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The Binding Properties and Functional Consequences of Ryr2-Cam Interaction

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LOYOLA UNIVERSITY CHICAGO

KINETICS OF CALMODULIN BINDING TO CARDIAC RYANODINE RECEPTORS AND FUNCTIONAL CONSEQUENCES

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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BY

YI YANG

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To My Parents and My Wife, Xuejing
# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS** ........................................................................................................ iii

**TABLE OF CONTENTS** ........................................................................................................ v

**LIST OF FIGURES** ........................................................................................................ viii

**LIST OF TABLES** ........................................................................................................ x

**CHAPTER I: INTRODUCTION** ............................................................................................. 1

**CHAPTER II: REVIEW OF RELATED LITERATURE** .......................................................... 6
  Cardiac Excitation – Contraction Coupling ................................................................. 6
  Cellular Structure Basis of Cardiac EC-Coupling....................................................... 7
    Myofibrils and sarcomere. ....................................................................................... 7
    Sarcolemma and transverse tubules. ................................................................. 8
    Sarcoplasmic reticulum (SR). ............................................................................. 10
  SR Ca$^{2+}$ release Channel (RyR) .......................................................................... 13
    RyR structure: ..................................................................................................... 13
  Regulation of RyR ................................................................................................... 14
    Ca$^{2+}$, Mg$^{2+}$, and ATP .................................................................................. 14
      FKBP12/12.6 ..................................................................................................... 15
    Calmodulin (CaM) ............................................................................................. 16
    Sorcin ................................................................................................................... 16
    PKA, CaMKII and phosphatases ........................................................................ 17
    Calsequestrin, Junctin and Triadin ...................................................................... 20
    Caffeine, Ryanodine and Tetracaine .................................................................. 20
  Ca$^{2+}$ Sparks, Ca$^{2+}$ transients and Ca$^{2+}$ waves ................................................. 21
  SR Ca$^{2+}$ leak .......................................................................................................... 22
  Main mechanisms for enhanced SR Ca$^{2+}$ leak ....................................................... 24
    FKBP12.6 dissociation by PKA phosphorylation ................................................... 24
    CaMKII and CaMKII phosphorylation .................................................................. 26
    Store Overload Induced Ca$^{2+}$ Release (SOICR) ............................................. 29
    Reduction / oxidation (Redox) Modification ....................................................... 32
    The interruption of interdomain interaction ....................................................... 34
  Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) ....................... 38
    Calmodulin .......................................................................................................... 40
    FK-506 Binding protein ....................................................................................... 43

--- 

v
Fluorescence resonance energy transfer (FRET) ......................................................... 45

CHAPTER III: MATERIALS AND METHODS .............................................................. 47
Heart Failure Model ............................................................................................... 47
Cardiac myocyte isolation.................................................................................... 47
Cell permeabilization ......................................................................................... 48
Fluorescent labeling of single-cysteine mutants of FKBP12.6 and CaM .............. 49
Fluorescence Recovery after Photobleaching (FRAP) ......................................... 51
FRET between F-CaMs and F-FKBP in permeabilized myocytes ..................... 53
Wash-in/off in myocytes .................................................................................... 55
Ca2+ signal measurement by fluo-4 with confocal microscopy ......................... 56
Ca2+ signal recording in intact myocytes ......................................................... 56
Ca2+ spark measurement and analysis ............................................................ 56
Statistics ........................................................................................................... 57

CHAPTER IV: HYPOTHESES AND AIMS ............................................................... 59
Aim 1. Measure the physical binding properties of RyR2-CaM in situ .............. 60
Aim 2. Define physiological consequences of RyR2-CaM binding in cardiac myocytes ........................................................................................................ 61
Aim 3. Define CaM-RyR2 binding affinity in heart failure myocytes .............. 61
Aim 4. Define CaM as a critical stabilizer for RyR2 through RyR2 unzipping peptide (DPc10). ............................................................... 62

CHAPTER V: KINETICS OF CALMODULIN BINDING TO CADIC RYANODINE RECEPTORS AND FUNCTIONAL CONSEQUENCES ................................. 63
INTRODUCTION ................................................................................................. 63
MATERIALS AND METHODS ........................................................................... 65
RESULTS ........................................................................................................... 68
Predepletion of endogenous CaM by suramin ................................................. 68
FRET between FKBP12.6 and CaM on the RyR ........................................... 71
Measurement of binding affinity (Kd) of CaM-RyR2 ................................ 73
Measurement of binding kinetics of CaM-RyR2 ........................................ 79
Estimating the percentage of Z-line total CaM that is RyR2-bound ....... 83
Ca2+ sparks in permeabilized RyR2 +/-ADA KI mouse myocytes ............. 87
Ca2+ transients for RyR2 +/-ADA KI mouse in intact myocytes ................ 92
Binding affinity (Kd) of CaM/FKBP12.6 to RyR2 in HF myocytes ........... 95
DISCUSSION ................................................................................................... 98

CHAPTER VI: DEFINE CAM AS A CRITICAL STABILIZER THROUGH RYR2 UNZIPPING PEPTIDE (DPc10) ................................................................. 108
INTRODUCTION ............................................................................................. 108
MATERIALS AND METHODS ........................................................................ 111
RESULTS ....................................................................................................... 114
Binding kinetics of F-DPc10 in permeabilized cardiac myocytes...... 114
Effect DPc10 on CaM and FKBP12.6 binding at the myocyte Z-line 120
Effect of DPc10 on FKBP12.6 and CaM binding in permeabilized
myocytes ........................................................................................................ 126
FRET between CaM and DPc10 ................................................................. 130
DISCUSSION .................................................................................................. 133

CHAPTER VII: SUMMARY .............................................................................. 138

REFERENCE ................................................................................................ 143

VITA ................................................................................................................. 170
LIST OF FIGURES

Figure 1: Schematic diagram of the mammalian cardiac muscle .................. 9
Figure 2: Schematic diagram of monomer RyR2 sequence ......................... 12
Figure 3: Domain unzipping .................................................................... 37
Figure 4: Suramin can predeplete endogenous CaM ............................... 70
Figure 5: FRET between F-FKBP12.6 (donor) and F-CaM (acceptor) in permeabilized rat myocytes .......................................................... 72
Figure 6: $K_d$ for RyR2 bound CaM .......................................................... 74
Figure 7: $K_d$ fitting from both donor and acceptor signals ....................... 76
Figure 8: Steady-state $K_d$ and Bmax for Z-line total CaM ....................... 78
Figure 9: CaM-RyR2 on- & off-rate measured by FRET + wash-in/off .......... 80
Figure 10: CaM-RyR2 on- & off-rates measured by FRAP ....................... 82
Figure 11: Confocal images for RyR2 bound FRET CaM and Z-line bound total CaM (RyR2ADA/+ vs WT) ......................................................... 84
Figure 12: Percentage of Z-line CaM that is bound to RyR2 ....................... 85
Figure 13: Total binding ($B_{max}$) of FKBP12.6 for WT and RyR2ADA/+ mice ........ 86
Figure 14: Effect of different time permeabilization .................................. 88
Figure 15: Diastolic Ca$^{2+}$ leakiness in RyR2ADA/+ mice ......................... 90
Figure 16: Ca$^{2+}$ spark characteristics for permeabilized myocytes .......... 91
Figure 17: Ca$^{2+}$ transients in intact myocytes ......................................... 93
Figure 18: Ca$^{2+}$ wave and triggered activities for RyR2$^{ADA/+}$ mice ...................... 94

Figure 19 K$_d$ for CaM/FKBP12.6 binding to RyR2 in HF myocytes ....................... 97

Figure 20: Fluorescence spectrum for FRET ............................................................. 113

Figure 21: F-DPc10 binding at the myocyte Z-line ............................................. 115

Figure 22: Wash-in time course for different [F-DPc10] ........................................ 117

Figure 23: Effect of RyR2 channel modulators on the kinetics of F-DPc10
          Z-line association ..................................................................................... 119

Figure 24: Effects of FKBP12.6 and CaM on F-DPc10 binding at Z-line .......... 121

Figure 25: Effects of FKBP12.6 and CaM on DPc10 binding and Ca$^{2+}$
          sparks in permeabilized cardiomyocytes ................................................. 124

Figure 26: The effect of DPc10 on FKBP12.6 binding to RyR2 in cardiac
          myocytes .................................................................................................. 128

Figure 27: The effect of DPc10 on CaM binding to RyR2 in cardiac myocytes 129

Figure 28: FRET between F-CaM and F-DPc10 ................................................. 132

Figure 29: Proposed model of the interaction between N-terminal and
          central domains .......................................................................................... 137
LIST OF TABLES

Table 1: Summary of CaM-RyR2 binding properties ................................................. 99
Table 2: Ca$^{2+}$ spark characteristics in saponin-permeabilized cardiomyocytes 125
Table 3: Comparison between CaM and FKBP12.6 as RyR2 stabilizers ........... 141
CHAPTER I

INTRODUCTION

Calcium (Ca\(^{2+}\)) functions as an important intracellular element that underlies essential cellular processes including muscle contraction, synaptic transmission, secretion of hormones, and gene expression (Fill and Copello, 2002; Zhang and Brown, 2004). In the heart, Ca\(^{2+}\) is the signaling massager for cardiac excitation-contraction coupling (E-C coupling) which is a chain reaction, and Ca\(^{2+}\) concentration fluctuation can be converted to cardiac contraction and relaxation. Moreover, the intracellular calcium concentration \([\text{Ca}^{2+}]\) should be finely adjusted to maintain its homeostasis, which helps maintain various cellular functions, such as: gene transcription (Atar et al, 1995) and post-translational protein modification, both of which are very critical for cell viability (Anderson et al, 2007, George et al, 2007). Alterations to this fine Ca\(^{2+}\) homeostasis could lead to abnormal gene transcription for hypertrophic remodeling, impaired contractile function, and arrhythmogenicity.

The cardiac ryanodine receptor (RyR2) is the Ca\(^{2+}\) release channel located in the sarcoplasmic reticulum (SR) membrane, and is the central link in cardiac EC coupling to control release of SR stored Ca\(^{2+}\), accounting for up to 90% of total Ca\(^{2+}\) in EC coupling cycle (Bers, 2001). Over the past ten years, diastolic Ca\(^{2+}\) leak through dysfunctional RyR2 has been recognized as an
important factor contributing to altered \( \text{Ca}^{2+} \) homeostasis in heart failure (HF). Evidence in several reports shows that abnormality within the RyR2 causes increased diastolic \( \text{Ca}^{2+} \) leak, leading to contractile and relaxation dysfunction (Yano et al 2000; Ai X, et al, 2005; Wehrens et al, 2006). Moreover, the abnormal \( \text{Ca}^{2+} \) leak through RyR2 could lead to lethal arrhythmia (Wehrens et al 2003). Now it is well known that RyR2-mediated SR \( \text{Ca}^{2+} \) leak could activate inward current through Na/Ca exchanger (NCX) (Vermeulen et al, 1994), and further evoke delayed after depolarizations (DADs), which has been proven to be the leading reason for ventricular tachyarrhythmia and sudden cardiac death (Pogwizd and Bers 2004). In HF there is an enhanced diastolic SR \( \text{Ca}^{2+} \) leak and other changes in electrophysiological substrate (like up-regulation of NCX) that greatly enhance the propensity for triggered cardiac arrhythmias (Pogwizd and Bers, 2002). Approximately 50% of heart failure (HF) patients die of DAD related sudden cardiac death (Noseworth et al 2008). All those properties now make RyR2 being a potential therapeutic target for anti-HF and anti-arrhythmia.

A leading hypothesis for explaining the RyR2 dysfunction, enhanced SR \( \text{Ca}^{2+} \) leakiness in HF and lethal arrhythmias, is defective interaction between the N-terminal (N: 0-600) and the central (C: 2000-2500) domains (Yamamoto and Ikemoto, 2002). According to this concept, in the resting state, the tight interaction between N-terminal and central domains acts as a regulatory switch for channel gating activity. This tight interdomain interaction, termed “domain zipping” can stabilize the channel in the fully closed state (Yamamoto and
Weakening of these interdomain interactions may be caused by mutations in either the N-terminal or central regions of RyR2 (Uchinoumi et al., 2010), resulting in an increased opening probability of the RyR2 and leakiness of Ca^{2+}. A synthetic peptide (DPc10) corresponding to the central domain (DP2460-2495) of RyR2, was reported to specifically associate with the N-terminal domain competing with its zipping to the central domain, and that the N-domain/DPc10 association can unzip and destabilize RyR2 (via domain unzipping), to increase Ca^{2+} leakiness (Oda T, et al, 2005).

Calmodulin (CaM) is a ubiquitous and highly conserved Ca^{2+} binding protein which regulates multiple cellular processes. With 148 highly conserved residues and ~16.7kDa molecular weight, CaM has two globular Ca^{2+} binding domains with E-F hands, connected by a flexible tether helix (Jurado et al, 1999). At high [Ca^{2+}], Ca^{2+} can bind cooperatively to CaM and result in a conformational change that translate intracellular Ca^{2+} signal to diverse targets. Working as a critical regulator for SR Ca^{2+} release channel, CaM binds to RyR2 stoichiometrically (four CaMs per tetrameric RyR2). Cryo-EM based 3D RyR reconstruction showed that Ca-free CaM (apoCaM) binds to a cleft in the cytosolic domain 3 (AA 3614-3643) (Moore et al, 1999; Wagenknecht et al 1994), with a distance of 60-70Å to FKBP12.6 on the same side (Cornea et al, 2009; Guo T et al 2011). FKBP12.6 was also reported to bind RyR2 tightly as an important regulator, but some aspects are still highly controversial (Brillantes AB, et al 1994; Marx SO et al 1998; Barg S et al, 1997; Guo T, et al 2010). Mutations
(RyR^{ADA}) to the highly conserved CaM binding region can severely inhibit CaM binding to RyR2 and cause severe hypertrophic cardiomyopathy and early death in animal models (Yamaguchi et al, 2007). In a non-ischemic rabbit HF model, CaM association to RyR2 was decreased with unchanged total CaM (Ai X, et al, 2005). A recent study indicates that defective CaM binding to RyR2 is involved in catecholaminergic polymorphic ventricular tachycardia (CPVT) associated RyR2 dysfunction and lethal arrhythmia (Xu X, et al, 2010). All these reports suggest that CaM-RyR2 interaction could be a critical molecular substrate for arrhythmias and HF pathogenic processes.

Here through FRET (fluorescent resonance energy transfer) detection, we for the first time identified RyR2-bound CaM from its numerous binding target proteins, and characterized CaM-RyR2 interaction properties in both normal and HF cardiomyocytes. The knock-in mice (RyR^{ADA}), with genetically disrupted CaM-RyR2 association while the interaction with other CaM targets are not affected, were used to estimate the percentage of Z-line bound CaM that is due to RyR2. Furthermore the patho-physiological consequences of CaM for RyR2 regulation were defined through function test.

DPc10 has been worked as useful molecular reagents to study RyR2’s function and structure. In this study, through fluorescent tagging we can characterize the binding kinetics of DPc10 to the RyR2 macromolecular complex in the relatively intact environment provided by saponin-permeabilized ventricular myocytes. Furthermore, we defined CaM as a critical stabilizer for RyR2 through
this RyR2 unzipping peptide and revealed the relationship between CaM and RyR2 domains interaction (domain zipping).

This accomplishment enriched our understanding about RyR2-CaM interaction and functional effects on RyR-mediated Ca\(^{2+}\) release in cardiac myocytes. This will also expand our knowledge of molecular mechanisms that underlie arrhythmogenesis and HF. Importantly, it could pinpoint a potential molecular target responsible for Ca\(^{2+}\) triggered arrhythmias and may translate into the development of novel therapeutic strategies.
CHAPER II

REVIEW OF RELATED LITERATURE

Cardiac Excitation – Contraction Coupling

Cardiac Excitation-contraction coupling (ECC) is an essential physiological process in which an electrical stimulus is converted to cardiac contraction, resulting in blood being pumped out to meet the need of the whole body. An action potential (AP), normally induced by pacemaker cells in Sinoatrial (SA) node, can propagate and reach the ventricular myocytes through cardiac conduction system (atrial muscle, atrio-ventricular node, His bundle, bundle branches and Purkinje fibers). Delivered AP can activate voltage-dependent Na channel and produce a very fast upstroke Na current, which brings resting membrane potential ($E_m$) from -80 mV up to $\sim$30-50 mV. Then voltage-dependent L-type calcium ($Ca^{2+}$) channels (LTCC) are activated and causing the $Ca^{2+}$ influx, which triggers a subsequent release of $Ca^{2+}$ stored in the sarcoplasmic reticulum (SR). This SR $Ca^{2+}$ release through the calcium-release channels (ryanodine receptors / RyRs), works as a positive feedback response and is termed $Ca^{2+}$-induce $Ca^{2+}$ release (CICR, Fabiato 1985). As total [$Ca^{2+}$], released from SR and entered through LTCC, rises to > 100 uM, the free $Ca^{2+}$ binds to troponin-C (TN-C) that is part of the regulatory complex attached to the thin filaments. Once bound with $Ca^{2+}$, TN-C undergoes a conformational change
and exposure of a binding site on the actins for myosin head. The physical contact of actin and myosin head results in ATP hydrolysis that supplies energy for a slide between the myosin and actin. This sliding shortens the length of cardiomyocyte and commits the cardiac contraction. Relaxation occurs after the sequestering of cytosolic Ca\textsuperscript{2+} via SR Ca\textsuperscript{2+}-ATPase (SERCA), sarcolemmal sodium-calcium exchanger (NCX), sarcolemmal calcium ATPase and mitochondrial Ca\textsuperscript{2+} uniporter. In mouse ventricular myocytes, \(~90\%\) Ca\textsuperscript{2+} needed for contraction is taken up by SERCA back into SR; \(~10\%\) Ca\textsuperscript{2+} is excluded from the cell by NCX; < 1% Ca\textsuperscript{2+} is excluded through sarcolemmal calcium ATPase or taken by mitochondrial uniporter (Bers 2001). The lowering of intracellular Ca\textsuperscript{2+} makes troponin C return to cover the active binding site and to separate myosin head from actin, which results in the end of contraction and causing relaxation.

**Cellular Structure Basis of Cardiac EC-Coupling**

1. **Myofibrils and sarcomere.**

Muscles are composed of tubular muscle cells (myocytes), and muscle cells are mainly (45\textendash60\%, Bers, 2001) composed of tubular myofibrils (Fig. 1), which is mainly constituted by myosin (thick filament) and actin (thin filament). Striated myofibrils, like skeletal and cardiac muscle, are composed of 1.8 um size repeating sections of sarcomere that is the fundamental contractile unit (Fig. 1). Z-line, where actin molecules (thin filaments) are attached (Fig 1), forms the border of the sarcomere (Bers, 2001).
2. Sarcolemma and transverse tubules.

The sarcolemma is the cell membrane of a muscle cell. In striated muscles, the cell membrane invaginates regularly into the cytoplasm of the cell, forming a special membranous tubule structure termed transverse tubules (T-tubules) (Fig. 1). These T-tubules allow a simultaneous penetration of sarcolemma depolarization into the interior of the cell for a synchronous Ca$^{2+}$ release and contraction. In cardiac muscle, T-tubule is typically located at Z-line and forms junctional coupling with terminal cisternae (enlarged bulbous ends of the SR at Z-line region), constituting an arrangement called a diad (Fig 1). T-tubules are the major sites for EC coupling; many essential components, like LTCC, RyR, and NCX, are concentrated at the T-tubule region. In the experiments named “detubulation”, low osmolarity can disrupt T-tubule. Through detubulation, myocyte’s capacitance proportional to the cell surface decreased by ~30% (Kawai M, et al, 1999), while ~87% of I$_{Ca}$ and almost all NCX current are lost due to detubulation (Kawai M, et al, 1999; Yang Z, et al, 2002). With β-adrenergic stimulation, the increase of LTCC current is greater for normal cells than detubulated cell, indicating a better coupling of LTCC in T-tubule to the second messenger than that on the surface of sacolemma (Brette F, et al, 2002; Brette F & Orchard C, 2003).
Fig 1: Schematic diagram of the mammalian cardiac muscle. Sarcomere refers to the myofibril between Z-lines. T-tubules are co-localized with Z-lines. Mitochondrias are filled into the zones between Z-lines. (Fawcett & McNutt, 1969)

The SR is an intracellular organelle and the main Ca\textsuperscript{2+} storage for cardiac myocytes. The structure is analogous to the endoplasmic reticulum present in other types of cells, with a more regular network structure, specialized for the efficient Ca\textsuperscript{2+} release required for CICR. The SR has a homogenous membrane geometry, which can be divided into junctional SR (JSR) and longitudinal SR (LSR) (Fig 1). The LSR is distributed throughout the myoplasm and is thought to be mostly concerned with Ca\textsuperscript{2+} uptake. The JSR, which is also called the terminal cisternae (Fig 1), exists in close proximity to T-tubules. In junctions between JSR and T-tubule, a bridging “foot” structure was found (Franzini-Armstrong, 1970). This junctional foot structure turned out to be highly specialized RyRs and function as Ca\textsuperscript{2+} release channels. In the SR lumen, there are three known Ca\textsuperscript{2+} binding proteins: (1) Calsequestrin is thought to bind the majority of Ca\textsuperscript{2+} in the SR and is highly localized to the JSR; (2) histidine-rich Ca\textsuperscript{2+}-binding protein (HRC) and (3) sarcalumenin, are distributed throughout the SR suggesting the role in LSR Ca\textsuperscript{2+} buffering (Bers 2004).

