 4:

S-nitrosylation of CaMKII Cys273 suppresses CaMKII Activation and Translocation within Cardiac Myocytes

4.1 Background

Nitrosylation is widely implicated in cardiac function and cell signaling in health and disease. Nitric Oxide Synthases (NOSs) regulate cardiac myocyte basal contractility and β-adrenergic responsiveness[1], mainly through the modulation of Ca^{2+} homeostasis or myofilament Ca^{2+} sensitivity. NOS protects the myocardium during ischemia–reperfusion (IR) via several mechanisms including inhibition of LTCC-induced Ca^{2+} currents to decrease Ca^{2+} overload[2,3], *S*-nitrosylation of RyR2 and SERCA2a[4,5]. The loss of endothelial or neuronal NOS function exacerbates myocardial damage due to ischemia–reperfusion or infarction[3,6-8], while overexpression reduces infarct size and improves LV function[2,9-11]. Notably, other studies have shown that inhibition of NOS during ischemia–reperfusion was associated with reduced myocardial injury[12-14]. NOSs activity can be regulated by kinases including Calcium-Calmodulin Kinase II (CaMKII). NOS1 activity is reduced by CaMKII dependent phosphorylation of Ser 847 and NOS3 activation is increased by phosphorylation at Ser1177[15,16]. Conversely, CaMKII is also regulated by nitrosylation in a potential positive feedback mechanism[17-19].

CaMKIIδ has been well-established as a nodal regulator of cardiac myocyte ion channel gating, calcium (Ca) regulation, contraction, transcriptional control, and is known to be chronically active in multiple pathological states[20,21]. Pre-clinical studies have shown that acute or genetic inhibition

of CaMKIIδ limits arrhythmias and progression of heart failure (HF), thus establishing the kinase as a therapeutic target[22-24]. These factors make it essential to understand the detailed mechanisms by which CaMKIIδ is regulated in adult cardiac myocytes. CaMKIIδ, as a holoenzyme, consists mainly of 12 subunits, where each subunit has 3 domains: an N-terminal catalytic domain, an autoinhibitory regulatory domain, and a C-terminal association domain[25]. The association domains direct multimeric assembly, binding together to form a central hub in the holoenzyme that is connected by a linker to the regulatory domain. The regulatory domain binds either the catalytic domain (in its inactive state), or calmodulin (CaM, in its active state). With increasing intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), the regulatory domain binds Ca-CaM with a K_D of 10-50 nM[26]. Once Ca-CaM is bound, CaMKIIδ adopts an open conformational state which allows it to phosphorylate molecular targets including Thr287 on neighboring CaMKII subunits[26]. This auto-phosphorylation raises Ca-CaM affinity and slows deactivation or re-association with the catalytic domain – also allowing the kinase to remain partially active independently of Ca-CaM[27,28]. Other posttranslational modifications (PTMs) of CaMKIIδ, including oxidation (Met281/Met282), GlcNAcylation (Ser280), and *S*-Nitrosylation (Cys290) in this same small region have been identified and can likewise trigger this autonomous activity[19,29,30].

Our group has previously shown that nitric oxide (NO) has dual effects on the activity of CaMKII: while *S*-Nitrosylation of Cys290 on CaMKIIδ promotes autonomous kinase activity, *S*-Nitrosylation of Cys273 suppresses activation by Ca-CaM[19]. The differential effect of *S*-nitrosylation is based on the timing of NO exposure relative to Ca-CaM binding. That is, at low $[Ca^{2+}]$ *S*-nitrosylation at Cys273 prevents Ca-CaM-dependent activation, but when CaMKIIδ is already in the open or active state, *S*-nitrosylation of Cys290 promotes autonomous CaMKIIδ activity (it may that Cys290 is more accessible in the active/open conformation and that Cys273

S-nitrosylation can either occur or reverse activation). This suggests a complex interaction between NO and CaMKII in cells with fluctuating [Ca^{2+}] – like the cardiomyocyte. The molecular mechanism by which *S*-nitrosylation occurs and alters myocyte Ca^{2+} cycling is suggested to be via NOS1 (nNOS, not NOS3 or eNOS) that is localized at the SR membrane where it activates local CaMKII to phosphorylate phospholamban (PLN) and ryanodine receptor (RyR), which can enhance the rate of SR Ca uptake by SERCA and sensitize SR Ca release[31,32]. The (patho)physiological role of CaMKII nitrosylation in the cardiomyocyte, however, remains poorly understood. It is also unknown to what extent inhibition at Cys273 can suppress SERCA or RyR activation. β-adrenergic stimulation can also activate CaMKII via mechanisms distinct from Ca/CaM activation[33-35]. Here, using mutant CaMKIIδ variants of our FRET-based CaMKII activity sensor 'Camui'[36] and GFP-tagged constructs of CaMKIIδ lacking the Cys273 *S*-nitrosylation site (CaMKIIδ-C273S), we assessed CaMKII activation and mobility after exposure to the NO donor *S*-nitrosoglutathione (GSNO). We show for the first time in ventricular cardiomyocytes, that nitrosylation of CaMKII at Cys273 prior to electrical field stimulation and activation by Ca^{2+} or β-adrenergic agonists suppress CaMKIIδ activity and mobility, whereas mutation of the Cys273 to Serine suppresses this effect.

4.2 Experimental Methods

Cardiomyocyte isolation and culture (adenoviral transfection)

Rabbit cardiomyocytes were isolated from young, 3–4-month-old male New Zealand White rabbits as previously described[37]. All animal handling and laboratory procedures were in accordance with the approved protocols (No. 19721 and No. 21064) of the Institutional Animal Care and Use Committee at University of California, Davis conforming to the Guide for the Care

and Use of Laboratory Animals published by the US National Institutes of Health (eighth edition, 2011). Freshly isolated rabbit myocytes were plated on sterile, laminin-coated glass coverslips (ibidi, polymer #1.5) in PC-1 (Lonza) media. Non-attached myocytes were washed away after an hour, and attached myocytes were then infected with recombinant adenoviruses - containing CaMKIIδc (WT, C273S or C290A) with green fluorescent protein (GFP) attached on the C-terminal or with Camui (WT or C273S mutant) and CaM as previously described[19]. The Camui sensor has red fluorescent protein (RFP) and GFP attached at the N-terminal and C-terminal of CaMKIIδ_C, respectively. Myocytes were subsequently cultured for 18 hours to allow sufficient expression for imaging and to limit potential overexpression artifacts. See supplement for CaMKII construct generation.

Fluorescence recovery after photobleaching (FRAP)

Cultured myocytes were used for FRAP experiments within 18-22 hours of adenovirus transfection. During imaging, they were maintained in Normal Tyrode's solution (NT; 135 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4, 23 °C) until protocols were begun. Some myocytes were pre-loaded with BAPTA, by a 30 min incubation with 50 μM BAPTA-AM, followed by a 30 min washout and de-esterification period prior to use in experiments. All FRAP measurements were performed on Olympus FLUOVIEW FV1000 confocal laser scanning microscope (inverted configuration) Magnification: 60x, NA: 1.49 (oil immersion), W.D.: 0.13 mm. Experiments were carried out at room temperature (20-25^{O}C). A frame size of 512 × 512 pixels at a zoom of 3x was scanned for a total of 50 frames. Using the tornado under stimulus setting in the FV-1000 software, a bleaching region (BR) with a diameter of 9 μm was selected in each myocyte. Software was set to activate in series: acquiring three images prior to bleaching and several post-bleach images, with image acquisition occurring every

2.71s. The BR fluorescence signal was bleached down to 30-40% of the initial value using 50% 488 nm and 22% 405 nm laser for 1-1.5s. The fluorescence intensity values from the pre-bleach images were averaged to normalize the post-bleach fluorescence recovery curve.

