CALCIUM SIGNALING IN CARDIAC MYOCYTES: CONTRIBUTIONS FROM MITOCHONDRIA, SODIUM-CALCIUM EXCHANGER, AND RYANODINE RECEPTOR

A Dissertation submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacology

By

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Sarah S. Haviland, M.S.

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ABSTRACT

This dissertation explores three critical players in the cardiac Ca\textsuperscript{2+} signaling pathway. First, I examined the role of mitochondria in Ca\textsuperscript{2+} signaling by generating a Ca\textsuperscript{2+} probe, mitycamE31Q, with 1.5\,µM affinity for Ca\textsuperscript{2+} directed to mitochondria. Using mitycamE31Q and Fura-2AM, in neonatal rat cardiomyocytes, I show that release of Ca\textsuperscript{2+} from the SR (caffeine-application) caused cytosolic Ca\textsuperscript{2+} rise, followed by slower mitochondrial uptake of calcium. Similarly, Ca\textsuperscript{2+} influx upon Na\textsuperscript{+} withdrawal increased both cytosolic and more slowly the mitochondrial Ca\textsuperscript{2+}. I also found two populations of mitochondria (central, peripheral) that on such interventions had differing patterns of Ca\textsuperscript{2+} release and uptake. My findings suggest that mitochondrial Ca\textsuperscript{2+} changes with changes in cytosolic Ca\textsuperscript{2+}, though with slower kinetics, and reveal Ca\textsuperscript{2+}-load dependent subpopulations of mitochondria.

The second Ca\textsuperscript{2+} signaling issue addressed was the shark-heart splice-variant of NCX, reported to have bimodal adrenergic regulation (protective mechanism against Ca\textsuperscript{2+}-overload induced arrhythmia). To test the anti-arrhythmic role of shark NCX, two approaches were employed: 1) creation of transgenic mice that express shark NCX-myc-tag; 2) construction of an adenovirus containing shark NCX-YFP. Although the transgenic mice failed to show biochemical and functional evidence for transgene overexpression, the adenoviral model showed 3-4 times greater expression of shark NCX current with shark-like bimodal regulation by 8-Br-
cAMP in neonatal rat cardiomyocytes. I conclude that shark NCX can be functionally expressed in the mammalian cardiomyocytes, but whether it can confer anti-arrhythmic properties to the mammalian heart remains undetermined.

The role of the 3rd major player (RyR2) in the Ca\(^{2+}\) signaling pathway in iPS cell-derived cardiomyocyte model expressing the wild and mutant RyR2-F2483I causing catecholaminergic polymorphic ventricular tachycardia, CPVT1, were evaluated. I found that while \(I_{\text{Ca}}\) and \(I_{\text{Ca}}\)-induced Ca\(^{2+}\)-transients were similar, caffeine-induced Ca\(^{2+}\)-release and \(I_{\text{NCX}}\) were smaller, confirming smaller Ca\(^{2+}\)-stores in CPVT. Adrenergic agonists enhanced \(I_{\text{Ca}}\), but differentially altered the CICR gain, diastolic Ca\(^{2+}\), and Ca\(^{2+}\)-sparks in mutant cells. We conclude that both iPS-CM lines express the adult cardiomyocyte Ca\(^{2+}\)-signaling phenotype. RyR2 F2483I-mutant myocytes have aberrant unitary Ca\(^{2+}\)-signaling, smaller Ca\(^{2+}\)-stores, higher CICR gains, and sensitized adrenergic regulation, consistent with functionally altered Ca\(^{2+}\)-release profile of CPVT.
ACKNOWLEDGMENTS

I would like to give my sincerest thanks to my mentor, Dr. Martin Morad, for teaching me much more than just being a scientist. Also, I would like to thank Dr. Lars Cleemann for being my savior when laboratory equipment would fail, which was often. Without their knowledge, assistance, and teachings, this dissertation would not be possible. In addition, I would like to thank Dr. Einsley Janowski, Dr. Hale Tufan, Dr. Xiaohua Zhang, Dr. Naohiro Yamaguchi, Susannah Stone, and Caitlin Hesch for their help and camaraderie along the way. Thank you to each of my committee members, Dr. Barry Wolfe, Dr. Baoji Xu, Dr. Gerard Ahern, and Dr. Don Menick who have provided me with guidance throughout this project. Lastly, I would like to thank my family for their love, support, and encouragement that kept me going through the many years it took to achieve this goal. Along the way my parents, grandparents, and husband have given me the push to keep going and believed in me more than I ever could. Most importantly, thank you to my husband, Chad, who endured the eighteen months spent apart so that I could earn my degree.

Many thanks,

SARAH S. HAVILAND
DEDICATION

This dissertation is dedicated to my parents, Susan and Douglas Haviland, grandparents, Patricia and Robert Krug, and husband, Chad Hilty. I am extremely grateful for your love and support.
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<td>8-bromoadenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>α-MHC</td>
<td>Alpha-Myosin Heavy Chain</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>Ad5</td>
<td>Adenovirus Serotype 5 Vector</td>
</tr>
<tr>
<td>AP</td>
<td>Action Potential</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1, 2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calmodulin Dependent Kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>Cadmium</td>
</tr>
<tr>
<td>CGP</td>
<td>7-Chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium Induced Calcium Release</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CPVT</td>
<td>Catecholaminergic Polymorphic Ventricular Tachycardia</td>
</tr>
<tr>
<td>CyA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>dB-cAMP</td>
<td>N(6),2'-O-dibutyryladenosine 3':5' cyclic monophosphate</td>
</tr>
<tr>
<td>DAD</td>
<td>Delayed After-Depolarizations</td>
</tr>
<tr>
<td>DHP(R)</td>
<td>Dihydropyridine (Receptor)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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</table>
eYFP  Enhanced Yellow Fluorescent Protein
EAD  Early After-Depolarizations
ECC  Excitation-Contraction Coupling
ECG  Electrocardiogram
EGTA  Ethylene Glycol Tetraacetic Acid
ER  Endoplasmic Reticulum
ET  Ejection Time
FBS  Fetal Bovine Serum
FCCP  Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FKBP  FK506 Binding Protein
gDNA  Genomic Deoxyribonucleic Acid
HCX  Hydrogen Calcium Exchanger
HR  Heart Rate
K⁺  Potassium
IMM  Inner mitochondrial membrane
IMFM  Intermyofibrillar mitochondria
IMS  Intermembrane space
iPSC  Induced Pluripotent Stem Cell
iPS-CM  Induced Pluripotent Stem Cell Derived Cardiomyocyte
ISO  Isoproterenol
IVCT  Isovolumic Contraction Time
IVRT  Isovolumic Relaxation Time
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>LTCC</td>
<td>L-Type Calcium Channel</td>
</tr>
<tr>
<td>LV</td>
<td>Left Ventricle</td>
</tr>
<tr>
<td>LVAWd</td>
<td>Left Ventricular Anterior Wall (diastole)</td>
</tr>
<tr>
<td>LVAWs</td>
<td>Left Ventricular Anterior Wall (systole)</td>
</tr>
<tr>
<td>LVEDD</td>
<td>Left Ventricular End Diastolic Diameter</td>
</tr>
<tr>
<td>LVESD</td>
<td>Left Ventricular End Systolic Diameter</td>
</tr>
<tr>
<td>LVPWd</td>
<td>Left Ventricular Posterior Wall (diastole)</td>
</tr>
<tr>
<td>LVPWs</td>
<td>Left Ventricular Posterior Wall (systole)</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>mNCX</td>
<td>Mitochondrial Sodium Calcium Exchanger</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>mRyR1</td>
<td>Mitochondrial Ryanodine Receptor Type 1</td>
</tr>
<tr>
<td>MCU</td>
<td>Mitochondrial Ca(^{2+}) Uniporter</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>Sodium</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-Essential Amino Acids</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium Calcium Exchanger</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>Ni(^{2+})</td>
<td>Nickel</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer Mitochondrial Membrane</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PLB</td>
<td>Phospholamban</td>
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<tr>
<td>PNM</td>
<td>Perinuclear Mitochondria</td>
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<td>PKA</td>
<td>Protein Kinase A</td>
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<tr>
<td>PTP</td>
<td>Permeability Transition Pore</td>
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<td>RaM</td>
<td>Rapid Mode of Ca^{2+} Uptake</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
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<td>RyR</td>
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<td>SERCA</td>
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<td>Sarcoplasmic Reticulum</td>
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<tr>
<td>SSM</td>
<td>Subsarcolemmal mitochondria</td>
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<td>TCA</td>
<td>Tricarboxylic Acid</td>
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<td>TG</td>
<td>Transgenic</td>
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<td>TIRF</td>
<td>Total Internal Reflection Fluorescence</td>
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<td>TMRE</td>
<td>Tetramethylrhodamine Ethyl Ester</td>
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<tr>
<td>TPK</td>
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<td>Voltage-Induced Ca^{2+} Release</td>
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<td>Wild Type</td>
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<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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INTRODUCTION
Calcium Signaling in Neonatal and Adult Cardiac Myocytes

Calcium is a universal signaling molecule that plays a critical role in many cellular processes throughout the body, including, but not limited to gene transcription, apoptosis, synaptic transmission, neurosecretion, and excitation-contraction coupling (ECC) in the heart. Although today numerous papers are published on these topics, it was not until Sidney Ringer was studying contraction in isolated rat hearts where he discovered that the beating of the heart weakened in the absence of Ca\(^{2+}\), and thus it was necessary to add Ca\(^{2+}\) to the solutions to maintain contraction (Ringer, 1883). With the development of voltage clamp technique for ventricular muscle (Morad and Trautwein, 1968) and calcium fluorescent dyes, the ability to directly study the role of this ion in cardiac cells became easier. In this dissertation, I will concentrate on the role of mitochondria, Na\(^{+}\)-Ca\(^{2+}\) exchanger, and ryanodine receptor in calcium signaling events of the heart. Since the heart is able to tightly control the amount of calcium entered, released, and extruded from its cells, this maintenance of calcium homeostasis is central to cardiac contractility.

Cardiac excitation-contraction coupling is initiated with the electrical stimulation of the individual myocyte and results with the synchronous contraction of the heart that sends blood out to the rest of the body. The process is tightly controlled and possesses feedback mechanisms to regulate its activity. As a result, myocyte mishandling of Ca\(^{2+}\) is a main cause in both contractile dysfunction and arrhythmias during pathophysiological conditions (Pogwizd et al., 2001). In the adult myocyte, during the cardiac action potential (AP), specific cellular events coincide with each phase of the action potential. Major channels, transporters, stores, and proteins associated with these cellular events are shown in Figure 1. These cells have a maintained resting
membrane potential, represented as Phase 4 of the AP that is dominated by high permeability to K⁺ keeping the membrane close to the highly negative electrochemical gradient for potassium, E_K (Deal et al., 1996). When these cells are depolarized, Na⁺ channels in the sarcolemma open and Na⁺ ions enter the cell, generating a current responsible for rapid upstroke of Phase 0 of the AP. At the peak of the AP, one set of K⁺ channels close while another set start to activate, simultaneously with inactivation of Na⁺ channel responsible for slight repolarization of Phase 1 of the AP before the opening of the L-type calcium channel (LTCC) during the plateau phase of the AP, Phase 2. During the plateau phase of the action potential, the sodium-calcium exchanger, NCX, is also activated and contributes to maintaining the duration of the AP. This phase is balanced by the net influx of Ca^{2+} and Na⁺ and by the efflux of K⁺. In Phase 3 of the action potential, the L-type calcium channel inactivate and repolarization of the membrane potential occurs when the potassium channels re-open and bring the cell back to its resting membrane potential. Once the AP is initiated in the cell, Phase 0, 1, 2, 3 represent the absolute refractory period of the cell and the cells is unable to be depolarized prior to returning to a resting potential. During Phase 2 of the action potential, important events occur inside the cell that allows the cardiac tissue to contract. Upon opening of the L-type calcium channel, Ca^{2+} enters the cell as inward Ca^{2+} current (I_{Ca}), and activates the ryanodine receptor (RyR) to release the contents of the internal calcium store, sarcoplasmic reticulum (SR), located at close proximity (20 nm) of the Ca^{2+} channel (Bers, 2002; Greenstein and Winslow, 2011). The Ca^{2+} released from the internal store, paired with Ca^{2+} entering through the channel causes a rise in intracellular free Ca^{2+} ([Ca^{2+}]_i) allowing Ca^{2+} to bind to troponin C initiating cross bridge motion that leads to the sliding of the actin and myosin filaments and contraction (Bers and Guo, 2005).
For relaxation to occur, \([\text{Ca}^{2+}]\), needs to decline back to resting conditions to allow \(\text{Ca}^{2+}\) to dissociate from troponin (Bers, 2002). A small fraction of \(\text{Ca}^{2+}\) is then extruded from the cell via the NCX, while the major fraction recirculates back into the SR via calcium-ATPase (SERCA2a) or into mitochondria via the mitochondrial \(\text{Ca}^{2+}\) uniporter (MCU) as the myocyte relaxes fully (Bers, 2002). In adult ventricular cells, this cascade begins with the cross-talk between two sets of \(\text{Ca}^{2+}\) transporting proteins, the RyR2 in the dyadic junctions, and L-type calcium-channels of the t-tubular membranes allowing for calcium-induced \(\text{Ca}^{2+}\) -release (CICR) to take place (Bers, 2008). In atrial cells that lack t-tubular networks, this occurrence is mostly limited to the peripheral junctions (Blatter et al., 2003).
Figure 1: Schematic of calcium signaling proteins and events in adult ventricular myocyte

Ca\(^{2+}\) enters through the L-type calcium channel, \((I_{\text{Ca}})\), and activates the ryanodine receptor (RyR) to release the contents of the SR. The Ca\(^{2+}\) released from the internal store, paired with Ca\(^{2+}\) entering from \(I_{\text{Ca}}\) causes a rise in intracellular free Ca\(^{2+}\) that can bind to troponin C (myofilaments) to cause contraction. Ca\(^{2+}\) is then extruded from the cell via the NCX, put back into the SR via calcium-ATPase (SERCA), or mitochondrial Ca\(^{2+}\) uniporter to return to basal Ca\(^{2+}\) levels.
Neonatal cardiac myocytes, like atrial cells, lack a t-tubular network. These cells are moving away from the embryonic state of calcium signaling which rely on both the SR and endoplasmic reticulum, ER, for their calcium release (Janowski et al., 2006). Although similar to the adult, the cascade of events can be seen in Figure 2 for the neonatal cardiac myocyte. It is believed that the changes from embryonic to adult phenotype can be attributed to various factors including, changes of protein expression, cellular localization and distribution of these calcium signaling proteins, and cellular morphology. Ca\textsuperscript{2+} stores in cardiac cells during embryogenesis and postnatal development before the maturation of the t-tubular system show considerable diversity with respect to the pharmacology of the release channels and that regional differences in Ca\textsuperscript{2+} signaling are observed centered in, at, and around the nucleus (Janowski et al., 2006).

Neonatal cardiomyocytes, unlike adult cells, have a diversity of calcium stores. Data from our laboratory has shown that some neonatal cardiomyocytes were primarily gated by ryanodine receptors while others gated by IP\textsubscript{3} receptors, and in many cells both types of Ca\textsuperscript{2+} release mechanisms were found to be expressed. In addition, it is important to note that in culture, neonatal cells are able to beat spontaneously. The cause of this spontaneous beating is currently being investigated in our laboratory. Neonatal cardiac myocytes provide a unique cell system for experimental work because these cells are able to be maintained in culture for a periods as long as a week, are flat with a high surface to volume ratio, and can be easily transduced to transiently express other exogenous proteins in culture. Figure 3 represents different pharmacological agents used in both adult and neonatal cardiomyocytes to modulate specific proteins. These agents include both blockers and activators.
Figure 2: Schematic of calcium signaling proteins and events in neonatal ventricular myocyte

Ca\textsuperscript{2+} enters through the L-type calcium channel, (I\textsubscript{Ca}), and activates the ryanodine receptor (RyR) to release the contents of the SR. The Ca\textsuperscript{2+} released from the internal store, paired with Ca\textsuperscript{2+} entering from I\textsubscript{Ca} causes a rise in intracellular free Ca\textsuperscript{2+} that can bind to troponin C (myofilaments) to cause contraction. Ca\textsuperscript{2+} is then extruded from the cell via the NCX, put back into the SR via calcium-ATPase (SERCA), or mitochondrial Ca\textsuperscript{2+} uniporter to return to basal Ca\textsuperscript{2+} levels. In neonatal cells, the Ca\textsuperscript{2+} that enters can directly activate the myofilaments and some of the Ca\textsuperscript{2+} release can also be attributed from the IP3 receptor store on the ER.
Figure 3: Schematic of calcium signaling proteins and pharmacological agents

Both blockers (nifedipine, nickel, cadmium, TTX, thapsigargin, Ru360, FCCP, cyclosporin A) and activators (isoproterenol, dB-cAMP, 8-Br-cAMP, and caffeine) are shown as they act on specific cardiac proteins.
Mitochondrial Calcium Signaling in Cardiac Myocytes