Since the SR is a single, physically continuous, and tubular intercellular compartment, it was first considered as a homogeneous Ca\textsuperscript{2+} storage. However, with some functional heterogeneity found, this simplistic view is not correct (Papps et al, 2003). Now the widely accepted notion is that: physically the SR is continuous tubular network and lumenally connected, but it is spatially and functionally heterogeneous. This heterogeneity is established by the nonuniform
distribution of SR Ca\(^{2+}\)-handling proteins: (1) the main SR luminal Ca\(^{2+}\)-buffer proteins, calsequestrin, are mainly in JSR; (2) the Ca\(^{2+}\) uptake pump, SERCA, is mainly in LSR; and (3) the SR Ca\(^{2+}\) release channels, ryanodine receptors (RyRs), is mainly in JSR (Papp s, et al, 2003). The nonuniform distribution of these Ca\(^{2+}\)-handling proteins provides the functional division of SR, rather than the structural divisions (JSR and LSR).
Fig 2: Schematic diagram of monomer RyR2 sequence. Associated important regulatory proteins and phosphorylation sites are indicated on cytosolic side. 6 transmembrane domains form the channel core with putative core region GIG. Triadin and Junctin are coupled with channel core and linked with calsequestrin (CSQ) (Bers, 2001).
SR Ca\textsuperscript{2+} release Channel (RyR)

RyRs, as intracellular Ca\textsuperscript{2+} release channels, play a central role in excitation-contraction coupling (ECC). This channel controls the release of SR stored Ca\textsuperscript{2+} accounting up to 90% total Ca\textsuperscript{2+} in EC coupling cycle (Bers 2001). As a family of Ca\textsuperscript{2+} release channels found on intracellular Ca\textsuperscript{2+} store/release organelles like SR, RyRs are expressed in many types of cells and three isoforms have been identified: RyR1 is primarily in skeletal muscle; RyR2 is the main isoform in cardiac muscle; RyR3 is expressed in various tissues at low level. In striated muscles, RyR1/RyR2 is concentrated at the Z-line or T-tubule region in the packed arrays. In a cardiac junction (at Z-line or T-tubule), it is estimated up to 200 RyRs are packed into 60 – 100 nM diameter circle region for a typical cardiac couplon, in which RyR tetramers are connected through corner-contact and implied an functional coupling for RyRs gating in the couplon (Marx et al 2001; Bers, 2001).

1. RyR structure:

As the biggest ion channel discovered so far, RyR is composed of four monomers that form a homotetramer and comprise a single RyR channel (Bers 2001). Each monomer has over 5000 amino acids and ~560 kDa molecular weight. The majority of the amino acid sequence folds to form the cytoplasmic domain, which provides docking sites for most channel regulators like CaM, FKBP, Ca\textsuperscript{2+}, kinases and phosphatases. Only a small part of the c-terminal sequence forms the transmembrane domain and channel pore (Fig.2), where the
loop between 5th and 6th transmembrane domain (Zhao M, et al, 1999) works as a putative pore loop (Fig 2, Bers 2004). 4 pore loops form the narrowest part of the channel pore that works as ion selection and mediates Ca$^{2+}$ passage (Bers 2004). Triadin and junctin are SR membrane proteins which interact with RyR transmembrane domain (Fig 2). Calsequestrin, the major intra-SR Ca$^{2+}$-binding protein, linked to triadin and junction in RyR (Fig 2). And three proteins form a complex that is thought for the sensation of SR lumenial Ca$^{2+}$ level and the gating of RyR (Caswell AH, et al, 1991; Knudson CM, et al, 1993; Jones LR, et al, 1993; Zhang L, et al, 1997).

2. Regulation of RyR

a. Ca$^{2+}$, Mg$^{2+}$, and ATP

Ca$^{2+}$ is the charge carrier conducted by RyR in EC coupling; it also works for RyR modulation from cytoplasmic and SR lumen sides. In single channel experiments, cytoplasmic Ca$^{2+}$ shows two-phase regulation for RyR1. Low Ca$^{2+}$ concentration (1-10 μM) activates RyR1 by binding to specific high-affinity sites; high Ca$^{2+}$ concentration (1-10 mM) inhibits RyR1 by binding to less selective low-affinity Ca$^{2+}$ sites (Copello et al., 1997; Meissner 1994; Meissner et al. 1997). Cytoplasmic Ca$^{2+}$ is also involved in RyR regulation through CaM and CaMKII (reviewed in CaMKII section). For the RyR regulation by Ca$^{2+}$ from the lumen side, it was shown that the increased luminal Ca$^{2+}$ level can enhance the sensitivity of RyR in single channel recording (Györke and Györke 1998; Laver et
al. 2004). The complex of calsequestrin, triadin, and junctin is reported as SR luminal Ca^{2+} sensor to regulate RyR from luminal side (Györke et al. 2004).

Mg^{2+} in the physiological concentration works as an inhibitor of RyR, probably by binding to the Ca^{2+} site. Mg^{2+} is believed to bind to Ca^{2+} high affinity site competitively to reduce opening of RyR; while for low-affinity Ca^{2+} site, Mg^{2+} binding presents a direct inhibition (Meissner et al, 1986; Laver et al; 1997).

ATP can activate both RyR1 and RyR2, but differently. Invitro studies showed that ATP can activate RyR1 in the absence of Ca^{2+}, but Ca^{2+} is needed for maximal activation (Meissner et al, 1984 and 1986; Laver et al, 2001). In the RyR2 case, the channel can’t be activated by ATP without Ca^{2+}, and ATP can sensitize RyR2 to Ca^{2+} but the effect is smaller than that for RyR1. Considering that under physiological conditions most ATP is in a complex with Mg^{2+}, so in cell experiments the activation of ATP is mixed with the inhibition of Mg^{2+} (Lanner et al, 2012), and therefore it is hard to differentiate those effects.

b. FKBP12/12.6

FKBP12 and FKBP12.6 are also termed as calstabin1 and calstabin2 respectively. They physically bind to RyR monomer at the same binding site in 1:1 manner (Timerman et al. 1993 and 1996). In skeletal muscle, FKBP12 associates with RyR1 with a relatively low affinity (~1uM) and stabilize the channel in the full closed state (Timerman et al., 1996, Brillantes et al., 1994). Removal of FKBP12 from RyR1 by pharmacologic depletion or genetic knock-
out, can increase channel open probability and prolong channel mean open time (Shou et al. 1998; Marx et al. 1998; Ahern et al. 1997).

In cardiac myocytes, FKBP12.6 preferentially binds to RyR2 with very high affinity (Timerman et al. 1993; Guo et al. 2010). Some studies showed that FKBP12.6 plays an important role for RyR2 gating to stabilize the channel in a fully closed state (Kaftan et al., 1996; Xiao et al., 1997, Xin et al., 2002). By contrast, other group’s study challenged this opinion (Timerman et al. 1996, Xiao et al. 2007a). The detail for FKBP12.6’s function on RyR2 will be reviewed in FKBP section.

c. Calmodulin (CaM)

CaM binds to RyR monomer in a 1:1 rate (Fruen et al. 2000). As an important RyR regulator, CaM regulates RyR1 and RyR2 activity differently and Ca^{2+}-dependently. For RyR1, CaM increases the open probability at low (<100nM) [Ca^{2+}] but inhibits channel activity at high (>1uM) [Ca^{2+}] (Rodney et al, 2000). However, for RyR2, CaM inhibits the channel activity at both low and high [Ca^{2+}] (Tripathy et al. 1995; Yamaguchi et al. 2005). Moreover, CaM also can regulate RyR indirectly through CaMKII. Details will be further reviewed in CaM section.

d. Sorcin

Sorcin is a 22 kDa Ca^{2+}-binding soluble protein and associates with RyR2 when Ca^{2+} is elevated (Meyers et al., 1995; Meyers et al., 1998). Lipid bilayer studies showed that sorcin reduced RyR open probability (Po) and this
depression could be relieved by PKA-dependent phosphorylation (Farrell et al., 2003). In intact myocytes, dialyzing sorcin into the cell can reduce Ca$^{2+}$ transient amplitude with no effect to LTCC current (Farrell et al, 2003). Furthermore, transient over expression of sorcin via adenoviral vector showed a decreased Ca$^{2+}$ transient with the unchanged SR Ca$^{2+}$ load (Seidler et al., 2003). All the studies suggest that sorcin, at high [Ca$^{2+}$], is similar to CaM which inhibits the Ca$^{2+}$ release from RyR2 in Ca$^{2+}$-dependent manner, but this inhibition can be relieved by low Ca$^{2+}$ and PKA activation.

e. PKA, CaMKII and phosphatases

Through muscle A kinase anchoring protein (mAKAP), PKA associates with RyR2 at 3003-3039 (Fig. 2) amino acids (Marx et al, 2001). In the classic physiological “fight or flight” situation, sympathetic nervous system (SNS) can cause ß-adrenergic stimulation, which results in larger and faster Ca$^{2+}$ transient and consequently stronger and faster muscle contraction. In this process, the activated PKA mainly phosphorlates 4 targets to cause this physiological respone: 1. phosphorylation of troponin I decreases myofilament Ca$^{2+}$ sensitivity causing the faster relaxation; 2. phosphorylation of LTCC increases $I_{Ca}$; 3. phosphorylation of phospholamban (PLB) increases SERCA Ca$^{2+}$ uptake rate and SR Ca$^{2+}$ load; 4, phosphorylation of RyR alters its gating (Bers 2001).

Regarding RyR, Marx et al found that PKA-dependent RyR phosphorylation at Ser-2809 can dissociate FKBP12.6 from the RyR and then enhanced single RyR’s open probability in bilayers experiment (Marx et al, 2000;
But other groups found that phosphorylation of RyR by cAMP-dependent PKA activation can not cause FKBP12.6 dissociation (Xiao et al, 2004; Guo et al, 2010). Moreover in PLB knock-out mouse, where SR Ca^{2+} load can not be increased by PLB-phosphorylation, the resting Ca^{2+} spark frequency, which worked as an index for RyR activity, is unchanged with PKA activation (Li et al, 2002). So another explanation is that the increased Ca^{2+} spark frequency by cAMP is largely due to increasing I_{Ca} and SR Ca^{2+} load by PKA activation (Bers 2004). Thus the RyR regulation by PKA phosphorylation is still controversial.

CaMKII is a multifunctional serine/threonine kinase and δ isoform is primarily expressed in heart (Edman et al, 1994). CaMKII can co-immunoprecipitate with RyR2, but the interaction sequence is still not known (Zhang et al, 2003). CaMKII has a catalytic domain, a central regulatory domain and an association domain. CaMKII can be activated through Ca^{2+}/CaM binding to regulatory domain and displacing the auto-inhibitory domain. Guanine nucleotide exchange protein (Epac) and oxidation are also reported to activate CaMKII through different pathways (Pereira et al, 2007; Erickson et al, 2008). In general, CaM is critical for CaMKII activation. Once CaM/Ca^{2+} is bound, the kinase retains 100% activity. Autophosphorylation can keep 20-80% maximal CaMKII activity despite of CaM dissociation (Meyer et al, 1992; Maier et al, 2003)

CaMKII can modify RyR through phosphorylation at up to 6 sites (Zucchi et al, 1997), including Ser-2809 that also is the target for both PKA and PKG (Witcher et al, 1991; Wehrens et al, 2004; Rodriguez et al, 2003; Huke and Bers
2007). Now Ser2814 is thought exclusively for CaMKII phosphorylation (Wehrens et al., 2004). Most studies on the effect of CaMKII phosphorylation on RyR are based on single channel and SR vesicle experiment with mixed results, which showed either increased (Wehrens et al., 2004; Hain et al., 1995) or decreased channel opening (Lokuta et al., 1995). In intact and permeabilized cells, Bers’ lab indicates that CaMKII can increase SR Ca\(^{2+}\) release for a given SR Ca load (Li et al., 1997; Guo et al., 2006). However, the data from Wu et al. (2001) showed decreased amplitude of Ca\(^{2+}\) transients after CaMKII activation. It could be that diasolic SR Ca\(^{2+}\) leak dramatically decreases the SR Ca\(^{2+}\) content and secondarily affects Ca\(^{2+}\) transient. So we think that under physiological conditions CaMKII phosphorylation exhibits a strong activating effect on RyR opening. CaMKII also plays an important pathological role, which is going to be reviewed in a later section.

Phosphates, PP1 and PP2a, are reported to associate with RyR2 indirectly through anchor protein spinophilin and PR130 respectively. The main function for phosphatases is to dephosphorylate the phosphorylated RyR2, thus keeping the channel in right balance between phosphorylation and dephosphorylation (Marx et al., 2001). PP1 is proposed mainly to dephosphorylate both Ser2809 and Ser2814, while Ser2814 is thought to be PP2a function site (Huke & Bers, 2008).
f. Calsequestrin, Junctin and Triadin

Calsequestrin (CSQ), as the major intra-SR Ca$^{2+}$ buffer, works with junctin and triadin to form a complex coupled with RyR (Fig 2). As the low-affinity but high-capacity Ca$^{2+}$-binding protein, two type of CSQ isoforms are expressed, CSQ1 is in skeletal muscle and CSQ2 is mainly in cardiac muscle. A current model hypothesizes that the elevation of luminal Ca$^{2+}$ weakens the interaction of CSQ complex causing SR Ca$^{2+}$ release to maintain SR Ca$^{2+}$ content (Zhang et al, 1997). Moderate over expression of CSQ can increase SR Ca$^{2+}$ load and Ca$^{2+}$ transient amplitude, thereby prolong SR Ca$^{2+}$ release time (Terentyev at al, 2003). CSQ2 knockout mouse showed normal SR Ca$^{2+}$ release and cardiac contractility, but exposure to catecholamines can cause an increase in SR Ca$^{2+}$ leak making this mouse susceptible to catecholaminergic ventricular arrhythmias (Knollmann et al, 2006).

g. Caffeine, Ryanodine and Tetracaine

These three pharmacological compounds are broadly used as exogenous modulators for RyR function studies. Caffeine can increase the sensitivity of RyR to Ca$^{2+}$ (Sitsapesan et al, 1990). Low concentration (2-5mM) worked as sensitizer for SR Ca$^{2+}$ release; high concentration (10mM) can completely open RyR and is used extensively for evaluation of SR Ca$^{2+}$ content. Ryanodine, a plant alkaloid, binds RyR with high affinity causing bimodal effects. In single channel recording, low dosage of ryanodine can increase SR Ca$^{2+}$ release; high concentration of ryanodine can block RyR completely (Meissner, 1986). The
binding site of ryanodine is proposed to be the channel pore (Callaway et al, 1994); it was reported that the diameter of RyR pore could be changed by ryanodine binding (Tinker and Williams, 1993; Tu et al, 1994); RyR opening can promote ryanodine binding and keep the channel in the open state (Chu et al, 1990). Tetracaine is a potent local anesthetic, which can bind to RyR in a reversible manner. At low concentrations, tetracaine causes an inhibition of SR Ca^{2+} release; while at high concentrations, tetracaine blocks SR Ca^{2+} release completely (Györke et al, 1997)

**Ca^{2+} Sparks, Ca^{2+} transients and Ca^{2+} waves**

The Ca^{2+} spark is a local increase in the concentration of intracellular Ca^{2+} and regarded as the elementary unit of SR Ca^{2+} release. It comes from the opening of a group of SR Ca^{2+} release channels (RyRs, 6-20) and the release normally does not propagate to surrounding sites (Blatter et al, 1997; Lukyanenko et al, 2000). It can be detected with a laser scanning confocal microscope and fluorescent Ca^{2+} indicator (Cheng et al, 1993). A prevailing concept stated that the whole cell global Ca^{2+} release (Ca^{2+} transient) triggered by L-type Ca^{2+} current is the summation of Ca^{2+} sparks spatially and temporally (Wier and Balke, 1999). When a Ca^{2+} spark is large enough to activate other Ca^{2+} release channels around through CICR (calcium induce calcium release), it can cause a propagation of Ca^{2+} sparks which is termed as “Ca^{2+} wave”. A “fire diffuse fire” mechanism on the basis of CICR is thought to underlie Ca^{2+} waves:
Ca\(^{2+}\) released during wave propagation at one site by “firing” diffuses to adjacent sites (Stern, 1992). Then corresponding rise in Ca\(^{2+}\) concentration induces another CICR which is the next firing and carries on the wave (Stern, 1992; Keizer et al., 1998). Ca\(^{2+}\) wave is therefore regarded as spontaneous SR Ca\(^{2+}\) release, which is thought the summation of not temporally synchronized Ca\(^{2+}\) sparks. Some studies showed that Ca\(^{2+}\) wave can greatly increase diastolic Ca\(^{2+}\) and then trigger cardiac arrhythmia.

**SR Ca\(^{2+}\) leak**

The SR, as the highly special intracellular Ca\(^{2+}\) store organelle, contains ~1 mM free Ca\(^{2+}\) and ~2.7 mmol/L total Ca\(^{2+}\) (Shannon and Bers, 1997; Shannon et al., 2003), while the diastolic cytosol [Ca\(^{2+}\)] is 50-100nM. Due to this large chemical driving force, there is a silent efflux of Ca\(^{2+}\) from SR to cytosol under resting conditions, which is termed as “SR Ca\(^{2+}\) leak” (Gomez et al., 1996; Zalk et al., 2007). This Ca\(^{2+}\) leak is primarily mediated by RyR and can be detected in intact cell through Ca\(^{2+}\) sparks, which indicate a local Ca\(^{2+}\) increase by ~200nM within 2 um spatial diameter and last about 50 ms (Bers, 2001; Cheng et al., 1993). These irregular and spontaneous leak events, as a comparison to synchronized SR Ca\(^{2+}\) release (Ca\(^{2+}\) transient) by stimulation, are only defined to happen in resting condition. Although Ca\(^{2+}\) spark is well-established intracellular local SR Ca\(^{2+}\) release event in cardiomyocytes, in adult mammalian skeletal muscle this phenomenon is rarely observed. The reason could be the property of
RyR1, which is activated very differently through physical interaction between L-type Ca\(^{2+}\) channel and RyR1, not through CICR. This section is going to focus on cardiac myocytes SR Ca\(^{2+}\) leak.

Under physiological condition, the normal SR Ca\(^{2+}\) leak helps maintain various cellular functions, such as: 1, gene transcription, a process termed as excitation-transcription coupling (ETC) (Atar et al, 1995); 2, post-translational protein modification, a process very critical for cell viability (Anderson et al, 2007, George et al, 2007); 3, enhancing local regenerativity and reliability of CICR (Keller et al, 2007; Lindegger et al, 2005); 4, protection by preventing SR Ca\(^{2+}\) overload in some extreme condition like β-adrenergic over-stimulation (Yang et al, 2007). Although Zima et al (2010) indicated importance of non-spark-mediated leak and that a complete block of RyR2 can’t totally abolish the leak in physiology condition, RyR2 mediation is still regarded as the major pathway for SR Ca\(^{2+}\) leak. RyR2 is normally closed at resting state under low cytosolic diastolic Ca\(^{2+}\) (~50-100nM). But in pathological states like heart failure and CPVT, due to pathogenetic modification on RyR2, there is a dramatically enhanced SR Ca\(^{2+}\) leak, primarily mediated through RyR2 (Shannon et al, 2003; Maier et al, 2003; Ai et al, 2005; Kohlhaas et al, 2006; Lehnart et al, 2006; Curran et al, 2007). The patho-physiological role of SR Ca\(^{2+}\) leak can work as 3 primary causes for progressive deterioration in cardiac function: 1, increased SR Ca\(^{2+}\) leak can work as abnormal Ca\(^{2+}\) signal which is involved in directly inducing gene transcription leading to hypertrophic remodeling (Frey et al, 2000; Wu et al,
2006; Molkentin et al, 2006; Mckinsey et al, 2002). 2, Increased SR Ca\[^{2+}\] leak combined with reduced SR Ca\[^{2+}\]–ATPase (SERCA) in HF (Bers, 2001), can predominantly reduce SR Ca\[^{2+}\] load and impair contractile function (Houser et al, 2000; Pogwizd et al, 2001). 3, Increased SR Ca\[^{2+}\] leak could activate inward current through Na\[^{+}\]/Ca\[^{2+}\] exchanger (Vermeulen et al, 1994), and further evoke delayed after depolarizations (DADs) and arrhythmogenicity (Pogwizd and Bers 2004; Shannon et al, 2003). All these properties now make RyR2 mediated SR Ca\[^{2+}\] leak a potential therapeutic target for anti-HF and anti-arrhythmia.

**Main mechanisms for enhanced SR Ca\[^{2+}\] leak**

1. **FKBP12.6 dissociation by PKA phosphorylation**

   This hypothesis was first proposed by Marx and colleagues (Marx et al, 2000). There are two main supportive points for this theory: (1), FKBP12.6 is the main stabilizer to keep RyR2 in a fully closed state, and (2), RyR2 hyperphosphorylation at Ser2808 by PKA can dissociate FKBP12.6 from RyR2 causing a leaky state. One of the main features for HF is the chronic hyperadrenergic state, causing a relatively high plasma catecholamine level and β-adrenergic over stimulation. Then PKA can be chronically over activated in a cAMP-dependent pathway and hyperphosphorylate RyR2 at Ser2808 to dissociate FKBP12.6 (Marx et al, 2000). Once in the hyperphosphorlated state that means 3-4 of 4 monomers are phosphorylated, RyR2 loses FKBP12.6 association and could not close completely. So that RyR2 is sensitized to
cytosolic Ca\textsuperscript{2+} leading to an enhanced open probability and diastolic SR Ca\textsuperscript{2+} leak (Lehnart et al, 2004). PKA hyperphosphorylation / FKBP12.6-depletion theory is supported by other studies about cAMP-specific PDE4D3, a local phosphodiesterase that could decrease cAMP though negative feedback (Lehart et al, 2005); PDE4D3 dissociation from RyR can cause elevated cAMP for chronic activation of PKA and RyR hyperphosphorylation. In some HF models, RyR2 complex is also found less associated with phosphatases (PP1, PP2a), which can decrease RyR2 dephosphorylation rate and keep the balance in favor of hyperphosphorylation (Reiken et al, 2001). Some knock-in mouse model studies also provide support for PKA hyperphosphorylation theory. In RyR2-S2808A knock-in mouse, RyR2 can’t be phosphorylated at Ser2808 and FKBP12.6 dissociation upon PKA activation is not observed. In post-myocardial infarction (MI) HF model, RyR2-S2808A mice exhibit stronger resistance to the progression of HF than wild-type (WT) mice (Wehrens et al, 2006). PDE4D3 knock-out mice also showed cardiomyopathy with hyperphosphorylated RyR2 at Ser2808 and FKBP12.6 depletion (Lehnart et al, 2005). Furthermore, the direct genetical depletion of FKBP12.6 showed that this mouse had an increased propensity for exercise-induced ventricular tachycardia (Wehrens et al, 2003).