Fluorescence Lifetime Imaging Microscopy (FLIM)

FLIM experiments were performed on a Leica Falcon confocal (SP8; 40x objective). A white light femtosecond frequency-pulsed laser excited the donor at 488 nm. Donor emissions from 490-550 nm were collected as were arrival times for time-correlated single photon counting (TCSPC). Leica Application Suite X software was used to analyze FLIM-FRET data. Curves were fit to TCSPC decay histograms to estimate donor lifetimes, and autofluorescence was accounted for. All cells had a minimum mean of 1,000 photons recorded per pixel to allow accurate curve fitting.

4.3 Results

Lifetime Imaging of CaMKII in different activation states

Figure 4.1A shows the regulatory region of CaMKIIδ with post-translational modification sites; the sites for nitrosylation include Cys273 and Cys290. We made a Cys273 to Ala mutation in the full length CaMKII that was also incorporated into the CaMKII Camui reporter for experiments below.

Camui, an activation-dependent FRET-based sensor assesses changes in CaMKII conformation from resting (auto-inhibited) to activated state of the kinase (Figure 4.1). Flanked on either terminus with a GFP (donor) and RFP (acceptor) fluorophore, fluorescence resonance energy transfer (FRET) levels between these tags are high in the closed, inactive CaMKII conformation (Figure 4.1A). In this state, energy from the excited GFP is transferred to the acceptor (RFP), decreasing the donor lifetime. When activated, the conformational change results in an increase in

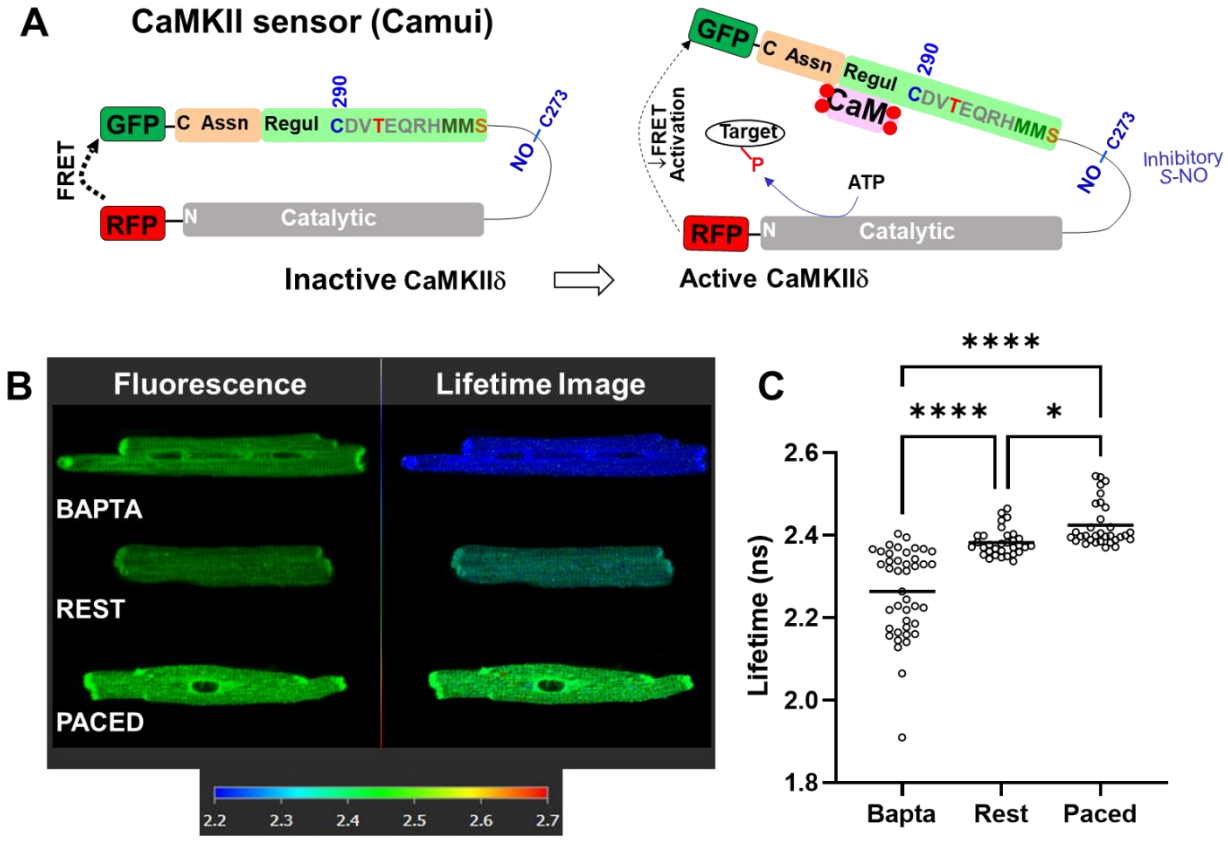


Figure 4.1: Visualizing CaMKII activation in cardiomyocytes. A, The FRET sensor Camui consists of the full-length CaMKII sandwiched between a fluorophore FRET pair. Activation results in a conformational shift that increases the distance between the N-terminal catalytic domain and the C-terminal regulatory/association domains, reducing FRET between the GFP/RFP pair and therefore increasing the GFP lifetime. **B**, Fluorescence intensity (left) and fluorescence lifetime (right) of cardiomyocytes in different activation states. The scalebar denotes lifetime in nanoseconds. **C**, Camui GFP lifetimes in cardiomyocytes at rest, electric field stimulated or pre-treated with BAPTA-AM.

distance between the GFP-RFP tags, which decreases FRET levels. This decrease in energy transfer results in longer lifetimes for GFP. Using the FLIM-FRET approach (described in Chapter 2, section 2.3), we assess the activation state of CaMKII dynamically in cells expressing Camui. Figure 4.1B shows both CaMKIIδc-GFP fluorescence intensity and lifetime values in the same rabbit ventricular myocyte for quiescent cells, paced cells, and myocytes pre-loaded with the calcium chelator, BAPTA (50 μM BAPTA-AM for 30 min followed by a 30 min de-esterification period). The color-coded lifetime values are relatively uniform spatially across the myocyte for the different groups.

With calcium chelated in the BAPTA group, the probability of Ca-CaM binding to and activating CaMKII is reduced, thus this group has shorter average lifetimes (2.26 ns) as seen in Figure 4.1C. In comparison, stimulated cells in the paced group have activated CaMKIIδ and exhibit longer average GFP lifetime values (2.43 ns), indicating a decrease in FRET/change in conformation of CaMKII to the open conformational state. Lifetime changes are in nanosecond range.

Resting NO exposure suppresses CaMKII activation in the presence of Cys273

Resting Control WT myocytes were incubated in NT containing 2 mM [Ca^{2+}] for 5 min and then paced at 0.5 Hz for 5 min and Camui lifetime was measured just before and at the end of the 5 min pacing period (Figure 4.2A). Paced Control myocytes uniformly exhibited an increase in Camui lifetime (in paired measurements), indicating that CaMKIIδ increased its more open active conformation, as expected[36]. This was also true for myocytes expressing Camui-C273S (Figure 4.2B; leftmost pairs of bars).

Separate myocytes were also exposed to isoproterenol (ISO) for 2 min prior to pacing for 5 min and Camui lifetime was measured again just before and after the 5 min stimulation period (Figure 4.2A). Once again, pacing increased Camui lifetime in ISO treated myocytes, expressing either WT or C273S CaMKIIδ containing Camui. Thus, pacing increased CaMKIIδ activation state in both the WT and C273S CaMKIIδ. Notably, ISO did not significantly increase Camui lifetime in the resting myocytes, which is not entirely surprising. However, we were surprised that pacing in ISO-treated myocytes did not produce a much greater increase in lifetime than in Control.