Mitochondria are often referred to as the powerhouse of the cell. They play an important role in energy production, metabolism, and apoptosis (Jouaville et al., 1999; Gustafsson and Gottlieb, 2008). They generate ATP through oxidative phosphorylation via the electron transport chain, produce reactive oxygen species (ROS), as well as play a role in the TCA cycle and β-oxidation of fatty acids. It was not until 1950 where researchers found that mitochondria could accumulate Ca\(^{2+}\) (Slater and Cleland, 1953). Initial studies by Carafoli and Lehninger showed that not only could mitochondria take up Ca\(^{2+}\) to a limited amount to maintain a dynamic state that could be returned to the cytosol, but they were capable of taking up larger amounts of Ca\(^{2+}\) when phosphate was also taken up (Carafoli and Lehninger, 1964). This early work on mitochondria was performed on isolated organelles, and it was not until the 1960s where work was done on mitochondria in intact cells in vivo. In this study, rats were injected with \(^{45}\text{Ca}\)\(^{2+}\) and the mitochondria in the heart were found to contain most of the radioactivity (Patriarca and Carafoli, 1968). Later it was shown that the Ca\(^{2+}\) taken up by the mitochondria can stimulate nitric oxide (NO) production that in turn modulates oxygen consumption, ATP production, ROS generation, and therefore may provide negative feedback for the regulation of mitochondrial Ca\(^{2+}\) production (Dedkova and Blatter, 2008). However, controversy in the field still remains and remains as yet unclear whether beat to beat mitochondrial Ca\(^{2+}\) regulation takes place in the heart and what role does \([\text{Ca}^{2+}]_m\) play in the overall myocyte calcium signaling profile. Since mitochondria make up between 30-40% of the cardiac cell volume, it is possible that this contribution could be substantial (Barth et al., 1992).
The structure and constitutive proteins of the mitochondria may play an important role in understanding their role in Ca^{2+} signaling. Mitochondria have two membranes: the outer mitochondrial membrane (OMM) and the inner mitochondria membrane (IMM), and both with different functions. The outer membrane faces on one side the cytosolic space and on the side the intermembrane space (IMS). There is, in addition, the mitochondrial matrix. The outer membrane contains many proteins and is highly permeable to small molecules that pass freely between the cell cytoplasm and the IMS due to a large number of porins (Patergnani et al., 2011). Conversely, the IMM, which contains complexes of the respiratory chain, ATP synthase, and other enzymes (Fernie et al., 2004), is almost completely impermeable even to small molecules such as Ca^{2+} and H^+. As a result, this allows complexes in the respiratory chain to build up a proton gradient that result in an electrochemical gradient and the basis for the $\Psi_m$ that is utilized to produce ATP (Patergnani et al., 2011). In normal hearts, $\Delta\Psi_m$ is highly regulated so that the production of ATP is maintained within the physiological range that matches the energy requirement (O'Rourke, 2007; Akar and O'Rourke, 2011). In order for Ca^{2+} to enter the mitochondria, it passes freely through the OMM but must pass the IMM into the matrix via one of three mechanisms. The first, and perhaps the most important, is the mitochondrial Ca^{2+} uniporter (MCU) (Gunter and Pfeiffer, 1990; Gunter et al., 1994; Baughman et al., 2011; De Stefani et al., 2011), the second is the rapid mode of Ca^{2+} uptake (RaM) (Sparagna et al., 1995; Gunter et al., 2000; Buntinas et al., 2001; Bazil and Dash, 2011), and lastly, the mitochondrial ryanodine receptor type 1 (mRyR1) (Beutner et al., 2001; Beutner et al., 2005; Altschafl et al., 2007; Ryu et al., 2011). The MCU is a channel on the IMM that transports Ca^{2+}, Sr^{2+}, but not Mg^{2+} with high selectivity and low affinity (Kirichok et al., 2004). The MCU is driven by the
electrochemical gradient ($\Delta\Psi = -180\text{mV}$) and activated by Ca$^{2+}$. There are many known modulators of MCU, such as ruthenium compounds, Ru360 (Matlib et al., 1998). RaM operates transiently and is controversial, but is based on experiments from isolated heart mitochondria that are exposed to repeated calcium pulses and at physiological levels are more efficient at taking it up and at rates several hundred times faster than MCU (Sparagna et al., 1995; Gunter et al., 2000; Buntinas et al., 2001; Murgia et al., 2009; Bazil and Dash, 2011). Lastly, and even more controversial, is the role of the mRyRs which when isolated from mitochondrial membranes and incorporated in lipid bilayers have similar properties to those of skeletal muscle RyR1, \textit{i.e.} large conductance, similar biophysical and pharmacological properties (Beutner et al., 2001; Beutner et al., 2005; Altschafl et al., 2007; Ryu et al., 2011).

Like the Ca$^{2+}$ influx pathways of mitochondrial matrix, three Ca$^{2+}$ efflux pathways have been identified: 1) the mitochondrial Na$^+$-Ca$^{2+}$ exchanger (mNCX), 2) H$^+$-Ca$^{2+}$ exchanger (HCX), and 3) the permeability transition pore (mPTP). The predominant form of Ca$^{2+}$ efflux of the mitochondria is the Na$^+$ dependent form, mNCX first discovered by Carafoli in 1974 (Carafoli et al., 1974). This transporter uses the Na$^+$ gradient across the IMM and can be blocked by such agents as CGP. There is some evidence that like the sarcolemmal NCX; the mNCX transports 3Na$^+$ molecules to 1Ca$^{2+}$ (Jung et al., 1995). The Na$^+$ independent efflux, HCX, is a transporter is the main efflux system in non-excitable cells (Saris and Carafoli, 2005), but plays little to no role in excitable cell membranes (Crompton et al., 1977). The PTP is a large pore on the IMM that has been shown to release Ca$^{2+}$ and cause matrix depolarization (Korge et al., 2011) and can be blocked by cyclosporin A (Weinbrenner et al., 1998). Although the long-lasting PTP opening is thought to be induced by Ca$^{2+}$ overload and a way to clear the
mitochondria of damaging and unneeded molecules (Murgia et al., 2009; Giorgi et al., 2012), Korge et al. also demonstrated transient openings of the PTP thought to be protective (Korge et al., 2011). These transient PTP opening allow the mitochondria to defend themselves against elevated \([\text{Ca}^{2+}]_c\) levels and stimulate ROS production which can provide the cell with cardio-protective signaling (Murgia et al., 2009).

There are three subpopulations of mitochondria. The first are known as subsarcolemmal mitochondria (SSM) and are easily seen in skeletal muscle fibers (Rambourg and Segretrain, 1980). This terminology, referred to all mitochondria that could be isolated by polytron treatment (Chemnitius et al., 1993) versus intermyofibrilliar mitochondria (IMFM) that are tightly packed within a cell that span the sarcomere from the couplon at one Z-disk to the couplon at the next Z-disk and between the junctional SR (Lukyanenko et al., 2009). It is thought that these IMFM are positioned closest to the microdomains that see the largest rise in cytosolic \(\text{Ca}^{2+}\), possibly interconnected to the SR network and myofibrils. Lastly, cardiac mitochondria are further divided between IMFM and perinuclear mitochondria (PNM). IMFM are larger in size with a complicated shape as compared to the small, round PNMs (Lukyanenko et al., 2009). Looking at the electron micrographs of Fawcett and McNutt, these PNMs are circular while the IMFM are oval and confined to the space between Z-lines of the myocyte (Fawcett and McNutt, 1969).

In adult rat ventricular myocytes using fluorescent dyes, beat to beat oscillations of \([\text{Ca}^{2+}]_m\) does not seem to be present (Miyata et al., 1991; Griffiths et al., 1997a), while biologically targeted probes, such as aequorin, appear to produce the opposite results (Bell et al., 2006). In neonatal rat cardiomyocytes, on the other hand, it has been shown that these \([\text{Ca}^{2+}]_m\)
oscillations do occur (Robert et al., 2001). As described earlier, the neonatal cardiomyocytes have multiple calcium signaling pathways, lack t-tubules, and are spontaneously active. Although their SR is less developed than adult myocytes, they express a robust response to caffeine and have a well developed CICR mechanism. In neonatal cardiomyocytes, cytosolic Ca\textsuperscript{2+} rises rapidly in the sub-cellular space during a Ca\textsuperscript{2+} transient, followed by a slower rise in the cell center, in contrast to the quasi-uniform elevation in the adult (Griffiths et al., 2010). Mitochondria in the neonatal cells also appear to look different as compared to the adult myocyte via our imaging techniques and occupying a slightly less percentage of the cell.

Even though mitochondria make up about one-third of the cardiac cell, controversy remains as to their contribution to calcium signaling, specifically uptake kinetics and the magnitude of Ca\textsuperscript{2+} rise (Dekkova and Blatter, 2013) into the mitochondria. Two different theories and models with supporting evidence for each have been thus far proposed. Model I proposed by F. Crompton, suggests that Ca\textsuperscript{2+} uptake into the mitochondria is slow and is paired with an even slower release of this Ca\textsuperscript{2+} (Crompton, 1990). Most of these studies were conducted with electron probe microanalysis or fluorescent techniques that demonstrate slow Ca\textsuperscript{2+} uptake with no beat to beat changes in [Ca\textsuperscript{2+}]\textsubscript{m} (Miyata et al., 1991; Griffiths et al., 1997a). Model II differs from the first in that it posits the faster cytosolic oscillations in [Ca\textsuperscript{2+}]\textsubscript{c} are efficiently translated into beat to beat changes in [Ca\textsuperscript{2+}]\textsubscript{m} (Huser et al., 2000). It seems that the difference in results depend on experimental technique, species used in experiments, and mitochondrial location (Dekkova and Blatter, 2008). In whole cell signaling with permeabilized cells incubated in Fura-2, Sharma et al. found that [Ca\textsuperscript{2+}]\textsubscript{m} rose with similar kinetics of SR Ca\textsuperscript{2+} release (Sharma et al., 2000). Additionally, in our laboratory, it was shown that the SR release of
Ca\textsuperscript{2+} loads the mitochondria which can then be released back into the cytoplasm with mechanical stress, or pressure flow pulses of solutions (Belmonte and Morad, 2008). O’Rourke argues that mitochondria need to match the energy supply with demand and therefore requires a rapid mitochondrial response (O’Rourke and Blatter, 2009).

Controversy regarding mitochondrial calcium metabolism has been, in part, fueled by lack of specificity in cellular distribution of fluorescent Ca\textsuperscript{2+} probes. Recently, efforts to develop more useful and mitochondrial-bound probes to measure [Ca\textsuperscript{2+}]\textsubscript{m} are well underway. To measure Ca\textsuperscript{2+} in the mitochondria, several problems need to be overcome, such as crossing the cell membrane while being retained in the cell, acting as a specific reporter for Ca\textsuperscript{2+} without altering cell function, and directing the probe to the mitochondria (Griffiths, 2009). This approach has yielded the Ca\textsuperscript{2+}-sensitive fluorescent probe called mitycam. In an adenovirus serotype 5 (Ad5) vector, Kettlewell et al. were able to express an inverse pericam based genetically engineered Ca\textsuperscript{2+}-sensitive indicator targeted to the mitochondria (Kettlewell et al., 2009). This probe uses a mitochondrial targeting sequence of the subunit VIII of human cytochrome c oxidase, yellow fluorescent protein (YFP) as the fluorophore, and calmodulin (CaM) mutated in the 2\textsuperscript{nd} EF hand as the Ca\textsuperscript{2+} sensor. This probe shows mitochondrial co-localization with MitoTracker dyes and beat to beat changes in [Ca\textsuperscript{2+}]\textsubscript{m} in cardiomyocytes (Kettlewell et al., 2009; Lu et al., 2013).

Although this biological probe seems promising, some, including our lab, have questioned the probe’s ability to sense the range of calcium encountered by mitochondria. One aim of our study is to improve this probe by genetically modifying the 1\textsuperscript{st} EF hand of calmodulin in hopes of improving its dynamic range. In previous work done by Ohkura et al., in vitro studies on CaM mutations could alter the affinity of the protein to Ca\textsuperscript{2+} (Ohkura et al., 2005). The mutation of
the 2\textsuperscript{nd} EF hand of calmodulin (original mitycam probe, E67K) produced an undetermined $K_d$ due to the presence of two or more different affinities for $\text{Ca}^{2+}$ and a $F_{\text{max}}$ (measured at 510)/$F_{\text{min}}$ (with EGTA) value of 1.7 (Ohkura et al., 2005). In comparison, mutation of the 1\textsuperscript{st} EF hand of calmodulin produced an $F_{\text{max}}$/$F_{\text{min}}$ ratio of 3.2 (Ohkura et al., 2005). From this data, we hypothesize that an altered form of the biological probe mitycam could improve its overall ability to sense $\text{Ca}^{2+}$ and provide novel and valuable information on mitochondrial $\text{Ca}^{2+}$ signaling in the heart.

Although progress is being made in this area of research, there are still difficulties to overcome. Some of the available inhibitors of mitochondrial channels are not easily permeable across the cell membrane. For example, some investigators report that Ru360, the blocker of MCU, does not permeate into either adult or neonatal cardiomyocytes (Robert et al., 2001; Bell et al., 2006). CGP, the blocker of the mNCX, was also a difficult drug to use for it had problems with solubility and may induce spontaneous $\text{Ca}^{2+}$ oscillations (Griffiths et al., 1997b). In addition to the lack of specific mitochondrial inhibitors, the $\text{Ca}^{2+}$ signals produced by the commercial dyes, such as Indo-1 or Rhod-2, are often contaminated by the signal in the cytosol, leading to a great deal of confusion and controversy in the field.
Evolution of Calcium Signaling Protein, NCX, in Cardiac Myocytes

Most living organisms have evolved in ways that require them to use a combination of their own genetic machinery and that of one or more other species if they are to survive and reproduce (Thompson, 1999). For example, voltage-gated ion channels and exchangers are relatively old proteins that exists in both bacteria and prokaryotes that as a result of major genetic events such as gene duplications and more minor ones involving numerous single base mutations, have caused in a plethora of channel types to be evolved from what was, presumably, a single or limited number of precursors (Anderson and Greenberg, 2001). Over time, species specialization of proteins has become a way to improve the function of the animal’s machinery. It is possible that one such protein, the Na\(^+\)-Ca\(^{2+}\) exchanger, is thought to have diverged in time with slight changes in the genetic sequence. Although highly conserved, these changes have developed for a reason.

The Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) proteins constitute a family of solute carriers (SLC8A) that is found throughout the biosphere, and in most cells, NCX serves as the major pathway for removal of Ca\(^{2+}\) ions from the cytosol in exchange for three times as many Na\(^+\) ions (Reuter H, 1968; Reeves J.P., 1984; Kimura J., 1986; Pott et al., 2004) that produces an ionic current (\(I_{NCX}\)). With 9 transmembrane domains and a large cytoplasmic loop between domain 5 and 6, the NCX contains two Ca\(^{2+}\) binding domains and other important regulatory domains (Brini and Carafoli, 2000). Interestingly, this loop can be deleted from the NCX in heterologous expression experiments without the total loss of exchanger activity and function (Matsuoka et al., 1993). NCX can work in both directions to extrude Ca\(^{2+}\) (inward \(I_{NCX}\)) or bring Ca\(^{2+}\) into the cell (outward \(I_{NCX}\)). Like other ion channels, NCX has an equilibrium potential dependent on E\(_{Ca}\)
and $E_{Na}$, where $E_{NCX} = 3E_{Na} - 2E_{Ca}$. As a result, the high cytosolic $Ca^{2+}$ would favor $Ca^{2+}$ efflux and high cytosolic $Na^+$ would favor $Ca^{2+}$ influx. Under physiological conditions at resting, NCX operates primarily in the $Ca^{2+}$ efflux mode with reversal potential of about -40mV. In cardiac cells, this electrogenic reversible process interacts in many ways with the other $Ca^{2+}$-transporting proteins and ion channels, that in concert generate the action potential and control the strength of the heartbeat through tidal changes in cytosolic $Ca^{2+}$ ($[Ca^{2+}]_i$). To maintain $Ca^{2+}$ homeostasis during sustained beating, the extrusion of $Ca^{2+}$ by NCX must match the entry of $Ca^{2+}$ via L-type $Ca^{2+}$ current ($I_{Ca}$) during systole. In addition, depending on cell type, species (Sham et al., 1995), and physiological and experimental conditions, NCX may also provide sufficient $Ca^{2+}$-influx during the positive phase of the action potential to aid the following: directly augment the development of contractile force, support or replace $I_{Ca}$ in triggering release of $Ca^{2+}$ from the SR (Mattiello et al., 1998; Hove-Madsen et al., 2003), and generate an outward $I_{NCX}$ that significantly changes the shape of the action potential (Le Guennec and Noble, 1994; Dipla et al., 1999; Gaughan et al., 1999). Conversely, during and following the repolarizing phase of the action potential, NCX may generate sufficient inward current to evoke aberrant early or late after-depolarizations, potentially triggering cardiac arrhythmias depending on how well that $Na^+$-pump is capable of maintaining $[Na^+]_i$ at normal low levels (Linask KK, 2001; Reuter H, 2002; Müller-Ehmsen J, 2003; Ruch SR, 2003).

Comparative studies in various species often reveal interesting similarities and differences in calcium signaling events. In the human alone, NCX has three different isoforms: NCX1, NCX2, and NCX3. While isoforms NCX1 and NCX2 are found ubiquitously distributed in various tissues, NCX3 is found only in the brain. Therefore, the isoforms of this protein allow
for specific protein specialization. It is also true for evolution between species. There is substantial evidence that the $\text{Ca}^{2+}$ influx transporting mode of NCX is more pronounced in cardiac cells without $\text{Ca}^{2+}$ release properties of an SR. Some examples of this are the frog and the shark where the SR is rudimentary, and devoid of $\text{Ca}^{2+}$-release machinery (Morad M, 1971; Nabauer M, 1992; Woo and Morad, 2001), embryonic ventricular cardiac myocytes of mammals where prior to the post-natal development of t-tubules appear to have a non-functional SR (Huynh TV, 1992), and hypertrophic ventricular cardiac myocytes where the functional coupling between dihydropyridine (DHP) and ryanodine receptors (RyR) is impaired (Cleemann et al., 1998b). Thus, the differential contribution from the NCX in different species appears to be related to the development of sarcoplasmic release pools (Morad M, 1973; Bers, 1985) or variation of internal $\text{Na}^+$ levels (Eisner et al., 1983; Sutko et al., 1986). It has been shown that non-mammalian species, such as frogs and sharks, rely solely on trans-mural $\text{Ca}^{2+}$ flux in order to induce contraction of the cardiac muscle (Morad and Orkand, 1971).