But in the past 10 years, this mechanism also met challenges constantly from other groups for both two main supporting points, focusing on whether PKA-mediated hyperphosphorylation at Ser2808 could dissociate FKBP12.6 from RyR2 and whether FKBP12.6 binding could alter RyR2 gating. Jiang et al (2002)
found that, in human and canine failing heart, FKBP12.6 exhibits unchanged binding affinity to hyperphosphorylated RyR2, compared with that from non-failing heart. In a recent paper, through direct measurement of binding affinity in permeabilized cardiomyocytes, PKA phosphorylation of RyR2 did not change binding properties between FKBP12.6 and RyR2 (Guo et al, 2010); the same study also showed that saturating RyR2 with FKBP12.6 only provides a mild RyR2 inhibition (~20%), suggesting a small relevance for RyR2 regulation in cardiomyocytes. Moreover, FKBP12.6 knock-out mice failed to demonstrate increased arrhythmia propensity by the combination of epinephrine and caffeine, which is the classical method to induce over SR Ca\(^{2+}\) leak (Xiao et al, 2007). Knock-in mice (RyR2-S2808A), where RyR2-2808 is nonphosphorylatable, are supposed to provide cardio-protection for PKA phosphorylation. But this knock-in mouse failed to produce relevant cardio-protective function to prevent HF development and progression both in the MI (myocardial infarction) and thoracic aortic constriction HF animal models (Berkusky et al, 2007; Zhang & Houser, 2012). Piece above information together, PKA hyperphosphorylation and FKBP12.6 dissociation mechanism is still highly controversial.

2. CaMKII and CaMKII phosphorylation

CaMKII attracted tremendous interest over the last decade in the cardiac field, because it is broadly involved in ETC and ECC in the heart. In this section, I am going to briefly review the impact of CaMKII on the whole cardiac system, especially on ETC and ECC. CaMKIIδ is the predominant isoform in heart with
two subtypes: δB (with a nuclear localization signal [NLS]) localized to nucleus and δC localized to the cytosol (Maier et al., 2003). CaMK δB has been reported to be broadly involved in ETC through its modulation of gene expression via different transcription factors (Zhang and Brown, 2004). For cardiac hypertrophic growth and gene expression, the Gq/PLC/InsP3 signaling pathway is extensively studied. In this signaling pathway, NE, PE or ET-1 could affect the expression of atrial natriuretic peptide (ANF), brain natriuretic peptide (BNP), myosin light chain-2 (MLC-2), and α, β-myosin heavy chain (Anderson, Brown & Bers 2011). The hypertrophy, caused by those fetal gene expressions, could be prevented by CaMKII inhibition in neonatal rat ventricular myocytes (Zhu et al, 2000; Ramirez et al, 1997). CaMKII also can alter cardiomyocyte hypertrophic gene expression through HDACs and MEF2 signaling. Phosphorylation of class II HDACs mediated by CaMKII can interrupt MEF2-HDAC interaction resulting in HDAC nuclear export while free MEF2 for the activation of downstream hypertrophic gene transcription (Berger et al, 2003; Frey et al, 2000). CaMKIIδc is also reportedly involved in the cardiomyocyte apoptosis via the mitochondria-dependent pathway. Increased CaMKIIδc activity enhances RyR2 mediated SR Ca\(^{2+}\) leak and consequently affect mitochondrial Ca\(^{2+}\) and further impacts on expression of p53 and the pro-apoptotic BAX protein (Zhu et al, 2003 & 2007; Toko et al, 2010). In HF, there is an upregulation of NCX, whose enhanced expression by ISO was thought had a link to the activation of CaMKII and transcription factor AP-1 (Mani et al, 2010).
In ECC, multiple proteins are regulated by CaMKII, including: L-type Ca\(^{2+}\) channel, phospholamban, RyR2, Na and K channels. CaMKII phosphorylates L-type Ca\(^{2+}\) channel causing a positive staircase of \(I_{\text{CaL}}\), representing an increase of \(I_{\text{Ca}}\) amplitude and prolonged LTCC inactivation over a series of pulses (Yuan et al, 1994; Xiao et al, 1994). This phenomenon is termed as Ca\(^{2+}\)-dependent \(I_{\text{CaL}}\) facilitation. Moreover CaMKII\(\delta\)c can cause modifications of Na channels and shift the channel availability to more negative membrane potentials, which means more Na channels can be activated in intermediate inactivated state (Wagner et al 2006). This can produce Brugada syndrome like effects (Bers and Grandi 2009). CaMKII also enhances late non-inactivating \(I_{\text{Na}}\), linked with ischemia / reperfusion injury, HF and long QT syndrome 3 (LQT3). For K channels, CaMKII modification can slow transient outward K current (\(I_{\text{to}}\)) and accelerate their recovery from inactivation, both of which can shorten AP duration and refractory period (Tessier et al, 1999). Over expression of CaMKII is reported to induce down-regulation of inwardly rectifying \(I_{\text{K1}}\), which stabilizes the resting membrane potential. The reduction of \(I_{\text{K1}}\) can increase excitability (Wagner et al, 2009).

Phosphorylation of PLB on Thr-17 by CaMKII can cause a release of PLB inhibition of SERCA, enhances SR Ca\(^{2+}\) uptake and increases SR load (Mattiazzi et al, 1994). In HF, the expression and activation of CaMKII are reportedly increased, resulting in increased RyR2 phosphorylation and diastolic SR Ca\(^{2+}\) leak (Ai et al, 2005). The wildly accepted view is that, RyR2 phosphorylation at Ser2814 by CaMKII activates RyR2 gating during both diastole and systole (Bers
& Grandi 2009; Wehrens et al, 2004; Li et al, 1997; Guo et al, 2006). In a knock-in mouse of RyR2-S2814D to mimic the RyR2 hyperphosphorilation by CaMKII, the mutation caused a significantly enhanced diastolic SR Ca$^{2+}$ leak and demonstrated increased arrhythmia propensity. While RyR2-S2814A mutants, where RyR2 can’t be phosphorylated at Ser2814 by CaMKII, shows resistance to ISO induced arrhythmia (Van Oort et al, 2010). Taken together, it is well accepted that CaMKII mediated RyR2 Ca$^{2+}$ leak is one of the major arrhythmogenic reasons in HF (Chelu et al, 2009).

3. Store Overload Induced Ca$^{2+}$ Release (SOICR)

Chen and colleagues first proposed this concept based on a phenomenon: spontaneous Ca$^{2+}$ release, which is the SR Ca$^{2+}$ release in absence of depolarization activating L-type Ca$^{2+}$ channel (Fabiato et al, 1983; Jiang et al, 2004). Spontaneous Ca$^{2+}$ release can be observed cellularly in the form of Ca$^{2+}$ wave, causing a dramatically local and global cytosolic Ca$^{2+}$ increase. Combined with NCX, Ca$^{2+}$ waves can trigger transient inward current and produce DADs. When the amplitude of DAD reaches the threshold for Na channel activation, an action potential can be triggered, leading to triggered arrhythmias (Bers 2001; Pogwizd & Bers 2004). Spontaneous Ca$^{2+}$ release has a strong dependence on the Ca$^{2+}$ load of SR. Some conditions, such as: β-adrenergic stimulation, digitalis toxicity, elevated extracellular Ca$^{2+}$ concentration, and fast pacing, lead to an increase in SR Ca$^{2+}$ content to an overload state which can subsequently cause inappropriate Ca$^{2+}$ spillover” (spontaneous Ca$^{2+}$ release) during diastole. And
this was termed as store overload-induced Ca\(^{2+}\) release (SOICR) (Xiao et al, 2007).

Since SOICR exhibits a strong link to SR Ca\(^{2+}\) load, it is suggesting that luminal Ca\(^{2+}\) concentration is the most important trigger for this spontaneous SR Ca\(^{2+}\) release. This is very different with the CICR, where the cytosolic Ca\(^{2+}\) underlies the activation of RyR2. So the sensor regulating SR Ca\(^{2+}\) release according to SR luminal Ca\(^{2+}\) is proposed to set a threshold for SR Ca\(^{2+}\) release (Ching et al, 2000). Diaz et al, (1997) found Ca\(^{2+}\) wave occurring at the threshold of SR Ca\(^{2+}\) load of \(~100\) μmol/L cytosol, which is similar to the normal SR Ca\(^{2+}\) content (\(1\sim1.5\) mmol/L) because SR volume is 18 times smaller than that in cytosol (Bers 2001). A SR Ca\(^{2+}\) release by Ca\(^{2+}\) wave could decrease SR Ca\(^{2+}\) load by \(~15\) μmol/L cytosol, which makes SR Ca\(^{2+}\) load fall below threshold (Bers, 2001). Evidence supports the proposition that activation of RyR2 is mediated by a luminal Ca\(^{2+}\) sensor that is distinct from cytosolic Ca\(^{2+}\) sensor, but the molecular identity of this luminal Ca\(^{2+}\) sensor is not yet identified.

Both RyR2 and CASQ2-complex are proposed as primary luminal Ca\(^{2+}\) sensor, and mutations on RyR2 and CASQ2 cause CPVT, characterized by increased SR Ca\(^{2+}\) leak and DAD triggered arrhythmia. So CPVT provide a very good model for SOICR study. In Chen’s model, with normal RyR2, the threshold for SOICR is higher than the free luminal [Ca\(^{2+}\)], even in SR Ca\(^{2+}\) overload condition, so there is none or little Ca\(^{2+}\) spillover. The CPVT mutation in RyR2 can lower the SOICR threshold, but which is still higher than resting luminal Ca\(^{2+}\)
level while below the level of SR Ca$^{2+}$ in overload state. Thus the increased free Ca$^{2+}$ in SR to the overload state, due to catecholamine stress or digitalis application, can exceed the lowered threshold causing SOICR, while in basal resting state this will not happen (MacLennan & Chen 2009). For mutations on CASQ2, two hypotheses are proposed. (1), the mutations, like R33Q or the reduction of CASQ2, could lower SOICR threshold and sensitize SR Ca$^{2+}$ release channel to luminal Ca$^{2+}$ with the similar effect as RyR2 mutation. (2), the mutation could reduce the level of CASQ2 and/or Ca$^{2+}$ buffering capability; this means that, for the same total Ca$^{2+}$, the free Ca$^{2+}$ could be much higher to exceed the normal SOICR threshold and thereby lead to Ca$^{2+}$ spillover.

In the bilayer experiments, where the sensitivity of RyR2 to luminal Ca$^{2+}$ can be gauged, Jiang and colleagues identified some RyR2 mutations that showed a consistent behavior characterized by an enhanced response of the channel to luminal Ca$^{2+}$ activation (Jiang et al, 2004 & 2005). Those mutations are: L433P and R176Q/T2504M located in the N-terminal region of the channel; mutations S2246L and R2474S located in the central region; and Q4201R, N4104K, R4496C, I4867M, N4895D, and V4653F located in the C-terminal region. In summary, SOICR described an aspect of SR Ca$^{2+}$ leak, the spontaneous Ca$^{2+}$ release (Ca$^{2+}$ wave); the most important consequence is DAD and triggered arrhythmia; the main reasons for SOICR are RyR2 sensitization to luminal Ca$^{2+}$ and enhanced SR Ca$^{2+}$ load.
4. Reduction / oxidation (Redox) Modification

The heart is an obligate aerobic organ with the highest oxygen consumption in the body (Giordano, 2005). As the myocardial O₂ level is low, hypoxia or ischemia could lead to an increased production of redox-active molecules, which can work as both a benevolent and dysfunctional role to cardiac system. Redox-active molecules, mainly including reactive oxygen species (ROS) and reactive nitrogen species (RNS), are widely characterized for their important role in the cardiac pathologies, especially in ischemia-reperfusion injury (Zima & Blatter 2006). ROS are the natural byproducts from normal metabolism of oxygen, which consist of hydroxyl radical (OH•), superoxide (O₂⁻) and hydrogen peroxide (H₂O₂). In hypoxia, ROS level can increase dramatically, therefore cause significant oxidative damage to the cell, which is known as oxidative stress. Reactive nitrogen species (RNS) are a family of active molecules mainly derived from nitric oxide (NO) metabolism. Peroxynitrite (ONOO⁻) is the main active component and has significant effects with various biological targets (Khan et al, 2004). Both ROS and RNS can cause protein alternations mainly by cysteine oxidation. Therefore, in protein oxidative modification, these two species of redox-active molecules are often collectively referred to as ROS/RNS. Catalase, glutathione peroxidase and superoxide dismutase (SOD) are known as the main defense system against this oxidative damage.
With 20 active cysteines that could be sensitive to redox modification per subunit, RyR is a well established redox-modulation channel. Mainly, 3 modifications by redox-active molecules could happen to RyR: (1), the oxidation through disulfide crosslinking: reagents specifically oxidize two spacially closed free SH groups and promote the formation of disulfide bonds between two cysteines within RyR (Eager et al, 1997; Haarmann et al, 1999; Xia et al, 2001); (2), S-nitrosylation which is the addition of a nitroso group to a sulfur atom of an amino acid, and (3), S-glutathionylation, the addition of a glutathione molecule to the amino acid through a disulfide linkage (like protein-SSG). Normally modifications by low concentration of ROS/RNS are reversible, while high level of ROS/RNS is able to cause irreversible modifications leading to defective channels (Ferdinandy and Schulz 2003). In RyR1 several specific cysteines are already identified for redox-modulation. Cys36 and Cys315 are located at N-terminal region and clamp domain (Liu et al, 2005; Hamilton and Serysheva; 2009 Amador 2009). Cys3635 is located in subdomain 3 which is primary CaM binding region (Moore et al, 1999; Sun et al, 2001), and S-nitrosylation of Cys3635 was showed to activate RyR1 by abolishing CaM’s inhibition (Moore et al, 1999). Oxidized glutathione (GSSG) also shows the ability to increase channel activity by dissociating CaM binding from RyR2 (Balshaw et al, 2001). Pathologically, high level nitrosylation to RyR1 has been reported in muscle dystrophy, and is thought to contribute to muscle weakness by increased SR Ca^{2+} leak (Bellinger et al, 2009). For RyR2, the main modification by ROS/RNS is
oxidation. Those modifications are believed to cause defective RyR2 and increase SR Ca\textsuperscript{2+} leak in chronic HF (Terentyev et al, 2008; Belevych et al, 2009). Unlike in RyR1, the specific redox-sensitive cysteine residues on RyR2 have not been identified. Another modification by ROS/RNS is S-nitrosylation modification, in which S-nitrosoglutathione is required (Sun et al, 2008). Notably, the high reactive free radicals (like OH\textsuperscript{·} and O\textsubscript{2}) only have very limited diffusion range which is within nanometers (Haugland RP, 1996). This implies that ROS modification would be predominantly proximal effect and highly compartmentalized (Zima & Blatter 2006).

5. The interruption of interdomain interaction

Based on the studies about RyR1 and RyR2 related mutations, Ikemoto and Yamamoto (2002) first proposed this inter-domain interaction concept. Missense mutations on RyR1 and RyR2 have been found related with two human inherited diseases: catecholaminergic polymorphic ventricular tachycardia (CPVT) and malignant hyperthermia (MH) (Brini 2004). The pattern of all known mutations on RyR is not a random distribution but clustered into 3 major regions: N-terminal domain (1-600 amino acids), central domain (2000-2500 amino acids) and channel core forming region (C-terminal domain, 4000-5000 amino acids). Mutations at either N-terminal or central regions seem to result in similar sensitization of Ca\textsuperscript{2+} release channel and leakiness, so they proposed an inter-domain interaction concept: after protein folding, N-terminal domain and central domain could get close in 3D structure and interact with each other to act as a
regulatory switch for channel-gating. A tight interaction between two domains serves to stabilize the channel in the full closed "zipping" state; a mutation in either domain can weaken this inter-domain interaction and cause the channel in the "unzipping" state (Fig 3b), resulting in channel hypersensitivity and Ca^{2+} leakiness (Yano & Matsuzaki 2005).

To prove this theory, some synthetic domain peptides are developed for inter-domain unzipping. In RyR1, DP4 corresponding to the Leu2442–Pro2477 in central domain, was shown to increase RyR1 open probability by enhanced [3H]-ryanodine binding and further induced Ca^{2+} release from the SR vesicles (Yamamoto et al, 2000). DP4 also can increase the frequency of Ca^{2+} sparks in skinned skeleton fibers, and open probability of RyR1 in bilayer experiment (Shtifman et al, 2002). The proposed mechanism is that DP4 can insert into the zipped domain pair causing domain unzipping and channel leakiness (Fig 3b). A similar cardiac-domain peptide (DPc10) is also synthesized, corresponding to the Gly2460–Pro2495 in the central domain of RyR2 (Yamamoto and Ikemoto 2002). DPc10 has been shown to produce significant activation of the RyR2 at low Ca^{2+} concentrations, which is similar as DP4’s effect to RyR1 (Yamamoto and Ikemoto 2002). In pathological canine HF model induced by rapid RV pacing, Oda et al (2005) showed the domain unzipping and abnormal Ca^{2+} leakiness in HF; furthermore they suggested a possible link between domain unzipping and FKBP12.6 dissociation. They hypothesized that, in the DPc10-induced domain unzipping model, the domain unzipping can facilitate both cAMP-dependent
RyR2 hyperphosphorylation and FKBP12.6 dissociation (Oda et al, 2005). However, DPc10 induced domain unzipping and SR $\text{Ca}^{2+}$ leaking could happen with no change to RyR2 phosphorylation and RyR2-bound FKBP12.6 (Oda et al, 2005), which revealed no or weak relation between domain unzipping and FKBP12.6 binding. Although the relation between PKA phosphorylation / FKBP dissociation and domain interaction are still not clear, Yano and colleagues think the domain unzipping is the key mechanism for the destabilization of RyR2 and $\text{Ca}^{2+}$ leakiness (Yano M, 2006).

With an insertion of green fluorescent protein (GFP) into defined position in the primary amino acid sequence and followed difference mapping, Liu et al (2005) showed a physical proximity between N- and central domains in the 3D structure of RyR2. They also find FRET interaction between these two regulatory domains in HEK cell with CFP/YFP labeled N- and central domains, However, the zipping pair (N- and central domains) is not from the same monomer but from neighbor RyR2 subunits. The treatment of DPc10 (Fig 3c) or caffeine-ryanodine (Fig 3a) can decrease the FRET interaction causing the unzipping condition in the visualized method; dantrolene, the effective medication for MH caused by RyR1 mutation, can prevent the domain unzipping caused by DPc10 insertion (Liu et al, 2010).
Fig 3: Domain unzipping. a, domains, in the tightly zipping state, was unzipped through agonists like high Ca$^{2+}$ and caffeine + ryanodine. b, unzipping by CPVT mutation. c, unzipping by synthetic domain peptide DPx. (Ikemoto 2002).
Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT)

CPVT, a primary electrical myocardial disease without structural cardiac abnormalities, is characterized by exercise and stress related ventricular tachycardia which may lead to ventricular fibrillation and sudden death (Priori SG & Chen SR, 2010). It is thought to affect as many as one in ten thousand people, and to cause as high as 15% of all unexplained sudden cardiac deaths in young people (Guy Katz et al, 2009). Bidirectional tachycardia, which is a ventricular arrhythmia with a 180° alternation for QRS axis (in EKG) on a beat-to-beat basis, is considered as a typical diagnostic marker for CPVT (Coumel P, et al, 1978; Priori SG, et al, 2001). But some individuals also have polymorphic VT instead of a "stable" QRS axis alternation. Mutations on the genes encoding RyR2 and CASQ2, are currently known to cause CPVT (Priori SG, et al, 2001; Lahat H, et al, 2001). It is accepted that CPVT requires a synergy between a genetic defect (i.e., mutation) to sensitize SR Ca²⁺ release channel, and an external stimulus (i.e., stress or exercise) which can dramatically increase the SR Ca²⁺ content for SR Ca²⁺ leak. All these conditions working together lead to aberrant Ca²⁺ release, which consequently activates NCX’s function, generating transient inward currents and triggering arrhythmias.

CPVT1 is caused by mutations on RyR2 in the autosomal-dominant form. Now 82 mutations are reported to cause CPVT1; 79 are point mutations and 3 are small deletion/insertion (Priori & Chen, 2010). Those mutations are considered to confer RyR2 an abnormal gain of function, assumed to cause
spontaneous Ca$^{2+}$ release due to altered sensitivity to luminal Ca$^{2+}$ or / and cytosolic Ca$^{2+}$, or increased response to phosphorylation by sympathetic agonist (Priori & Chen, 2010; Guy Katz & Michael, 2009). These mutations are not evenly distributed across the entire RyR2 coding region, but clustered into well-defined domains: domain I (amino acid 77 to 466), domain II (amino acid 2246 to 2534), domain III (amino acid 3778 to 4201), domain IV (amino acid 4497 to 4959) (Guy Katz & Michael, 2009). Two mechanisms are proposed for CPVT1. The First one is FKBP12.6 related; Wehrens et al (2003) first hypothesized that CPVT mutations could impair FKBP12.6 binding to RyR2 and cause channel leaking. In bilayer experiments for some CPVT1 mutations on central region (S2246L, P2328S, R2474S) and C-terminal region (Q4201R, R4494C, V4653F), they found a common decreased affinity of FKBP12.6 to RyR2 with any those mutation (Wehrens et al, 2003; Lehnart et al, 2008). However, some studies found different results that CPVT1 mutations had no effect on FKBP12.6 binding (Jiang et al, 2005; George CH et al, 2003). So both in HF and CPVT, the role of FKBP12.6 is highly controversial, at least it won’t be considered as the sole causative reason for all CPVT1 mutation (Priori & Chen, 2010). The other alternative mechanism is the so called “domain unzipping” proposed by Ikemoto and Yano, which was supported by recent RyR2 3D structure study (Amador FJ, et al, 2009; Lobo PA, et al, 2009; Tung CC, et al, 2010), indicating that most CPVT mutations are located on the interface of domains where domain-domain interaction could be disrupted.
Mutations in the gene for cardiac calsequestrin isoform 2 (CASQ2), can cause an autosomal recessive form of CPVT (CPVT2). 8 missense mutations are reported until now. R33Q and D307 are first reported to reduce the level of CASQ2 to 5% and 45% respectively (Rizzi N, et al 2008; Song L, et al 2007); along with other missense mutations (K206N, L167H), those four were also showed to decrease the Ca^{2+} binding capacity or/and Ca^{2+}–dependent polymerization of CASQ2 (Rizzi N, et al 2008; Bal NC, et al, 2010; Kirchhefer U, et al 2010; Valle G, et al 2008). With the same amount of total Ca^{2+}, reduced SR Ca^{2+} buffering will result in a fast SR fill-up of free Ca^{2+} during diastole and potentially higher level of SR free Ca^{2+}. Both conditions can increase the propensity for SOICR and consequently DAD. Beside the reduction of SR Ca^{2+} buffering capacity, R33Q alters RyR2 sensitivity to luminal Ca^{2+} lowering the threshold for SR Ca^{2+} spontaneous release (Qin J, et al 2008; Terentyev D, et al 2008). However, since CASQ2 works as the major intra-SR Ca^{2+} buffer, the reduction of CASQ2 protein and Ca^{2+} buffering capacity is thought the main mechanism for CPVT2 muatation (Knollmann BC, 2009).