To induce C273 nitrosylation on inactive CaMKII in live myocytes expressing Camui, we first pretreated cells with BAPTA-AM to limit $[Ca^{2+}]_i$ and deactivate the kinase. This deactivating condition should optimize *S*-nitrosylation of CaMKIIδ C273 upon treatment with the NO donor, GSNO (150 μM) for 10 min[19].

After GSNO exposure myocytes were placed in NT with the usual 2 mM [Ca^{2+}] and subjected to the same protocols as above (Figure 4.2, pre-GSNO panels). In WT myocytes, GSNO pretreatment did not alter resting Camui lifetime, but prevented the increase in WT Camui lifetime observed upon pacing (Figure 4.2B, top left). In contrast, GSNO pretreatment in the C273S Camui (that cannot be *S*-nitrosylated at that site), failed to prevent the pacing-induced increase in CaMKII activation, and even the resting level may have been slightly increased, based on lifetimes (Figure 4.2B, bottom left). Thus, the presence of the C273 *S*-nitrosylation site appears to limit CaMKII activation in rabbit ventricular myocytes.

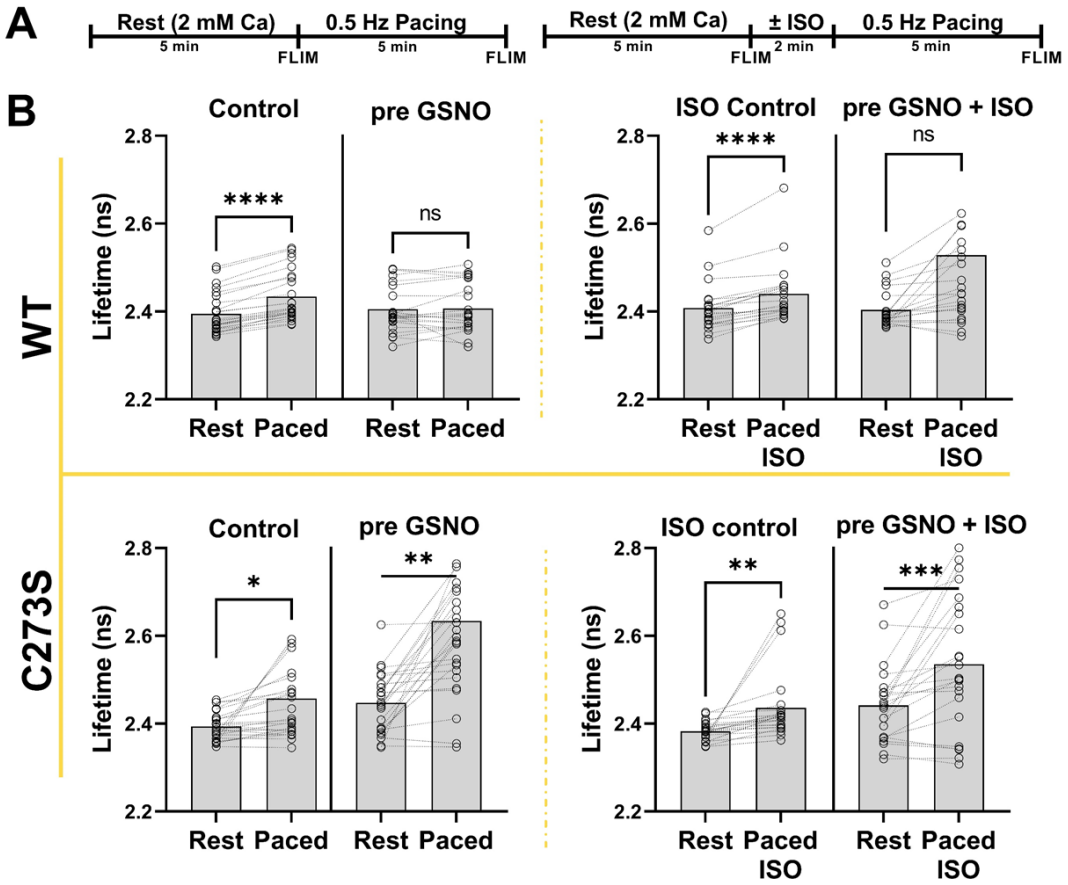


Figure 4.2: Pre-treatment with GSNO suppresses CaMKII activation in the presence of Cys273. **A**, Experimental protocol. **B**, In wildtype Camui, the nitric oxide (NO) donor, GSNO, suppressed activation of CaMKII (top), while in the Camui variant lacking the Cys273 site (bottom), NO exposure did not limit CaMKII activation.

In ISO-treated myocytes, GSNO pretreatment again prevented a significant increase in WT Camui lifetime, although some individual myocytes exhibited an increase (Figure 4.2B, top right). This would be consistent with GSNO blunting the pacing-induced CaMKIIδ activation (even in ISO), as may be expected for *S*-nitrosylation at CaMKIIδ C273. Moreover, when C273 is unavailable in Camui-C273S, that limiting effect of GSNO on CaMKIIδ activation was not observed (Figure 4.2B, bottom right). Taken together, this data supports the idea that *S*-nitrosylation of CaMKIIδ at C273 limits the activation of the kinase in intact beating adult ventricular myocytes. Moreover, the highest Camui lifetimes by far occurred in Camui-C273A vs. WT (i.e. many more cells showed Camui lifetimes greater than 2.6 ns; bottom vs. top panels in Figure 4.2B).

GSNO slows CaMKII-GFP Fluorescent recovery after photobleaching (FRAP)

Our group recently showed that pacing-induced activation of CaMKIIδ in cardiomyocytes enhances its mobility and rapid translocation to extra-dyadic targets, providing a mechanism for broad myocyte target phosphorylation[38]. Indeed, dynamic spatiotemporal regulation of CaMKIIδ is integral to achieving the widespread functional impact of CaMKIIδ signaling in the heart. Here, we assessed the effect of GSNO-exposure to CaMKII mobility using the same experimental protocols as in Figure 4.2, but using GFP-tagged CaMKIIδ protein and fluorescent recovery after photobleaching (FRAP).

Using FRAP in living myocytes, I studied the real-time dynamics of CaMKIIδ mobility in WT and mutant CaMKIIδ-GFP that lack either the inhibitory or activating *S*-nitrosylation sites (C273S and C290A, respectively). Figure 4.3A shows an adult rabbit ventricular myocyte expressing CaMKIIδ-GFP paced at 0.5 Hz, in which GFP is photobleached in a 9 μm diameter region of interest (ROI; Bleach panel) and FRAP is recorded over the next two minutes (lower images).