Additionally, the activation of the $\beta$-adrenoreceptor/adenylate cyclase/cAMP dependent pathway leads to down-regulation of NCX in ventricular cells of the frog and shark in a manner that is quite different from the effects on mammalian cardiac cells where NCX is either insensitive to such interventions or enhanced. Specifically, in the frog ventricle, NCX was shown to have a unique insertion of 9 amino acids (27 base pairs, known as exon X) with a Walker A motif, or P-loop, which conferred a unique cAMP suppressive effect to the clone (Iwata et al., 1996; Shuba et al., 1998). Interestingly, this suppressive effect was able to be conferred onto the dog NCX via a chimera (He et al., 2003). Thus, although the difference in species in ionic milieu, heart rate, temperature, or absence or presence of the SR, the regulatory
effect of this amino acid sequence was dependent on its presence (Page and Niedergerke, 1972; Janowski et al., 2009). Similarly to the frog ventricle, the shark NCX was also shown to possess a unique regulatory domain. Their findings showed that with the deletion of this shark-specific, second proline/alanine-rich amino acid insertion within the P-loop eliminated the species specific bimodal regulation where there exists a differential regulation of the Ca$^{2+}$ influx and efflux pathways (Janowski et al., 2009). The β-adrenergic stimulation in frog and shark ventricular cardiomyocytes, in addition to enhancement of $I_{Ca}$ and thereby twitch force, has relaxant effects that are manifested in faster relaxation of the twitch, and suppression of the tonic force seen during sustained depolarization by KCl and long lasting voltage clamp pulses (Morad et al., 1981). Whereas similar effects in adult mammalian ventricular cells may be attributed, in part, to activation of the Ca$^{2+}$ release channel (RyR) and calcium-ATPase (SERCA) of the SR, it is proposed that the NCX may provide an alternate target essential for achieving both the inotropic and relaxant effects of epinephrine in certain vertebrates like sharks, and possibly in embryonic mammalian hearts. To examine these possibilities, it is my aim to study and characterize the transgenic mouse hearts and adenoviral expression system that overexpresses the shark NCX and probe its cAMP regulation.
Modulation and Regulation of Important Calcium Signaling Proteins

The normal cardiac cycle involving contraction and relaxation, also known as systole and diastole, is modulated by both the parasympathetic and sympathetic nervous system. In both physiological and pathological cases where adrenergic stimulation occurs, the clinical manifestation includes increased chronotropic, inotropic, and lusitropic effects on the myocardium. These effects are modulated primarily by the neurohomonal agonist, epinephrine, binding to the β-adrenergic receptor that induces intracellular changes through the Gs subunit of the g-coupled protein to stimulate increased production of cAMP and activation of protein kinase A (PKA) to phosphorylate many important calcium signaling proteins. The proteins that are phosphorylated by PKA include L-type Ca\(^{2+}\) channel, phospholamban, ryanodine receptor, and troponin. Through these actions, the heart is able to beat more efficiently with increased heart rate, contractility, and relaxation rate.

In mammals, the ryanodine receptor has three isoforms, RyR1 found in skeletal muscle, RyR2 in the heart, and RyR3 expressed in the brain. In cardiac muscle, the ryanodine receptor serves as the primary calcium release channel for the SR. Since it provides a large portion of the Ca\(^{2+}\) released for contraction, it plays an important role in CICR. Specifically, RyR2, is found organized in dyads of a group of about 30-250 RyRs (Franzini-Armstrong et al., 1999) that are also in close relation to the t-tubular network forming a microdomain with the L-type Ca\(^{2+}\) channel. Due to the large number of RyRs located in one small area, small Ca\(^{2+}\) release events can occur that give rise to Ca\(^{2+}\) sparks when only a few of these RyRs open. When these RyRs act in unison, they form the Ca\(^{2+}\) transient responsible for myocyte contraction. When
spontaneous Ca\(^{2+}\) sparks last longer or re-ignite repeatedly, they may contribute to SR Ca\(^{2+}\) leak (Lipp and Niggli, 1996; Sobie et al., 2006).

On the molecular level, RyR2 is a large macromolecular complex made up of a homotetramer with 4 subunits and a molecular weight of 2.2MDa. It is not only a Ca\(^{2+}\) release channel on the SR, but also a scaffolding protein that localizes key regulatory proteins to the junctional complex including: calmodulin, FKBP, PKA, phosphatases 1 and 2A, and sorcin (Bers, 2002). The tethering of the PKA and phosphodiesterase PDE4D allows for homeostatic regulation of the balance between phosphorylation and dephosphorylation (Niggli et al., 2013). Calmodulin dependent kinase II (CaMKII) has been also shown to have a role in post-translational modification of the RyR2. It has been proposed that this protein contains various serine sites as possible phosphorylation sites for both PKA and CaMKII. In single channel experiments, CaMKII modification increases the RyR open probability (Wehrens et al., 2004) and in single cardiomyocytes an increase of spark frequency (Guo et al., 2006). Conversely, PKA phosphorylation remains less clear. In transgenic mouse models, there have been conflicting reports, but in cases of heart failure, it has been proposed that phosphorylation dissociates the stabilizing protein, calstabin 2, resulting in diastolic Ca\(^{2+}\) leak and SR Ca\(^{2+}\) depletion (Marx et al., 2000; Niggli et al., 2013). Therefore, patients carrying a mutation in RyR2, such as patients with catecholaminergic polymorphic ventricular tachycardia (CPVT), often have a proclivity for destabilizing of the complex and show abnormal gating behavior. One way to evaluate these mutations is through our laboratory’s work on induced pluripotent stem cells (iPS cells). We have established cells lines of both control RyR and mutant RyR that expresses the CVPT specific mutation. The study of such cell lines will not only provide us with direct information.
on the iPS-CM phenotype, but also provide insight into the clinical implication and treatment of this disease.

Like the RyR, the NCX is also controversial in its PKA regulation. The mammalian Na\(^+\)-Ca\(^{2+}\) exchanger (NCX1, SLC8A1) contributes significantly to the Ca\(^{2+}\) fluxes that regulate the heartbeat, maintains long-term Ca\(^{2+}\)-homeostasis, and is likely to be subjected to cAMP-mediated, \(\beta\)-adrenergic regulation (Sham et al., 1995; Maxwell et al., 1999; Pott et al., 2004). As mentioned earlier, the mammalian NCX has three tissue specific subtypes, NCX1, NCX2, NCX3, and is further extended to the variable splicing subtypes such as NCX1.1, NCX1.2, and NCX 1.4. These subtypes demonstrate subtle changes in regulation caused by transportation of cations or adrenergic stimulation (Morad et al., 2011). In addition to regulation of NCX by thermodynamics of ion gradients, there is also allosteric regulation mediated by Ca\(^{2+}\) and Na\(^+\) where high concentrations inactivate the protein (Bers, 2008). In adult mammalian cardiac myocytes, our laboratory and others have shown that the activity of NCX1 to be unchanged by cAMP, perhaps because Ca\(^{2+}\)-ATPase (SERCA) of the SR has evolved as an important alternative \(\beta\)-adrenergic regulatory mechanism (Wei et al., 2003; Reppel et al., 2007). However, some groups have reported that \(\beta\)-adrenergic stimulation/cAMP enhanced NCX activity (Perchenet et al., 2000).

As mentioned in the previous section, intracellular elevation of cAMP suppresses NCX1 activity in frog and shark cardiac myocytes, which lack significant Ca\(^{2+}\) release stores, SERCA2, or phospholamban (Morad M, 1971; Woo and Morad, 2001). The frog NCX sequence has revealed a 9 amino acid Walker A motif, known as exon X (Iwata et al., 1996) that proved to be necessary for cAMP-dependent regulation, such that expression of a frog mutant sequence that
lacked this motif eliminated cAMP-dependent regulation (Shuba et al., 1998). Additionally, the shark NCX has been shown to display bimodal regulation that may provide an interesting pharmacological target for the regulation of the exchanger either in the Ca\(^{2+}\)-efflux (forward) or influx (reverse) mode (Woo and Morad, 2001; Cleemann et al., 2006). This bimodal regulation is defined as differential regulation of the Ca\(^{2+}\)-efflux and influx pathways where the outward current is suppressed while the inward current is enhanced or unchanged. This effect, in addition to a larger shift in \(E_{NCX}\) reversal potential with isoproterenol, may reflect the protective effect of NCX against cellular Ca\(^{2+}\) overload against adrenergically induced enhancement of Ca\(^{2+}\) channel current and its depolarizing effect on the plateau potential of the action potential (Woo and Morad, 2001).

To better understand this controversy, it has been suggested that PKA-mediated regulation of NCX can occur when a number of sizable unstructured linker sequences allow a more rigid \(\beta_1\), \(\beta_2\), and \(\alpha_{CAT}\) to form an open formation to expose important serine and threonine residues to PKA (Morad et al., 2011). The understanding of these alternative modes of regulation is important to the arrhythmogenic potential of the inward current generated by NCX1 (\(I_{NCX}\)), especially in hypertrophied mammalian myocardium and the pathophysiology of heart failure, where NCX1 is overexpressed and the SR function is impaired.

It is possible that overexpression of the shark NCX1 may prevent Ca\(^{2+}\) overload and arrhythmias in cardiac myocytes subjected to adrenergic stimulation when such transgenic mice are challenged with heart failure. This hypothesis is based on the following observations: 1) NCX is up-regulated in patients with heart failure (Munch et al., 2006a), resulting in the generation of delayed after-depolarizations (DADs), 2) Cardiac glycosides, which indirectly
regulate NCX through suppression of Na\(^+\)/K\(^+\) ATPase, are still the drug of choice in treating heart failure, 3) Unlike the human NCX1, the shark NCX1 has a unique bimodal cAMP-mediated regulation, that is, the Ca\(^{2+}\)-influx mode is strongly suppressed while the Ca\(^{2+}\)-efflux is enhanced or unchanged (Woo and Morad, 2001; Cleemann et al., 2006), reducing the proclivity for calcium overload and arrhythmogenesis, both common manifestations of human heart failure. Based on these findings, it is possible that overexpression of shark NCX1 gene in mice maybe highly protective in preventing calcium overload and arrhythmias in heart failure.
Diseased States of Calcium Signaling Proteins

The strict regulation of calcium flux in the cardiomyocyte is vital to proper cardiac electrical and mechanical function. Abnormalities in calcium handling, cycling, and signaling have been implicated in cardiovascular disease and are particularly well-characterized in heart failure and certain cardiac arrhythmias, including the rare, but life-threatening ventricular arrhythmia such as catecholaminergic polymorphic ventricular tachycardia (CPVT).

As mentioned earlier, the dependence upon calcium (Ca\(^{2+}\)) for cardiac contraction was initially identified by Sydney Ringer in 1883 (Ringer, 1883), and subsequently, Ca\(^{2+}\) was demonstrated to link myocardial contraction and excitation by Locke and Rosenheim in 1907 in their experiments on dextrose handling in the cardiomyocyte (Locke FS, 1907). The initiation of cardiac contraction in a healthy heart begins with the electrical depolarization of the sinoatrial (SA) node, a compact area of cardiac tissue in the high right atrium at the lateral part of the sulculus terminalis (the junction of the inferior vena cava and the atrium). The SA node is comprised mostly of specialized cardiac cells known as pacemaker cells, which display automaticity and are sensitive to many stimuli including changes in temperature, pressure, shear stress, and various chemical and electrical stimuli (Boineau et al., 1988). In addition, the SA node is highly innervated by parasympathetics (vagal nerve) and sympathetics (paravertebral chain ganglia) of the autonomic nervous system and therefore subject to regulation by neurotransmitters such as acetylcholine (ACh) and norepinephrine (NE). The SA node action potential is divided into three phases. Spontaneous depolarization (Phase 4) triggers the action potential when the cell membrane potential reaches a threshold which is typically between -40 and -30 mV. This is followed by Phase 0, which represents the depolarization phase of the
cardiac action potential. Next is repolarization (Phase 3), which is predominantly characterized by inward rectifying potassium ($I_K$) currents. Once the cell is completely repolarized at about -60 mV, the cycle is then repeated without dependence on external electrical stimuli, a pacemaker cell trait known as automaticity. Sinoatrial node automaticity is thought to be dependent on sarcolemmal voltage and sarcolemmal calcium clocks, as described by Lakatta, Maltsev and colleagues (Maltsev and Lakatta, 2008, 2009), which are responsible for the SA node action potential. The “voltage clock” is a hyperpolarization-activated, cAMP-modulated primarily inward current called $I_f$ (“funny”). Early in Phase 4 of the cardiac action potential, $I_f$ current activation depolarizes the sarcolemmal membrane leading to Ca$^{2+}$ influx which activates the cardiac ryanodine receptor (RyR2), in turn releasing intracellular Ca$^{2+}$ that is stored in the sarcoplasmic reticulum. Intracellular Ca$^{2+}$ is transported back to the SR by the SR Ca$^{2+}$-ATPase (SERCA), which reloads the cell for the next action potential. SA node depolarization is not entirely dependent upon the “voltage clock”. In addition, there is an equally important sarcolemmal “calcium clock”, which is active in late Phase 3 and early Phase 4. This “calcium clock” is driven by $I_{NCX}$, whereby 3 extracellular sodium (Na$^+$) ions are exchanged for 1 intracellular Ca$^{2+}$ ion (Chen et al., 2010). This process, which is collectively referred to as atrial depolarization, lasts between 80 and 110 milliseconds, spreading from the left to right atria via Bachmann’s bundle and to the atrioventricular (AV) node through internodal pathways. Signal is then typically delayed slightly through the AV node, and conducted down the bundle of His, left and right bundle branches and ultimately through a system of Purkinje fibers to depolarize the ventricular myocardium. Upon the arrival of the action potential wave front in the myocardium, calcium which is stored in vesicles within the SR, is released and binds to troponin C (TnC),
thereby enabling troponin I (TnI) to release from actin. This allows binding of actin and myosin filaments that result in shortening of the sarcomere and ultimately myocardial contraction (Phillips et al., 1986).

Abnormal Ca\(^{2+}\) cycling has been implicated in the development of many of the clinical cardiomyopathies. Studies of cardiomyocytes from patients with advanced heart failure have demonstrated abnormal calcium handling. In particular, there was reduced Ca\(^{2+}\) transient amplitude, prolonged Ca\(^{2+}\) transient decay time and increased Ca\(^{2+}\) transient duration as well as reduced SR Ca\(^{2+}\) levels (Hobai and O'Rourke, 2001). Proposed explanations of these changes may be due to alterations in SERCA expression and function, abnormalities in NCX expression, alterations in L-type calcium channels and mutations and/or hyperphosphorylation of the ryanodine receptor (Kho et al., 2012).

The decreased systolic contractile function that characterizes clinical heart failure can, in part, be attributed to abnormal Ca\(^{2+}\) handling in the sarcoplasmic reticulum. A failing human heart is characterized by reduced SERCA Ca\(^{2+}\) transport rate (Limas et al., 1987; Mercadier et al., 1990; Pieske et al., 2002), in part due to reduced SERCA protein expression, increased inhibitory action of unphosphorylated phospholamban, and altered β-adrenergic signal transduction, therefore reducing the major source of calcium release from the SR for contractile proteins in the failing heart (Schlotthauer et al., 1998; Munch et al., 2006b). SERCA down-regulation has been particularly implicated in contractile dysfunction, including evidence for partial normalization of contraction after adenoviral overexpression of SERCA in failing human cardiomyocytes (del Monte et al., 1999).
NCX expression has been shown to be upregulated in patients with clinical heart failure, possibly as a result of elevated diastolic Ca\(^{2+}\) levels in the failing cardiomyocyte (Zaugg and Buser, 2001). As a result, there have been numerous studies that utilize transgenic models of NCX overexpression. In these models, the mice developed normally and did not produce any heart conditions such as hypertrophy or heart failure (Adachi-Akahane et al., 1997; Baumer et al., 1998). The effects of chronic overexpression of the Na\(^{+}\)-Ca\(^{2+}\) exchanger in isolated mouse myocytes were evaluated in several different publications and the NCX proved to be functionally active. In all of the transgenic data examined, there was no change in other protein levels such as SERCA, phospholamban, and calsequestrin but there was an increase in exchanger current density, \(I_{NCX}\) (Morad M, 2004). Additionally, there was an increase in calcium content of the SR with no changes in dP/dt and enhanced \(-dCa/dt\) as compared to control littermates (Adachi-Akahane et al., 1997). Furthermore, data suggest that transgenic overexpression of NCX was accompanied by enhanced inotropic responsiveness upon agonist stimulation (Adachi-Akahane et al., 1997).

LTCCs, which are responsible for entry of Ca\(^{2+}\) into the cytosol, facilitate activation of the RyR which release stored Ca\(^{2+}\) from the SR (Ca\(^{2+}\)-dependent Ca\(^{2+}\)-release) to initiate myocyte contraction (Benitah et al., 2010; Kho et al., 2012). In cardiac hypertrophy and heart failure, \(I_{Ca}\) density remains unchanged, however, the kinetics are clearly altered. There are measurable delays in AP duration, increasing LTCC Ca\(^{2+}\) entry which favors the development of early after-depolarizations (EADs) which can be pro-arrhythmic (Benitah et al., 2010).

Diastolic “leak” of calcium through abnormally functioning RyR is another important part of the abnormal calcium cycling in the failing heart (Marx et al., 2000). One proposed
mechanism of abnormal RyR function in heart failure has to do with β-adrenergic driven PKA hyperphosphorylation of 3-4/4 of the RyR monomers at Ser2809 (Marx et al., 2000; Reiken et al., 2003b). In fact, it has been shown that with the use of mechanical left ventricular assist devices (LVADs), which decrease left ventricular stroke-work and left ventricular end-diastolic pressure (LVEDP) in the failing heart, levels of hyperphosphorylated RyR are reduced along with levels of circulating catecholamines (Estrada-Quintero et al., 1995). The beneficial effect of β-blockers in heart failure has been attributed to this observation. In 2003, Reiken and colleagues demonstrated reduced RyR hyperphosphorylation at Ser2809 in heart failure patients taking β-blockers (Reiken et al., 2003a).