**Calmodulin**

Calmodulin (CaM) is a ubiquitous and highly conserved Ca^{2+} binding protein which regulates multiple cellular processes, such as channel gating, Ca^{2+} release, and cell cycle progression. With 148 highly conserved residues and ~16.7kDa molecular weight, CaM has two globular Ca^{2+} binding domains with E-
F hands, connected by a flexible tether helix (Jurado, Chockalingam & Jarrett, 1999). At high [Ca\(^{2+}\)], Ca\(^{2+}\) binds cooperatively to CaM and results in a conformational change that translate intracellular Ca\(^{2+}\) fluctuation signal to diverse targets, including more than 30 different target proteins, such as: phosphodiesterase (PDE), myosin light chain kinase (MLCK), protein phosphatase calcineurin (CaN), adenylyl cyclase (AC), nitric-oxide synthase (NOS), Ca\(^{2+}\)-activated potassium channels, Ca/CaM-dependent kinase (CaMK), L-type Ca\(^{2+}\) channels (LTCC) and RyRs (Means et al, 1991; Vogel et al, 1994; James P, et al 1995; Jurado, Chockalingam & Jarrett, 1999; Maier & Bers, 2002). CaM is widely involved not only in EC-coupling by L-type Ca\(^{2+}\) channel inactivation (Zuhlke et al, 1999; Peterson et al, 2000) and RyR2 regulation, but also in ET-coupling for hypertrophic signaling transduction especially mediated through CaMKII. The total cellular CaM concentration is in the range of 2-25 μM, which varies according to different tissues and species (Kakiuchi et al, 1982). In cardiomyocytes, the total CaM is about 2-5 μM depending on the species (Maier 2006). It was estimated that, in resting conditions, ~90-95% cellular CaM is bound to target protein, and free CaM concentration is about 50-75 nM (Wu et al, 2006)

CaM binds to RyR2 stoichiometrically (four CaMs per tetrameric RyR2), and works as a critical regulator for SR Ca\(^{2+}\) release. The primary CaM binding region on RyR2 is the residues 3583-3603 (Yamaguchi et al 2003). CaM binds to RyR in a Ca\(^{2+}\)-dependent way. It is proposed that CaM can switch between two
binding positions on RyR due to oscillating intracellular Ca\(^{2+}\) concentration (Jurado, Chockalingam & Jarrett, 1999; Samso et al, 2002). However, this position switch can not be found by FRET measurements (Cornea et al, 2009; Guo et al, 2011). Mutations to highly conserved CaM-binding region on critical residues can nearly abolish CaM binding to RyR2. Meissner group bioengineered a knock-in mouse in which three amino acids in CaM binding region are mutated (RyR2-w3587A/L3597D/F3603A). This triple-mutation greatly impairs CaM binding to RyR2 is greatly impaired without interference to other CaM signaling pathways and lead to severe hypertrophic cardiomyopathy at early stage (Yamaguchi et al, 2007). In Cryo-EM based 3D RyR reconstruction, Ca\(^{2+}\)-free CaM (apoCaM) binds to a cleft in the cytosolic domain 3 (AA 3614-3643) (Moor et al 1999; Wagenknecht et al 1994), with a distance of 60-70Å to FKBP12.6 on the same face of RyR2 (Cornea et al, 2009; Gao et al, 2011). For the functional meaning, CaM association can inhibit RyR2 activity both for single channel recording and permeabilized cells. It was reported that CaM could stabilize the RyR2 in closed state during diastole and could facilitate the termination of Ca\(^{2+}\) release by decreasing the probability of channel reopening, thereby prolonging the closed time in bilayer experiment (Xu et al, 2004). In permeabilized cardiomyocytes, increasing CaM binding to RyR2 can decrease Ca\(^{2+}\) spark frequency by ~70% (Guo et al 2006). In the non-ischemic HF model, CaM binding affinity to RyR2 and associated CaM was decreased without changing total CaM, and increasing CaM association to RyR2 can reduce SR Ca\(^{2+}\) leak in
HF cardiomyocytes (Ai et al, 2005; Ono et al 2010). A recent study indicates that defective CaM binding to RyR2 is involved in CPVT-associated RyR2 dysfunction (Xu et al, 2010). Taken together, all these reports suggest that CaM-RyR2 interaction could be a critical molecular substrate for arrhythmias and HF pathogenic processes.

**FK-506 Binding protein**

FKBP is a family of intracellular protein named for its binding to immunosuppressant FK506. Two isoforms of FKBP are expressed in the heart, FKBP12 and FKBP12.6 (~12 kDa and 108 residues). They are very similar in sequence (85% homology, 18 residues different) and 3-D structure (Standaert RF ea al, 1990; Lam E et al, 1995). The main structure of FKBP12/12.6 consists of a large antiparallel five-stranded, amphiphilic β-sheet which wrapped with its hydrophobic face against an amphiphilic α-helix, and thus forms a well-ordered hydrophobic core, providing a binding site for FK506/Rapamycin (Van Duyne et al, 1993; Griffith et al, 1995; Huse et al, 1999). Residues 81 to 96 in FKBP12&12.6 form a small flexible subdomain that interacts with FK506 and other target proteins (Huse et al 1999; Ke et al 2003). FKBP12 and 12.6 were found to associate with RyRs primarily and stoichiometrically (i.e., 4 FKBP: 1RyR tetramer / 1 FKBP12: 1 RyR monomer) (Timerman et al, 1993 & 1996). Guo et al (2010) described FKBP binding kinetics in permeabilized cardiac myocytes. FKBP12.6 preferentially binds to RyR2 with very high affinity (K_d=~1nM), while
FKBP12’s binding affinity is much lower (K_d=\sim 200\text{nM}). However, the expression of FKBP12.6 in mouse cardiac myocytes is low, so that only 10-20\% of RyR2s are associated with FKBP12.6, and the remaining RyRs are associated with FKBP12 (Guo et al, 2010).

The function for FKBP12/RyR1 interaction is well understood, with some consensus that FKBP12 stabilizes the full conductance state (Brillantes et al. 1994; Ahern et al. 1997), the closed state (Timerman et al. 1993; Gaburjakova et al. 2001), and coupled gating between neighboring RyR1 channels (Brillantes et al. 1994; Marx et al. 1998), thus preventing spontaneous channel openings, and subconductance states. In addition, FKBP12 is proposed to enhance the gain of EC coupling in skeletal muscle (Lamb et al. 1996; Avila et al. 2003). But in cardiac myocytes, FKBP12 association to RyR2 could not reduce RyR2 activity (Ca^{2+} sparks) (Guo, et al, 2010). A similar role for FKBP12.6 as RyR2 channel-stabilizing regulator has been supported by evidence from some groups (Kaftan et al. 1996; McCall et al. 1996; Xiao et al. 1997; Prestle et al. 2001; Xin et al. 2002; George et al. 2003; Kohno et al. 2003; Yano et al. 2003) but are opposed by others (Timerman et al. 1996; Barg et al. 1997; Xiao et al. 2007; Jones et al. 2008), so the function for FKBP12.6 as the RyR2 stablizer is still controversial (reviewed in “Main mechanisms for enhanced SR Ca^{2+} leak”).
Fluorescence resonance energy transfer (FRET)

FRET is a phenomenon in which one excited chromophore (donor) can transfer its excitation energy to a nearby neighbor (acceptor). The energy transferred between the donor and acceptor is through nonradiative dipole–dipole coupling (Volkhard 2007), which is a “radiationless” mechanism and termed "Förster resonance energy transfer". This name is from its discoverer, a German scientist Theodor Förster. In most cases, the FRET happened between fluorophores, where the energy transfering can be detected through fluorescence change. So it is also called "fluorescence resonance energy transfer". There are two requirements for fluorescence resonance energy transfer (FRET): one is the spectrum requirement, which is a significant overlap between the donor emission spectrum and acceptor absorption spectrum; the other one is proximity requirement, which means the distance between donor and acceptor must be minimal (usually less than 10nm).

FRET has a broad application for protein studies, especially worked as a sensitive molecular ruler for an accurate distance measurement between two labeled proteins and further defining the potential interaction between those two proteins (LeTilly and Royer, 1993). Another one of most important applications is to detect conformation changes within a protein complex, in which two domains are labeled with fluophores. The conformation change can be detected through FRET efficiency response which reflects the subtle change of two labeling fluophores' orientation and distance (LeTilly and Royer, 1993). Additionally,
FRET is applied for many other studies like: protein synthesis and turnover, manipulation of protein activity, and monitoring enzyme activity, et al (Giepmans, Adams, Ellisman, and Tsien, 2006).
CHAPTER III
MATERIALS AND METHODS

Heart Failure Model

In Sprague-Dawley rats, heart failure was induced and developed by coronary ligation as Tanonaka approach (Tanonaka et al, 2001; Lin et al, 2007). The rat HF model, developed by Anne Knowlton’s lab (UC Davis), produces HF in 12 weeks after surgery. The animal protocol was approved by the University of California, Davis Animal Research Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Cardiac myocyte isolation

Isolation of ventricular myocytes was carried out as previous description (Li et al, 2002). RyR2^{+/ADA} KI mice were provided by Dr G. Meissner (University of North Carolina). All procedures were performed according to the Guiding Principles in the Care and Use of the Animals approved by the Council of American Physiological Society. Hearts were excised from WT or RyR2^{+/ADA} KI mice, rats (normal and HF). After the animal was anesthetized by 3-5% isoflurane for 1 min, hearts were excised out and hung on to a Langendorff perfusion apparatus (37 °C) via aortic cannulation and secured with a stainless steel alligator clip and tied with silk suture to the cannula. Hearts were perfused
at constant pressure with 0 Ca\(^{2+}\) NT through the coronary circulation for approximately 5 minutes. This assured that all blood was washed off from the coronary system. The perfusion solution was then switched to 0 Ca\(^{2+}\) NT solution containing 0.8 mg/ml collagenase (type B, Boehringer Mannheim) for 7-12 min, until the heart became flaccid. Hearts were then removed from the perfusion system and the atria were discarded. The ventricular tissue was chopped into small pieces and incubated for 10 min at 37°C in a shaking water bath in the presence of both collagenase and protease. Myocytes were subjected a dissociation by gentle agitation and filtered through two layers of gauze. Extracellular [Ca\(^{2+}\)] was progressively raised to 1 mM in three successive washes, each 5 minutes. Myocytes were placed finally in NT solution (in mM): NaCl 140, KCl 4, MgCl\(_2\) 1, CaCl\(_2\) 1, glucose 10, HEPES 5, pH 7.4. All experiments were performed at room temperature (23°C).

**Cell permeabilization**

Cell permeabilization provides a useful tool to control the intracellular environment and to introduce exogenous agents into cells while keeping relative intact cellular surroundings (Lukyanenko and Gyorke, 1999). Saponin permeabilization has been used in intracellular Ca\(^{2+}\) signal recording, such as Ca\(^{2+}\) spark/wave measurements through confocal microscopy. In this project, through cell permeabilization, I can keep the [Ca\(^{2+}\)] tightly controlled and prevent the complication from the L-type Ca\(^{2+}\) channels which localize on the...
sarcolemmal membrane. The potential disadvantage of this method is that some cellular contents can be lost, which can be partially compensated by experiment design.

All myocytes originally suspended in NT solution were transferred to relaxation solution, and then were permeabilized by saponin (50µg/ml). For fluorescent protein required experiments, myocytes were permeabilized for 3 minutes. For only fluorescent dye required experiments, myocytes were permeabilized for 20-30 seconds. Relaxation and permeabilization solution contain (mM): EGTA 0.1, HEPES 10, K-aspartate 120, MgCl₂ 1, ATP 5, reduced glutathione 10, phosphocreatine di-Tris 5 and saponin 50 µg/ml (only in permeabilizing solution), pH 7.4. After permeabilization, myocytes were superfused with internal solution (mmol/L): EGTA 0.5, HEPES 10, K-aspartate 120, ATP 5; free MgCl₂ 1, reduced glutathione 10, [Ca²⁺] 50 nmol/L (calculated using MaxChelator), creatine phosphokinase 5 U/ml, phosphocreatine 10, dextran (MW: 40,000) 4%, pH 7.2.

**Fluorescent labeling of single-cysteine mutants of FKBP12.6 and CaM**

The fluorescent FKBP12.6 and CaM were provided by our collaborators: Dr Cornea and Dr Fruen (University of Minnesota). The cDNA of the human FKBP12.6 and CaM were subcloned into the *NdeI/NheI* site of the pRSET expression vector (Invitrogen). This hFKBP12.6/CaM clone was used as a site-directed mutagenesis starting point to create null-Cys FKBP12.6 (C22A/C76I-
FKBP12.6). Site-directed mutagenesis was carried out on these clones using quick change site-directed mutagenesis kits (Stratagene). Expression was carried out in the E. coli BL21 (DE3) pLysS cells, transformed with the mutagenized hFKBP-pRSET vectors.

Recombinant FKBP/CaM variants were purified by a procedure adapted from previous reports (Guo et al, 2010). Briefly, the cell lysate was subjected to 40-60 % ammonium sulfate fractionation. The 40-60% pellet was resolubilized in 10-15 ml of 10 mM HEPES (pH 8.0), 1 mM DTT, 0.1 mM PMSF (Buffer A), dialyzed against 4 L of Buffer A, then subjected to isocratic anion exchange chromatography on DEAE Sephacel (35 ml gel, 3 cm diameter) pre-equilibrated in Buffer A. This column was developed with Buffer A at 0.5 ml/min and FKBP/CaM elutes in the first peak, which was then concentrated to 30 mg/ml, and NaCl was adjusted to 150 mM. A 1 ml volume of this concentrate was then applied to a Sephacryl-200 HR (70 x 1.5 cm) pre-equilibrated with Buffer A supplemented with 150 mM NaCl (Buffer B) at a rate of 0.2 ml/min. Fractions (1ml) were collected and analyzed by electrophoresis. The fractions containing >95% pure FKBP/CaM were pooled, and the purity of FKBP/CaM in this sample was further confirmed by SDS-PAGE. The typical yield was ~30 mg of pure proteins per liter of cell culture. The concentration of purified WT protein used as standard was determined by using their calculated extinction coefficients.

The FKBP/CaM mutant was labeled at its single cysteine using the maleimide derivatives of Alexa Fluor 488 or Alexa Fluor 568. The FKBP/CaM
sample was treated with TCEP (1mM) for 1 hr in buffer containing 20 mM MOPS, 150 mM NaCl, pH 7.5. The fluorophore was applied then from a DMF stock to a final concentration of 1.6 mM, DMF was adjusted to 20% by volume, and the reaction was allowed to proceed for 4 hrs at room temperature, with tumbling, protected from light. Unreacted dye was removed by chromatography on DEAE Sephacel and the sample was dialyzed and concentrated using Amicon device (Millipore, Billerica, MA) into 20 mM MOPS, 30 mM NaCl, pH 7.0 at a protein concentration of 60-100 µM.

**Fluorescence Recovery after Photobleaching (FRAP)**

FRAP is a method that can be used to define protein binding kinetics through optical techniques. First, a protein binding region was photobleached by strong laser illumination, and then fluorescence recovery of this region is recorded. The fluorescence recovery in photobleached region is plotted vs time to yield a curve that reflects the kinetic coefficients for the protein's reversible binding. Diffusion and binding/unbinding are needed to be considered for the fluorescence recovery. If the diffusion is very fast and can be ignored in the whole process, the analysis is very simple since the fluorescence recovery is determined by the rate at which bleached bound proteins dissociate from their binding sites and are replaced by free fluorescent protein in the bath. Note that the recovery depends on dissociation rate ($k_{off}$) only, not the association rate.
(k\textsubscript{on}). Some assumptions are necessary for the data interpretation (Bulinski, 2001):

1. The concentration of bound protein region (like Z-line) is much higher than free protein concentration in the bath, so that the fluorescent contribution of the free protein can be neglected.
2. There is no significant movement for bound proteins during recovery.
3. Compared with binding/unbinding, the free diffusion is much faster. So that the contribution of fluorescence recovery by diffusion can be ignored, and recovery rate reflects the process in which the fluorescent free protein binds and replaces the bound photobleached protein and so recovers the fluorescence.

After three assumptions are satisfied, we can fit the recovery curve with single exponential function and find the on- and off-rate constants for protein binding. In my experiments, all these assumption are statisfied.

Permeabilized myocytes were incubated with FRET pair: 100nM fluorescent FKBP12.6 (F-FKBP12.6, donor) and a range of fluorescent CaM [F-CaM (acceptor)]s. F-CaM bound on RyR2 was detected through FRET by sensitized emission. After steady state was reached, F-CaM was photobleached at Z-lines though 543nM excitation with high laser power (90%) for 50s, resulting in >95% exposed F-CaM fluophore destruction. Thereafter the patterned striation recovery of photobleached region was recorded in the FRET acceptor channel.
(488nM excitation, >600nM recording). This reflects recovery of RyR2 bound CaM through reversible binding, which can be modeled as a first order reaction. The observed recovery rate constant $k_{\text{FRAP}}$ was fitted by a single-exponential function. The observed recovery rate constant, $k_{\text{FRAP}}$, is an integrated measurement depending on both $k_{\text{on}}$ and $k_{\text{off}}$, which can be described by the equation:

$$k_{\text{FRAP}} = k_{\text{on}}[\text{F-CaM}] + k_{\text{off}}.$$ 

Since $k_{\text{FRAP}}$ is F-CaM concentration dependent, measured $k_{\text{FRAP}}$ at various [F-CaM] allows measurement of $k_{\text{on}}$ (slope) and $k_{\text{off}}$ (intercept) in a linear fitting (Guo et al, 2010). Three conditions are important for accurate measurement. 1) There should be no pre-bound endogenous CaM, which was predepleted by suramin. 2) The initial F-CaM application should be around the ~K_d range. 3) The diffusion of F-CaM should be much faster than binding/unbinding process, such that it is not the rate-limiting step.

**FRET between F-CaMs and F-FKBP in permeabilized myocytes**

For the FRET between FKBP12.6 and CaM, we used fluorescent FKBP12.6, labeled at position 14 with green-fluorescent Alexa 488 (AF488-14-FKBP12.6), and F-CaM labeled with red-fluorescent Alexa 568 attached at the position 34 (AF568-34-CaM) (Cornea et al, 2009; Guo et al, 2010 & 2011). After permeabilization, myocytes were placed in internal solution. Fluorescent FKBP12.6 and CaM were added to the bath solution. F-FKBP12.6 (donor) was
excited at 488 nm, and emission was recorded through a 500-530nm band pass filter (donor channel). F-CaM (acceptor for FRET) fluorescence emission was recorded by sensitized emission through a >600nm filter (acceptor channel). We utilized two experimental approaches, (1) comparing the donor fluorescence intensities before and after equilibrating with the acceptor (donor quenching) and (2) monitoring the increase in donor fluorescence after acceptor photobleaching (acceptor photobleaching), to detect and measure FRET signals in the permeabilized cardiomyocytes.

For the donor quenching method, FRET is indicated by a decrease in the donor fluorescence at wavelengths 500-530 nm (AF488-FKBP12.6), after acceptor is added and reaches equilibrium. Both donor and acceptor saturate RyR binding sites. The FRET efficiency is calculated based on the equation:

\[ E = 1 - \frac{F_{DA}}{F_D} \]

\( F_D \) is for the fluorescence intensities of the donor-only state, \( F_{DA} \) stands for the fluorescence recorded through donor channel after adding the acceptor.

For Acceptor photobleaching, permeabilized myocytes were first incubated with AF488-FKBP12.6 and AF568-34-CaM to reach equilibrium. The acceptor photobleaching was achieved by repeated scans with maximum laser power at 543 nm for 60 sec to completely photobleach the acceptor (AF568-34-CaM). There is an increase in donor fluorescence signal when FRET is disrupted by photobleaching of acceptor fluorophore. The FRET efficiency was calculated according to the equation:
\[
E = \left( \frac{I_{\text{donor-post}} - I_{\text{donor-pre}}}{I_{\text{donor-post}}} \right) \times 100\%,
\]

where \(I_{\text{donor-post}}\) and \(I_{\text{donor-pre}}\) are donor fluorescence intensity after and before photobleaching of acceptor fluorescence.

Donor-acceptor distances, \(R\), were calculated from the equation:

\[
R = R_0 (E^{-1}-1)^{\frac{1}{6}},
\]

where the Förster distance, \(R_0\) is defined as the distance at which FRET=0.5. Here \(R_0\) for AF-488 and AF568 is 62Å (Invitrogen.com).

Two control experiments are needed for FRET measurement to detect potential bleeding through or cross talk between donor and acceptor. 1), with donor alone (AF488-FKBP12.6), acceptor channel whether can detect any signal due to the excitation of donor. 2), with acceptor alone (AF568-34-CaM), donor channel whether can detect any signal when acceptors were directly excited through 543nM laser illumination.

**Wash-in/off in myocytes**

Fluorescence changes, after a rapid wash-in and wash-off of fluorescent protein (F-CaM or F-DPc10), can be used to estimate protein binding kinetics in permeabilized myocytes. After permeabilization, pretreatment was applied to predeplete possible endogenous bound CaM and made the binding site available for fluorescent protein. Then I washed in fluorescent protein and recorded the fluorescence increase in myocytes over time. After the fluorescence reached steady state, I washed fluorescent protein off by internal solution and recorded
fluorescence decrease, which was fitted with a single-exponential function. For wash-off, I can establish $k_{\text{off}}$ by fitting the equation: specific-binding = $A \times \exp (-k_{\text{off}} \times t)$, here $A$ stands for the specific binding (fluorescent value) at equilibrium. For wash-in experiment, I can fit the association rate constant ($k_{\text{ob}}$) by the equation: specific binding = $A \times (1 - \exp (-k_{\text{ob}} \times t))$, from which $k_{\text{on}}$ can be calculated through $k_{\text{ob}} = k_{\text{off}} + k_{\text{on}}[F-CaM]$.