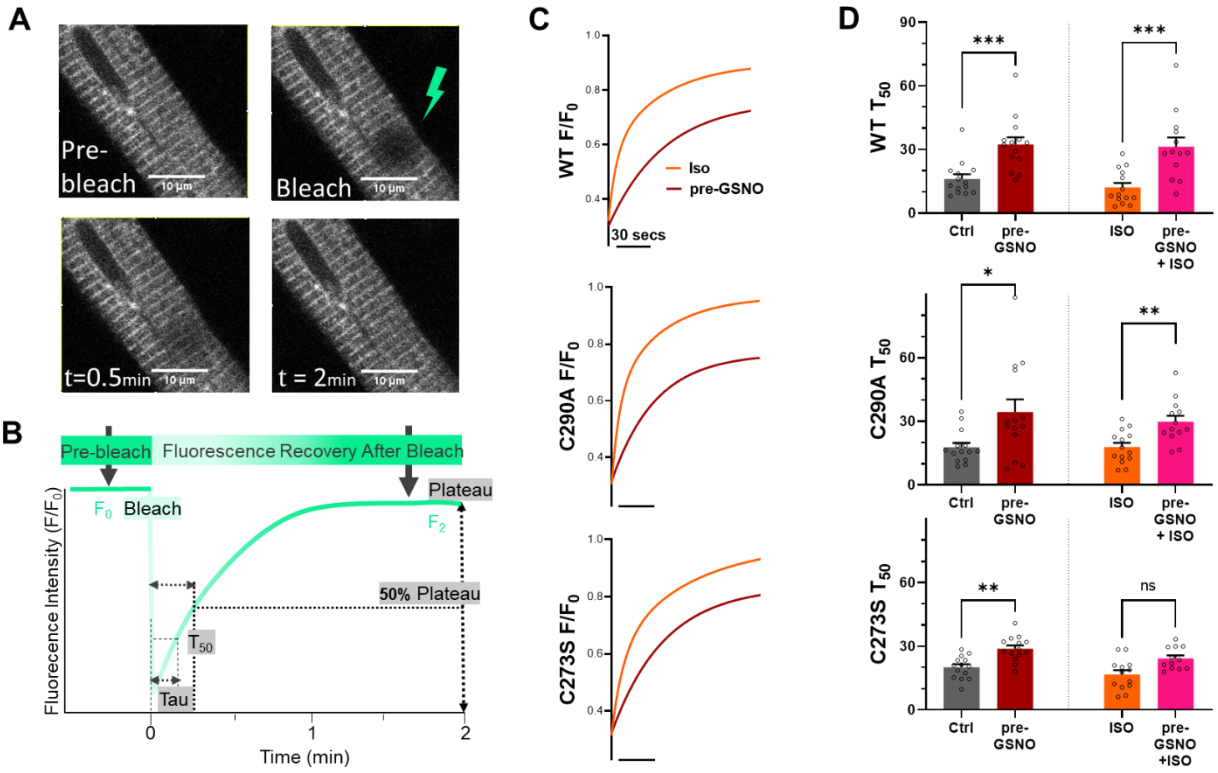


Figure 4.3: Fluorescence recovery after photobleaching. **A**, A small circular region (~9 µM diameter) is photobleached (Bleach) and recovery within this region is shown monitored over time (lower images). **B**, FRAP example and analysis, showing Pre-bleach level (F_0), extent of bleach and recovery over 2 min (to F_2) which in this case is to steady state. Time to 50% (T_{50}) and time constant of recovery (τ) are shown. **C,** Recovery curves for 0.5 Hz stimulated myocytes exposed to GSNO (maroon), and myocytes treated with ISO (orange) in WT, C290A, and C273S CaMKIIδ variants. **D**, Time to 50% recovery for all three CaMKIIδ variants under control (Ctrl) and ISO-treated, with or without GSNO pretreatment.

Figure 4.3B shows an exemplar FRAP trace how these were analyzed. The bleaching illumination was adjusted to rapidly photobleach ~70% of the GFP in the ROI for these experiments. The nearly full eventual fluorescence recovery in the ROI indicates that CaMKIIδ-GFP is largely mobile, even if some CaMKII is bound to fixed targets. If CaMKIIδ were mostly immobile, FRAP would be more incomplete. The FRAP kinetics are increased by both higher CaMKIIδ mobility and faster CaMKIIδ dissociation rate from any relatively fixed binding partners. That is, a bleached CaMKIIδ-GFP would have to dissociate before an unbleached CaMKIIδ-GFP could replace it. We assessed the time to 50% recovery (T_{50}) as a simple FRAP index (Figure 4.3B).

Mean FRAP time courses are shown in Figure 4.3C for the extreme cases (fastest was with ISO and slowest was for pre-GSNO treatment without ISO). Traces (top to bottom) are shown for WT CaMKII and C290A and C273S mutants that prevent *S*-nitrosylation at these sites. Figure 4.3D shows that in the WT group, pre-exposure to GSNO increased the average FRAP T_{50} by 100%, reflecting a decrease in mobility in comparison to untreated paced myocytes. ISO treatment caused faster FRAP (by 25%, P=0.06), but again GSNO pretreatment slowed that significantly (by 159%). In the absence of C273, GSNO pretreatment produced much less slowing of FRAP (by 44% for Ctrl and 45% for ISO) and none of the mean T_{50} values was above 30 s in CaMKII-C273S. In contrast, the CaMKII-C290A mutant behaved largely like the WT CaMKII, showing substantial slowing upon GSNO pretreatment. Thus, pretreatment with GSNO slowed CaMKIIδ mobility, but this effect was largely abrogated in the C273S Camui, implicating CaMKIIδ C273 *S*-nitrosylation as the mediator of pre-GSNO-induced reduction of CaMKIIδ mobility in intact ventricular myocytes.

Single exponential recovery is often used in FRAP studies, but similar to other proteins[39,40], CaMKII FRAP is often better fit by a two-phase exponential recovery. In our case here, FRAP for GSNO-pretreated myocytes in the absence of ISO are typically well-fit by a single exponential recovery, Control and ISO usually required an additional faster component (with τ values of 8 s and 6 s respectively). To explore this, I analyzed all FRAP data using two-phase exponentials. Figure 4.4A shows that in WT CaMKII-GFP, nearly half of the FRAP occurs in this fast phase, but that pretreatment with GSNO dramatically reduces the fraction of FRAP that was in this fast phase. The C290A mutant behaved similarly to WT. Notably, in the C273S mutant, the fast FRAP phase was not significantly reduced by GSNO pretreatment, either in control or ISO groups. These data suggest that the C273 site is critical for GSNO-induced slowing of CaMKIIδ mobility in

myocytes, just as it was for limiting the activation of CaMKIIδ in the FLIM conformational studies.

A final FRAP comparison using the exponential analysis showed that in WT CaMKIIδ the slow FRAP tau was also made slower by GSNO pretreatment, in both Control and ISO treatment groups Figure 4.4B, top). In the C273S mutant, by contrast, GSNO pretreatment did not significantly alter the slow τ value of FRAP. Taken together, our data is consistent with the idea that CaMKIIδ-