Catecholaminergic polymorphic ventricular tachycardia is a familial, exercise-induced, potentially fatal ventricular arrhythmia that is not caused by ischemia. In the late 1990s, two research groups demonstrated several mutations in the ryanodine receptor in families of patients with CPVT (Swan et al., 1999; Priori et al., 2001). Currently there have been 155 mutations identified in 3 distinct regions of RyR2 linked to CPVT. Several theories have been postulated to explain the arrhythmogenic potential of the mutated ryanodine receptor.

The first, as observed in several mutations (S2246L, R2474S and R4497C) demonstrates a reduced affinity of the RyR to calstabin2, a ryanodine receptor regulatory protein, which increases diastolic Ca$^{2+}$ leak from the SR leading to EADs (Wehrens et al., 2003). Engineered mice with the Ryr-R2474S human CPVT mutation develop exercise-induced polymorphic ventricular tachycardia which can be prevented by administering the drug S107, which binds calstabin2 to mutant CPVT channels and prevents diastolic Ca$^{2+}$ leak from the SR (Lehnart et al., 2008).
Another theory by Chen and colleagues is that CPVT mutations make RyR sensitized to baseline $\text{Ca}^{2+}$ levels, thereby preventing any normal $\text{Ca}^{2+}$ leak (Chen et al., 2010). However, when $\beta$-adrenergic receptors are stimulated above threshold, there is increased diastolic $\text{Ca}^{2+}$ leak and increased risk of EADs. This is supported by work in HEK cells expressing the recombinant CPVT-mutant ryanodine receptor, which had higher extracellular calcium levels upon stimulation compared to WT cells (Jiang et al., 2005).

Additional mutations have been shown to induce unzipping of the central and N-terminal domains of the RyR leading to diastolic $\text{Ca}^{2+}$ leak (Uchinoumi et al., 2010) and have been implicated in the development of CPVT. This mechanism, first proposed by Tateishi et al. (Tateishi et al., 2009), used the domain peptide DPc10, which matches the Gly (2460)-Pro (2495) region of RyR, interfering with the normal interaction of two domains of the RyR receptor. Addition of DPc10 induced domain unzipping, increasing SR $\text{Ca}^{2+}$ leak and $\text{Ca}^{2+}$ spark frequency in failing cardiomyocytes. In addition, calmodulin (CaM) binding to the RyR is disrupted by channel unzipping in mutant myocytes with the DPc10 peptide but not in WT cells, which suggests a possible mechanism for increased diastolic $\text{Ca}^{2+}$ leak and the development of arrhythmias. In fact, studies of the drug dantrolene, which is used to prevent anesthetic-induced malignant hyperthermia in patients with abnormal RyR1 (not RyR2), has been studied and may stabilize unzipping in a pacing-induced failing dog heart model (Kobayashi et al., 2009).

Clinically, the $\text{Na}^+$ channel antagonist flecainide is used to treat patients with CPVT, as was demonstrated by Watanabe and colleagues (Watanabe et al., 2009) in 2009, to prevent lethal arrhythmias in mice and 2 human volunteers who were recalcitrant to standard therapy. The mechanism of action has been recently proposed to be a direct effect of flecainide on the
ryanodine receptor to reduce Ca\textsuperscript{2+} spark frequency by briefly closing the open ryanodine receptor, reducing their bursting pattern burst mass (Hilliard et al., 2010). However, Liu and colleagues have recently offered evidence that flecainide may simply work through Na\textsuperscript{+}-channel blockade, by reducing DADs from activating action potential (Liu N, 2010). Others have looked at the role of Ca\textsuperscript{2+} and calmodulin-dependent protein kinase II (CaMKII) in the generation of spontaneous Ca\textsuperscript{2+} waves from the SR after β-adrenergic stimulation, implicating its role in the development of arrhythmias and possibly CPVT (Curran et al., 2010). In fact, inhibition of CaMKII has been shown to prevent CPVT (Liu et al., 2010) in the RyR2 (R4496C+/−) knock-in mouse model of CPVT (Liu et al., 2010).
CHAPTER I

A New Approach to Evaluation of Mitochondrial Calcium Signaling in Cardiac Myocytes
Abstract

Cardiac EC coupling is mainly controlled by $I_{Ca}$-gated $Ca^{2+}$ release, but the extent to which mitochondria contribute to the overall $Ca^{2+}$ signaling remains controversial. To examine the possible role of mitochondria in $Ca^{2+}$ signaling, we used a lower $Ca^{2+}$ affinity mitochondrial probe, mitycamE31Q (300-500 MOI, 48-72h) in conjunction with Fura-2AM in cultured neonatal rat cardiomyocytes using a dual wavelength TIRF imaging system (< 150nm resolution depth, 10-70Hz). $Ca^{2+}$ was simultaneously measured in cytosol and mitochondria using mitycamE31Q and Fura-2AM. TMRE was used to monitor mitochondrial membrane potential. MitycamE31Q and TMRE staining of feline cardiomyocytes showed typical mitochondrial longitudinal fluorescent bands, while neonatal cardiomyocytes show a more disorganized punctate pattern of expression. In these cells, caffeine application caused a rapid decline in Fura-2 signal (increased cytosolic $Ca^{2+}$) that recovered slowly while average mitycamE31Q signal decreased (increase of mitochondrial $[Ca^{2+}]_m$) before decaying slowly back to baseline levels. Withdrawal of Na$^+$ decreased both the Fura-2AM and mitycamE31Q signals, consistent with rise of cytosolic and mitochondrial $Ca^{2+}$, however, in some mitochondrial populations mitycamE31Q signals suggests a decrease in $[Ca^{2+}]_m$. Similar experiments in neonatal cardiomyocytes identified peripheral and central mitochondria as responding differently to cellular $Ca^{2+}$ overload conditions. Our findings suggest that $Ca^{2+}$ in the mitochondria not only shows transients that are generally delayed and attenuated compared to the cytosolic $Ca^{2+}$ signals, but also identified conditions and mitochondrial subpopulations that spontaneously regulate their own local calcium concentrations. When synchronized, such local mitochondrial $Ca^{2+}$ releases may generate large global cytosolic $Ca^{2+}$ alterations that may interfere with normal $Ca^{2+}$ signaling.
Introduction

Cardiac excitation-contraction coupling (ECC) is a series of events that allows the electrical signal of the action potential to trigger cellular events to generate a contraction. These cellular events have been widely studied and are known as Ca\(^{2+}\) induced Ca\(^{2+}\) release, where influx of Ca\(^{2+}\) through the L-type calcium channel triggers a large release of Ca\(^{2+}\) from the SR to be used by the myofilaments for activation of contraction. The Ca\(^{2+}\) in this process needs to also be taken back into the SR by SERCA and extruded out via the NCX to maintain proper Ca\(^{2+}\) homeostasis. In addition, this cytosolic Ca\(^{2+}\) released by the SR is used for the control of other processes, such as gene transcription and stimulation of mitochondrial ATP production (Denton and McCormack, 1990; Bers, 2008). Since it is known that ECC consumes large amount of ATP, it is possible that mitochondria play a role in this process as well, and given the fact that mitochondria make up between 30-40% of the cardiac cell volume, it is possible that this contribution could be substantial (Barth et al., 1992). It was not until 1950s where researchers found that mitochondria could accumulate Ca\(^{2+}\) (Slater and Cleland, 1953). Initial studies by Carafoli and Lehninger showed that not only could mitochondria take up Ca\(^{2+}\) to a limited amount to maintain a dynamic state that could be returned to the cytosol, but also they were capable of taking up larger amounts of Ca\(^{2+}\) when phosphate was also taken up (Carafoli and Lehninger, 1964). Now it is known that this Ca\(^{2+}\) taken into the mitochondria can stimulate nitric oxygen production (NO), which in turn can modulate oxygen consumption, ATP production, ROS generation, and therefore provides negative feedback for the regulation of mitochondrial Ca\(^{2+}\) production (Dekdova and Blatter, 2008). Nevertheless, controversy in the field still remains primarily because there is little consensus on whether there is a beat to beat rise and fall
of mitochondrial Ca$^{2+}$ in the heart and what role, if any, [Ca$^{2+}$]$_{m}$ plays in the overall calcium signaling.

The structure and proteins of the mitochondria may play an important role in understanding their role in Ca$^{2+}$ signaling. The mitochondria have two membranes, known as the outer mitochondrial membrane (OMM) and the inner mitochondria membrane (IMM), and both have different functions. Around the outer membrane is the cytosolic side of the OMM and the intermembrane space (IMS) found between the two membranes. Lastly there is the mitochondrial matrix. The outer membrane contains many proteins and can pass small molecules freely between the cell cytoplasm and the IMS due to a large number of porins (Mihara et al., 1982; Towbin et al., 1989; Paterniani et al., 2011). Recently, other evidence have suggested that the OMM is not passively permeable to Ca$^{2+}$ and actually serves as a permeability barrier to both Ca$^{2+}$ influx and Ca$^{2+}$ efflux (Moran et al., 1992; Crompton et al., 2002; Baines et al., 2007). This research suggests that the OMM may have an important role in controlling Ca$^{2+}$ cycling and mitochondria function (Bathori et al., 2006). Conversely, the IMM, which contains complexes of the respiratory chain, ATP synthase, and other enzymes (Fernie et al., 2004), is impermeable even to small molecules such as Ca$^{2+}$ and H$^{+}$. As a result, this allows complexes in the respiratory chain to build up a proton gradient that result in an electrochemical gradient and the basis for the Ψ$^{m}$ which is utilized to produce ATP (Paterniani et al., 2011). In normal hearts, ΔΨ$^{m}$ is highly regulated so that the production of ATP is maintained within a physiological range that matches energy production to metabolic demand (O'Rourke, 2007; Akar and O'Rourke, 2011). In order for Ca$^{2+}$ to enter the mitochondria, it is generally supposed to pass freely through the OMM, but its entry into the matrix must be mediated through one of three mechanisms. The
three mitochondria calcium entry pathway thus far identified are: MCU, RaM, and mRYR1. It is the MCU that is thought to be the most important Ca$^{2+}$ influx pathway into the matrix. Ca$^{2+}$ efflux pathways have also been proposed for the mitochondria: Na$^+$.Ca$^{2+}$ exchanger (mNCX), H$^+$.Ca$^{2+}$ exchanger (HCX), and the permeability transition pore (mPTP).

Complicating the calcium transporting role of mitochondria are also the findings that three subpopulations of mitochondria are recognized in a single cardiac myocyte; the subsarcolemmal mitochondria (SSM) (Rambourg and Segretain, 1980); the perinuclear mitochondria (PNM); and the inter myofibrillar mitochondria (IMFM) that are tightly packed within a cell that span the sarcomere from the couplon at one Z-disk to the couplons at the next Z-disk between the junctional SR (Lukyanenko et al., 2009). It is thought that the IMFMs are positioned closest to the microdomains that are subject the largest rise and fall in cytosolic Ca$^{2+}$ especially where they were interconnected to the SR network and myofibrils. IMFMs are larger in size with a complicated shape as compared to the small, round PNMs (Lukyanenko et al., 2009). Looking at the electron micrographs of Fawcett and McNutt, these PNMs have circular cross sections while the IMFM are oval found between Z-lines of the myocyte (Fawcett and McNutt, 1969).

Even though mitochondria make up about one-third of the cardiac cell volume, controversy remains on their contribution to calcium signaling, specifically uptake kinetics and magnitude (Dedkova and Blatter, 2013) into the mitochondria. There are two different theories with supporting evidence for both. Model I proposed by Crompton et al. that Ca$^{2+}$ uptake into the mitochondria is slow and is paired with an even slower release of this Ca$^{2+}$ (Crompton, 1990). Most of these studies were conducted with electron probe microanalysis or fluorescent
techniques that demonstrate slow Ca\(^{2+}\) uptake with no beat to beat changes in [Ca\(^{2+}\)]\(_m\) (Miyata et al., 1991; Griffiths et al., 1997a). Model II differs in that fast oscillations in [Ca\(^{2+}\)]\(_c\) are efficiently translated into beat to beat changes in [Ca\(^{2+}\)]\(_m\) (Huser et al., 2000). It seems that the difference in results depend on experimental technique, species used in experiments, and mitochondrial location (Dedkova and Blatter, 2008). Additionally, in our laboratory, we found that CICR loads the mitochondria and could be released rapidly (~300ms) back into the cytoplasm with mechanical stress, or pressure puff (Belmonte and Morad, 2008). O’Rourke argues that mitochondria need to match the energy supply with demand and therefore requires a rapid mitochondrial response (O'Rourke and Blatter, 2009).

Recently, there has been a push to develop more useful probes to measure [Ca\(^{2+}\)]\(_m\). Both biologically targeted Ca\(^{2+}\)-sensitive photoprotein aequorin and ratiometric pericams have been developed. To measure Ca\(^{2+}\) in the mitochondria, several problems need to be overcome; such as crossing the cell membrane while being retained in the cell, acting as a reporter for Ca\(^{2+}\) without disturbing cell function, and specifically locating to the mitochondria (Griffiths, 2009). This alternative approach has yielded the Ca\(^{2+}\)-sensitive fluorescent probe called mitycam (Kettlewell et al., 2009). In an adenovirus serotype 5 (Ad5) vector, Kettlewell et al. expressed an inverse pericam based genetically engineered Ca\(^{2+}\)-sensitive indicator targeted to the mitochondria (Kettlewell et al., 2009). This probe uses a mitochondrial targeting sequence of the subunit VIII of human cytochrome c oxidase, yellow fluorescent protein (YFP) as the fluorophore, and calmodulin (CaM) mutated in the 2\(^{nd}\) EF hand as the Ca\(^{2+}\) sensor. This probe shows mitochondrial co-localization with MitoTracker dyes and beat to beat changes in [Ca\(^{2+}\)]\(_m\) in cardiomyocytes (Kettlewell et al., 2009; Lu et al., 2013). Although this biological probe
seems promising, some, including our lab, criticize the probe’s ability and limited range to sense calcium. In the work presented here, we aimed to alter this probe by genetically modifying the 1st EF hand of calmodulin, as shown previously in *in vitro* studies of CaM mutations that altered CaM affinity to Ca$^{2+}$ (Ohkura et al., 2005), and thereby improving its dynamic range. The mutation of the 2nd EF hand of calmodulin (original mitycam probe, E67K) produced a $F_{\text{max}}$(measured at 510)/$F_{\text{min}}$(with EGTA) value of 1.7 (Ohkura et al., 2005). In comparison, mutation of the 1st EF hand of calmodulin produced an $F_{\text{max}}$/ $F_{\text{min}}$ of 3.2 (Ohkura et al., 2005). From this data, we hypothesize that an altered form of the biological probe mitycam could improve its overall ability to sense Ca$^{2+}$ and provide valuable information to the debate on mitochondrial Ca$^{2+}$ signaling in the heart.

With this new probe, mitycamE31Q, we determined the $K_d$ of the probe to be 1.5-1.8μM as compared to the original mitycam $K_d$ of 197nM (Lu et al., 2013). In addition, we were able to study the role of Ca$^{2+}$ signaling in the mitochondrial while simultaneously measuring Ca$^{2+}$ flux in the cytosol using Fura-2AM. We determined that subsequent to Ca$^{2+}$ release from the SR upon exposure to caffeine stimulation or spontaneous Ca$^{2+}$ transients, mitochondria take up Ca$^{2+}$ into their matrix. Conversely, when cells are loaded with Ca$^{2+}$, the mitochondria operate as separate populations where the central mitochondria initially pick up Ca$^{2+}$ before releasing their contents quickly, where the peripheral mitochondria seemingly only pick up Ca$^{2+}$. It is possible that this large release from the central mitochondria is due to triggered opening of the PTP. Although the long-lasting PTP opening is thought to be induced by Ca$^{2+}$ overload and a way to clear the mitochondria of damaged and unneeded molecules (Murgia et al., 2009; Giorgi et al., 2012), Korge *et al.* also demonstrated transient openings of the PTP thought to be protective. These
transient PTP opening allow the mitochondria to defend themselves against elevated $[\text{Ca}^{2+}]_c$ levels and stimulate ROS production which can provide the cell with cardio-protective signaling (Murgia et al., 2009). Our findings suggest that Ca$^{2+}$ in the mitochondria not only show transients that are generally delayed and attenuated compared to the cytosolic Ca$^{2+}$ signals, but at intervals can also be released locally and spontaneously. When synchronized, the local mitochondrial Ca$^{2+}$ releases may trigger generate large cellular cytosolic Ca$^{2+}$ releases that may interfere with normal Ca$^{2+}$ signaling. Lastly, our method used to detect mitochondrial Ca$^{2+}$, pairing the biological probe with our powerful imaging technique of TIRF, could potentially offer insight into many mitochondrial functions.
Materials and Methods