**Ca²⁺ signal measurement by fluo-4 with confocal microscopy**

1. **Ca²⁺ signal recording in intact myocytes**

    Ca²⁺ transients were recorded using 5 µM fluo-4-AM dye loading, a single-wavelength Ca²⁺ indicator, and the laser scanning confocal microscope (BioRad, Carl Zeiss). The confocal microscope is equipped with a 40x oil immersion objective lens (n.a. = 1.3). Fluo-4 was excited with the 488 nm line of an argon laser. Emitted fluorescence was collected through a 500-530 nm band pass emission filter. Fluo-4 images were recorded in the line-scan mode with 512 pixels per line at 166 Hz. Ca²⁺ transients were obtained by field stimulation at 1 Hz in normal Tyrode’s solution; the amplitude of Ca²⁺ transient was presented by $F/F_0$; the time constant was fitted by one exponential; SR Ca²⁺ load was assessed by the rapid application of 15 mM caffeine in the normal tyrode (NT).

2. **Ca²⁺ spark measurement and analysis**

    Ca²⁺ sparks were recorded in saponin-permeabilized ventricular cardiomyocytes, which were superfused by internal solution with free 50nM /
100nM \([\text{Ca}^{2+}]\). Sparks were counted and characterized by an algorithm in Interactive Data Language (IDL 5.3 computer software) (Cheng et al., 1999). The program detects \(\text{Ca}^{2+}\) sparks as areas of increased fluorescence compared to the standard deviation (SD) of the background of the fluorescence image. We used the \(\text{Ca}^{2+}\) spark measurement threshold \(3.8 \times \text{s.d.}\), with human verification of \(\text{Ca}^{2+}\) spark detection. \(\text{Ca}^{2+}\) spark amplitudes were normalized \((F/F_0)\) to fluorescence baseline \((F_0)\). Duration of \(\text{Ca}^{2+}\) sparks was taken from the dwell time at > 50% of the peak level (full-duration-half-maximum, FDHM). Width of the \(\text{Ca}^{2+}\) sparks was indicated by the spatial size at > 50% of the peak level (full-width-half-maximum, FWHM). \(\text{Ca}^{2+}\) spark frequency (CaSpF) was normalized to time and spatial size of the volume elements (or voxels) of the cell from which \(\text{Ca}^{2+}\) sparks are measured. It is based on point spread function of the confocal microscope. Under our conditions (zoom = 3.0), the width and length of one voxel is 0.2 \(\mu\text{m}\) & 0.2 \(\mu\text{m}\), and the depth (assumed) is 1 \(\mu\text{m}\), so the voxel is 0.04 \(\mu\text{m}^3\). The fluorescence images were recorded in longitudinal line-scan mode (512 pixels per line), such that the volume of one scan line corresponds to 0.04 \(\mu\text{m}^3\) times the number of pixels that are within the cell length.

**Statistics**

Data are expressed as mean +/- SEM. Significance is evaluated by using paired or unpaired Student’s t test. One way analysis of variance (ANOVA) was
used for multiple comparison. P < 0.05 was considered to be statistically significant.
CHAPTER IV

HYPOTHESES AND AIMS

Cardiac ryanodine receptors (RyR2), intracellular Ca\(^{2+}\) release channels, play a central role in excitation-contraction coupling (ECC). A dysfunctional RyR2, exhibiting enhanced diastolic Ca\(^{2+}\) leak, has been implicated in arrhythmogenesis and heart failure (HF). Excessive diastolic sarcoplasmic reticulum (SR) Ca\(^{2+}\) leak can reduce SR Ca\(^{2+}\) load and generate cardiac arrhythmia. Therefore, the RyR2 leakiness regulation (without affecting normal ECC) is a potentially valuable modality for the treatment of cardiac arrhythmias and HF. As an important regulator, calmodulin (CaM) binds and stabilizes RyR2. Single channel studies suggest that CaM inhibits the RyR2 during diastole and may regulate the shut off of Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR) during systole. And defective CaM-RyR2 interaction is broadly implicated in HF and CPVT models. All these indicate CaM’s essentially patho-physiological meaning for RyR2 regulation. However, direct measurements of RyR2-CaM interaction in myocytes are lacking despite its potential clinical importance. My goal here is to define the physical RyR2-CaM interaction and its functional consequences in situ. The central hypothesis is that the stoichiometry and degree of RyR-CaM binding are key determinants in local SR Ca\(^{2+}\) release. Using fluorescent FKBP12.6 and CaM as well as transgenic mice with defective RyR2-CaM
interaction, I combine FRET and FRAP, Ca\textsuperscript{2+} sparks measurement, and domain unzipping peptide (DPc10) to assess both the RyR2-CaM physical interaction and its functional importance in the native myocyte environment.

**Aim 1. Measure the physical in situ binding properties of RyR2-CaM.**

CaM has numerous downstream target proteins beside the RyR2, such as the L-type Ca\textsuperscript{2+} channel (LTCC), calcineurin (CaN), and Ca\textsuperscript{2+}-CaM dependent protein kinase II (CaMKII). To minimize interfering nonspecific signals from other targets, we can use FRET detection to define the RyR2 bound CaM. Studies indicate that FKBP12.6 and CaM specifically bind to RyR2 in a physically close enough proximity to produce measurable FRET which is not expected at other sites. I apply F-FKBP12.6 and F-CaM in saponin-permeabilized cardiac myocytes and provide original direct measurements of: 1) FRET between CaM and FKBP12.6 on RyR2 in permeabilized myocytes; 2) RyR2-CaM binding properties (steady-state affinity, on-& off-rates) from three independent methods; 3) the percentage of Z-line bound CaM is on RyR2 through using knock-in mice, where a triple-mutation (RyR2\textsuperscript{ADA}) prevents CaM binding to RyR2. The differences of binding stoichiometry and degree of RyR2-CaM interaction between WT and knock-in mice would be correlated with functional attributes of SR Ca\textsuperscript{2+} release in specific aim 2.
Aim 2. Define the physiological consequences of RyR2-CaM binding in cardiac myocytes.

In recent years, CaM has emerged as a critical regulator of SR Ca\(^{2+}\) release. Specifically, CaM helps maintain the RyR2 in a closed state during diastole and facilitates termination of Ca\(^{2+}\) release by decreasing the probability of channel reopening and prolonging the closed times. In non-ischemic canine and rabbit HF models, there is a decreased CaM binding to RyR2 without changes in total CaM. Based on previously published results and our preliminary data, I hypothesize that disrupting the RyR2-CaM interaction will lead to aberrant activation of RyR2 during diastole and systole that could be arrhythmogenic and contributing to HF. This could be manifested as aberrant diastolic and systolic Ca\(^{2+}\) release. Using knock-in mice having defective RyR2-CaM interaction (where interaction with other CaM downstream targets is not affected), I will measure the diastolic Ca\(^{2+}\) leak in permeabilized myocytes where [Ca\(^{2+}\)]\(_i\) is tightly controlled, and systolic Ca\(^{2+}\) transient and fractional release in intact myocytes. The outcome will provide critical new information concerning how RyR2-CaM interaction influences SR Ca\(^{2+}\) release in myocytes.

Aim 3. Define RyR2-CaM binding affinity in heart failure myocytes.

The affinity of RyR2 binding to both CaM and FKBP12.6 are reported to be decreased in HF animal model (Ai et al, 2005, Ono et al, 2010). However, most measurements have been done by using either SR vesicles or cell lysates.
Here for the first time, we use coronary ligation induced rat HF model to define RyR2 binding affinity to CaM and FKBP12.6 in situ.

**Aim 4. Define CaM as a critical RyR2 stabilizer through RyR2 unzipping peptide (DPc10).**

One hypothesis for elevated Ca$^{2+}$ leak through cardiac ryanodine receptors (RyR2) in heart failure (HF) is interdomain “unzipping” that can enhance aberrant channel activation. A peptide (DPc10) corresponding to RyR2 central domain 2460-2495 mimics this arrhythmogenic RyR2 leakiness by unzipping N- and central-domains. CaM binds to RyR2 and stabilizes the closed channel. Little is known about DPc10 binding to the RyR2 and how that may interact with binding (and effects) of CaM with RyR2. There are 3 small aims to reveal DPc10 binding kinetics and the relation between CaM and domain unzipping: 1). DPc10 could bind to RyR2 in a conformation-dependent manner; 2). CaM can prevent RyR2 unzipping caused by DPc10 binding; 3). RyR2 unzipping by DPc10 can decrease CaM binding affinity to RyR2.

All those aims can lead to a better understanding about RyR2-CaM interaction and functional effects on RyR-mediated Ca release in cardiac myocytes.
CHAPTER V

Kinetics of Calmodulin Binding to Cardiac Ryanodine Receptors and Functional Consequences

INTRODUCTION

Approximately 50% of heart failure patients die of ventricular arrhythmia and sudden cardiac death (Noseworthy & Christopher, 2008). Now it is well known that RyR2 mediated SR Ca^{2+} leak could activate inward current through Na/Ca exchanger and evoke delayed after depolarizations (Vermeulen et al., 1994). In HF there is enhanced diastolic SR Ca^{2+} leak via RyR2 and other electrophysiological changes (e.g. up-regulation of NCX) that greatly enhance the propensity for DADs and triggered cardiac arrhythmias (Pogwizd and Bers, 2002; Bers, Pogwizd and Klaus 2002), which is proved to be the leading reason for ventricular tachyarrhythmia and sudden cardiac death (Pogwizd & Bers, 2004; Shannon, Pogwizd and Bers, 2003). Because of RyR2 has emerged as a potential therapeutic target for treating HF and arrhythmia. Therefore, stabilizing RyR2 and preventing abnormal Ca^{2+} leak (without affecting normal excitation contraction coupling) is a potentially valuable modality for the treatment of cardiac arrhythmias and HF.
Calmodulin is a very important RyR2 regulator, but also has many cellular binding partners involved in different cellular processes. With 148 highly conserved residues, CaM has two pairs of globular Ca\(^{2+}\) binding E-F hand domains connected by a flexible tether helix (Jurado, Chockalingam and Jarret, 1991). At high [Ca\(^{2+}\)], Ca\(^{2+}\) binds cooperatively to CaM and result in conformational change that translate intracellular [Ca\(^{2+}\)] signals to diverse targets, including: myosin light chain kinase (MLCK), protein phosphatase calcineurin (CaN), nitric-oxide synthase (NOS), phosphodiesterase (PDE), adenylyl cyclase (AC), Ca\(^{2+}\)/CaM-dependent kinase (CaMK), Ca\(^{2+}\)-activated potassium channels, L-type Ca\(^{2+}\) channels (LTCC) and RyRs (Means et al, 1991; Vogel 1994; James et al, 1995; Maier and Bers, 2002).

CaM binds to RyR2 stoichiometrically (four CaMs per tetrameric RyR2), and works as a critical regulator for SR Ca\(^{2+}\) release. Cryo-EM based 3D RyR reconstruction showed that Ca\(^{2+}\)-free CaM (apoCaM) binds to a cleft in the cytosolic domain 3 (AA 3583-3603) (Yamaguchi et al, 2003), with a distance of 60-70Å to FKBP12.6 bound to the same RyR2 face (Moore et al, 1999; Wagenknecht et al, 1994), and FRET can occur between these two partners (Cornea et al, 2009; Guo et al, 2011). FKBP12.6 was reported to bind RyR2 tightly as an important regulator in RyR2 gating but some aspects are still controversial (Brillantes et al, 1994; Marx et al, 1998; Barg et al, 1997; Guo et al, 2010). It was reported that CaM could stabilize the RyR2 in closed state during diastole and could facilitate the termination of Ca\(^{2+}\) release by decreasing the
probability of channel reopening and prolonging the closed time (Xu et al, 2004). Additionally, defective RyR2-CaM interaction has potentially broad implication in cardiac pathology. Mutations of critical residues in the highly conserved CaM binding region can severely abolish CaM binding to RyR2 and cause severe hypertrophic cardiomyopathy and early death in animal models (Yamaguchi et al, 2007). In non-ischemic HF animal models, CaM binding to RyR2 was decreased (Ai et al, 2005; Ono et al, 2010). A recent study indicates that defective CaM binding to RyR2 is also involved in CPVT-associated RyR2 dysfunction (Xu et al, 2010). All these studies suggest that CaM-RyR2 interaction is a critical molecular substrate for arrhythmias and HF pathogenesis.

However, due to its multiple binding targets, CaM-RyR2 binding affinity is measured in isolated systems, SR vesicles, lipid bilayers or cell lysates, rather than in native cells. Here by using FRET, we can identify RyR-bound CaM vs its numerous other target proteins, and for the first time we characterize CaM-RyR2 interaction properties both for the normal and HF cardiomyocytes in the native cellular environments. The knock-in mice (RyR2+/ADA) (Yamaguchi et al, 2007), with genetically disrupted CaM-RyR2 association while the interaction with other CaM targets are not affected, are used to estimate the percentage of Z-line bound CaM that is on RyR2, and further define pathophysiological consequences of CaM for RyR2 regulation in cardiomyocytes.

MATERIALS AND METHODS

Pretreatment with suramin to predeplete bound endogenous CaM.
In resting cardiomyocytes, endogenous CaM is already prebound to most binding sites. We used suramin to predeplete endogenous bound CaM and make binding sites accessible for F-CaM, allowing accurate measurement of association kinetics. After myocytes permeabilization, suramin (5μM) was applied for 5min, and then followed the wash-off with internal solution for 20 min.

**FRET measurement for F-FKBP12.6 and F-CaM on RyR2 in permeabilized myocytes.**

See details in chapter III

**FRET + steady-state concentration -dependent CaM binding in myocytes.**

Myocytes were first superfused with relaxing solution, and then permeabilized with saponin. Once permeabilized, myocytes were incubated with 5 μM suramin solution for 5min to predeplete endogenous bound CaM and then washed for 20min by internal solution. Then 100nM AF488-FKBP12.6 was washed in to saturate FKBP binding site on RyR2. After that AF543-CaM in different concentrations was washed in, with binding allowed to reach steady state. Excitation wavelengths is 488 nM for FRET and 543 nM for direct Z-line CaM measurement. For FRET, emission is recorded at 500-530nM for donor channel and >600nM for acceptor channel. Thus we have two sets of measurements, one for CaM binding at the Z-line (direct fluorescence) and one that is specific for RyR2 bound CaM (FRET).

**In situ FRET + FRAP, to measure $k_{on}$ & $k_{off}$ for CaM-RyR2 interaction.**
Permeabilized myocytes are incubated with 100nM F-FKBP12.6 and a range of [F-CaM]s, each until steady state is reached. Z-line bound F-CaM will be photobleached by 543nM excitation with high laser power (90%) for 50s, resulting in >95% fluophore destruction. Thereafter the recovery of striation pattern in photobleached region is recorded through the FRET acceptor channel (488nM excitation, >600nM recording).

**FRET + wash-in/off for the measurement of $k_{on}$ and $k_{off}$ for CaM-RyR2 interaction.**

FRET fluorescence changes were monitored in rapid wash-in and wash-off of F-CaM in permeabilized myocytes. After pretreatment with suramin, the permeabilized myocytes were first saturated with F-FKBP12.6 (100 nM). This gave a strong striation pattern in the donor channel. While keeping RyR2 saturated with F-FKBP12.6, F-CaM was washed in and the fluorescence changes over time in both donor and acceptor channel were recorded. After the fluorescence reaching steady state, F-CaM was washed out with keeping RyR2 saturated with F-FKBP12.6 (with excess non-fluorescent CaM to prevent rebinding of F-CaM), and the fluorescence got recorded in acceptor channel.

**Confocal Ca$^{2+}$ imaging**

Ca$^{2+}$ sparks were measured as a readout for diastolic RyR2 activity in saponin-permeabilized (20-30 seconds) ventricular cardiomyocytes, which were superfused by internal solution with free 50nM/100nM Ca$^{2+}$. For details see section III.
For systolic RyR2 function test, intact ventricular myocytes were loaded with Fluo-4 AM (5 μM, Molecular Probes) and Ca²⁺ transients were recorded as previously described (Van Oort et al, 2010). Ca²⁺ transients were obtained by field stimulation at 1 Hz in normal Tyrode’s solution. SR Ca²⁺ load was evaluated by Ca²⁺ transient upon rapid caffeine application (10 mM). Experiments were performed on a confocal microscope (BioRad, Radiance 2100, 40x objective) in line scan mode with argon laser (λex 488 nm, λem >505 nm). Image analysis was done with ImageJ, Ca²⁺ spark master and custom made software implemented in LabVIEW® (National instrumental lve).

**Heart Failure Model**

In Sprague-Dawley rats, heart failure was induced and developed by coronary ligation described as the approach of Tanonaka (Tanonaka et al, 2001; Lin et al, 2007). The rat HF model, developed by Ann Knowlton’s lab (UCD), produces HF in 12 weeks after surgery.

**RESULTS**

**Predepletion of endogenous CaM by suramin**

RyR2s are concentrated along the Z-line, and previous studies showed that CaM is highly concentrated within the same Z-line region and >90% apoCaM binding sites are occupied by endogenous CaM (Wu et al, 2007). Fig 4A(i) shows the striation image of fluorescent CaM (F-CaM) concentrated on Z-line, after 60nM [F-CaM] was washed in. Suramin (5μM) can totally abolish the striations
within 1 min (Fig 4A (ii)); after the wash-off of suramin, F-CaM can rebind at the Z-line with the same striation pattern (Fig. 4A (iii)). Before suramin treatment, F-CaM washed in and yield a moderately slow increase of F-CaM fluorescence at the Z-line ($\tau = 11.5$ min, Fig. 4B). This slow phase represents F-CaM replacing endogenous bound CaM (Wu et al, 2007). After suramin depleted F-CaM binding and was washed off for 20 min (hydrophilicity allows rapid reversibility), F-CaM rebinds much faster at Z-line (Fig. 4C). Now F-CaM striations appeared with $\tau_{\text{fast}} = 2$ min, which represents the time constant for F-CaM binding to unoccupied binding sites at Z-line. Since $B_{\text{max}}$ before and after suramin treatment are the same, we think suramin may completely deplete endogenous CaM bound at Z-line which are then totally washed off.
**Fig 4: Suramin can predeplete endogenous CaM.** A, confocal images of saponin-permeabilized myocytes incubated with 60nM F-CaM in different conditions. (i), after 40min incubation, fluorescent striations reached B_max. (ii), 10μM suramin can totally abolish fluorescent striations within 1 min. (iii), after suramin treatment and rewash-in F-CaM, fluorescent striation reached B_max much faster. B, time course for 60nM F-CaM wash-in, immediately after permeabilization. C, time course for 60nM F-CaM rewash-in, after suramin treatment.
FRET between FKBP12.6 and CaM on the RyR

Both CaM and FKBP12.6 bind RyR1 at a very close proximity and get FRET interaction (Cornea et al, 2009). In cardiac myocytes CaM and FKBP12.6 are concentrated at the Z-line (Guo et al, 2010; Xu et al, 2007). Here, using acceptor photobleach, we investigate the efficiency of FRET pair (F-FKBP and F-CaM) which reflects the distance between these two proteins on RyR2 in cardiac myocytes. After F-FKBP12.6 and F-CaM are preequilibrated, we observed striation both at donor channel (green, Em = 500-530 nM) and acceptor channel (red, Em > 600 nM). Acceptor (F-CaM) was selectively photobleached by powerful 543nM excitation in the central region, while the donor fluorescence got enhanced only in this region (Fig 5A). The average FRET efficiency is 0.35 ± 0.04 (n=8) reflecting a distance of 69 ± 0.7 Å (Fig 5B) between this FRET pair, which matches well with the result on RyR1 (Cornea et al, 2009). As a valuable control, repeating the photobleaching with only AF488-FKB12.6 present had no effect on donor emission (Fig. 5C). Based on a RyR1 EM-model, the distance between FKBP and ApoCaMs on the same side of RyR is 50-60 Å (Samso et al, 2002), which is well within the FRET range (10-100 Å). The distance between FKBP12.6 and CaMs on other RyR faces could be: R2=122 Å, R3=232 Å and R4=207 Å (Fig 5D), which are all out range of FRET with respect to AF488 and AF568 (R0 = 62 Å). Therefore, we think 69 ± 0.7 Å is the distance for the same side FKBP12.6 and CaM, and there is no possibility of FRET interference from the other side of RyR2.
Fig 5: FRET between F-FKBP12.6 (donor) and F-CaM (acceptor) in permeabilized rat myocytes. A, permeabilized myocytes were incubated with 100nM F-FKBP12.6 and 1uM F-CaM to reach steady-state. After acceptor photobleaching, there is a fluorescence enhancement in donor channel. B, the average FRET efficiency and reflected distance for F-FKBP12.6 and F-CaM on RyR2. C, acceptor photobleaching had no effect on donor emission. D, based on RyR1 EM-model, the distance between F-FKBP and F-CaM on the same face of RyR (R₁) is 50-60Å (Samso et al, 2002); the distances to other side potential FRET partner (R₂, R₃, R₄) are out of FRET range.
Measurement of binding affinity ($K_d$) of CaM-RyR2

The strong FRET interaction between F-FKBP12.6 and F-CaM provides us with an effective approach to distinguish CaM bound to RyR2 vs other CaM binding partners (Guo et al, 2011). After suramin pretreatment, 100nM AF488-FKBP12.6 (donor) first was washed in to saturate FKBP binding site on RyR2 (Guo et al, 2011), then AF543-CaM (acceptor) at different concentrations was washed in, with binding allowed to reach steady state. The RyR2 bound CaM can be detected through FRET by sensitized emission, where excitation wavelengths is 488 nM for FRET measurement and emission is recorded at 500-530nm for donor and >600nm for acceptor. Fig. 6A shows the fluorescent cross striation pattern at 2 different [F-CaM]s in both donor channel (green, Em= 500-530nm) and acceptor channel (red, Em>600nm). With high [F-CaM] there is a reduced donor and an increased acceptor (FRET) signal. In the acceptor channel, the heights of the FRET signal peaks (difference between Z-line and M-line) represent the amount of CaM specifically bound to RyR2, which are well fitted with single specific binding curve with $K_d$=18.2 ± 2 nM (Fig 6B). To test whether these FRET measurements reflect 1:1 transfer between F-CaM and F-FKBP12.6, we plotted the donor fluorescence change upon acceptor binding increase. A linear relationship reveals a 1:1 ratio for donor and acceptor (Fig 6C). Thus the source of FRET appears to be exclusively between FKBP12.6 and CaM bound to the same face of the RyR2 (e.g. not on another nearby protein).
Fig 6: $K_d$ for RyR2 bound CaM. A, the FRET images for 2 different acceptor [F-CaM]s, while keeping the donor saturated. There is a decreased donor signal but increased signal acceptor for high [F-CaM] incubation. B, signal in acceptor channel was fitted with single saturable binding revealed the binding affinity for CaM-RyR2 ($n=11-13$). C, the donor fluorescence decreased upon the acceptor binding, a linear relationship revealed a 1:1 donor and acceptor ratio ($n=11-13$).
Since in FRET the fluorescent energy transferred between donor and acceptor, and the ratio of donor and acceptor is 1:1, then we can fit the $K_d$ value from both donor and acceptor signals. We plotted the mean fluorescence value of donor or acceptor fluorescence versus CaM concentration. The acceptor fluorescence was fitted by equation:

$$F_{(acceptor)} = B_{\text{max}} \times \frac{[\text{CaM}]}{K_d + [\text{CaM}]}, \quad K_d = 18.2 \text{ nM (Fig 7)}$$

And donor fluorescence was fitted by equation:

$$F_{(donor)} = C - B_{\text{min}} \times \frac{[\text{CaM}]}{K_d + [\text{CaM}]}, \quad C \text{ stands for the donor max fluorescence without the acceptor, and } K_d = 19.4 \text{ nM (Fig 7)}$$

We can see that both $K_d$ values fitted from the donor and acceptor signals are very similar, testing that the $K_d$ value is correctly estimated.
Fig 7: $K_d$ fitting from both donor and acceptor signals. $C$ is the max donor fluorescent value without the acceptor.