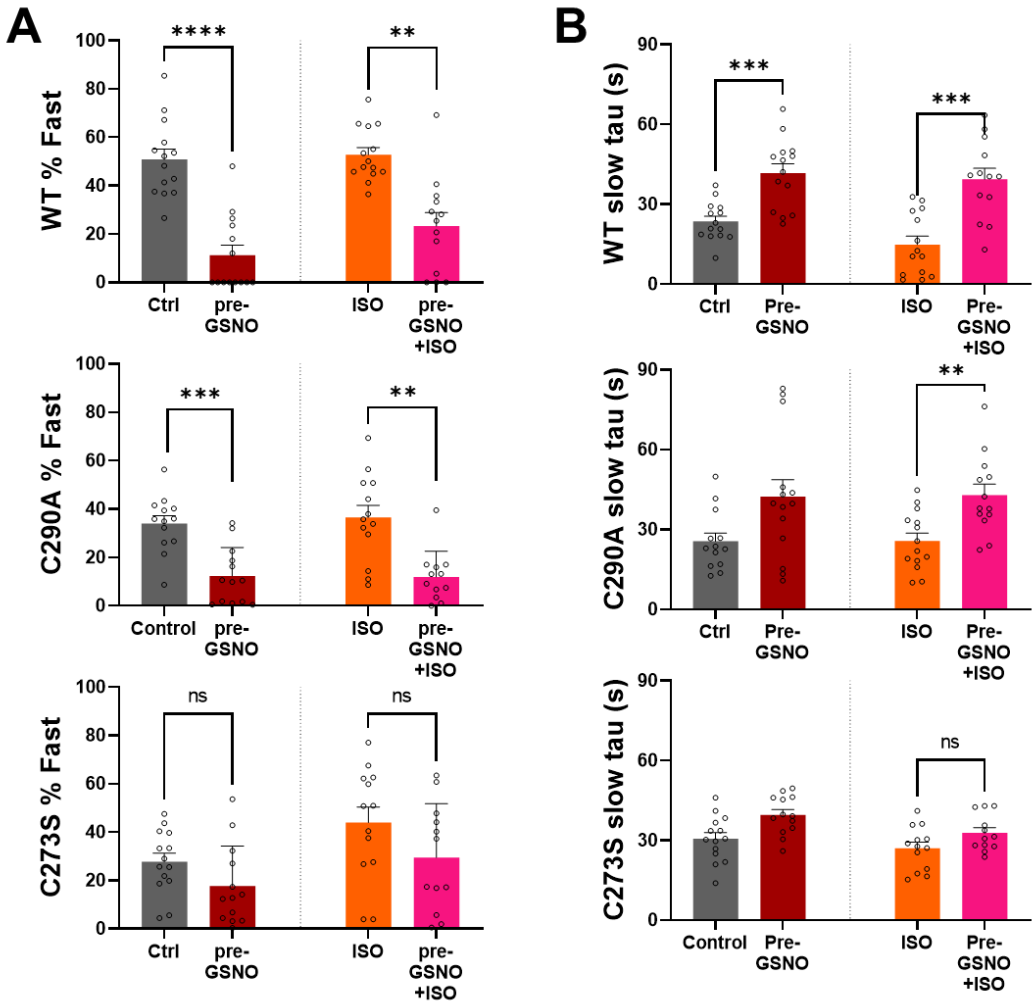


Figure 4.4: GSNO Pre-treatment suppresses CaMKII activation in the presence of Cys273. In WT and Cys290→Ala mutant form, NO exposure limits activation, and therefore mobility. **A**, The percentage of recovery accounted for by the faster of the two CaMKII components. **B**, Time constant or tau values in WT, Cys290→Ala, and Cys273→Ser Camui variants.

C273 is essential in mediating a GSNO-induced suppression of both CaMKIIδ activation state and its mobility in adult cardiac myocytes.

4.4 Discussion

Data here demonstrate that the Cys273 site on CaMKIIδ is critical for NO-induced suppression of CaMKIIδ activation and mobility in adult ventricular myocytes. We determined this by measuring Camui lifetime values in cardiomyocytes in the presence and absence of pre-treatment with GSNO using the fluorescent biosensor Camui WT and C273S, and by assessing changes in CaMKII-GFP mobility. Camui enables real time monitoring of CaMKII activation state in intact cardio-myocytes[36]. After GSNO pretreatment, WT Camui showed no pacing-induced increase in fluorescence lifetime, indicating that nitrosylation prior to Ca-CaM binding suppresses CaMKII activation. In the mutant Camui lacking the Cys273 site, GSNO exposure did not prevent pacing-induced CaMKII activation – serving as confirmation that nitrosylation at the 273 site is necessary to inhibit activation. Experiments testing changes in CaMKII mobility, which has been shown to be increased by activation[38], confirmed the same trend in the CaMKIIδ mutant form lacking the Cys273 site. That is, the mutation of C273S in CaMKIIδ prevented the GSNO-induced suppression of CaMKIIδ mobility assessed by FRAP.

Given that the Cys290 site that promotes CaMKIIδ autonomous activation[19] is situated in the regulatory region between the CaM-binding and autophosphorylation site of CaMKII (Figure 4.1A), it may be less available for nitrosylation in the basal, inactive CaMKII form due to steric hindrance by the association between the regulatory and catalytic domains. But like other post-translational modifications at neighboring regulatory domain sites (S280, M281, M282 and T287) *S*-nitrosylation at Cys290 may require the open active conformation of CaMKIIδ. In contrast, the

Cys273 site, is just outside the autoinhibitory regulatory domain, and appears to be available for *S*-nitrosylation upon pretreatment via GSNO when CaMKIIδ is in a CaM-free inactive conformation[19]. The differential effect of nitrosylation at Cys273 and Cys290 on CaMKIIδ implies a complex interaction for the cardiomyocyte that exhibit regular changes in $[Ca^{2+}]_i$ at each heartbeat.

These findings support the suggestion that CaMKII nitrosylation at Cys273 could protect from the overactivation of CaMKIIδ in heart that has been implicated in numerous pathological states, including progression form hypertrophy to heart failure, cardiac fibrosis, diabetic hyperglycemia and upon reperfusion after ischemia. In addition, females exhibit their higher basal NOS and nitrosylation levels in heart[41], and that may provide some degree of intrinsic protection against pathological CaMKIIδ activation.

It merits mention that the Cys273 *S*-nitrosylation site on CaMKIIδ is highly conserved among CaMKII isoforms CaMKIIβ, -γ and -δ in mammals and in the homologous CaMKIIδ gene in Drosophila and Xenopus, but remarkably that cysteine is absent in the major neuronal isoform CaMKIIα[19]. In CaMKIIα the widely conserved Cys-Gln at that site is replaced by Ser-His, which may confer both a different local conformation and a lack of *S*-nitrosylation potential. Another clue that this Cys273 site is functionally important is that when our lab made the C273A mutation in CaMKIIδ the kinase activity was greatly diminished[19], even at saturating Ca-CaM conditions.

Inhibition of CaMKIIδ has been suggested as a potential therapeutic strategy for preventing or limiting the progression of heart disease [23,42]. For the development of future therapies, it is crucial to understand the basic mechanisms that underlie CaMKIIδ inhibition. We have demonstrated in ventricular myocytes, that *S*-nitrosylation alters the CaMKIIδ activation state and mobility in adult cardiac myocytes. In the intact myocyte setting, we have confirmed that NO exposure before Ca-

CaM binding can suppress both CaMKII activation and mobility in the cytosol. Notably, NO-induced effect on CaMKIIδ may also be affected by the presence or absence of other post-translational modifications in the complex regulatory domain of CaMKIIδ, including autophosphorylation (T287), oxidation (MM281/282), and *O*-GlcNAcylation (S280). Further studies will be required to investigate the interplay between these sites. Further studies would also be required to demonstrate the role of CaMKII nitrosylation in pathological signaling.

Limitation and Caveat

To optimize GSNO-induced S-nitrosylation of Cys273 and reveal its suppression of CaMKII activation, we first pretreated cells with BAPTA-AM to limit $[Ca^{2+}]_i$ and deactivate the kinase. This BAPTA loading is expected to also suppress pacing-induced Ca^{2+} signals in the pre-GSNO myocytes and that Ca^{2+} may be important in activating intracellular CaMKII or Camui in the myocytes. We do not know the BAPTA concentration in the myocytes or how severe this limitation was for our experiments. We were encouraged because the level of Camui-lifetimes and CaMKII-GFP mobility were not far from the non-BAPTA loaded resting myocytes, and that pacing and ISO were still able to activate Camui and CaMKII mobility. Moreover, the effects observed are consistent with our expectations based on prior work[19,36,38]. Consider Figure 4.2B, where in WT cells (without BAPTA) pacing activated Camui, but that activation was prevented by pre-GSNO (where BAPTA had been used). Part of that suppression might have been due to pre-GSNO, but part might also have been due to low $[Ca^{2+}]_i$ (due to the BAPTA). As a counterpoint to that, in the C273S myocyte (where the pre-GSNO effect should be blocked), pacing now induced a robust increase in Camui activation (which a serious BAPTA issue should have prevented). Thus, we do not think the use of BAPTA invalidates our results. Nevertheless, to be rigorous before submitting this work for journal publication, I will repeat key experiments

using a different pre-GSNO protocol that does not include intracellular BAPTA loading. While we expect heavily BAPTA-loaded myocytes to have only weak global Ca^{2+} transients, it is possible that our BAPTA loading was modest and that local rises in cleft $[Ca^{2+}]_i$ during pacing could have sufficed to activate CaMKII in the pretreated group.