Adenovirus Production

MitycamE31Q plasmid was a generous gift from Dr. Godfrey Smith, University of Glasgow. This plasmid contained the site specific mutation of amino acid 31 of the calmodulin portion of the mitycam probe as confirmed by sequencing. A compatible cloning site, as well as Kozak sequence, was then introduced to the mutated mitycam prior to the start codon in order to allow future cloning compatibility with the viral shuttle vector needed for viral production. This was accomplished through PCR of the 1.4kb mitycamE31Q sequence using fast start high fidelity polymerase (Roche) with proofreading capabilities. The primers used for PCR were sense, 5’-AGTACTACCATGTCCGTCTGACGACCGCTGC-3’, and antisense primer, 5’-GCGGCCGCTCACTTTGCTGTCATCATTTGTAC-3’. The PCR was run on the C1000 thermal cycler (Bio-Rad) with the following protocol: 95°C for 5 minutes, [95°C for 30s, 55°C for 1 minute, 72°C for 3 minutes]x35 cycles, 72°C for 10 minutes. The product was then run on a 1% TAE agarose gel and extracted using the method described in the Gel Extraction Kit (Qiagen). Ligation into the pGEM-T-easy vector (Promega) was then preformed using JM109 (Promega) cells and correct colonies were selected using blue and white colony screening on ampicillin resistant LB agar plates treated with 20mM IPTG and 80μg/mL X-Gal (both Promega). DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen) and QIAfilter Plasmid Midi Kit (Qiagen) and the construct was sequenced to ensure no mutations. Once confirmed, the construct was ligated into the EcoRV and Not I (both New England Biolabs) sites of the multiple cloning site of the viral shuttle vector Dual-CCM(+) (Vector Biolabs) which contains a CMV promoter and poly-A termination signal and transformed into DH5α
(Invitrogen) cells according to the manufacturer’s protocol and screened using kanamycin treated LB agar plates. Colonies were selected and DNA was again extracted. DNA concentration was calculated using the 260nm absorbance on a BioMate 3S UV-Viable Spectrophotometer (Thermo Scientific) and later used for transfection of HEK 293 and adenoviral production by Vector Biolabs. The final viral product was titered to $5.2 \times 10^{10}$ PFU/mL.
Cell Culture and Transfection

HEK 293 cells (ATCC) were maintained in culture at low passage for all experiments (passage 4-30). Cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 1% non-essential amino acids (Invitrogen). When growth reaches about 90% confluency, cells were passaged for continued growth in culture. HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. In summary, cells were split into 18mm coverslips at a density of $0.25 \times 10^5$ cells per well where 70% confluency was achieved the following day. Cells were then transfected with a total of 3µg plasmid DNA. Eight hours later, the medium was changed to fresh complete medium and allowed to express the protein 48 hours post-transfection.
**Isolation and Culture of Neonatal Cardiomyocytes**

Hearts of 5-10 Sprague Dawley rats between postnatal day 1-5 were excised, and the main vessels and atria removed according to the Institutional Animal Care and Use Committee. The ventricles were minced with a razor blade and incubated in Hank's Balanced Salt Solution (HBSS, Worthington Biochemical) with trypsin (50 μg/ml, Worthington Biochemical) for 15 h at 4 °C. The digestion was halted by trypsin inhibitor (200 μg/ml, Worthington Biochemical) for 20 minutes at 37°C. Collagenase (100 U/ml, Worthington Biochemical) was used for 30 min to isolate single neonatal cardiomyocytes, which were then filtered and centrifuged at 1000 rpm for 3 min, re-suspended in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen) with 1% penicillin–streptomycin (Invitrogen) and 1% non-essential amino acids (Invitrogen), plated on 100-mm dishes and placed in the incubator for 90 minutes to eliminate fibroblasts. Isolated single neonatal cardiomyocytes were plated onto either glass coverslips (Fisher Scientific) or WillCo Dishes (Ted Pella) and used for immunocytochemistry or TIRF experiments.
Adenoviral Transduction

Twenty-four hours after the neonatal cells were plated on WillCo Dishes, the complete medium was removed and the cells were infected with the mitycamE31Q adenovirus at a multiplicity of infection (MOI) of 300-500 virus particles per cell (vp/cell). The following day (after 12 hours) the virus medium was removed and cells were supplemented with normal neonatal medium and kept in the incubator at 37°C and 5% CO₂ until ready for experiments 48-72 hours post infection.
**Immunocytochemistry**

MitycamE31Q infected rat neonatal cardiomyocytes were grown on 18mm glass coverslips and stained with 100nM MitoTracker DeepRed (Invitrogen) for 30 minutes. Cells were then fixed with 4% paraformaldehyde for 10 minutes at room temperature and mounted on Superfrost Slides (VWR) using ProLong Gold with DAPI (Invitrogen) according to manufacturer’s directions. Once cured, the cells were imaged sequentially to avoid bleed through using Diode 405nm, Argon 514nm, and HeNe 633nm on a Leica SP5 Confocal microscope with a 63x oil objective and images were collected in LAS AF (Leica Microsystems).
**Calibration of MitycamE31Q**

MitycamE31Q was calibrated in permeabilized adult rat cardiomyocytes. Adult rat cells were isolated according to previously published method (Mitra and Morad, 1985). Once Langendorf-perfused and enzymatically digested, the ventricle was removed and cut into pieces and washed with O₂-saturated 0.2mM CaCl₂ normal Tyrodes consisting of (in mM): 137 NaCl, 10 Glucose, 10 HEPES, 5.4 KCl, 1 MgCl₂. This tissue was then incubated at 37°C with gentle shaking for 5 minutes. After rinsing tissue in the same buffer, a gentle trituration was applied to release myocytes. The cellular suspension was then plated on 25mm coverslips (Fisher Scientific) coated with extracellular matrix proteins (ECM gel from Engelbreth-Holm Swarm murine sarcoma, (Sigma) diluted to 12.5% with M199 (Invitrogen)). Cells were allowed to settle for about 1 hour in Medium 199 supplemented with 1% penicillin/streptomycin (Invitrogen) and 870nM insulin, 65nM transferrin, and 29nM Na⁺-selenite (ITS, Invitrogen), and 0.5µM cytochalasin D (Sigma). One hour after plating, fresh supplemented medium was replaced and allowed 1 hour before mitycamE31Q transduction. For calibration measurements, Na⁺-free internal solutions to enable the measurement of the Ca²⁺ sensitivity of mitycamE31Q had the following composition (mM): 5 BAPTA, 20 HEPES, 100 K-aspartate, 40 KCl, 1 MgCl₂, 2 maleic acid, 2 glutamic acid, 5 pyruvic acid, 0.5 KH₂PO₄, pH 7.4 adjusted with Trizma base. CaCl₂ was added to create a range of Ca²⁺ from < 1 nM to 400µM and free [Ca²⁺] was confirmed with Fura-2 calibration for each solution at 340nm and 380nm as previously reported (Cleemann and Morad, 1991). AdMitycamE31Q transduced cardiomyocytes were mounted on the stage of an inverted microscope and permeabilized with saponin (100 µg/ml) and ionomycin (1 µM) and thapsigargin (5µM) before perfusion with the intracellular solutions containing the range of Ca²⁺.
concentrations (< 1 nM to 400 μM) and 5μM FCCP and 1 μM oligomycin to dissipate mitochondrial membrane potential. Epi-fluorescence measurements were taken with excitation at 488 nm on LAS AF (Leica). Fluorescence measurements were normalized to the baseline fluorescence using the following equation: F=1-(F/F_{max}).
Total Internal Reflection Fluorescence (TIRF) Imaging

Intact infected neonatal cardiomyocytes were maintained in normal bath Tyrode (in mM): 137 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 10 Glucose and 10 HEPES (titrated to pH 7.4 with NaOH). Spontaneous Ca²⁺ release events were measured after rapid application of 5 mM caffeine or exposure to depolarizing solution containing in mM: 5 NaCl, 40 KCl, 100.4 TeaCl, 2 CaCl₂, 1 MgCl₂, 10 Glucose and 10 HEPES (titrated to pH 7.4 with TeaOH). In certain experiments, cells were exposed to depolarizing solution containing specific mitochondrial agents either: 400nM Cyclosporin A or 5µM Ru360. All experiments were carried out at room temperature (22–24 °C). Cells were loaded with Fura-2AM + Pluronic (5µM for 30 minute, both Invitrogen) in order to measure subsarcolemmal cytosolic calcium. The cells were imaged using a Leica multicolor total internal reflection fluorescence (TIRF) imaging system (Leica Microsystems, Buffalo Grove, IL) fitted with a 63x oil-immersion objective lens and an Andor iXon3 camera with 512 x 512 pixels. An argon ion laser was used for excitation at 405 nm (Fura-2AM) and 488 nm (mitycamE31Q) and fluorescence emission was measured at wavelengths >515 nm. Cells were imaged at 10-70 Hz with a depth of penetration less than 150 nm into the cell, focus on sub-sarcolemmal Ca²⁺ release in regions where the cell membrane was attached to the underlying glass cover slips. The pixel size in the object plane was 0.25 µm square, or 0.157 µm with 1.5 zoom (Cleemann et al., 1998b; Woo et al., 2005).
Image analysis

The obtained image sequences were collected and surveyed with Leica software (LAS AF), but displayed and analyzed in detail using a custom-designed program (Con2i). The average resting fluorescence intensity ($F_0$) was calculated from several frames measured immediately before drug application or intervention with little or no indication of Ca$^{2+}$ release activity and after initial mitycam bleaching. Images were filtered and the amplitudes of the Ca$^{2+}$-dependent cellular fluorescence signals were quantified as $\Delta F/F_0$ where $F_0$ is the average of several frames before the Ca$^{2+}$ releases. $\Delta F$ and $F_0$ were either integrated over the entire cell (Fura-2AM) or were measured in specific regions of mitochondrial expression.
Statistical Analysis

Average values are presented in histograms and in the text as the mean ± the standard error of the mean for “n” cells. The distribution of data in individual cells is shown in separate panels. T-test was used to determine statistical significance. Significant findings are labeled with one (p <0.05, *) or two (p<0.005, **).
Chemicals

All chemicals used in experiments were from Sigma Aldrich unless otherwise stated and prepared fresh daily.
Results

Production of a Modified Mitochondrial Calcium Probe

Figure 4A shows a confocal image of adult feline myocyte that has been transduced with the mitochondria specific Ca\(^{2+}\) probe, mitycam, as previously reported (Kettlewell et al., 2009; Lu et al., 2013). In these panels, there is representative baseline fluorescence (F\(_0\)), two ratiometric images (F\(_0\)/<F\(_0\>) and F/F\(_0\)), and the regions of interest (Mask) to demonstrate the two separate mitochondria population. These binucleated (shown in blue) myocytes contain mitochondria in the periphery as well as the perinuclear region. Figure 4B shows that upon application a pressurized puff solution (Belmonte and Morad, 2008), there is a 5% change in fluorescence in both the perinuclear and peripheral populations of mitochondria, but while the perinuclear population shows a slight release of Ca\(^{2+}\), the peripheral mitochondria appear to take up Ca\(^{2+}\). Figure 4C shows a TIRF image of adult feline cardiomyocyte exposed to caffeine that demonstrates heterogeneous responses from mitochondria where some gain Ca\(^{2+}\) exclusively, while others spontaneously release Ca\(^{2+}\) at different time points. These results obtained with the original mitycam were small, generally showing signal magnitudes in the range of 5% calling for improved strategies to enhance the reliability of detection. Figure 5A shows the schematic of the original probe by Kettlewell \textit{et al.} where the inverse pericam (Terhzaz et al., 2006) is flanked by calmodulin with a mutation in the second EF hand (E67Q), the M13 peptide domain, and the mitochondrial pre-sequence of the human cytochrome oxidase subunit VIII (Rutter et al., 1993). Figure 5B shows where we have altered this probe so that it is only mutated in the first EF hand of calmodulin (E31Q) in hope of lowering the affinity of the probe for Ca\(^{2+}\).
Figure 4: Original mitycam probe sensitivity to Ca\(^{2+}\)

A: Shows confocal images of typical adult feline myocyte expressing mitycam. Baseline fluorescence (F\(_0\)) is shown in the top image, ratiometric images (F\(_0\)/<F\(_0\>) and F/F\(_0\)) in the middle, and the regions of interest (mask) are shown at the bottom. This cell shows mitochondria distribution of strong perinuclear fluorescence and additional peripheral fluorescence. B: The top graph show the “puff” induced response by mitochondria where perinuclear (red) show a slight increase of fluorescence or Ca\(^{2+}\) release, while the peripheral mitochondria show a slight decrease in fluorescence or Ca\(^{2+}\) gain. The bottom graph shows the effect of contraction on the myocyte (purple/green region of the mask). C: Heterogeneous population of responses from mitochondria during caffeine application. Note that signals are small, about 5%.
Figure 5: Comparison of original and modified mitycam probe

A: Mitycam schematic. The inverse pericam (EYFP, enhanced yellow fluorescent protein) is flanked by *Xenopus* calmodulin mutated at E67Q, the M13 peptide domain, and the mitochondrial pre-sequence (MPS) of the human cytochrome oxidase subunit VIII. B: MitycamE31Q schematic. The inverse pericam (EYFP, enhanced yellow fluorescent protein) is flanked by *Xenopus* calmodulin mutated at E31Q, the M13 peptide domain, and the mitochondrial pre-sequence (MPS) of the human cytochrome oxidase subunit VIII.
Figure 6 shows confocal images of HEK 293 cells expressing the mitycamE31Q plasmid. MitycamE31Q fluorescence measured at 514nm (Figure 6B) shows specific mitochondrial expression when co-localized with the mitochondrial marker MitoTracker Deep Red (Figure 6C) imaged at 633nm. Figure 6A shows nuclear stain DAPI measured at 405nm, and the overlay of these three channels is shown in Figure 6D. This confirmation of mitochondrial expression was performed in HEK 293 cells prior to production of the adenovirus.

Once confirmed by transfection of HEK 293 cells, the mitycamE31Q adenovirus was produced and titered to $5.2 \times 10^{10}$ PFU/mL. This virus was then used to transduce both adult (rat and feline) and neonatal rat cardiomyocytes. Figure 7A shows a TIRF image of a typical adult feline cardiomyocyte expressing AdMitycamE31Q taken at 488nm. The pattern of mitochondria shows longitudinal bands consistent with sarcomeric banding. Unlike the organized structure of mitochondria in adult myocytes, the mitochondria in neonatal cardiomyocyte shown in the TIRF image of Figure 7B appears disorganized with a combination of streak-like and punctate staining. In Figure 7C, the same neonatal cardiomyocyte from 7B shows uniform cytosolic staining of Fura-2AM distinct from the mitochondrial staining in 7B taken with TIRF. Lastly, the membrane potential dye, TMRE, which stains mitochondria based on membrane potential, is shown in Figure 7D. Although it is not the same cell as in 7B and 7C, this neonatal cardiomyocyte shows similar structure of mitochondria as in the mitycamE31Q transduced cell where there exists both punctate and streak-like fluorescence and disorganized pattern.
Figure 6: The mutant mitochondrial probe mitycamE31Q co-localizes with MitoTracker

Confocal images of HEK 293 transfected with MitycamE31Q and stained with MitoTracker Deep Red using 63x objective. A: DAPI nuclear stain taken with 405nm diode, B: MitycamE31Q [514nm Argon], C: MitoTracker Deep Red [633nm HeNe], D: Overlay of all channels. Modified mitycamE31Q co-localizes with MitoTracker DeepRed and demonstrates expected mitochondrial staining pattern in HEK293 cells.
Figure 7: Expression of mitycamE31Q in feline adult cardiomyocytes (A) and neonatal rat myocytes (B-D)

TIRF images taken at 63x. A: Feline cardiomyocyte infected with MitycamE31Q shows longitudinal mitochondrial bands consistent with sarcomeric banding. B: Neonatal cardiomyocyte infected with mitycamE31Q show disorganized mitochondrial staining with streak-like and punctate pattern. C: Same neonatal cell from panel B stained with Fura-2AM shows uniform cytosolic staining. D: TMRE staining of neonatal cardiomyocyte show similar mitochondrial pattern as panel B.
To calibrate the new probe, we first assessed the affinity of the mitycamE31Q for Ca\(^{2+}\) in adult rat cardiomyocytes that were saponin permeablized. These myocytes were pretreated with both thapsigargin (to block SR release) and ionmycin (the Ca\(^{2+}\) ionophore) and maintained in a Ca\(^{2+}\) and Na\(^{+}\) free internal solution containing both FCCP and oligomycin to eliminate the mitochondrial membrane potential, similarly to previously published method (Lu et al., 2013). Ca\(^{2+}\) changes were then visualized with epi-fluorescence over increasing free Ca\(^{2+}\) concentrations buffered with BAPTA. These free Ca\(^{2+}\) concentrations were confirmed using Fura-2 calibration as previously reported, assuming a K\(_d\) of 220nM for Fura-2 (Cleemann and Morad, 1991). Figure 8A shows a typical experimental calibration for mitycamE31Q where the cell is rapidly exposed to solutions of differing free Ca\(^{2+}\) concentrations and resulting fluorescence is measured. Peak fluorescence (F\(_{\text{max}}\) at 0 Ca\(^{2+}\)) is shown in the top image, and the minimum fluorescence (F\(_{\text{min}}\)) at a saturating concentration of Ca\(^{2+}\) is shown in bottom cellular image. The resulting hyperbolic curve generated over increasing Ca\(^{2+}\) concentrations at pH 8.0, producing a K\(_d\) for mitycamE31Q of 1.8\(\mu\)M (see \(\frac{1}{2}\) maximal value of the curve) is shown in Figure 8B. At a pH 7.4 (Figure 8C), the K\(_d\) for mitycamE31Q was calculated to be 1.5\(\mu\)M at \(\frac{1}{2}\) maximal value of the curve, demonstrating that pH has a minimal effect on the K\(_d\) for the new probe.
Figure 8: Calibration of mitycamE31Q

A: Representative experimental calibration of mitycamE31Q where the cell has been exposed to various concentrations of Ca\(^{2+}\). Graph shows rapid changes of fluorescence in response to changes of [Ca\(^{2+}\)]. F\(_{\text{max}}\) is shown in the top image compared to F\(_{\text{min}}\) shown in the bottom image.