$F_{\text{(donor)}} = C - \{B_{\text{min}} \times [\text{CaM}] / (K_d + [\text{CaM}])\}$

$F_{\text{(acceptor)}} = B_{\text{max}} \times [\text{CaM}] / (K_d + [\text{CaM}])$

$K_d = 19.4 \pm 3.3 \text{ nM}$

$K_d = 18.2 \pm 2 \text{ nM}$
Furthermore, by using direct 543nm F-CaM excitation (not FRET), we measured the $K_d$ for Z-line total CaM (CaM bound to RyR2 plus other sites along the Z-line). Fig 8A shows the Z-line striated pattern by direct excitation at different $[F\text{-CaM}]$s. Similarly, the plot of peak height reveals a single saturable binding component for F-CaM at the Z line with the $K_d$ of $17.2 \pm 1.8$ nM (Fig. 8C). The $K_d$ values for RyR2-CaM and Z-line CaM are very close. One possible explanation is that RyR2 is the quantitatively dominant CaM binding site in the Z-line. The other is that several CaM binding sites have almost the same apparent affinity. Through linear bath $[F\text{-CaM}]$ calibration (Fig 8B), we can infer the concentration of Z-line bound F-CaM in permeabilized myocytes. The CaM ($B_{max}$) reflects the total amount of binding of CaM on Z-lines, (1.2 μM). These CaM binding values are similar to our prior work (Song et al, 2009) and consistent with FKBP12.6 binding in rat myocytes (Guo et al, 2010).
Fig 8: Steady-state $K_d$ and $B_{max}$ for Z-line total CaM. A, confocal Z-line images for two different [F-CaM]. B, calibration upon bath fluorescence on different F-CaM concentration (n=11-13). C, binding affinity fitting for CaM on Z-line, reflecting the $K_d$ and total CaM ($B_{max}$) on Z-line (n=11-13).
Measurement of binding kinetics of CaM-RyR2

Measurement of $k_{on}$ and $k_{off}$ for CaM-RyR2 by FRET + wash-in/off in myocytes. After pretreatment with suramin, the permeabilized myocytes are saturated with F-FKBP12.6 (100 nM). Using FRET, we can characterize CaM-RyR2 association ($k_{on}$) and dissociation ($k_{off}$) rate in wash-in/off experiment. Fig 9A illustrates a representative time course of FRET intensity in the 20nM [F-CaM] continuous wash-in/off experiment. For wash-off, the fluorescent striation pattern gradually declined and followed the single exponential decay with a time constant of 4.5 min, which means $k_{off} = 0.22 \pm 0.01$ min$^{-1}$ (n=5). For wash-in, the association rate is dependent on the on-, off-rate and [F-CaM] ($k_{washin} = k_{on} [CaM] + k_{off}$). Since $k_{washin}$ can be fitted, we can calculate $k_{on} = 18.9 \pm 1.6 \times 10^6$ min$^{-1}$ M$^{-1}$ (n=5). Based on $k_{on}$ and $k_{off}$, $K_d$ can be calculated ($K_d = k_{off} / k_{on} = 12 \pm 0.9$ nM), and is consistent with the result from the steady-state binding experiment.
Fig 9: CaM-RyR2 on- & off-rate measured by FRET + wash-in/off (n=5). Representative time course of FRET CaM fluorescence intensity in 20nM [F-CaM] continuous wash-in/off.
Using FRET + in situ FRAP, to measure $k_{on}$ & $k_{off}$ for CaM binding to RyR2 in myocytes. Permeabilized myocytes were first equilibrated with saturating F-FKBP12.6 (100nM) and [F-CaM] in $K_d$ range. After photobleaching the acceptor by 543nM excitation, there is a recovery of photobleached striation pattern. The recovery of FRET allows a measurement of RyR2-bound CaM recovery rate ($k_{FRAP}$). This recovery reflects reversible CaM-RyR2 binding, in which the photobleached RyR-bound F-CaM was replaced by fresh F-CaM from the bath. Fig 10A shows the recovery of FRET in 20nM bath [F-CaM] at different times. In Fig 10B, $k_{FRAP}$ was fitted by single-exponential function in two different bath [F-CaM]s, on which the fluorescence recovery is dependent. According to the equation: $k_{FRAP} = k_{on}[F-CaM] + k_{off}$, $k_{on}$ (slope) and $k_{off}$ (intercept) were fitted by linear regression (Fig 10C). Both $k_{on} = 14.5 \pm 2.5 \times 10^6 \text{ min}^{-1} \text{ M}^{-1}$ and $k_{off} = 0.144 \pm 0.05 \text{ min}^{-1}$ agree well with wash-in/off experiment, and calculated $K_d = 10 \pm 2 \text{ nM}$ also matches results above.
Fig 10: CaM-RyR2 on- & off-rates measured by FRAP. A, recovery of photobleached striation image in 20nM [F-CaM] over different time. B, $K_{FRAP}$ was fitted with single-exponential function in two different bath F-CaM concentrations. C, $k_{on}$ (slope) and $k_{off}$ (intercept) are fitted through linear regression (n=8-10), and $K_d$ is calculated.
Estimating the percentage of Z-line total CaM that is RyR2-bound

Knock-in mice with genetically disrupted CaM-RyR2 interaction are used here (along with WT mice) to test the percentage of Z-line total CaM that is due to RyR2, vs other CaM binding targets, like LTCC. These knock-in mice express RyR2 with a triple-mutation (RyR2-W3587A/L3597D/F3603A), where the binding of CaM to RyR2 is dramatically impaired without interfering with other CaM signaling pathways (Yamaguchi et al, 2007). Notably, these mice, when homozygous, die very soon after birth; however the heterozygous mice survive long enough to be useful experimental animals (Yamaguchi et al, 2007). In steady state binding, we detect a decrease both for CaM binding on RyR2 by FRET, and Z-line total bound CaM by direct F-CaM exitation (Fig 11A, B). For RyR2^{ADA/+}, there is a 50% reduction in the FRET with unchanged binding affinity (Fig. 12A), which fits the expectation that in heterozygous mice 50% RyR2 monomers have the triple-mutation. But for the Z-line total CaM, there is a 46% reduction (Fig. 12B), which is due to 50% decrease in CaM binding to RyR2. This means ~92% of Z-line localized CaM is on RyR2. In control experiments (Fig 13 A & B), we tested that the total binding (B_{max}) of FKBP12.6 to RyR2^{ADA/+} and WT myocytes is unchanged, indicating that the total number of RyR2 monomers is the same, which matches previous [^3H] ryanodine binding experiments in these mice (Yamaguchi et al, 2007).
Fig 11: Confocal images for RyR2 bound FRET CaM and Z-line bound total CaM (RyR2^{ADA+} vs WT). A, FRET images in saturating condition for WT and RyR2^{ADA+}, B_{max} (FRET CaM) decreased for RyR2^{ADA+} mutant. B, Z-line total bound F-CaM images for WT and RyR2^{ADA+}.
Fig 12: Percentage of Z-line CaM that is bound to RyR2. A, for RyR2\textsuperscript{ADA/+} in steady state binding, B\textsubscript{max} (FRET CaM) decreased ~50% with a similar K\textsubscript{d} compared with WT (n=12-20). B, for Z-line bound total CaM in RyR2\textsuperscript{ADA/+}, B\textsubscript{max} decreased 46% (n=14-20), which is due to RyR2 bound CaM 50% reduction, so ~92% of Z-line bound CaM is on RyR2.
Fig 13: Total binding (B\textsubscript{max}) of FKBP12.6 for WT and RyR2\textsuperscript{ADA/+} mice. A, The confocal images of F-FKBP12.6 binding for WT and RyR2\textsuperscript{ADA/+}. B, B\textsubscript{max} (100nM) is the same for RyR2\textsuperscript{ADA/+} and WT (n=20).
Ca\(^{2+}\) sparks in permeabilized RyR2\(^{+}/\text{ADA KI}\) mouse myocytes

Saponin-permeabilized myocytes are powerful tools for the evaluation of diastolic SR Ca\(^{2+}\) leak. Through cell permeabilization, we can tightly control the \([\text{Ca}\(^{2+}\)]_i\) and prevent complications from L-type Ca\(^{2+}\) channels which also can trigger RyR2 mediated Ca\(^{2+}\) release. However, the potential disadvantage of this method is that some cellular contents (like endogenous proteins) can be lost due to wash-off. Since the endogenous CaM associated with RyR2 may be washed off within 5 min (Fig 9A), we modified the permeabilizing protocol for Ca\(^{2+}\) spark measurement to partially compensate this disadvantage. For the Ca\(^{2+}\) spark measurement here, myocytes were exposed to 50μg/ml saponin for only 20-30 s’ instead of the 3 min used above for CaM wash-in. Fig. 14 shows that after 20-30 s’ permeabilization, Ca\(^{2+}\) sparks were detected in myocytes after 20 min exposure to F-FKBP12.6, F-CaM and fluo-4. In this case there is no visible striated pattern observable for F-FKBP12.6 or F-CaM or FRET. This indicates that fluo-4 can enter the cell but that CaM and FKBP12.6 can not. For the 3 min permeabilization, both FRET striation pattern and Ca\(^{2+}\) sparks were detected indicating that all 3 probes entered the cell. This experiment indicates that 20-30 s’ permeabilization can effectively prevent FKBP12.6 and CaM wash-off during the time of Ca\(^{2+}\) spark measurement. Thus, we can make Ca\(^{2+}\) spark measurement in the relatively native cellular environment with respect to CaM and FKBP12.6 presence.
Fig 14: Effect of different time permeabilization. A, 30 sec permeabilization only fluo4 can enter the cell. B, 3 min permeabilization can make F-FKBP12.6, F-CaM and fluo4 enter the cell.
Using this approach, we evaluated diastolic SR Ca$^{2+}$ leak by recording Ca$^{2+}$ sparks within 15-20 min after permeabilization. Fig 15A-B shows that, at baseline ([Ca$^{2+}$] = 50nM), Ca$^{2+}$ spark frequency was significantly increased in RyR2$^{+/ADA}$ mice versus WT (8.85 ± 0.26 vs 6.4 ± 0.51 /s per 100 μm), while SR Ca$^{2+}$ content appeared slightly but insignificantly decreased for RyR2$^{+/ADA}$ mutants (Fig 15B). The increase of Ca$^{2+}$ spark frequency could not be inhibited by AIP, the specific CaMKII inhibitor (Fig 15B). This is consistent with the reduced CaM binding to RyR2 allowing higher RyR2 activity, and rule out the potential contribution of CaMKII activity to this observation. Ca$^{2+}$ spark properties (Ca$^{2+}$ spark amplitude, full duration at half-maximum, full width at half-maximum, and maximum Ca$^{2+}$ release) were unchanged (Fig. 16). Then we increased intracellular [Ca$^{2+}$] slightly to 100nM, close to the threshold for Ca$^{2+}$ wave production, to further test the propensity for DADs or arrhythmogenesis. The increased [Ca$^{2+}$] mimics a Ca$^{2+}$ loading stress in permeabilized cells, since both cytosolic and SR Ca$^{2+}$ load are increased. We can see that for RyR2$^{+/ADA}$ mutant mice ~90% of myocytes produced Ca$^{2+}$ waves, but for WT mice only ~10% of myocytes produce Ca$^{2+}$ waves (Fig 15C, D) while no significant difference in Ca$^{2+}$ SR load was detected (Fig 15D). All these data indicate that the ADA mutation in RyR2, which caused CaM dissociation from RyR2, increases diastolic SR Ca$^{2+}$ leak under resting conditions and leads to a significantly higher propensity for arrhythmogenic Ca$^{2+}$ wave production under moderate Ca$^{2+}$ loading condition.
**Fig 15: Diastolic Ca^{2+} leakiness in RyR2_{ADA/+} mice.**

A, at 50nM [Ca^{2+}], representative confocal line-scan Ca^{2+} spark images for WT and RyR2_{ADA/+} mice. B, quantification of Ca^{2+} spark frequency and SR Ca^{2+} content for WT and RyR2_{ADA/+} mice (+AIP). C, at 100nM [Ca^{2+}], representative confocal line-scan Ca^{2+} wave images for RyR2_{ADA/+} mice, but not for WT. D, % of cells (n=8) that produced Ca^{2+} waves and the SR Ca^{2+} content for WT and RyR2_{ADA/+} mice.
Fig 16: Ca$^{2+}$ spark characteristics for permeabilized myocytes. 

A. Ca$^{2+}$ spark full duration at half-maximum (FDHM). B. Ca$^{2+}$ spark maximum release. C. Ca$^{2+}$ spark amplitude. D. Ca$^{2+}$ spark full width at half-maximum (FWHM).
**Ca^{2+} transients for RyR2^{+/ADA} KI mouse in intact myocytes**

We also measured Ca^{2+} transients in intact ventricular myocytes (Fig 17A) with or without isoproterenol (ISO, 50 nM) present, and SR Ca^{2+} content was evaluated through rapid caffeine application. In 1 Hz field stimulation for the basal level, the myocytes from RyR2^{+/ADA} mutant behave as WT in Ca^{2+} transient amplitude, time constant of Ca^{2+} transient decline, SR Ca^{2+} content and fractional SR Ca^{2+} release (Fig 17B-E). However, exposure to 50nM ISO revealed differences including prolonged time constant of Ca^{2+} transient decline, decreased SR Ca^{2+} content and increased fractional SR Ca^{2+} release (Fig 17C-E). At the same time, for the RyR2^{+/ADA} mutant, there is a major increase of propensity for Ca^{2+} wave and triggered activity under ISO conditions (Fig 18). Indeed, the induction of Ca^{2+} waves that can trigger action potential in the presence of ISO indicates significant arrhythmogenic risk when CaM binding to RyR2 is inhibited. With Ca^{2+} loading via 50nM ISO, in RyR2^{+/ADA} KI myocytes, the highly sensitized RyR2 can contribute to the prolonged Ca^{2+} transient decline, SR Ca^{2+} depletion and higher fractional release, but resulting in relatively normal Ca^{2+} transient amplitude (similar as WT).
Fig 17: Ca$^{2+}$ transients in intact myocytes. A, representative Ca$^{2+}$ transient ($\Delta$[Ca$^{2+}$]i) under 1 Hz stimulation and SR Ca$^{2+}$ load with/without isoproterenol (50nM) for WT and RyR2$^{ADA/+}$ mice. B, average twitch transient amplitude (F/F0). C, time constant of twitch [Ca$^{2+}$]i decline. D, SR Ca$^{2+}$ content. E, fractional Ca$^{2+}$ release expressed as the ratio for twitch Ca$^{2+}$ transient over caffeine Ca$^{2+}$ release in absence or presence 50nM isoproterenol.
Fig 18: Ca$^{2+}$ wave and triggered activities for RyR2$^{\text{ADA/}+}$ mice. A, representative line-scan image of Ca$^{2+}$ wave and triggered activity in intact cardiomyocytes with 50 nM isoproterenol. B, the percentage of cells that exhibited Ca$^{2+}$ waves and triggered activities for WT (n=24) and RyR2$^{\text{ADA/}+}$ (n=22) mouse myocytes under 50nM isoproterenol.
**Binding affinity (Kd) of CaM/FKBP12.6 to RyR2 in HF myocytes.**

To further examine the CaM binding affinity under a pathological condition, we measured the CaM-RyR2 binding affinity in a rat HF model induced by coronary ligation. In this rat HF model, HF developed in 12 weeks post-surgery. In this HF model an increased heart/body weight ratio, increased LV diastolic dimensions (LVDD) and decreased fractional shortening were observed (Lin et al, 2007). Other HF molecular markers, like ANP, BNP, and TNF-α were also found significantly increased in this HF model (Lin et al, 2007). Here we only used HF rats with LVDD > 1.1 cm (normal: 0.9) and fractional shortening < 20% (normal: 43%) for experiments. Through FRET detection and steady-state concentration-dependent binding, the Kd for CaM-RyR2 measured in HF myocytes is 50.9 ± 4.4 nM (Fig 19A). Compared to the Kd for normal myocytes, there is a ~3 fold decrease in CaM affinity for the rat HF model. To exclude the possible strain/species difference, we performed additional control experiments. We tested the CaM-RyR binding in control vs HF rats in the Kd range of [CaM] and at saturating [CaM] (B_max) (Fig 19C). In the Kd range, which is sensitive to detect affinity changes, we see a significant decrease in CaM binding to RyR2 for HF myocytes, indicating a decreased binding affinity, but unchanged Bmax for CaM binding. FKBP12.6 has been suggested to also be a critical RyR2 stabilizer whose affinity changes in HF, despite the intensive controversy (Bers, 2012). We, for the first time, examined the FKBP12.6-RyR2 binding in situ affinity in HF myocytes. Through steady-state binding measurement, the Kd for FKBP12.6-
RyR2 is \(0.84 \pm 0.07 \text{nM}\) (Fig 19B). This is almost unchanged in comparison to our previous control \(K_d\) value (\(K_d = 0.7 \pm 0.07\text{nM}\) measured in the same conditions (Guo et al, 2010)). To more carefully test for differences, the same paired control experiments as done for CaM were performed. Fig 19D shows that FKBP12.6 binding was unaltered when \([\text{FKBP12.6}]\) was in either the \(K_d\) range or saturating condition in control vs HF rat myocytes.
Fig 19: $K_d$ for CaM/FKBP12.6 binding to RyR2 in HF myocytes. A, Using FRET detection (as in Figure 1) and steady-state binding isotherms, we measured an increase in the $K_d$ (decrease in affinity) of CaM-RyR2 binding in HF myocytes, but we detected no significant change in the FRETmax. B, Using direct excitation of FKBP12.6, and steady-state binding isotherms, we detected no significant differences in the FKBP12.6-RyR2 binding in HF and normal myocytes. C, At sub-saturating [A-CaM] ($\approx K_d$), there is a significant decrease in CaM bound to RyR2 in HF myocytes, but there is no significant change in FRETmax (at saturating A-CaM). D, for $K_d$ range or saturated condition (Bmax), the FKBP12.6-RyR2 associate rate are unchanged for normal and HF myocytes.
DISCUSSION

In this study through fluorescent protein and FRET detection, we for the first time identify CaM bound to RyR2 vs its other targets and characterize CaM-RyR2 interaction properties (Kd and on/off rates) in permeabilized ventricular myocytes by 3 independent methods (Table 1). The knock-in mice, with genetically disrupted CaM-RyR2 association but no effects on other CaM binding targets, are used to estimate the percentage of Z-line CaM that is bound to RyR2. And the patho-physiologically functional consequences of CaM for RyR2 regulation are defined through confocal Ca2+ measures at myocytes in RyR2ADA/+ KI mice. We further define the in situ CaM-RyR binding affinity in HF myocytes to reveal a potential pathological role of reduced CaM binding in RyR2 regulation. All these results could lead to a crucial improvement for understanding and definition of CaM-RyR2 interaction in cardiac myocytes in health and disease.
Table 1. Summary of CaM-RyR2 binding properties

<table>
<thead>
<tr>
<th></th>
<th>$k_{on}$ ($\times 10^6 \text{min}^{-1} \text{M}^{-1}$)</th>
<th>$k_{off}$ (min$^{-1}$)</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash-in/off</td>
<td>18.9 ± 1.6</td>
<td>0.22 ± 0.01</td>
<td>12 ± 0.9</td>
</tr>
<tr>
<td>FRAP</td>
<td>14.5 ± 2.5</td>
<td>0.144 ± 0.05</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Steady-state</td>
<td>--</td>
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<td>18.2 ± 2</td>
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</table>
CaM-RyR2 binding properties in the myocytes

CaM, as an important RyR2 regulator, may be important in HF, cardiac hypertrophy and CPVT. But CaM-RyR2 in situ binding properties are unknown. Through 3 independent methods, we showed that CaM can bind to RyR2 with high affinity ($K_d = 10-20 \text{ nM}$) in resting condition, where $[\text{Ca}^{2+}]_i$ is 50nM. In this condition, according to the measured free [CaM] in myocytes (50-75 nM) (Wu X, et al, 2007), ~70-90% of RyR2 monomers are associated with CaM, which makes CaM a potential important RyR2 stabilizer, because CaM is associated with the majority of RyR2 monomers and inhibits RyR2 opening at all physiological $[\text{Ca}^{2+}]$. Our measured binding affinity is higher than the result from cell lysates and SR vesicles (Guo et al, 2011; Ono et al, 2010). We think that could be due to differences of experiment conditions. In lysates and SR vesicles experiments, there is a higher risk of losing essential partners from the cellular environment or causing subtle modification to RyR2 (such as: phosphorylation or oxidation). And phosphorylation, oxidation, or conformation change can dramatically decrease CaM-RyR2 binding affinity (Xu et al, 2010; Balog et al, 2006). Our results for CaM-RyR2 binding affinity, from 3 independent methods, matched each other quite well, and fit with CaM’s physiological function. So we believe the $K_d$ (10-20 nM) better reflects the binding affinity for CaM-RyR2 in the native cardiac myocyte environment.