Addendum

This data also shows for the first time that myocyte CaMKII has at least two different populations, definitively and quantitatively. When Ca^{2+} is chelated with BAPTA, a CaMKIIδ population with much shorter fluorescence lifetime is prominent than that observed in resting myocytes (Figure 4.1C), but also another population that exhibits lifetimes closer to the main group of lifetimes in resting myocytes. It is possible that the shorter lifetime cluster in BAPTA-loaded myocytes represents and even more compact CaMKIIδ conformation than that seen in resting myocytes, a point suggested by some neuronal CaMKIIα work[43]. Measuring CaMKIIδ mobility with photobleach and recovery showed that CaMKII Cys273 nitrosylation suppresses CaMKIIδ mobility. Put together, the data in this chapter sheds light on the regulation of CaMKII via NO signaling.

Additionally, to confirm that the CaMKII recovery was not solely diffusion-limited (a diffusion-coupled recovery), I carried out the 'diffusion-limited FRAP test'. The test is based on the principle that diffusion is very fast, and independent of spatial scale - so performing several FRAP measurements while varying the size of the bleaching region, the rate of recovery maintains a nonlinear relationship with the bleach size. Doubling the radius of the bleaching region therefore, should make recovery four times slower[44]. In principle this is not the case for mainly reaction-limited FRAP recovery, where the rate should not significantly change[45]. To test this, I used FRAP

measurements with different bleach ROI sizes (4.5 µm, 9 µm) which decreased the recovery time for cardiomyocytes treated with BAPTA (Figure 4.5C left), but the longer τ at 9 µm diameter did not increase by 4-fold and for paced myocytes there was no consistent change in FRAP τ (Figure 4.5C right). This indicates that CaMKII is not just freely diffusing but is also actively participating in binding/unbinding interactions in the heart.

Figure 4.5 shows some background information on CaMKII FRAP recovery curves fitted using a single- or dual-phase exponential association curve. I found that CaMKII FRAP recovery curves

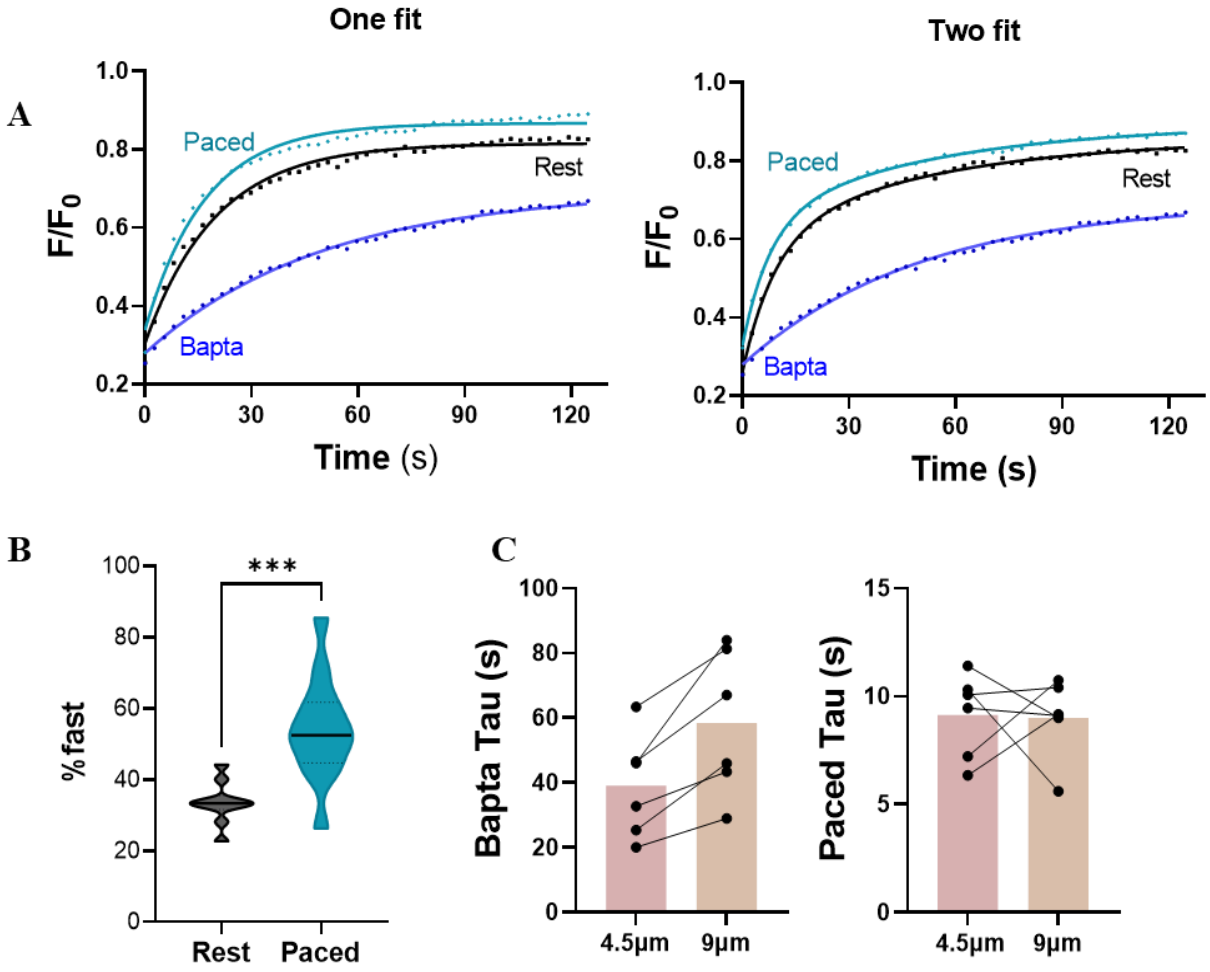


Figure 4.5: CaMKII FRAP recovery curves for rest and paced cardiomyocytes were more accurately fit by two exponential fits (A) and the fast population in paced myocytes (52%) is higher than in resting myocytes (35%, B). Tau of recovery in paced myocytes does not increase when the bleaching region is doubled (C).

for rest and paced cardiomyocytes were more accurately fit by two exponential fits (Figure 4.5A), indicating there are at least two populations contributing to FRAP recovery, which is similar to other proteins[39,40]. Notably, cells treated with the Ca^{2+} chelator, BAPTA-AM, were accurately fit by a single exponential curve. Further analysis as seen in Figure 4.5B, showed cardiomyocytes at rest had a smaller fast population (35%) compared to paced cardiomyocytes (52%).

References

1 Balligand, J. L., Kelly, R. A., Marsden, P. A., Smith, T. W. & Michel, T. Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. *Proc Natl Acad Sci U S A* **90**, 347-351, doi:10.1073/pnas.90.1.347 (1993).

2 Brunner, F. *et al.* Attenuation of myocardial ischemia/reperfusion injury in mice with myocyte-specific overexpression of endothelial nitric oxide synthase. *Cardiovasc Res* **57**, 55-62, doi:10.1016/s0008-6363(02)00649-1 (2003).

3 Burger, D. E. *et al.* Neuronal nitric oxide synthase protects against myocardial infarction-induced ventricular arrhythmia and mortality in mice. *Circulation* **120**, 1345-1354, doi:10.1161/CIRCULATIONAHA.108.846402 (2009).