B: Hyperbolic curve generated by the normalized peak response of mitycamE31Q to increasing concentrations of free Ca\(^{2+}\) at pH 8.0. K\(_{\text{d}}\) of mitycamE31Q was calculated to be 1.8 \(\mu\)M. C: Hyperbolic curve generated by the normalized peak response of mitycamE31Q to increasing concentrations of free Ca\(^{2+}\) at pH 7.4. K\(_{\text{d}}\) of mitycamE31Q was calculated to be 1.5 \(\mu\)M.
Mitochondrial Calcium Movement During CICR

Cytosolic and mitochondrial Ca\(^{2+}\) movement were recorded in spontaneously beating neonatal cardiomyocytes using Fura-2AM and AdMitycamE31Q using TIRF imaging. A short, 5s caffeine puff was rapidly applied to each cell and both cytosolic and mitochondrial Ca\(^{2+}\) transients were recorded. Figure 9A top panels show a single cardiomyocyte that is expressing mitycamE31Q. The left image is the mitochondrial regions of interest shown in blue, followed by the baseline fluorescence (F\(_{0}\)) and the peak ratiometric response (dF/F\(_{0}\)). The three panels below represent the cytosolic Fura-2AM staining of the same cells with the regions of interest defined in white and the baseline and peak ratiometric images. The graph in Figure 9A shows a typical response to caffeine-induced Ca\(^{2+}\) release. The black line is the cytosolic Ca\(^{2+}\) transient that shows a rapid release of Ca\(^{2+}\) from the SR that decays back to baseline levels. Notice that Fura-2AM, which is often used with dual wavelength excitation for calibrated ratiometric measurements, is used here with excitation only at 405 nm where the fluorescent intensity decreases with increasing Ca\(^{2+}\) concentrations. Thus, both the mitycamE31Q and the Fura-2AM measurements are plotted so that a downward deflection represents an increase in cytosolic or mitochondrial Ca\(^{2+}\) respectively. The blue traces represent the mitochondrial response to the caffeine-induced Ca\(^{2+}\) release which follows the SR Ca\(^{2+}\) release with significant delay. The mitochondrial release had slower kinetics with magnitudes generally about half of that of the cytosolic transient. After washout of Ca\(^{2+}\), the cytosolic Ca\(^{2+}\) rapidly returned to baseline while the mitochondrial Ca\(^{2+}\) remained elevated to the end of the recording, 20 sec later. The inset on this graph shown in the blue box emphasizes the time course of mitochondrial Ca\(^{2+}\) uptake which is delayed and attenuated compared to the cytosolic Ca\(^{2+}\) signals. Figure 9B shows another
typical neonatal cardiomyocyte that is expressing mitycamE31Q and stained with Fura-2AM. The top three panels of 6B show the mitycamE31Q regions of interest (blue) followed by the baseline and peak fluorescence. Similarly, the bottom three images represent the Fura-2AM images of ROI shown in white, baseline, and peak fluorescence. The graph below represents a cell that has been exposed to caffeine-induced Ca$^{2+}$ release decays back to baseline and then generates a spontaneous Ca$^{2+}$ transient at the time marked with a red star. The blue mitochondrial signal is again delayed and attenuated as compared to the cytosolic transient but occurs in both caffeine-induced Ca$^{2+}$ release as well as the spontaneous Ca$^{2+}$ transient consistent with mitochondrial Ca$^{2+}$ uptake. Figure 9C show a quantification of the Ca$^{2+}$ uptake in the mitochondria and Ca$^{2+}$ release from the SR to the cytosol. On average, the percent change of fluorescence in mitochondria is about 9.55 ± 1.5% while the Fura-2AM cytosolic signal changes about 31.3 ± 4.5%. This indicates that the during CICR, mitochondria are able to pick up a percentage of Ca$^{2+}$ released from the SR and only slowly divest themselves of this Ca$^{2+}$ load.
Figure 9: Mitochondrial uptake of Ca$^{2+}$ (mitycamE31Q) is delayed and sustained compared to the cytosolic Ca$^{2+}$ transients (Fura-2AM) measured during brief exposure to caffeine or spontaneous Ca$^{2+}$ releases.

TIRF imaging. A: During caffeine application, [Ca$^{2+}$]$_c$ rapidly rises due to Ca$^{2+}$-release from the SR and then decays back to baseline, while [Ca$^{2+}$]$_m$ increases subsequent to the SR release and with slower kinetics. B: At the end of the caffeine transient, there is a spontaneous Ca$^{2+}$-release where [Ca$^{2+}$]$_m$ also increases subsequent to the SR release and with slower kinetics. Notice that...
Figure 9 legend continued:

Fura-2AM was excited at 405 nm and at this wavelength resembled mitycamE31Q in that fluorescence decreased in response to elevated Ca\(^{2+}\). C: Average % change of fluorescence in neonatal rat cardiomyocytes in response to caffeine induced Ca\(^{2+}\) release from the SR (cytosolic Ca\(^{2+}\) increase) and mitochondrial Ca\(^{2+}\) uptake in 8 cells.
Central vs. Peripheral Mitochondrial Calcium Changes

In addition to the uptake of Ca\(^{2+}\) during CICR, mitochondria are also able to release Ca\(^{2+}\) when the cell is loaded with Ca\(^{2+}\). Figure 10 shows a cell that has been exposed to a depolarizing solution containing a low concentration of Na\(^{+}\). This low Na\(^{+}\) solution activates the NCX of the neonatal cell and brings in a large, sustained Ca\(^{2+}\) across the sarcolemma. Figure 10A top panels show the mitycam regions of interest represented by two colors, red and blue due to the heterogeneity of response in these regions. The blue mitochondria are found around the periphery of the cell, while the red are found in the central portion of the cell. The following two images represent the baseline and peak fluorescence of the mitochondria, while the right three images show the ROI of the cytosol (white) and the respective baseline and peak fluorescence. The graph of Figure 10B shows the response of the cell to a 50s long pulse of a solution containing low concentrations of Na\(^{+}\). Initially, all mitochondria take up Ca\(^{2+}\), but quickly the central mitochondria appear to release Ca\(^{2+}\) (shown in red). Unlike the central mitochondria, the peripheral mitochondria continue to uptake Ca\(^{2+}\) throughout the low Na\(^{+}\) exposure. Upon termination of the low Na\(^{+}\) solution all mitochondria return toward baseline levels. In addition, the graph also shows the cytosolic changes in Ca\(^{2+}\) as measured with Fura-2 AM signal (black trace). The Fura-2AM signal shows the initial rapid increase of cytosolic Ca\(^{2+}\) and the sustained Ca\(^{2+}\) flux throughout the low Na\(^{+}\) application. Also, similarly to the mitochondria, upon termination of the low Na\(^{+}\) solution, the cytosolic Ca\(^{2+}\) returns to baseline level. Figure 10C show the inset blue box of 10B where we focus on the time course of these signals. Like the CICR-induced uptake of Ca\(^{2+}\) in mitochondria, these transients are also delayed as compared to the cytosolic signal. Figure 10D quantifies the percentage change of peripheral mitochondria,
central mitochondria, and the cytosol. The average percent change of the gain of Ca\(^{2+}\) in the cytosol was 52.8 ± 2.76%. In numerous cells, the peripheral mitochondria demonstrate the prolonged uptake of Ca\(^{2+}\), with a percent change on average about 24.3 ± 2.72%, while the central mitochondria release Ca\(^{2+}\) with a percent change of fluorescence on average of about 37.4 ± 6.74%. These results show that there are regional differences that exist between mitochondria populations in neonatal rat cardiomyocytes.

Figure 11A shows a neonatal cell expressing mitycamE31Q (peripheral, colored blue and central, colored red) and stained with Fura-2AM (white). The graph of Figure 11B shows the cell being loaded with Ca\(^{2+}\) due to low Na\(^{+}\) exposure, and during the low Na\(^{+}\) a rapid application of caffeine is given to release the SR Ca\(^{2+}\). This caffeine puff shows how when loaded with Ca\(^{2+}\), central mitochondria release Ca\(^{2+}\) and the peripheral mitochondria pick up even more Ca\(^{2+}\). This is consistent with Figure 10 where central mitochondria release Ca\(^{2+}\). Once the cell returns to baseline Ca\(^{2+}\) levels, the cell is then activated again with low Na\(^{+}\) and caffeine solution. Here, where the mitochondria are not pre-loaded with Ca\(^{2+}\), both sites, peripheral and central mitochondria, are able to take up Ca\(^{2+}\). Figure 11C shows inset from 11B where the central mitochondria can be separated into three different responses shown in purple, orange, and green. The orange mitochondria show a slower time course of release, while the purple and green have both a greater magnitude and faster kinetics.
Figure 10: Heterogeneity of uptake and release of Ca\textsuperscript{2+} by mitochondrial populations

TIRF imaging of regional mitochondrial differences. A: Representative images of ROI, baseline, and peak fluorescence changes. B: Upon application of depolarizing solution, cytosolic Ca\textsuperscript{2+} (black) quickly rises and maintains elevation until solution is removed, while peripheral mitochondria (blue) take up Ca\textsuperscript{2+} and central mitochondria (red) initially take up Ca\textsuperscript{2+} and then quickly release Ca\textsuperscript{2+}. At the end of the transient both central and peripheral mitochondria return toward baseline Ca\textsuperscript{2+} levels. C: Expanded region from graph B shows time course and kinetics
Figure 10 legend continued:

of Ca\(^{2+}\) uptake in both the cytosol and mitochondria. D: Average % change of fluorescence in neonatal rat cardiomyocytes in response to low Na\(^+\) (Ca\(^{2+}\) loading and cytosolic Ca\(^{2+}\) increase, black) and mitochondrial Ca\(^{2+}\) uptake (peripheral mitochondria, blue) and release (central mitochondria, red) in 9 cells.
Figure 11: Centrally located, preloaded mitochondria can release Ca$^{2+}$

TIRF imaging. A: Representative images of region of interest (ROI), baseline, and peak fluorescence changes. B: Regional differences are seen in mitochondria upon application of both depolarizing solution and with rapid double application of caffeine, both regions gain Ca$^{2+}$. C: Central mitochondria grouped by response time and magnitude during region expanded from graph B show different release kinetics.
Pharmacology of Mitochondrial Calcium Transporters

Although the pharmacological agents are difficult to penetrate the cell, with pre-incubation of the compound, it is possible to get a percentage of these blockers into the cell. Figure 12 shows representative traces before and after treatment with the mitochondrial permeability transition pore (PTP) blocker, cyclosporin A, and the mitochondrial Ca\(^{2+}\) uniporter (MCU), Ru360. Figure 12A shows a cell expressing mitycamE31Q that has been subject to Low Na\(^{+}\) for 50s. In this cell the peripheral mitochondria (blue) show a continued uptake of Ca\(^{2+}\) while the central mitochondria (green) show an initial uptake of Ca\(^{2+}\) followed by a spontaneous Ca\(^{2+}\) release. To test if the spontaneous release of Ca\(^{2+}\) is due to opening of the PTP, we incubated this cell with cyclosporin A before repeating the experiment. In the green region, cyclosporin A blocked the release of Ca\(^{2+}\) from the mitochondria (shown on the right graph of 12A). Additionally, to test the role of the uniporter during this Ca\(^{2+}\) uptake, Figure 12B shows the effect of low Na\(^{+}\) on peripheral and central mitochondria before and after incubation of Ru360. As shown in the graph on the right of 12B, Ru360 blocked a portion of both the uptake and release of Ca\(^{2+}\) from the mitochondria. This effect is quantified in right panel of Figure 12B where the % change of fluorescence before and after treatment of Ru360 was measured. On average it appears that Ru360 partially blocked both the uptake and release of Ca\(^{2+}\).
Figure 12: Pharmacology of mitochondrial Ca\textsuperscript{2+} transporters

Application of mitochondrial agents, Cyclosporin A and Ru360.  A: Cyclosporin A, a blocker of the mitochondrial permeability transition pore, blocks the release of Ca\textsuperscript{2+} from the central mitochondria. B: Ru360, a blocker of the mitochondrial Ca uniporter, MCU, reduces both the uptake of Ca\textsuperscript{2+} in the peripheral mitochondria and release of Ca\textsuperscript{2+} from the central mitochondria.
Figure 12 legend continued:

Right panel shows quantification of percent suppression of both Ca$^{2+}$ uptake (peripheral mitochondria) and release (central mitochondria) by Ru360 in 4 cells.
Discussion

The major objective of this work was to improve the sensitivity and reliability of mitochondrial Ca$^{2+}$ measurements in order to address a number of issues that have clouded the understanding of the Ca$^{2+}$ signaling pathways of this important organelle. Building on an existing probe, we succeeded in expanding its dynamic range and proceeded to evaluate the contributions of mitochondrial Ca$^{2+}$ uptake and release to the tidal Ca$^{2+}$ signals and overall Ca$^{2+}$ homeostasis that not only control the strength heartbeat, but also regulates mitochondrial energy production and cellular maintenance processes such as protein synthesis. In agreement with others, we found that the mitochondrial Ca$^{2+}$ signals often were attenuated and delayed compared to the cytosolic Ca$^{2+}$ transients (Robert et al., 2001; Bell et al., 2006; Lu et al., 2013). In part, the slower kinetics were seen as an accumulation of mitochondrial Ca$^{2+}$ that was maintained for 10s of seconds so that sustained or repeated elevation of cytosolic Ca$^{2+}$ had a cumulative effect on the mitochondrial Ca$^{2+}$ content. Taking advantage of the improved sensitivity of the new probe and the clear mitochondrial imaging of mitochondria provided by TIRF microscopy, we also found that peripheral and central mitochondria had different Ca$^{2+}$ handling properties. In particular, it was striking that the centrally located, perinuclear mitochondria that normally took up Ca$^{2+}$ during brief cytosolic Ca$^{2+}$ transients produced the opposite response when the cells were overloaded with Ca$^{2+}$ by sustained influx of Ca$^{2+}$ via the Na$^+$/Ca$^{2+}$ exchanger. These findings suggest that the mitochondria may normally contribute relatively little to the tidal Ca$^{2+}$ signals that regulate the heartbeat, but that they may accumulate Ca$^{2+}$ under conditions of Ca$^{2+}$ overload and, in this case, may assume different, more active roles of potential pathological or cardio-protective importance.
**Improving the Mitochondrial Calcium Probe**

The study of mitochondrial function, including their Ca\(^{2+}\) handling capabilities, has generally relied on extraction of isolated mitochondria or on positively charged Ca\(^{2+}\) indicator dyes that were driven to accumulate in the mitochondrial matrix space by its highly negative potential. The isolation of the mitochondria eliminates the need to measure through the cytosolic compartment, but thereby also eliminates the ability to evaluate the function of mitochondria in their native environment and study their interactions with other cellular components. The Ca\(^{2+}\)-indicator dyes, Rhod-2, Indo-1, and Fura-2, have been used with some success, but have relied on complicated loading procedures and has generally been subject to the criticism that they also accumulated in other organelles. Contamination of the cytosol appears to have been effectively eliminated by permeabilization of the cell membrane, but that, in turn, exposed the mitochondria to an artificial environment. In this light, the use of biologically targeted probes has been a definite advancement and has pioneered Ca\(^{2+}\) measurements in mitochondria with the use of the original mitycam probe (Kettlewell et al., 2009). However, it appeared to us that the initial effort could be improved upon by adjusting the Ca\(^{2+}\) binding characteristics of the probe to the inter-mitochondrial environment. Specifically, we hypothesized that the calmodulin which serves multiple purposes in the cytosolic environment might bind Ca\(^{2+}\) too tightly to be well suited as a Ca\(^{2+}\) sensor within the mitochondria. Similarly, it was of concern that since Ca\(^{2+}\) on many probes displaces H\(^+\) there may be an additional confounding pH effect that may be highly relevant in the alkaline mitochondrial environment.

Our first aim was therefore to improve the Ca\(^{2+}\) affinity of an existing mitochondrial Ca\(^{2+}\) probe, mitycam, as to be able to reliably measure mitochondrial Ca\(^{2+}\) signals. Since recent work
in our laboratory involving the high affinity mitycam probe suggested that this probe may be potentially underestimating the mitochondrial Ca\(^{2+}\) signal, we thought to modify the mitycam probe by mutating the first EF hand of calmodulin. By doing so, we hoped to lower the affinity of the probe to Ca\(^{2+}\) and thereby increasing the dynamic range of Ca\(^{2+}\) that could be measured by this probe. The modified plasmid was first tested in HEK 293 cells in order to determine whether this plasmid could maintain its ability to co-localize with the commercially available dye, MitoTracker Deep Red. It was also desirable to make a probe that would be easily expressed in primary cells such as adult and neonatal cardiomyocytes. In order to do so, we produced AdMitycamE31Q, an adenovirus that we could use in primary cells such as cardiomyocytes. We tested this new probe for mitochondrial expression and found that we were indeed able to lower the affinity of the probe for Ca\(^{2+}\) where the K\(_d\) of the new probe ranged around 1.5-1.8\(\mu\)M, as compared to the 197nM found by Lu et al. for the original mitycam (Lu et al., 2013). In addition, the expression of this probe is significantly different in adult versus neonatal cardiomyocytes. While adult cardiomyocytes demonstrate punctate longitudinal mitochondrial bands consistent with sarcomeric banding, neonatal cardiomyocytes show a very different profile. These cells produce mitochondrial fluorescence that ranges from punctate to streak-like expression. Lastly, we utilize a new technique for imaging mitochondrial calcium by using high resolution, total internal reflection fluorescence (TIRF) microscopy. Using this method, we were able to visualize only sub-sarcolemmal mitochondria that exist less than 150nm into the cell. As compared to using confocal imaging, this technique allows us to see distinct mitochondria and clearly differentiate them from the cytosolic Ca\(^{2+}\) signals. As shown in Figure 7, the mitochondrial signal imaged at 488nm and the Fura-2AM signal imaged at 405nm are
clearly separated and avoid the problems emanating from signal crossovers as encountered when measuring a combination of dyes such as Rhod-2 and Fura-2AM. Further, by targeting to the mitochondrial matrix by cytochrome C oxidase subunit VIII, we avoided concerns arising from distribution of the probe in the cytosol as is the case with most commercial Ca^{2+} indicator dyes. The ability of mitycamE31Q to sense much larger range of Ca^{2+} may make it possible to obtain critical data for the field of mitochondrial Ca^{2+} signaling, especially under pathological conditions.