According to our data, CaM-RyR2 has a relatively slow off rate ($k_{off} = \sim 0.2 \text{ min}^{-1}$), meaning the average dwell time for CaM on RyR2 is ~5 min in resting
condition. In addition, high [Ca\(^{2+}\)]\(_i\) (500 nM) favors CaM binding to Z-line and greatly prolongs CaM dissociation time and increases the affinity (Guo et al, 2011; Wu et al, 2007). So the increased CaM-RyR2 affinity during the Ca\(^{2+}\) transient (< 1 second) during which intracellular [Ca\(^{2+}\)] could be as high as hundreds micromolar; this would further enhance CaM saturation at all RyR2 monomers in the physiological beat-to-beat situation. Furthermore, it was proposed that CaM can switch between two binding sites with 33Å distance on RyR during each cycle of contraction (Tripathy et al, 1995; Samso and Wagenknecht 2002). The two-site switch involves two sets of association and dissociation process, which might be unlikely to occur within the very short systolic Ca\(^{2+}\) time according to our measured kinetics. Taken together, we think that CaM is a residently associated protein to RyR2 and is anchored to at least residues 3583-3603. But we can not exclude the possibility of beat-to-beat RyR2 regulation by CaM, because in RyR2\(^{ADA/ADA}\) mutant sustained peak time for Ca\(^{2+}\) transient were observed, indicating the possible importance of resident CaM for RyR2 shut-off (Yamaguchi et al, 2007). It is possible that, during Ca\(^{2+}\) transient, RyR opening and conformation change could alter anchored CaM function, where binding of Ca\(^{2+}\) to CaM would facilitate the termination of Ca\(^{2+}\) release.

**Nearly all Z-line bound CaM is on RyR2**

One of the important aspects in this study is that, through knock-in mice (RyR2\(^{ADA/+}\)) we estimate that > 90% of total Z-line associated CaM is on RyR2, meaning ~1.1 μM CaM is associated with RyR2. This result is consistent with
FKBP12.6 $B_{\text{max}}$ measurements (Guo et al, 2010) and the estimated concentration of RyR2 monomers in rat ventricular myocytes (Bers and Stiffel, 1993), revealing that other CaM binding sites at the Z-line are $\sim 0.07\mu\text{M}$. Since LTCC is also known to bind CaM and at the same location as RyR2, and there are $\sim 32$ RyR2 monomers per LTCC in rat myocytes (Bers and Stiffel, 1993), the calculated LTCC-bound CaM could be $\sim 0.04 \mu\text{M}$ consistent with our result. We can see that RyR2 is the quantitatively dominant CaM binding site at the Z-line. The total cellular CaM concentration in cardiac myocytes is about 2-5 $\mu\text{M}$ in different species (Maier et al, 2006). From this we can see that RyR2 is also an overall major binding site for CaM, and that any binding affinity variation in RyR2, like in HF, could cause changes in cellular free [CaM] and consequent distribution.

**Defective RyR2-CaM interaction and SR Ca$^{2+}$ release**

Yamaguchi (2007) explained the important link between CaM-RyR2 interaction and abnormal SR Ca$^{2+}$ leak, and cardiac hypertrophy in RyR2$^{\text{ADA}}$ homozygous mice. But, due to the early death of RyR2$^{\text{ADA}}$ homozygous mice (Yamaguchi et al, 2007), the relation between defective CaM-RyR2 interaction and arrhythmogenesis is not clear. However, RyR2$^{\text{ADA}}$ heterozygous mice can survive to provide us an excellent model for our study. We notice a similarity between RyR2$^{\text{ADA}}$ KI mice and CPVT1 mutation KI mice (RyR2-R2474S) (Stephan et al, 2008), that is both homozygous KI mice do not survive but heterozygous can survive similar to WT. Furthermore, decreased CaM-RyR2 binding affinity has been reported in CPVT1 KI mice (Xu et al, 2010). So for
functional tests, we focused on the SR Ca$^{2+}$ release under stress conditions in RyR2$^{ADA}$ heterozygous mice. In permeabilized cells, when [Ca$^{2+}$]$_i$ is tightly controlled at 50 nM, we only observed a moderate Ca$^{2+}$ spark frequency increase for RyR2$^{ADA/+}$ mice. This moderate Ca$^{2+}$ leakiness explains why the heterozygous mice could survive as long as WT, unlike homozygous RyR2$^{ADA}$ mice which developed severe cardiac hypertrophy and early death. For the CPVT1 mutant, spontaneous Ca$^{2+}$ releases (Ca$^{2+}$ waves) which can trigger DADs, are a typical phenomena. In our case the diastolic Ca$^{2+}$ leak at resting condition in RyR2$^{ADA/+}$ is insufficient to produce Ca$^{2+}$ waves and triggered arrhythmias unless SR Ca$^{2+}$ load is driven up by external stimulus (i.e., stress or exercise). Then we increased intracellular [Ca$^{2+}$] to 100 nM to load the SR and this produced Ca$^{2+}$ waves significantly in RyR2$^{ADA/+}$ mice, which indicates the hypersensitized RyR2. There are two reasons which could be behind this phenomenon. 1), the increased SR Ca$^{2+}$ content over the threshold releases more Ca$^{2+}$ than in resting condition during the SR Ca$^{2+}$ leak; 2), possible endogenous CaMKII activation, due to increased free [CaM] (released by 50% defective CaM-RyR2 interaction) and high Ca$^{2+}$, which could activate endogenous CaMKII then further phosphorylate and sensitize RyR2 to amplify the leakiness.

For the Ca$^{2+}$ transient measurements in intact cells, ISO makes a big difference in RyR2$^{ADA/+}$ mice. We observe a prolonged Ca$^{2+}$ transient decline time constant. Since Yamaguchi (2007) reported that there is no change for Ca$^{2+}$ uptake by the SR CaATPase for RyR2$^{ADA/+}$ mice (Yamaguchi et al, 2007), we
think that the prolonged Ca\textsuperscript{2+} decline might be due to delayed RyR2 closure, which is consistent with single channel recording where CaM-RyR2 interaction facilitates termination of Ca\textsuperscript{2+} release (Xu et al, 2004). Under ISO stimulation, there is a decreased SR Ca\textsuperscript{2+} content and an increased fractional SR Ca\textsuperscript{2+} release for RyR2\textsuperscript{ADA/+} mice, indicating the sensitized RyR2 by CaM dissociation could increase systolic SR Ca\textsuperscript{2+} release. Additionally, with the presence of ISO, there is an increase of the propensity for Ca\textsuperscript{2+} waves and triggered activity (Fig 18). This phenomenon is very similar to the situation in CPVT1 KI mice (Uchinoumi H, et al, 2010). In resting condition, there is a mild diastolic Ca\textsuperscript{2+} leak (Fig 17B) with unchanged Ca\textsuperscript{2+} transients and fractional release. But under ISO stimulation, we see a dramatically increased propensity for Ca\textsuperscript{2+} wave and trigger activity. The ADA mutation on RyR2 is not a known human disease linked mutation, but it causes very similar phenotype as CPVT mutations. These results imply a strong link between defective RyR2-CaM interaction and arrhythmogenesis, and the potential mechanism underlying arrhythmia.

**CaM-RyR2 binding affinity in HF myocytes**

One of the most important aims for this study is to define the in situ binding affinity for RyR2-CaM in HF myocytes and access potential pathological impact of that. Our result indicates a 3-fold decreased binding affinity for RyR2-CaM interaction in failing heart induced by MI. HF causes a reduced RyR2-CaM association, from 70-90\% to 50-70\%, assuming unchanged free myocyte [CaM]. Using IP other studies have also reported less CaM bound to RyR2 in HF in
rabbits (Ai et al, 2005) and dogs (Ono M, et al, 2010), so this seems like a
general finding in HF. Less CaM association can decrease RyR2 gating
threshold, causing abnormal Ca$^{2+}$ release and triggered activation. In RyR$^{\text{ADA}/+}$
mice, we revealed that 50% reduction of RyR2 association leads to dramatically
increased propensity for Ca$^{2+}$ wave and trigger activity in the presence of
isoproterenol. In our HF model, the RyR2-CaM association rate is close to 50%
level, revealing a strong link between CaM-RyR2 disassociation to
arrhythmogenesis in HF. Furthermore, the attenuated RyR2-CaM association in
HF could increase intracellular free [CaM], since RyR2 is a major cellular binding
site for CaM. This could change CaM cellular distribution extensively; the CaM
lost by RyR2 could be driven to the binding targets with a lower affinity than
RyR2, such as: CaMKII and adenylyl cyclase (Maier and Bers, 2002). Further
study is needed to assess such CaM redistribution and its effect in HF.

Overall we think that HF associated SR Ca$^{2+}$ leak is a complicated process
in which many factors may be involved (e.g. CaM, CaMKII, FKBP12.6, redox
modification etc). Even though in normal myocytes saturating RyR2 with
FKBP12.6 only slightly reduces Ca$^{2+}$ sparks (Guo et al, 2010), we could not
exclude the possibility that FKBP12.6 might inhibit pathological leaky RyRs.
Here, we for the first time have directly measured the in situ binding affinity of
RyR2-FKBP12.6 in failing heart myocytes, which revealed unchanged binding
affinity. We conclude that CaM binding to RyR2 is an important physiological
regulator of RyR2 gating in cardiac myocytes, and that defects in this binding in
HF or CPVT may be an important molecular mechanism of triggered arrhythmias.
CHAPTER VI

Define CaM as a critical stabilizer through RyR2 unzipping peptide (DPc10)

INTRODUCTION

A leading hypothesis explains the RyR2 dysfunction in HF and lethal arrhythmias, such as catecholaminergic polymorphic ventricular tachycardia (CPVT), by RyR2 mutations that result in defective interaction between the N-terminal (N: 0-600) and the central (C: 2000-2500) domains (Yamamoto T & Ikemoto 2002). According to this concept, in the resting state, the N-terminal and central domains interact with each other to act as a regulatory switch for channel gating activity. This tight interdomain interaction, termed “domain zipping” serves to stabilize the closed channel. Weakening of these interdomain interactions may be caused by mutations in either the N-terminal or central regions of RyR2 (Uchinoumi et al, 2010) or via synthetic competition by cardiac domain peptides (Ikemoto & Yamamoto 2002; Ikemoto 2004; Yamamoto et al, 2000) (domain unzipping), resulting in an increased opening probability of the RyR2 and leakiness of Ca$^{2+}$. DPc10 is a synthetic peptide corresponding to 36-residue stretch of the central domain (Gly$^{2460}$-Pro$^{2495}$) of RyR2 (Yamamoto & Ikemoto 2002). It has been proposed that DPc10 can specifically associate with the N-terminal domain, and thus compete with its zipping to the central domain, and
that the N-domain/DPc10 association can destabilize RyR2 (via domain unzipping), to increase Ca\(^{2+}\) leakiness (Oda et al, 2005). A single R2474S point mutation in DPc10 (DPc10-mut) inhibits all of the effects that would have been produced by DPc10. Liu et al (2005), through GFP insertion and mapping, showed a physical proximity between N- and central domains in the 3D structure of RyR2. They also find FRET interaction between these two regulatory domains for CFP/YFP insertion in HEK cell, but the zipping pair (N- and central domains) is not from the same RyR2 monomer, but from two different neighbor subunits in one RyR2, indicating the interaction of neighboring subunits plays important role to stabilize RyR2 (Liu et al, 2010). Taken together, these data suggest that synthetic domain peptides bind to key subdomains of RyR2 and are capable of mimicking disease conditions of the RyR2 channel by interfering with interdomain interactions. The interaction between neighbor subunits is important for the RyR2 stabilization.

FKBP12 and FKBP12.6 are expressed in cardiac myocytes and can form tight complexes with RyR2, at a stoichiometry of four FKBPs per tetrameric RyR2 channel (Bers, 2004). As such, these FKBP isoforms are considered important RyR2 subunits, and have been reported to promote the closed channel state, but this role is controversial in the heart. In myocytes from normal rat hearts, we found that FKBP12 does not significantly alter Ca\(^{2+}\) sparks, whereas FKBP12.6 is slightly inhibitory, and PKA-dependent RyR2 phosphorylation does not alter FKBP12.6 binding affinity (Guo et al, 2010). Furthermore, a small fraction of
RyR2 in native myocytes has FKBP12.6 bound (Guo et al, 2010). Two previous studies, in which RyR2 was treated with domain peptides to mimic pathologic Ca^{2+} leakage, found no direct effect of DPc10 on FKBP12.6 co-immunoprecipitation with RyR2 (Oda et al, 2005; Tateishi et al, 2009). But it is unknown whether FKBP12.6 influences binding of DPc10 to RyR2 or the ensuing increased Ca^{2+} leakage.

CaM is a ubiquitous Ca^{2+} binding protein that binds to the RyR2 and modulates its channel function (Yamaguchi et al, 2003). Binding of CaM within the cytosolic domain of RyR2 (at a site partly formed by residues 3583-3603) inhibits channel activity both at physiological and pathophysiological cellular [Ca^{2+}] (Fruen et al, 2000; Balshaw et al, 2002). This implies that CaM stabilizes the closed state of RyR2 in the resting state (Guo et al, 2006). In the 3D RyR2 structure, the CaM binding site is very close to the boundary between adjacent subunits (Hamilton & Serysheva II, 2009). There is the suggestion that CaM binds to two adjacent subunits of RyR (Zhang et al, 2002), and contributes to stabilization. Interestingly, concurrent-addition of a high concentration of CaM with DPc10 in WT cardiomyocytes reduced the Ca spark frequency compared to addition of DPc10 alone (Ono et al, 2010). Furthermore, in the CPVT mutant myocytes where beta-adrenergic stimulation leads to defective interdomain interaction, the affinity of CaM binding to the RyR2 is lower than that of WT myocytes (Xu et al, 2010). In addition, Ono et al (2010) also reported that the
CaM binding affinity to RyR2 in the failing heart is significantly reduced compared to that of normal RyR2.

DPc10 and related RyR2 peptides may, therefore, serve as useful molecular probes to study the channel's function-structure relationship. However, the details of DPc10 binding to RyR2, including affinity and kinetics, are still unknown. In this study, our goal was to characterize the binding of DPc10 to the RyR2 in the relatively intact environment of saponin-permeabilized rat ventricular myocytes. We used fluorescent DPc10 to measure the affinity and kinetics of DPc10 binding to RyR2, and its influence on CaM and FKBP12.6 binding and function, and how DPc10 alters CaM and FKBP12.6 binding.

MATERIALS AND METHODS

Domain peptide

DPc10 peptides unlabeled and labeled with 5-carboxyfluorescein or HiLyte FluorTM 647 at N-terminal were synthesized at AnaSpec (Fremont, CA). The DPc10 sequence is:

2460-GFCPDHKAAMVLFLDRVYGIEVQDFLLHLLEVGFLP-2495.

Laser scanning confocal microscopy

Confocal images were measured using a Biorad Radiance 2100 laser scanning confocal microscope equipped with an Argon ion laser, Green HeNe laser and a Nikon Fluo 40 x oil objective lens. FRET between CaM and DPc10 were performed on Olympus FV1000 confocal microscope. All experiments were done at room temperature
F-DPc 10 washin/off in permeabilized myocytes

See details in chapter IV

Ca$^{2+}$ sparks in permeabilized cells using confocal microscopy

See details in chapter IV

FRET measurements

For the FRET between CaM and DPc10, we used Alexa Fluor 568 attached at the C-lobe of CaM (AF568-110-CaM) or Alexa Fluor 488 at N-lobe (AF488-34-CaM) as the donor, and HiLyte Fluor™647-DPc10 (HF647-DPc10) as the acceptor. AF488, AF568- and HF647- were excited with separate lasers of 488 nm, 543 nm and 635 nm, respectively. Emission fluorescence intensity data were obtained at 500-530 nm for AF488-34-CaM, 560-620 nm for AF568-110-CaM and 655-755 nm for HF647-DPc10. Fig 20 shows the spectrum of FRET probes.
Fig 20: Fluorescence spectrum for FRET (adopted from invitrogen.com)
RESULTS

Binding kinetics of F-DPc10 in permeabilized cardiac myocytes

To characterize DPc10 binding kinetics at the Z-lines, we performed F-DPc10 wash-in (500 nmol/L), wash-off experiments in permeabilized myocytes (Fig 21A). We observed very slow association ($\tau_{\text{wash-in}} = 79.0 \pm 3.2$ min) and dissociation processes ($\tau_{\text{wash-out}} = 149.8 \pm 4.4$ min), compared to FKBP12.6 measurements (Guo T, et al, 2010). From the wash-in/wash-off measurements, we calculated the association and dissociation rates constants, $k_{\text{on}}$ and $k_{\text{off}}$, according to:

$$k_{\text{wash-in}} = [\text{F-DPc10}]k_{\text{on}} + k_{\text{off}}$$

Eq 1

where $k_{\text{wash-out}} \approx k_{\text{off}}$ and $k = 1/\tau$ (s). Accordingly, $k_{\text{on}} = 188 \pm 37$ (s$^{-1}$ M$^{-1}$), and $k_{\text{off}} = 0.12 \pm 0.01$ (10$^{-3}$ s$^{-1}$). Based on these values and $K_d = k_{\text{off}}/k_{\text{on}}$, F-DPc10 binds at the Z-line with $K_d = 644 \pm 149$ nmol/L.
Fig 21: F-DPc10 binding at the myocyte Z-line (collaboration with Oda Tetsuro). A, striation pattern of saponin-permeabilized myocytes incubated in 5 μmol/L F-DPc10 for 3 hours, and illustration of DPc10 binding pocket between N-(blue) and central (red) domains in RyR2. B, Time course of F-DPc10 (0.5 μmol/L) wash-in and wash-out.
We were intrigued by the slow $k_{in}$, and conducted measurements to further understand the structural basis of this slow association. Thus, we tested the hypothesis that, at resting $[Ca^{2+}]_i$, normally RyR2 is in closed situation, where N- and central domains are in the tightly zipped condition, which sterically hindered DPc10 access to its RyR2 binding site. If the N-terminal and central domains are tightly “zipped” to each other, this interaction may occlude the DPc10 binding site on the RyR2, thus limiting the DPc10 $k_{on}$. Alternatively, a limiting factor may be the rate at which DPc10 adopts a conformation that is competent to bind to RyR2. To discern between these mechanisms, we determined the effect of [F-DPc10] on $k_{in}$ which should increase linearly with [F-DPc10], according to Eq 1. This is not what was observed, however. Instead, a 10-fold increase in [F-DPc10] (from 0.5 to 5μmol/L) had no significant effect on the $\tau_{wash-in}$ of F-DPc10 but significantly increased $B_{max}$ (Fig 21A, B). This result suggests that F-DPc10 association at its RyR2 binding site is restricted by a factor residing on RyR2, which is probably the binding pocket opening rate.
Fig 22: Wash-in time course for different [F-DPc10] (collaboration with Oda Tetsuro). **A**, Time course wash-in curve for different [F-DPc10] (0.5 and 5μmol/L). **B** $\tau_{\text{wash-in}}$ and $B_{\text{max}}$ for different [F-DPc10]. Data are reported as mean ± SE.
In our working model, under resting conditions, the RyR closed state is stabilized by the interaction between the N-terminal and central domain in the "zipped" state. We hypothesized that conditions that promote RyR opening will result in a higher rate of unzipping, and a faster F-DPc10 wash-in rate. To test this, we first monitored F-DPc10 wash-in at elevated Ca\textsuperscript{2+} (500 nmol/L) and found slightly faster association vs. 50nmol/L [Ca\textsuperscript{2+}], (Fig 23A). Next, we pre-incubated the myocytes with ryanodine (100 μmol/L) plus caffeine (5 mmol/L), which has been reported to cause RyR2 domain unzipping in HEK293 cells (Liu Z, et al, 2010), and found a significantly faster F-DPc10 association (Fig 23B). However, the most significant effect was seen after pre-incubating myocytes with a saturating concentration of non-fluorescent DPc10 (NF-DPc10). This treatment significantly accelerated F-DPc10 association by a factor of ~2 (Fig 23A). Neither treatment significantly altered $B_{\text{max}}$ (Fig 23A & B). Assuming that $k_{\text{off}}$ of NF-DPc10 is the same as for F-DPc10 and using Eq 1, the $k_{\text{on}}$ is increased by 3.2-fold by unzipping by prebinding NF-DPc10 to the RyR2. These results are consistent with a small fraction of channels being in the unzipped state even at 500 nmol/L Ca\textsuperscript{2+} or with caffeine +ryanodine. Moreover, it would seem that the RyR2 open state (favored by caffeine-ryanodine) differs from the unzipped state (with DPc10 bound).
Fig 23: Effect of RyR2 channel modulators on the kinetics of F-DPc10 Z-line association (collaboration with Oda Tetsuro). A, Time course of F-DPc10 (0.5 μmol/L) Z-line binding in low [Ca\(^2+\)], high [Ca\(^2+\)], or after a 3 hr pre-equilibration with saturating [NF-DPc10] (2 μmol/L) in low [Ca\(^2+\)]. B, Time course of F-DPc10 (5 μmol/L) Z-line binding after a 3 hr pre-equilibration in internal solution containing ryanodine (100 μmol/L) and caffeine (5 mmol/L). Data are reported as mean ± SE.
Effect of DPc10 on CaM and FKBP12.6 binding at the myocyte Z-line

Both FKBP12.6 and CaM bind to the RyR2 and can reduce channel opening, which might inhibit DPc10 binding. Fig 24A shows representative confocal images of FKBP12.6 (100 nmol/L) and CaM (1 μmol/L) effects on F-DPc10 binding, as detected after a 200 min incubation with F-DPc10. While pre-equilibration with CaM (1 μmol/L) greatly reduced F-DPc10 binding, pre-treatment with FKBP12.6 (100 nmol/L) did not alter F-DPc10 binding in permeabilized myocytes. Fig 24B shows the time course of F-DPc10 wash-in with or without pretreatment of 100 nmol/L FKBP12.6 or 1 μmol/L CaM. Saturation of RyR2 with FKBP12.6 (100 nmol/L) did not alter either F-DPc10 maximal binding (B_max) or τ_{wash-in}. In contrast, saturation of RyR2 with CaM dramatically reduced F-DPc10 B_max and dramatically slowed DPc10 access to its binding site, as indicated by the increase in τ_{wash-in} (Fig 24C). We infer that CaM stabilizes the domain interaction between N-terminal and central domains in the "zipped" state, and may thereby reduce DPc10 access to its binding site.
Fig 24: Effects of FKBP12.6 and CaM on F-DPc10 binding at Z-line. **A**, Representative confocal images illustrating the effect of FKBP12.6 (100 nmol/L), and CaM (1 μmol/L) on the F-DPc10 (5 μmol/L) binding at the Z-lines. **B**, Time course of F-DPc10 (5 μmol/L) wash-in (circles), and in the presence of FKBP12.6 (100 nmol/L, diamonds) or CaM (1 μmol/L, triangles). **C**, Summary of fitting parameters ($B_{max}$ and $\tau_{wash-in}$).
Next, we asked whether RyR channel activation by DPc10 is inhibited by FKBP12.6 or CaM. We assessed Ca\(^{2+}\) sparks in permeabilized myocytes perfused with internal solution containing 50 nmol/L free Ca\(^{2+}\) plus 1 μmol/L AIP (to inhibit CaMKII activity). Line-scan images were recorded after 3 hr incubations under control conditions, and in the presence of 5 μmol/L DPc10, with or without 1 μmol/L CaM or 100 nmol/L FKBP12.6 (Fig 25A). DPc10 robustly increased Ca\(^{2+}\) spark frequency (CaSpF) vs. control, an effect almost completely blocked by CaM (Figure 25B). However, CaSpF activation by DPc10 is only slightly decreased by FKBP12.6 (Fig 25B). In DPc10-treated permeabilized myocytes, Ca\(^{2+}\) spark full width at half maximum (FWHM) and full duration at half maximum (FDHM) is significantly increased compared to control, and decreased when pre-treated with CaM (Table 2).