4 Sun, J., Morgan, M., Shen, R. F., Steenbergen, C. & Murphy, E. Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. *Circ Res* **101**, 1155-1163, doi:10.1161/CIRCRESAHA.107.155879 (2007).

5 Sun, J. *et al.* Ischaemic preconditioning preferentially increases protein S-nitrosylation in subsarcolemmal mitochondria. *Cardiovasc Res* **106**, 227-236, doi:10.1093/cvr/cvv044 (2015).

6 Yasmin, W., Strynadka, K. D. & Schulz, R. Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc Res* **33**, 422-432, doi:10.1016/s0008-6363(96)00254-4 (1997).

7 Saraiva, R. M. *et al.* Deficiency of neuronal nitric oxide synthase increases mortality and cardiac remodeling after myocardial infarction: role of nitroso-redox equilibrium. *Circulation* **112**, 3415-3422, doi:10.1161/CIRCULATIONAHA.105.557892 (2005).

8 Dawson, D. *et al.* nNOS gene deletion exacerbates pathological left ventricular remodeling and functional deterioration after myocardial infarction. *Circulation* **112**, 3729-3737, doi:10.1161/CIRCULATIONAHA.105.539437 (2005).

9 Burkard, N. *et al.* Conditional overexpression of neuronal nitric oxide synthase is cardioprotective in ischemia/reperfusion. *Circulation* **122**, 1588-1603, doi:10.1161/CIRCULATIONAHA.109.933630 (2010).

10 Elrod, J. W. *et al.* Cardiomyocyte-specific overexpression of NO synthase-3 protects against myocardial ischemia-reperfusion injury. *Arterioscler Thromb Vasc Biol* **26**, 1517-1523, doi:10.1161/01.ATV.0000224324.52466.e6 (2006).

11 Szelid, Z. *et al.* Cardioselective nitric oxide synthase 3 gene transfer protects against myocardial reperfusion injury. *Basic Res Cardiol* **105**, 169-179, doi:10.1007/s00395-009-0077-4 (2010).

12 Wang, P. & Zweier, J. L. Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. *J Biol Chem* **271**, 29223-29230, doi:10.1074/jbc.271.46.29223 (1996).

13 Flögel, U., Decking, U. K., Gödecke, A. & Schrader, J. Contribution of NO to ischemia-reperfusion injury in the saline-perfused heart: a study in endothelial NO synthase knockout mice. *J Mol Cell Cardiol* **31**, 827-836, doi:10.1006/jmcc.1998.0921 (1999).

14 Csonka, C. *et al.* Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. *Circulation* **100**, 2260-2266, doi:10.1161/01.cir.100.22.2260 (1999).

15 Komeima, K., Hayashi, Y., Naito, Y. & Watanabe, Y. Inhibition of neuronal nitric-oxide synthase by calcium/ calmodulin-dependent protein kinase IIalpha through Ser847 phosphorylation in NG108-15 neuronal cells. *J Biol Chem* **275**, 28139-28143, doi:10.1074/jbc.M003198200 (2000).

16 Fleming, I., Fisslthaler, B., Dimmeler, S., Kemp, B. E. & Busse, R. Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res* **88**, E68-75, doi:10.1161/hh1101.092677 (2001).

17 Hegyi, B., Bers, D. M. & Bossuyt, J. CaMKII signaling in heart diseases: Emerging role in diabetic cardiomyopathy. *J Mol Cell Cardiol* **127**, 246-259, doi:10.1016/j.yjmcc.2019.01.001 (2019).

18 Coultrap, S. J. & Bayer, K. U. Nitric oxide induces Ca2+-independent activity of the Ca2+/calmodulin-dependent protein kinase II (CaMKII). *J Biol Chem* **289**, 19458-19465, doi:10.1074/jbc.M114.558254 (2014).

19 Erickson, J. R. *et al.* S-Nitrosylation Induces Both Autonomous Activation and Inhibition of Calcium/Calmodulin-dependent Protein Kinase II δ. *J Biol Chem* **290**, 25646-25656, doi:10.1074/jbc.M115.650234 (2015).

20 Said, M. *et al.* Calcium-calmodulin dependent protein kinase II (CaMKII): a main signal responsible for early reperfusion arrhythmias. *J Mol Cell Cardiol* **51**, 936-944, doi:10.1016/j.yjmcc.2011.08.010 (2011).

21 Vila-Petroff, M. *et al.* CaMKII inhibition protects against necrosis and apoptosis in irreversible ischemia-reperfusion injury. *Cardiovasc Res* **73**, 689-698, doi:10.1016/j.cardiores.2006.12.003 (2007).

22 Hoeker, G. S., Hanafy, M. A., Oster, R. A., Bers, D. M. & Pogwizd, S. M. Reduced Arrhythmia Inducibility With Calcium/Calmodulin-dependent Protein Kinase II Inhibition in Heart Failure Rabbits. *J Cardiovasc Pharmacol* **67**, 260-265, doi:10.1097/FJC.0000000000000343 (2016).

23 Pellicena, P. & Schulman, H. CaMKII inhibitors: from research tools to therapeutic agents. *Front Pharmacol* **5**, 21, doi:10.3389/fphar.2014.00021 (2014).

24 He, Q., Cheng, J. & Wang, Y. Chronic CaMKII inhibition reverses cardiac function and cardiac reserve in HF mice. *Life Sci* **219**, 122-128, doi:10.1016/j.lfs.2019.01.010 (2019).

25 Hoelz, A., Nairn, A. C. & Kuriyan, J. Crystal structure of a tetradecameric assembly of the association domain of Ca2+/calmodulin-dependent kinase II. *Mol Cell* **11**, 1241-1251, doi:10.1016/s1097-2765(03)00171-0 (2003).

26 Gaertner, T. R. *et al.* Comparative analyses of the three-dimensional structures and enzymatic properties of alpha, beta, gamma and delta isoforms of Ca2+-calmodulin-dependent protein kinase II. *J Biol Chem* **279**, 12484-12494, doi:10.1074/jbc.M313597200 (2004).

27 Lai, Y., Nairn, A. C., Gorelick, F. & Greengard, P. Ca2+/calmodulin-dependent protein kinase II: identification of autophosphorylation sites responsible for generation of

Ca2+/calmodulin-independence. *Proc Natl Acad Sci U S A* **84**, 5710-5714, doi:10.1073/pnas.84.16.5710 (1987).

28 Meyer, T., Hanson, P. I., Stryer, L. & Schulman, H. Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science* **256**, 1199-1202, doi:10.1126/science.256.5060.1199 (1992).

29 Erickson, J. R. *et al.* Diabetic hyperglycaemia activates CaMKII and arrhythmias by O-linked glycosylation. *Nature* **502**, 372-376, doi:10.1038/nature12537 (2013).

30 Erickson, J. R. *et al.* A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell* **133**, 462-474, doi:10.1016/j.cell.2008.02.048 (2008).

31 Burgos, J. I. *et al.* Nitric oxide and CaMKII: Critical steps in the cardiac contractile response To IGF-1 and swim training. *J Mol Cell Cardiol* **112**, 16-26, doi:10.1016/j.yjmcc.2017.08.014 (2017).

32 Jian, Z. *et al.* Mechanochemotransduction during cardiomyocyte contraction is mediated by localized nitric oxide signaling. *Sci Signal* **7**, ra27, doi:10.1126/scisignal.2005046 (2014).

33 Gutierrez, D. A., Fernandez-Tenorio, M., Ogrodnik, J. & Niggli, E. NO-dependent CaMKII activation during β-adrenergic stimulation of cardiac muscle. *Cardiovasc Res* **100**, 392-401, doi:10.1093/cvr/cvt201 (2013).