We conclude that mitycamE31Q probe has definite advantages and provides a dynamic range for Ca^{2+} encountered by mitochondria. Additional experiments are required to take full advantage of this new biological tool, especially to understand the intriguing roles of mitochondrial Ca^{2+} release and the heterogeneous mitochondrial populations. Based on our study, where mitycamE31Q was used in conjunction with cytosolic Fura-2AM measurements, it appears that simultaneous use of spectrally distinct probes may provide a powerful approach yielding new insight. It may be suggested, for instance, that the mitycamE31Q Ca^{2+} measurements might be corroborated by simultaneous measurements with spectrally distinct mitochondrially targeted probes measuring its membrane potential ($\Psi_m$) and/or pH.
Mitochondrial Ca\(^{2+}\) Uptake and Release

Electron probe experiments and measurements with several fluorescent Ca\(^{2+}\) sensitive probes (\(i.e.\) Rhod-2 and the original mitycam probe) have shown that mitochondria in cardiac cells take up Ca\(^{2+}\) to an extent that vary with the level of activity (Gallitelli et al., 1999; Mackenzie et al., 2004; Lu et al., 2013). With adequate temporal resolution the mitochondrial Ca\(^{2+}\) signals were reported as being delayed and attenuated compared to the cytosolic Ca\(^{2+}\) transient, and central issues remaining were whether the mitochondrial Ca\(^{2+}\) signals were contaminated by a relatively fast cytosolic component and whether they were of sufficient magnitude to play a significant role in the modulation of the cardiac contraction. On this background, we found that the mitochondrial Ca\(^{2+}\) signals were significantly delayed compared to the cytosolic Ca\(^{2+}\) transients produced during spontaneous beats, by caffeine-induced release of SR Ca\(^{2+}\), or by sarcolemmal Ca\(^{2+}\) influx by “reverse” Na\(^+\)-Ca\(^{2+}\) exchange. Any small and fast component (<100-200 ms) that occasionally was present appeared to be a local motion artifact and not representative of cytosolic Ca\(^{2+}\) signals. Like, Lu et al., who used the original mitycam probe (Lu et al., 2013), we conclude that the mitochondrial targeting of mitycamE31Q is very effective and eliminates the cytosolic contamination that appears to have produced artifactual signals with probes like Indo-1 (Miyata et al., 1991; Di Lisa et al., 1993; Griffiths et al., 1997a). Unfortunately Lu et al. did not report the overall change in the fluorescence intensity (\(F_{max}\) vs. \(F_{min}\)), and their calibration procedure using EGTA at pH 8 is somewhat mysterious (Lu et al., 2013), but we found a large response (43%, Figure 8), and a \(K_d\) (1.5-1.8\(\mu\)M) that appears to have prevented saturation of the new mitycamE31Q under physiological conditions. Nevertheless, we agree with Lu et al. that the mitochondrial uptake during a single beat is fairly modest and that
stronger measures are needed to produce large mitochondrial Ca\(^{2+}\) uptake (Lu et al., 2013). Such cumulative effects may occur, in part, because the mitochondria re-equilibrate extremely slowly by releasing sequestered Ca\(^{2+}\) over long periods of time (~30s, Figure 9). In turn, this raises the question of the responses that may be evoked by conditions of Ca\(^{2+}\) overload, and brings to mind the relatively rapid mitochondrial Ca\(^{2+}\) releases that have been found to be triggered by mechanical stimulation in mitochondria preloaded with Ca\(^{2+}\) (Belmonte and Morad, 2008). As yet, we have not attempted to reproduce this exact protocol using mitochondrial Ca\(^{2+}\) imaging, but we did find that centrally located mitochondria released Ca\(^{2+}\) when preloaded and that this might occur gradually during prolonged loading (Figure 12A) or could be triggered by caffeine as a rapid response (Figure 11).

The slow mitochondrial uptake and release of Ca\(^{2+}\) are likely to involve the mitochondrial Ca\(^{2+}\) uniporter (MCU) and Na\(^+-\)Ca\(^{2+}\) exchange, but clarification of the mechanism of the fast mitochondrial release is challenging. Possible mechanisms include: 1) Transient opening of the mitochondrial permeability transition pore (PTP); 2) Indirect effects of MCU blockage; 3) Na\(^+-\)Ca\(^{2+}\) exchange; and 4) H\(^+-\)Ca\(^{2+}\) exchange all of which would depend on changes in \(\Psi_m\) and/or ionic gradients. Involvement of PTP is indicated by the finding that the fast mitochondrial Ca\(^{2+}\) release can be blocked by cyclosporin A (Figure 12A). If indeed PTP is involved, it is unclear whether or not these PTP openings are transient or long lasting since the Ca\(^{2+}\) release timing is variable in each cell. While in some cells the central mitochondria release Ca\(^{2+}\) very quickly in response to low Na\(^+\), Ca\(^{2+}\) loading, other cells show central mitochondria initially take up Ca\(^{2+}\) before releasing its contents above baseline Ca\(^{2+}\) levels. It is possible that the PTP opening allows the cardiac mitochondria to protect themselves from Ca\(^{2+}\) overload and may even
stimulate ROS production which may engage reactive-oxygen species-dependent cardioprotective signaling (Korge et al., 2011). We also found that the MCU blocker, Ru360 partially blocks both Ca\(^{2+}\) uptake and release (Figure 12 B & C). Here, the block of Ca\(^{2+}\) uptake is easily understood, but is unclear how the MCU might transport Ca\(^{2+}\) out of the mitochondria unless \(\Psi_m\) has completely collapsed and dissipated. However, MCU might indirectly block the fast mitochondrial Ca\(^{2+}\) release by preventing the preloading. To clarify the involvement of PTP or MCU in the fast mitochondrial Ca\(^{2+}\) release, it would be advantageous if the mitycamE31Q measurements were performed with simultaneous measurements of \(\Psi_m\). Such experiments with dual probes might also involve parallel measurements of both \(\Psi_m\) and H\(^+\), which in turn would also facilitate evaluation of the possible involvement of Na\(^+-\)Ca\(^{2+}\) exchange and H\(^+\)-Ca\(^{2+}\) exchange.
Distribution of Mitochondria: Central vs. Peripheral

We find that subsarcolemmal mitochondria in neonatal cardiomyocytes contain two distinct populations. Although we refer to them as peripheral and central, it is possible that they are distinct types of mitochondria and further structural investigation would be necessary to determine this information. In Figure 10 we show that when the cell is loaded with Ca\(^{2+}\), peripheral mitochondrial sites continue to take up Ca\(^{2+}\), while central mitochondrial sites release Ca\(^{2+}\). To further evaluate the idea of Ca\(^{2+}\) load-dependent activity of mitochondria, we used the experiment in Figure 11 to see if, when the mitochondria return to baseline levels of Ca\(^{2+}\), we will see the same differential response or whether the central mitochondria will only release Ca\(^{2+}\) when they are subject to potential Ca\(^{2+}\) overload. Previously, different mitochondrial populations have been recognized based on their location (perinuclear, subsarcolemmal, intermyofibrillar) shape (elongated vs. rounded) (Fawcett and McNutt, 1969; Rambourg and Segretain, 1980; Chemnitius et al., 1993; Lukyanenko et al., 2009), but we find only a few reports assessing functional differences in Ca\(^{2+}\) signaling (Gallitelli et al., 1999; Mackenzie et al., 2004). Using rapid freezing of guinea pig ventricular cells and subsequent electron probe analysis, Gallitelli et al. found that trains of rapid stimulation caused loss of Ca\(^{2+}\) in central mitochondria, but uptake in at the periphery (Gallitelli et al., 1999). Similarly, using Rhod-2 loaded rat atrial myocytes, Mackenzie et al. found differential Ca\(^{2+}\) signals in peripheral and central mitochondria (Mackenzie et al., 2004). Both reports appear to be consistent with our finding, but that central mitochondria accumulate less Ca\(^{2+}\) than peripheral ones and even may release Ca\(^{2+}\) but mechanism is by no means clear. Both groups suggest that preferential mitochondrial Ca\(^{2+}\) accumulation may depend on the existence of μ-domains linking local cytoplasmic hot spots of
Ca\textsuperscript{2+} to mitochondrial Ca\textsuperscript{2+} uptake, but they do not describe any rapid mitochondrial Ca\textsuperscript{2+} release, nor do they ascribe any functional implications for the different mitochondrial populations. It appears that future experiments should be conducted with the triple objectives of: 1) Identification of conditions where mitochondria play an active role in cellular Ca\textsuperscript{2+} signaling (e.g. Ca\textsuperscript{2+} oscillations in neonatal cardiomyocytes, generation of large Ca\textsuperscript{2+} transients of potential arrhythmogenic importance); 2) Identification of the Ca\textsuperscript{2+} transporters that play a dominant role in mitochondria, in particular the release; and 3) Clarification of the roles of different mitochondrial populations.
CHAPTER II

Bimodal Regulation of Shark Sodium-Calcium Exchanger Overexpressed in Cardiac Myocytes

Using Transgenic and Adenoviral Models
Abstract

Inward currents generated by the mammalian cardiac Na\(^+\)-Ca\(^{2+}\)-exchanger (NCX1.1) have arrhythmogenic potential, especially in the failing heart where expression of NCX is up-regulated. We hypothesize that arrhythmogenesis might be alleviated if NCX were subject to the same cAMP mediated regulation (suppression of Ca\(^{2+}\)-influx, but enhancement of Ca\(^{2+}\)-efflux on NCX) as found in the native shark ventricle. To first test this hypothesis, we tested whether we could confer the shark NCX specific bimodal regulation on the mammalian system. Two approaches were employed to achieve this goal: 1) We created heterozygous transgenic mice that express the shark NCX protein with a myc-tag, under the control of the alpha-myosin heavy chain promoter (\(\alpha\)-MHC); 2) An adenovirus shark NCX with YFP color gene in the P-loop was constructed, to see if we could confer the shark specific NCX regulation onto the mammalian myocardium.

In transgenic mice genotyped to be carrying the shark NCX gene, echocardiography and ECG studies showed no remarkable cardiac phenotype, and voltage-clamp studies in single isolated myocytes showed little change in cell capacitance and exchanger current. In the small fraction of cells that demonstrated \(I_{\text{NCX}}\) overexpression, we measured the typical bimodal regulation of NCX by adrenergic stimulation. It is likely that these data arise from paucity of gene penetrance in the transgenic model as also supported also by absence of c-myc expression. In the adenoviral model where YFP fluorescence tag could be visualized, we found 3-4 times greater expression of the construct in neonatal myocytes and equivalent increase in \(I_{\text{NCX}}\) as compared to the endogenous wild NCX. In addition the larger \(I_{\text{NCX}}\), unlike the endogenous of neonatal myocytes, was significantly more sensitive to regulation by 8-Br-cAMP. Our findings
show that shark NCX is functional in the mammalian expression system, but it remains to be determined as yet whether shark NCX can confer anti-arrhythmic properties to the mammalian heart due to limitations of expression in the transgenic model.
Introduction

The Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) proteins constitute a family of solute carriers (SLC8A) that are found throughout the biosphere. In most cells, NCX serves as the major pathway for removal of Ca\(^{2+}\) ions from the cytosol in exchange for three times as many Na\(^+\) ions (Reuter H, 1968; Reeves J.P., 1984; Kimura J., 1986; Pott et al., 2004) generating an inward ionic current \(I_{\text{NCX}}\). Like other ion channels and transporters, the equilibrium potential of NCX depends on \(E_{\text{Ca}}\) and \(E_{\text{Na}}\), where \(E_{\text{NCX}} = nE_{\text{Na}} - 2E_{\text{Ca}}/n-2\). As a result, high cytosolic Ca\(^{2+}\) would favor Ca\(^{2+}\) efflux and high cytosolic Na\(^+\) would favor Ca\(^{2+}\) influx. Under physiological conditions at resting conditions, NCX operates primarily in the Ca\(^{2+}\) efflux mode with reversal potential of about -40mV. In cardiac cells, this electrogenic process interacts with the other Ca\(^{2+}\) transporting proteins and ion channels, that in concert, generate the action potential and control the strength of the heartbeat through tidal changes in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\text{c}).

NCX has 9 transmembrane domains, with a large cytoplasmic loop between transmembrane domain 5 and 6 which contains two Ca\(^{2+}\) binding motifs in addition to other important regulatory domains (Brini and Carafoli, 2000). Interestingly, this large cytoplasmic loop can be deleted from the NCX in heterologous expression systems without the loss of exchanger activity and function (Matsuoka et al., 1993). In adult mammalian cardiac myocytes, our laboratory and others have shown that the activity of NCX1 to be unaffected by cAMP, probably because evolutionarily Ca-ATPase (SERCA2a) and Ca\(^{2+}\) release stores of the SR evolved as an alternative \(\beta\)-adrenergic regulatory site of action (Wei et al., 2003; Reppel et al., 2007). Nevertheless, some groups have reported \(\beta\)-adrenergic-mediated enhancement of NCX
activity, but the data has been criticized experimentally and has not been verified (Perchenet et al., 2000).

In contrast, there is substantial evidence that the Ca\(^{2+}\) influx mode of NCX is more pronounced in cardiac cells that have not as yet developed SR Ca\(^{2+}\) release stores. Some examples of this are the frog and the shark hearts where the SR is rudimentary (Morad M, 1971; Nabauer M, 1992; Woo and Morad, 2001) and embryonic ventricular cardiac myocytes of mammals where prior to the post-natal development of t-tubules appear to have a low functional SR (Huynh TV, 1992). Additionally, the activation of the β-adrenoreceptor/adenylate cyclase/cAMP dependent pathway leads to down-regulation of Ca\(^{2+}\) influx mode of NCX in ventricular cells of the frog and shark in a manner that is quite different from the effects of adrenergic hormones on mammalian cardiac cells. Specifically, in the frog ventricle, NCX was shown to have a unique insertion of 9 amino acids (27 base pairs, known as exon X) with a Walker A motif which conferred a unique cAMP suppressive effect to the clone (Iwata et al., 1996; Shuba et al., 1998). Interestingly, this suppressive effect could be conferred onto the dog NCX using a chimera containing exon X (He et al., 2003). Thus, although the difference in species, ionic milieu, heart rate, temperature, or extent of expression of SR, regulatory effect of this amino acid sequence was dependent on its presence (Janowski et al., 2009). Similar to the frog heart, the shark cardiac NCX was also shown to have a unique regulatory domain and a bi-modal regulation where the outward current is suppressed and the inward current is enhanced in response to adrenergic stimulation (Woo and Morad, 2001). Data from our lab also showed that with the deletion of this shark-specific, second proline/alanine-rich amino acid insertion
within the P-loop eliminated the species specific bimodal regulation and the differential regulation of the Ca\(^{2+}\) influx and efflux transport modes (Janowski et al., 2009).

In an attempt to further understand this regulation, we have created two unique models of shark cardiac NCX overexpression. The first method was the creation of transgenic mouse model, utilizing an α-myosin heavy chain promoter to express the shark NCX that was e-myc tagged at the C-terminus. This mouse model showed no difference in cardiac function as measured by ECG and echocardiography at baseline and with an isoproterenol challenge. In addition, we could not locate the tagged protein via western blotting and immunocytochemistry, and electrophysiological measurements proved to be too variable from cell to cell. While the overall effect of isoproterenol or directly elevating the cAMP levels on TG \(I_{NCX}\) regulation at first did not appear to be different from WT, in a small percentage of cells an up-regulation of \(I_{NCX}\) did in fact occur which demonstrated the typical shark-like bimodal regulation. Our findings suggest that this TG mouse model was not sufficiently dependable to test the role of shark heart NCX as an anti-arrhythmic protein. We believe that this may be related to gene penetrance of the shark NCX into all cells and potential cleavage or degradation of the c-myc tag, and find that continued work requires a more sophisticated approach to develop a better transgenic model.

For this reason we developed a second model to study the regulation of shark NCX protein in mammalian myocardium. We created an adenovirus with the shark NCX that was tagged with eYFP in the intracellular P-loop of the protein away from any possible important regulatory domains. This protein’s presence in cells was confirmed with both western blotting and immunocytochemistry. Expression of this virus in neonatal cardiomyocytes demonstrates a
typical shark-like NCX function upon activation of PKA signaling pathway with cAMP. This bimodal regulation was recorded in the transduced mammalian cardiomyocytes. It is likely that this YFP-tagged construct could now be used to generate a novel transgenic mouse line in which shark NCX expression could easily be identified in isolated single cardiomyocytes.
Materials and Methods

Generation of Transgenic Mice

The transgenic construct was created by introducing both a SalI and HindIII restriction enzyme site on the N- and C-terminus respectively of the shark NCX gene via PCR. In addition, at the C-terminus prior to the stop codon we introduced a c-myc tag. Using the following primers: sense, 5’-ATTAGTCGACATGAGGTCGTGGAAAACGGCGCC-3’, and anti-sense, 5’-ATTAAACGTTTTACAGATCCTCTTTTGTTCGAAGCCCCTTTATGTAGCAATAG-3’ and the GC-RICH PCR System (Roche) with the following PCR program: 95°C for 3 minutes, [95°C for 30s, 50°C for 30s, 68°C for 3 minutes]x20 cycles, 68°C for 10 min. The resulting PCR product was then ligated into the pBS II SK+ bacterial vector that contained the α-myosin heavy chain clone 26 promoter (gift from Dr. Jeff Robbins) using SalI and HindIII (both, New England Biolabs). The plasmid was then amplified in STBL2 (Invitrogen) cells and selected on ampicillin resistant 2XYT agar plates and purified using QIAfilter Plasmid Midi Kit (Qiagen) and the construct was sequenced to ensure no mutations. To prepare for transgenic microinjection, the transgene was excised from the bacterial vector backbone and isolated from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen) diluted in TE buffer (Qiagen), pH=7.4. The resulting sample was then sent to the transgenic core at Medical University of South Carolina for microinjection. Three transgenic founders were identified using genotyping procedures (see below) with three resulting transgenic lines used for experimentation.
Identification of Transgenic Mice

Genomic DNA (gDNA) of mice 21 days old were isolated from 0.5cm of tail tissue using proteinase K (Roche, 0.5mg/mL) digestion. Samples were allowed to digest overnight at 55°C and the following day, 8M potassium acetate and chloroform were added to the samples and inverted 4-5 times. The samples were then placed on ice for 15 minutes and then centrifuged for 10 minutes at 14000 rpm. The aqueous upper layer was then removed and the gDNA was precipitated out of solution using 100% ethanol by inversion. The gDNA was then pelleted by centrifugation for 2 minutes at 14000 rpm and dried to remove all traces of ethanol from the sample. The pellet was redissolved in TE buffer and used for PCR. Primers for both shark NCX and SMAD 6 were used to identify transgenic (Tg) versus wild-type (WT) animals from the isolated gDNA. The following primers sequences were used:

shark NCX, sense, 5’-TGAGAATGTTTCCCCCCTGTAGACAGCA-3’ and anti-sense, 5’-GACTTGGTGTAGGTTTGAAATCGG-3’; SMAD 6, sense, 5’-CCCCCTCTCCCCCCAGCAATAA-3’ and anti-sense, 5’-GCGCCGCACCGACTCAC-3’.