Since Ca\(^{2+}\) spark frequency strongly depends on the SR Ca\(^{2+}\) content, we also assessed SR Ca\(^{2+}\) content as the amplitude of caffeine-induced Ca\(^{2+}\) transient (Fig 25B, right). With DPc10 alone or with FKBP12.6, SR Ca\(^{2+}\) load was significantly lower than under control conditions. In contrast, treatment with CaM plus DPc10 resulted in no significant decrease in SR Ca\(^{2+}\) content vs control. Thus, the increased CaSpF in the presence of FKBP12.6 plus DPc10 cannot be secondary to increased SR Ca\(^{2+}\) content, which actually decreased. These results are consistent with a DPc10-induced increase in RyR2 channel activity resulting from defective interaction between N-terminal and central domains. This also agrees with the lack of FKBP12.6 effect on F-DPc10 binding kinetics (Fig
25B) and the potent inhibition of DPc10 binding by CaM, which may promote the 
“zipped” state and inhibit DPc10 access.
Fig 25: Effects of FKBP12.6 and CaM on DPc10 binding and Ca^{2+} sparks in permeabilized cardiomyocytes (collaboration with Oda Tetsuro). A, Ca sparks measured using Fluo-4 as Ca^{2+} indicator. Representative line-scan images acquired after addition of DPc10 (5 μmol/L), DPc10 (5 μmol/L) plus FKBP12.6 (100 nmol/L), and DPc10 (5 μmol/L) plus CaM (1 μmol/L). [Ca^{2+}]_i = 50 nmol/L. B, Summary of Ca^{2+} spark frequency and SR Ca^{2+} content. Data are reported as mean ± SE.
Table 2. Ca$^{2+}$ spark characteristics in saponin-permeabilized cardiomyocytes

<table>
<thead>
<tr>
<th></th>
<th>No. of Sparks</th>
<th>Peak $(F/F_0)$</th>
<th>FWHM (µm)</th>
<th>FDHM (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1761</td>
<td>0.499 ± 0.004</td>
<td>1.28 ± 0.01</td>
<td>55.7 ± 0.67</td>
</tr>
<tr>
<td>+DPc10</td>
<td>1467</td>
<td>0.475 ± 0.004*</td>
<td>1.34 ± 0.01*</td>
<td>58.5 ± 1.00*</td>
</tr>
<tr>
<td>+FKBP12.6/DPc10</td>
<td>1921</td>
<td>0.439 ± 0.002*</td>
<td>1.21 ± 0.01*</td>
<td>60.5 ± 0.73*</td>
</tr>
<tr>
<td>+CaM/DPc10</td>
<td>2195</td>
<td>0.467 ± 0.003*</td>
<td>1.23 ± 0.01*</td>
<td>56.9 ± 0.74</td>
</tr>
</tbody>
</table>
Effect of DPC10 on FKBP12.6 and CaM binding in permeabilized myocytes

To examine the converse influence that DPC10 may have on FKBP12.6 and CaM binding to RyR2 in situ, we used fluorescent FKBP12.6 and CaM variants labeled with Alexa Fluor 488 or 568 (AF488 and AF568, respectively). These fluorescent proteins were added to saponin permeabilized myocytes with or without pre-equilibration with saturating DPC10 concentration. First, we found that AF488-FKBP12.6 at a 1 nmol/L (near its K_d) (Guo T, et al, 2010), forms a striated pattern that is not affected by pre-incubation of 5 μmol/L DPC10. (Fig 26A & B). Thus, DPC10 does not influence FKBP12.6 binding to RyR2. Next, we measured FRET between AF488-FKBP12.6 (donor) and AF568-34-CaM (acceptor in the N-domain) at a [CaM] near the K_d (20 nmol/L), in order to only detect CaM that is specifically bound to the RyR2 (Fig 27A). Using direct excitation at 543 nm (emission at >600 nm) we detected total CaM at the Z-lines (Fig 27A). We also did this with high [CaM] (500 nmol/L) which saturates RyR2 with CaM under control conditions. Fig 27B shows that pre-treatment with DPC10 significantly reduced CaM binding (at 20 nmol/L CaM) both at the RyR2 and overall at the Z-lines, and by similar proportions. Even at high AF568-34-CaM levels (500 nmol/L) DPC10-treated myocytes exhibited reduced CaM binding at the RyR2 (FRET) and at the Z-lines (Total) vs. control (Fig 27B). Thus, once F-DPC10 binds to the RyR2 and decreases N-terminal-central domain interactions, it reduces the CaM affinity for RyR2. Taken together, these results show that DPC10 and CaM binding to RyR2 are mutually inhibitory. To test whether DPC10
and CaM bind at the same or nearby RyR2 sites we measured FRET between CaM and DPc10.
Fig 26: The effect of DPc10 on FKBP12.6 binding to RyR2 in cardiac myocytes (collaboration with Oda Tetsuro). A. Representative image for FKBP12.6 binding (100nM) with or without DPc10 (5μM) pretreatment. B. Average fluorescence for FKBP12.6 binding with/without DPc 10.
**Fig 27: The effect of DPc10 on CaM binding to RyR2 in cardiac myocytes** (collaboration with Oda Tetsuro). **A.** Representative confocal image of the effect of DPc10 on AF568-CaM binding at the Z-lines (total CaM, Ex =543 nm) and at the RyR2 detected by FRET between AF488-FKBP12.6 (donor) and AF568-CaM (acceptor) (Ex =488 nm). Myocytes were incubated with 5 μmol/L DPc10 (3 hrs) before adding CaM. **B.** Summary of RyR2-bound CaM and Z-line total CaM binding of DPc10 pretreatment.
FRET between CaM and DPc10

We used FRET to assess the distance between a fluorescence donor probe (AF568) at the C-lobe of CaM (AF568-110-CaM) and HiLyte Fluor 647 (HF647) as acceptor probe on the N-terminus of DPc10 (HF647-DPc10). We utilized the acceptor photobleaching approach with measurement of the resultant increase in donor (AF568-110-CaM) fluorescence in saponin permeabilized myocytes. To use this approach quantitatively, all acceptor sites must be loaded, so that all donors can participate in FRET.

Our results show that it is impractical to saturate RyR2 with both CaM and DPc10 (Fig 24B and 27B). To overcome this challenge, we pre-equilibrated the myocytes with saturating HF647-DPc10, thus loading all DPc10 binding sites on RyR2. Then when we add AF568-110-CaM (500 nmol/L) ~50% of RyR2s have donor, but all have acceptor, allowing quantitative analysis of enhanced donor fluorescence upon acceptor photobleach. Fig 28A shows selective photobleach of HF647-DPc10 (at 635 nm) in only the central region of the myocyte, and donor fluorescence was enhanced only in that region, indicating that donors and acceptors are within FRET range.

To rule out the possibility that there is energy transfer between a donor and multiple acceptors, we measured the relationship between donor fluorescence enhancement and acceptor photobleach, and found a linear relationship (Fig 28B), which indicates that there is a 1:1 stoichiometry for the CaM-DPc10 FRET. We interpret this result as clear evidence that the FRET
efficiency (E) between AF568-110-CaM and HF647-DPc10 reflects the proximity of one CaM to one DPc10. E and donor-acceptor distance calculations are described in the methods section (chapter IV).

The FRET efficiency between AF568-110-CaM and HF647-DPc10 upon 98.2 ± 0.2 % acceptor photobleach was E = 0.89 ± 0.01 (n=8). This corresponds to a distance of 53 ± 1 Å (Fig 28C), based on $R_0 = 75$ Å for the AF568-HF647 donor-acceptor pair. With an alternative donor probe (AF488), this time attached at the N-lobe of CaM, and the same acceptor (HF647) on DPc10, we measured E = 0.27 ± 0.02 which corresponds to an interprobe distance of 63 ± 1 Å (Fig 28C). Thus, this result shows that the donor probes on CaM are 53-63 Å from the acceptor on DPc10, suggesting that CaM and DPc10 can simultaneously bind at distinct, yet nearby sites within the RyR2 structure. This again favors an allosteric rather than competitive basis for the mutual inhibition seen between CaM and DPc10 binding to the RyR2.
Fig 28: FRET between F-CaM and F-DPc10. A, Confocal images show FRET between AF568-110-CaM (donor) and HF647-DPc10 (acceptor). B, AF568-110-CaM fluorescence intensity increase on HF647-DPc10 photobleach. Data is fitted by a linear function, indicating that the ratio between donor and acceptor is 1:1. C, FRET efficiency and distances between AF568-110-CaM and HF647-DPc10, and between AF488-34-CaM and HF647-DPc10.
DISCUSSION

We used fluorescent DPc10, FKBP12.6, CaM and confocal microscopy of permeabilized cardiomyocytes and found that (1) DPc10 access to its binding site is sterically hindered in resting (zipped) RyR2, (2) F-DPc10 wash-in kinetics provide a sensitive measure of the RyR2 unzipped state in permeabilized myocytes, (3) DPc10 and CaM binding to RyR2 are mutually inhibitory (via allosteric rather than competitive interaction), and (4) DPc10 and CaM are physically 50-60 Å from each other.

Access of DPc10 to Its RyR2 Binding Site Is Restricted

We found that both the wash-in and wash-off kinetics of F-DPc10 binding (kwash-in and kwash-off) are extremely slow (Fig 21B). The calculated kon for F-DPc10 is about 1800-fold slower than that we measured for FKBP12.6 under similar conditions (Guo T, et al, 2010). This suggests either that DPc10 very slowly adopts a conformation that can bind RyR2, or that the DPc10 binding site on RyR2 becomes available only very slowly. The insensitivity of kwash-in to higher F-DPc10 concentration (Fig 22A), is most consistent with the latter interpretation, indicating that kon is limited by RyR2 properties that restrict the access of DPc10 to its binding site. Further supporting this hypothesis, pretreatment with NF-DPc10 (Fig 23A) robustly increased kon (~320%). We infer that the bound NF-DPc10 shifted RyR2 to the unzipped state allowing better access and exchange with F-DPc10. We also found that enhancing RyR2 open state (by elevated [Ca$^{2+}$] or caffeine+ryanodine) hastened the F-DPc10 association (Fig 23A, B).
However, these effects on $k_{\text{wash-in}}$ were small compared to that of pre-binding NF-DPc10 (~15%), despite the very strong channel activation expected. From this and other work (Liu et al, 2010), we suggest that the unzipped and open states differ, although unzipping may enhance opening and opening may enhance the unzipping transition (and DPc10 access).

**Relationship between FKBP12.6 and DPc10 binding to RyR2**

FKBP12.6 has been found to quiet RyR2 channel opening (Prestle et al. 2001; Xin et al. 2002; George et al. 2003; Yano et al. 2003), but this is an intensely controversial issue (Timerman et al. 1996; Barg et al. 1997; Xiao et al. 2007; Jones et al. 2008; Bers 2012) and FKBP12.6 may only inhibit pathologically leaky RyRs (Yano M, et al, 2000). Since more than 80% of the RyRs in cardiomyocytes have no natively-bound FKBP12.6 (Guo T, et al, 2010), adding saturating concentrations of exogenous FKBP12.6 ought to decrease Ca$^{2+}$ leak caused by DPc10-induced unzipping. Here, we found that FKBP12.6 has no effect on either DPc10 binding ($B_{\text{max}}$ or $\tau_{\text{wash-in}}$) or vice-versa (Fig 24B and Fig 26) and does not quiet the activating effect of DPc10 on the CaSpF (Fig 25B). That is similar to our previous myocyte studies, where FKBP12.6 had very minor effects on Ca$^{2+}$ sparks (Guo et al, 2010). Taken together, these results suggest that DPc10 and FKBP12.6 modulate RyR2 function through independent mechanisms.

**Relationship between CaM and DPc10 Binding to RyR2**
In myocytes containing a CPVT-linked RyR2 mutation β-adrenergic stimulation decreases CaM binding at the Z-lines, and this effect is mimicked in healthy myocytes by treatment with DPc10 (Ono et al, 2010). Here, we used methods designed to monitor CaM and DPc10 binding specifically at RyR2, aiming to understand the structural basis of the inhibition of CaM-RyR binding by DPc10. One important finding in the present study is that saturating CaM binding at the RyR2 dramatically reduced F-DPc10 binding and Ca²⁺ spark activation (Fig 24 & 25), presumably by stabilizing the zipped RyR2 state.

Our novel FRET-based method allows direct assessment of CaM-RyR binding in the native cardiac myocyte environment (using FKBP12.6-CaM FRET) (Guo et al, 2011). Using this method, we found that unzipping the RyR by treatment with saturating [DPc10], reciprocally inhibits CaM binding to RyR2 (Fig 26B, right panel). There are two possible explanations for this reciprocal binding inhibition: (1) DPc10 and CaM compete to bind at overlapping sites (orthosteric mechanism) or (2) the DPc10 and CaM binding sites are separate but coupled in a mutually inhibitory interaction (allosteric mechanism).

To discern between these possibilities we assessed if CaM and DPc10 can coexist on RyR2. In Fig 27A, we show strong FRET between donor-labeled CaM and acceptor-labeled DPc10 at the Z-lines, indicating that CaM and DPc10 binding sites in neighboring regions are simultaneously occupied. This conclusion is further supported by FRET measurements using two different donor-acceptor pairs and two different labeling sites on CaM, which indicate
distances of $63 \pm 1$ and $53 \pm 1$ Å between DPc10 and the N- and C-lobes of CaM, respectively (Fig 28C). Taken together, these results strongly support the conclusion that DPc10 and CaM bind at separate sites on RyR2, and they interact through an allosteric mutually inhibitory mechanism.

Our working hypothesis (Fig 29) is as follows. The resting zipped RyR2 does not readily allow DPc10 access to its site (i) and CaM binding at a different site may stabilize this zipped state (iii). We suppose the RyR2 can transition spontaneously between this zipped and the unzipped state (i-ii) but that the low probability at rest cause the slow, but eventual access of DPc10 to its site. This transition may be favored by channel opening ($Ca^{2+}$, caffeine, ryanodine) and also in pathological conditions (e.g. HF). Once the central domain-mimicking DPc10 gains access and binds, it stabilizes the unzipped state (ii) which reciprocally facilitates channel opening and inhibits CaM binding (iv).
Fig 29: Proposed model of the interaction between N-terminal and central domains. Kinetics results (Figures 2-4) suggest that F-DPc10 access to its binding site is controlled by inter-domain interaction within RyR2. (i) The F-DPc10 access is sterically hindered in resting normal RyR2 (“zipped” state). (ii) Pre-treatment of RyR2 with physiological, pharmacological, or disease-mimetic agents that promote unzipping increase the F-DPc10 association rate. (iii) CaM inhibits the F-DPc10 binding to RyR2. (iv) DPc10 binding to RyR2 inhibits CaM binding to RyR2.
CHAPTER VII

SUMMARY

The aim of my dissertation is to understand the regulation of RyR2. The whole dissertation is composed of two parts. The first part focused on RyR2-CaM interaction. The second focused on synthetic RyR2 domain peptide (DPc10), which worked as a powerful molecular tool for RyR2 functional and structural studies.

CaM has been long identified as an important cardiac RyR regulator. Broad studies suggest CaM is a critical RyR2 stabilizer (Xu L, et al, 2004, Guo T, et al, 2006) and CaM-RyR2 interaction is a critical molecular substrate for arrhythmias and HF pathogenesis (Yamaguchi N, et al, 2007; Ai X, et al, 2005; Ono M, et al, 2010; Xu X, et al, 2010), but the in situ binding properties for CaM-RyR2 are still unknown. Here we, Using FRET detection and permeabilized myocytes, identified RyR2-bound CaM from other potential targets and revealed that CaM binds to RyR2 with high affinity ($K_d = 10^{-20}$ nM) in myocytes. Therefore physiologically CaM is bound to > 70% of RyR2 monomers and inhibits SR Ca$^{2+}$ release. Using RyR2$^{ADA^+/}$ knock-in mice in which half of the RyR2-CaM binding is suppressed, we estimated that >90% of Z-line CaM is RyR2-bound and identify RyR2 as one of main CaM cellular binding sites. In consistence with binding
properties studies, functional tests indicated a higher propensity for Ca\textsuperscript{2+} waves in RyR2\textsuperscript{ADA/+} mice upon ISO challenge. In a post MI rat HF model, I detected a decrease binding affinity for CaM-RyR2 ($K_d = \sim51\text{nM}$, $\sim3$ fold increase) and unchanged binding affinity for FKBP12.6-RyR2 ($K_d = \sim0.8\text{nM}$).

The defective interaction between interdomains (the N-terminal and the central domains) is a leading hypothesis to explain the RyR2 dysfunction in HF and lethal arrhythmias, such as CPVT. And DPc10 can bind to RyR2 and recapitulate this arrhythmogenic RyR2 leakiness by unzipping N- and central-domains. In this study fluorescently-labeled DPc10, FKBP12.6, and CaM were used to characterize the binding properties of DPc10 to RyR2 in permeabilized rat ventricular myocytes. DPc10 access to its binding site is extremely slow in resting RyR2, but accelerated by promoting RyR2 opening or unzipping (by unlabeled DPc10). RyR2-bound CaM (but not FKBP12.6) drastically slowed DPc10 binding. Conversely, DPc10 binding significantly reduced CaM (but not FKBP12.6) binding to the RyR2. FRET measurements indicate that DPc10 and CaM binding sites are separate.

Since CaM and FKBP12.6 are both regarded as important RyR stabilizer and implicated broadly in HF, hypertrophy and arrhythmia genesis. And FKBP12.6 is still a highly controversial issue in cardiology (this was reviewed in chapter II, FK-506 Binding Protein section). Here, mainly based on the data from our lab, I made a brief comparison between these two important RyR2 stabilizers, which suggested CaM has more prominent effect for RyR2 gating
than FKBP12.6 does. However, I can't exclude the possibility that FKBP12.6 may modulate RyR2 activities in a subtle way which is undetectable by our experiment, like non-spark Ca^{2+} leak by RyR2.
Table 3. Comparison between CaM and FKBP12.6 as RyR2 stabilizer

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<tr>
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<th>CaM</th>
<th>FKBP12.6</th>
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<tbody>
<tr>
<td><strong>K(_d)</strong> and RyR2 monomer association rate</td>
<td>High affinity (K(_d) = 10-20nM), intracellular [CaM] = 50-70nM, 70-90% RyR2 monomers are associated with CaM.</td>
<td>High affinity (K(_d) = 1nM), but low endogenous FKBP12.6, 10-20% RyR2 monomers are associated with FKBP12.6.</td>
</tr>
<tr>
<td><strong>Phenotype of bioengineered mice</strong></td>
<td>RyR2(^{ADA}) knock-in mice, in homozygous, severe hypertrophy and early death.</td>
<td>FKBP12.6 knock-out mice, no obvious phenotype at resting condition.</td>
</tr>
<tr>
<td><strong>K(_d)</strong> in HF myocytes</td>
<td>Increased</td>
<td>No change</td>
</tr>
<tr>
<td><strong>Ca\textsuperscript{2+}</strong> spark inhibition in myocytes in saturated condition</td>
<td>50-70% decrease (e)</td>
<td>~20% decrease (b)</td>
</tr>
<tr>
<td>Association can keep RyR2 in zipping condition</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>If RyR2 is in unzipping state</td>
<td>K(_d) (RyR2-CaM) increased</td>
<td>No change for K(_d) (RyR2-FKBP12.6)</td>
</tr>
</tbody>
</table>

(a), Wu x, et al, 2006  
(b), Guo T, et al, 2010  
(c), Yamaguchi N, et al, 2007  
(d), Wehrens X, et al, 2003  
(e), Guo T, et al, 2006
Future studies

For the future studies, these projects can be taken further to address the specific reasons for pathologically decreased CaM-RyR2 affinity, like: (1) phosphorylation by PKA or CaMKII, because the phosphorylation sites (S2808 by PKA and S2814 by CaMKII) are close to CaM docking pocket in RyR2 3D structure (Xing M, et al, 2007), (2) RyR2 modification by redox. For DPc10 studies, F-DPc10 wash-in kinetics can be used in the more native environment of permeabilized cardiomyocytes to evaluate domain interaction between the N-terminal and central domains of RyR2. This could serve as a powerful and versatile investigative tool in pre-clinical and clinical studies with respect to the domain unzipping hypothesis. For example, the time course of F-DPc10 wash-in can be monitored in myocytes from failing hearts in which “unzipped” state had already taken place (Oda T, et al, 2005; Ono M, et al, 2010), or to gauge RyR2 function under pathological conditions (e.g., oxidative stress, phosphorylation state etc) and in the evaluation (or validation) of drug candidates that act to stabilize the RyR2 “zipped” state (Oda T, et al, 2005).
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