34 Curran, J. *et al.* Nitric oxide-dependent activation of CaMKII increases diastolic sarcoplasmic reticulum calcium release in cardiac myocytes in response to adrenergic stimulation. *PLoS One* **9**, e87495, doi:10.1371/journal.pone.0087495 (2014).

35 Pereira, L. *et al.* The cAMP binding protein Epac modulates Ca2+ sparks by a Ca2+/calmodulin kinase signalling pathway in rat cardiac myocytes. *J Physiol* **583**, 685-694, doi:10.1113/jphysiol.2007.133066 (2007).

36 Erickson, J. R., Patel, R., Ferguson, A., Bossuyt, J. & Bers, D. M. Fluorescence resonance energy transfer-based sensor Camui provides new insight into mechanisms of calcium/calmodulin-dependent protein kinase II activation in intact cardiomyocytes. *Circ Res* **109**, 729-738, doi:10.1161/CIRCRESAHA.111.247148 (2011).

37 Hegyi, B. *et al.* Altered Repolarization Reserve in Failing Rabbit Ventricular Myocytes: Calcium and β-Adrenergic Effects on Delayed- and Inward-Rectifier Potassium Currents. *Circ Arrhythm Electrophysiol* **11**, e005852, doi:10.1161/CIRCEP.117.005852 (2018).

38 Wood, B. M. *et al.* Cardiac CaMKII activation promotes rapid translocation to its extra-dyadic targets. *J Mol Cell Cardiol* **125**, 18-28, doi:10.1016/j.yjmcc.2018.10.010 (2018).

39 Phair, R. D., Gorski, S. A. & Misteli, T. Measurement of dynamic protein binding to chromatin in vivo, using photobleaching microscopy. *Methods Enzymol* **375**, 393-414, doi:10.1016/s0076-6879(03)75025-3 (2004).

40 Phair, R. D. *et al.* Global nature of dynamic protein-chromatin interactions in vivo: three-dimensional genome scanning and dynamic interaction networks of chromatin proteins. *Mol Cell Biol* **24**, 6393-6402, doi:10.1128/MCB.24.14.6393-6402.2004 (2004).

41 Murphy, E. & Steenbergen, C. Gender-based differences in mechanisms of protection in myocardial ischemia-reperfusion injury. *Cardiovasc Res* **75**, 478-486, doi:10.1016/j.cardiores.2007.03.025 (2007).

42 Rokita, A. G. & Anderson, M. E. New therapeutic targets in cardiology: arrhythmias and Ca2+/calmodulin-dependent kinase II (CaMKII). *Circulation* **126**, 2125-2139, doi:10.1161/CIRCULATIONAHA.112.124990 (2012).

43 Myers, J. B. *et al.* The CaMKII holoenzyme structure in activation-competent conformations. *Nat Commun* **8**, 15742, doi:10.1038/ncomms15742 (2017).

44 Carnell, M., Macmillan, A. & Whan, R. Fluorescence recovery after photobleaching (FRAP): acquisition, analysis, and applications. *Methods Mol Biol* **1232**, 255-271, doi:10.1007/978-1-4939-1752-5_18 (2015).

45 Wu, E. S., Jacobson, K., Szoka, F. & Portis, A. Lateral diffusion of a hydrophobic peptide, N-4-nitrobenz-2-oxa-1,3-diazole gramicidin S, in phospholipid multibilayers. *Biochemistry* **17**, 5543-5550, doi:10.1021/bi00618a033 (1978).

Conclusion and Future Directions

Summary

The results presented in this dissertation represent a significant contribution to the study of CaMKIIδ regulation in cardiomyocytes. In chapter 3, we investigate the role of CaMKII nitrosylation in mechano-chemo-transduction. Data in this chapter identify CaMKIIδ Cys290 as the critical mediator of the intrinsic afterload-induced increase in cardiac Ca^{2+} transient enhancement that allows the heart to contract more strongly in response to greater circulatory load. We see that CaMKIIδ activation by nitrosylation at the Cys290 site is essential to acute mechanical-stress induced Ca^{2+} upregulation in cardiomyocytes. In Chapter 4, we investigate the effect of the post translational modification - nitrosylation - on CaMKII. We observed that nitric oxide (NO) alters CaMKIIδ activation state and mobility in adult cardiomyocytes. We then investigate the relevance of the Cys273 site on CaMKIIδ for NO-induced suppression of CaMKIIδ activation and mobility in adult ventricular myocytes. The data derived shows for the first time in ventricular cardiomyocytes, that nitrosylation of CaMKII at Cys273 prior to electrical field stimulation and activation by Ca^{2+} or β-adrenergic agonists, suppress CaMKIIδ activity and mobility – whereas mutation of the Cysteine residue at 273 to Serine eliminates this effect.

Future Directions

Heart failure remains a major health burden around the world, and continued investigation of mechano-transduction in cardiac pathologies can reveal opportunities for therapeutic approaches. Further understanding of MCT mechanisms at the cellular level can provide the basis for translation to the tissue and organ level. Like nitric oxide synthase (NOS), nicotinamide adenine

dinucleotide phosphate oxidase 2 (NOX2) also mediates mechano-chemo-transduction. Given the effect of the Cys290 nitrosylation PTM on CaMKII, investigating the effect of other CaMKII PTMs on mechano-chemo-transduction – especially Oxidation – may provide a more complete understanding of the mechano-chemo-transduction pathway. In this dissertation studies where a hydrogel was used to impose mechanical load, investigating the effect of the mechanical load on SR Ca^{2+} content proved technically challenging. The Ca^{2+} gradient obtained from 20 mM caffeine treatment occurred over longer periods instead of instantly. This was probably because the gel was not thin enough, which delayed caffeine in the applied bathing solution to diffuse through the gel (unlike the immediate access caffeine has in the gel-free condition). Further experiments to investigate the changes in SR Ca with afterload would shed light on molecular mechanisms behind mechano-chemo-transduction regulation. Investigating possible sex-differences in the regulation of MCT could also hold more insight for therapeutic approaches.

Another ideal experiment would be to determine if cardiac output varies in the absence of CaMKII Cys290 nitrosylation with changes in aortic pressure. The same increase in aortic pressure induced acutely in the WT and Cys290A mice might result in a greater reduction in cardiac output in the Cys290A mice compared to WT. We saw a decrease in contractility when WT cardiomyocytes were contracting under load. Further experiments on the effect of load on contractility in the cardiac-specific CaMKII knock out mice and the Cys290A mice would be necessary to ascertain CaMKII effect on contractility.

The results from the CaMKII nitrosylation studies raised additional questions that merit further investigation. As mentioned in Chapter 4, a different protocol for suppressing $[Ca^{2+}]_i$ and

deactivating CaMKII would be ideal to confirm the effect of GSNO-induced S-nitrosylation of Cys273. Filling these gaps in knowledge will help to fully understand the molecular mechanisms of CaMKII as a finetuned multi-functional modulator. We know that neuronal isoforms of CaMKII regulate GPCRs in the brain. And while I show here how GPCR (like β-adrenergic receptor) stimulation influences CaMKII activation, we do not know how CaMKII activation influences GPCR expression and function. This would be an interesting area for further studies. Measuring CaMKIIδ mobility with photobleach and recovery showed that GPCR stimulation further increases CaMKII mobility. This confirms that in addition to Ca^{2+} activation, CaMKII can be further activated via other mechanisms. However, the specific pathways for activation by Angiotensin II and Isoproterenol remain yet to be identified completely.

The multifunctional signaling molecule CaMKII has received considerable attention over recent years for its central role in maladaptive remodeling and arrhythmias in the setting of chronic disease. Hopefully, with continued research, these basic science discoveries will contribute to a greater understanding of cardiac function and heart disease and ultimately translate into new therapies for human patients.