The amplification profile used was as follows: 94°C for 2 minutes, [94°C for 30s, 55°C for 30s, 72°C for 1 minute]x35 cycles, 72°C for 5 minutes. PCR products were then run on a 1.2% agarose gel (Fisher Scientific) for the presence of positive bands.
RT-PCR

Total cellular RNA was extracted from both WT and TG hearts using RNeasy fibrous tissue mini kit (Qiagen) according to the manufacturer’s instructions. First strand cDNA synthesis was performed using SuperScript III First Strand Synthesis System for Reverse Transcriptase PCR (Invitrogen) with the supplied random hexamers. The following primers were used: β-actin, sense, 5’-GACGAGGCCAGAGCAAGAG-3’ and anti-sense, 5’-GTGGTGGTGAAGCTTAGCC-3’; mouse NCX, sense, 5’-CCCAGGACCAGATGCAGAT-3’ and anti-sense, 5’-CATGGTAGATGGCAGCAATG-3’; shark NCX, sense, 5’-GGTTTTAGGGACAATCCG-3’ and anti-sense, 5’-GCCGTTCTTCTCATCTTGC-3’. Real-time amplifications were performed using the CFX96 Real-Time PCR Detection System (BioRad) and SYBR Green PCR Master Mix (Applied Biosystems). The amplification profile included initial denaturation at 95°C for 10 minutes, [95°C for 30s, 55°C for 30s, 72°C for 30s]x40 cycles, 72°C for 3 minutes. Melt curve analysis was performed at the end of each run to ensure and monitor for non-specific products. The specificity of each primer set was further confirmed for the predicted base pair length by running the reaction products on 2% agarose gel. The comparative threshold cycle (C_T) was used to determine the relative amounts of target RNA. The threshold cycle number was normalized to an endogenous reference.
Western Blotting

Western blots were carried out on the lysates of WT and TG hearts. Tissues were harvested and homogenized in lysis buffer and protein concentrations were determined by the Bio-Rad protein assay. 20 µg of sample were then loaded and separated by SDS-PAGE on 7% Tris-Acetate gel (Invitrogen). The separated protein was transferred onto polyvinylidene difluoride membranes (PVDF) by electrophoresis. Following transfer, PVDF membranes were incubated for 1 hr in blocking buffer (20 mM Tris-HCl, 147 mM NaCl, 1% bovine serum albumin and 0.1% Tween 20, pH 7.4). Depending on experiment, primary antibodies used were selected from: c-myc (mAb, 9E10, 1:1000, Santa Cruz Biotechnology), NCX ( polyclonal Ab, π, 1:1000, Dr. Kenneth Philipson), NCX (mAb, R3F1, 1:1000, Dr. Ken Philipson), YFP ( polyclonal, 1:1000, Clontech), GAPDH (mAb, 1:1000, Abcam) and were kept in block. PVDF membranes were incubated with appropriate antibodies overnight at 4°C while gently rocking. The following day, the membrane was washed several times and then incubated in secondary antibody anti-mouse IgG (or anti-rabbit for polyclonal) conjugated to horseradish peroxidase in blocking buffer. Membranes were washed extensively with TBST before adding ECL (Millipore). Bands were visualized on autoradiography film and scanned to a computer for evaluation. For western blotting of infected neonatal cardiomyocytes, isolated cardiomyocytes were used instead of homogenized whole cell lysates.
**Echocardiography**

Mice were anesthetized with isoflurane (2-3% induction, 1-25 maintenance) via inhalation and eye lubricant, boric acid, was used to protect their eyes for the duration of experimentation. As an additional precaution, a heating lamp and anal temperature probe was used to prevent hypothermia of the animal during the experiment. Before beginning, mice foot pads were taped to sensors that simultaneously measured electrocardiogram (ECG) during the echocardiogram. Once the fur of the mouse chest was removed, ultrasound gel was applied and the transducer of the Visual Sonics VEVO 707 imaging system was placed at different angles to establish baseline m-mode and Doppler measurements (short axis and long axis). After recording baseline measurements, isoproterenol (0.1mg/kg) was given i.p. to the animal and duplicate measurements were taken at peak drug response. Analysis of measurements was performed on the Visual Sonics acquisition system with the guidance of pediatric cardiologist Dr. Tim McQuinn.
Isolation of Adult Mouse Cardiomyocytes

Cells were isolated from WT and TG mice based off two previously published langendorf perfusion system (Mitra and Morad, 1985) and AfCS Procedure Protocol PP00000125. Mice were anesthetized using sodium pentobarbital (50mg/kg, i.p.) and the heart was excised from the chest cavity and placed into perfusion solution containing (in mM): 10 HEPES, 0.33 Na$_2$HPO$_4$, 110 NaCl, 1.5 MgCl$_2$, 30 taurine, 15.4 L-glutamic acid potassium salt monohydrate, 10 glucose, 2 CaCl$_2$. The heart was allowed to pump out blood and cannulated on a blunted 25 gauge needle. The cannulated heart was then hung on the Langendorf apparatus (37°C, 3.0 mL/minute flow rate) and perfused with 0 Ca$^{2+}$ perfusion buffer with 100mM blebbistatin (TOCRIS) for 6 minutes. Next, the buffer was switched to digestion buffer that contained collagenase type II (657.39 u/ml, Worthington Biochemical), trypsin (0.6 mg/ml, Invitrogen), 100mM blebbistatin, and 12.5 µM CaCl$_2$ for 8-15 minutes depending on the softness of the heart tissue. Once digested the heart was washed with perfusion solution with 0 Ca$^{2+}$ plus blebbistatin for an additional 5 minutes to wash out residual enzyme. The heart ventricle was then cut from the cannula into stopping buffer that contained (in mM): 10 HEPES, 0.33 Na$_2$HPO$_4$, 110 NaCl, 1.5 MgCl$_2$, 30 taurine, 15.4 L-glutamic acid potassium salt monohydrate, 10 glucose, 12.5 µM CaCl$_2$, and 10% FBS. In this buffer, the heart is carefully minced up into smaller pieces and warmed to 37°C. CaCl$_2$ is then added incrementally every 4 minutes until the Ca$^{2+}$ concentration is physiological (1mM). The cells are allowed to settle by gravity at 37°C and resuspended in plating solution of (in mM): 10 HEPES, 0.33 Na$_2$HPO$_4$, 110 NaCl, 1.5 MgCl$_2$, 30 taurine, 15.4 L-glutamic acid potassium salt monohydrate, 10 glucose, 1 CaCl$_2$ for use in electrophysiology and immunocytochemistry experiments.
Cell Culture and Transfection

HL-1 cells (gift from Dr. William Claycomb, Louisiana State University School of Medicine) were maintained in culture at low passage for all experiments (passage 20-40). Cells were grown in Claycomb Media (Sigma) supplemented with 10% fetal bovine serum (Lot#11A568, Sigma), 1% norepinephrine [+-arterenol] (Sigma), 1% penicillin/streptomycin (Sigma), 1% L-glutamine (Sigma) and grown on 0.02% gelatin (Fisher Scientific), fibronectin (1mg, Sigma) coated dishes. HeLa cells (ATCC) were maintained in culture at low passage for all experiments (passage 4-30). Cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) 1% penicillin-streptomycin (Invitrogen), and 1% non-essential amino acids (Invitrogen). When growth reaches about 90% confluency, cells were passaged for continued growth in culture. Both HL-1 and HeLa cells were plated on 18mm coverslip at a density of $2 \times 10^5$ and transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions and 3μg of plasmid DNA.
**Immunocytochemistry**

HL-1 cells were rinsed with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde for 10 minutes at room temperature. After washing 3 times with PBS, cells were blocked overnight at 4 °C in 1% bovine serum albumin and 0.1% Triton in PBS. Next, the block was removed and the cells were incubated with anti-c-myc-FITC mAb (5mg/mL, Sigma) in block for 2 hours. Lastly, cells were rinsed before being mounted on a slide using a drop of ProLong Gold with DAPI and allowed to cure for 24 h. Imaging was performed on a Olympus confocal microscope system using a 405 diode and 488 nm argon ion laser and 63x objective to examine c-myc and DAPI expression.

Immunocytochemical approaches were used to detect both c-myc and NCX in isolated adult cardiomyocytes of both WT and TG animals. Media were removed and cells were rinsed with PBS and then fixed with -20 methanol for 10 min at 4°C. After washing the cells 3 times with PBS, the cells were blocked overnight at 4 °C in 1% bovine serum albumin and 0.1% Triton in PBS. Then, the blocking solution was removed and primary antibody (1:100 c-myc mAb (Santa Cruz Biotechnology) and 1:100 π polyclonal (Dr. Kenneth Philipson) was applied for 2 h at room temperature in block. After washing the cells 3 times with PBS, anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 568 (1:1000, both Invitrogen) in PBS was applied for 2 h at room temperature. The secondary antibody dilution was removed and the cover slip was washed before being mounted on a slide using a drop of ProLong Gold and allowed to cure for 24 hours. Imaging was performed on a Leica TCS SP5 AOBS confocal microscope system using a 488 nm argon ion and 543 HeNe laser and 63x objective to examine c-myc and NCX expression respectively.
**Adenovirus Production**

The original full-length shark NCX protein was mutated in the intercellular P-loop at amino acid 262 using QuikChange site-directed mutagenesis kit (Stratagene) to introduce a HindIII restriction enzyme site. The following primers were used for the amplification of the mutated plasmid: sense, 5’-GGACGGCAAGATGATAAGCTTCACGAAGCCGCAGG-3’ and anti-sense, 5’-GCCGCCGCTTCGTGAAGCTTAGCATCTTGCCGTCC-3’. The following PCR profile was used: 95°C for 30 s, [95°C for 30 s, 55°C for 1 minute, 68°C for 5 minutes]x16 cycles, 68°C for 3 minutes and the reaction was allowed to cool to less than 37°C before DpnI (New England Biolabs) treatment for 1 hour before transformation in XL1-Blue supercompetent cells (Agilent) according to manufacturer’s instruction. Colonies were then screened on amicillin-resistant LB agar plates treated with 80μg/mL X-gal and 20mM IPTG. Colonies were selected and purified using QIAprep Spin Miniprep Kit (Qiagen) and sequenced to confirm mutation. Next, the mutated plasmid was opened at the HindIII site and the enhanced yellow fluorescent protein (eYFP) color gene flanked by a HindIII restriction site was ligated into the shark NCX mutated plasmid. This plasmid was then amplified using STBL2 cells (Invitrogen) selected from ampicillin resistant 2XYT agar plates. Once confirmed by sequencing, the construct was ligated into the NheI and EcoRI (both New England Biolabs) sites of the multiple cloning site of the viral shuttle vector Dual-CCM(+) (Vector Biolabs) which contains a CMV promoter and poly-A termination signal and transformed into DH5α (Invitrogen) cells according to the manufacturer’s protocol and screened using kanamycin treated LB agar plates. Colonies were selected and DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen) and QIAfilter Plasmid Midi Kit (Qiagen). DNA concentration was calculated using the 260nm absorbance on
a BioMate 3S UV-Viable Spectrophotometer (Thermo Scientific) and later used for transfections of HeLa cells and adenoviral production by Vector Biolabs. The final viral product was titered to $1.1 \times 10^{10}$ PFU/mL.
Isolation and Culture of Neonatal Cardiomyocytes

Hearts of 5-10 rats between postnatal day 1-5 were excised, and the main vessels and atrias removed. The ventricles were minced with a razor blade and incubated in Hank's Balanced Salt Solution (HBSS, Invitrogen) with trypsin (50 μg/ml) for 15 h at 4 °C. The digestion was then stopped by trypsin inhibitor (200 μg/ml) for 20 minutes at 37°C. Collagenase (100 U/ml) was used for 30 min before trituration of tissue to isolate single neonatal cardiomyocytes, which were then filtered and centrifuged at 1000 rpm for 3 min, re-suspended in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen) with 1% penicillin–streptomycin (Invitrogen) and 1% non-essential amino acids (Invitrogen), plated on 100-mm dishes and placed in the incubator for 90 minutes to eliminate fibroblasts. Isolated single neonatal cardiomyocytes were onto either 18mm or 12mm glass coverslips (Fisher Scientific) and used for immunocytochemistry or electrophysiology experiments respectively. For western blotting, cells were plated on tissue culture treated 6-well plates.
Adenoviral Transduction

Twenty-four hours after the neonatal cells were plated, the complete medium was removed and the cells were transfected with shark YFP-NCX adenovirus at a multiplicity of infection (MOI) of 500 virus particles per cell (vp/cell). The following day the virus medium was removed and cells were supplemented with normal neonatal medium and kept in the incubator at 37°C and 5% CO₂ until used in experiments 48-72 hours post infection.
Electrophysiology

Acutely isolated adult WT and TG mice cardiomyocytes were plated on 12mm glass coverslips coated with laminin. Cells were bathed in Tyrode solution containing (in mM): 137 NaCl, 5.4 KCl, 2 CaCl₂, 10 HEPES, 1 MgCl₂, and 10 Glucose (titrated to pH 7.4 with NaOH). Borosilicate glass pipettes (World Precision Instruments, Inc) were prepared using a horizontal pipette puller (Model P-87, Sutter Instruments, CA) with a resistance around 5MΩ. Whole-cell patch-clamp recordings were performed using a Dagan voltage-clamp amplifier and pClamp (Clampex 10.2) software. The voltage-clamped cells were dialyzed with a Cs⁺-based, moderately Ca²⁺-buffered pipette solution for NCX 5(mM): 14 EGTA, 94 CsCl, 15 Na⁺, 10 TEACl, 5 MgATP, 10 HEPES and 6.2 mM CaCl₂, (titrated to pH 7.2 with CsOH; measured osmolarity: 294 mOsm). $I_{\text{NCX}}$ was measured by a repolarizing ramp protocol: holding potential -60mV, descending from +80 to −120 mV in 200–400 ms, 5 mM Ni²⁺ as a NCX blocking agent, 10μM nifedipine to block Ca²⁺ channels, and 5μM isoproterenol as β-adrenergic receptor agonist. All experiments were carried out at room temperature (22–25 °C).

HeLa cells transfected with the sharkNCX YFP plasmid and rat neonatal cardiomyocytes infected with shark NCX YFP virus were voltage-clamped in the whole-cell configuration after 48-72h post transfection. Cells were bathed in Tyrode's solution containing (in mM): 137 NaCl, 5.4 KCl, 2 CaCl₂, 10 HEPES, 1 MgCl₂, and 10 Glucose (titrated to pH 7.4 with NaOH). Borosilicate glass pipettes (World Precision Instruments, Inc) were prepared using a horizontal pipette puller (Model P-87, Sutter Instruments, CA). The resistance of the pipette was around 5MΩ. A LED-based illuminator (Prismatix, Modiin Ilite, Israel) and GFP-dependent fluorescent light (>500 nm) was detected with a photomultiplier tube that placed behind a moveable,
adjustable diaphragm. Only the green beating cells were picked for experimentation (for HeLa cells, green cells were selected). Whole-cell patch-clamp recordings were performed using a Dagan voltage-clamp amplifier and pClamp (Clampex 10.2) software. The voltage-clamped cells were dialyzed with a Cs$^+$-based, moderately Ca$^{2+}$-buffered pipette solution (mM): 0.2 EGTA, 100 Cs-Aspartic, 15 Na$^+$, 20 TEACL, 5 MgATP, 0.5 MgCl$_2$, 10 Glucose, 10 HEPES and 0.1 mM CaCl$_2$, (titrated to pH 7.2 with CsOH; measured osmolarity: 295 mOsM), [Ca$^{2+}$]$_i$ =100 nM).

$I_{NCX}$ was measured by a repolarizing ramp protocol: holding potential -60mV, descending from + 80 to − 120 mV in 200–400 ms, 1 mM Cd$^{2+}$ as a NCX blocking agent, 10µM nifedipine to block Ca$^{2+}$ channels, and 50-100µM 8-Br-cAMP as β-adrenergic agonist. All experiments were carried out at room temperature (22–25 °C).
**Statistical Analysis**

Average values are presented in histograms and in the text as the mean ± the standard error of the mean for “n” cells. The distribution of data in individual cells is shown in separate panels. T-test was used to determine statistical significance. Significant findings are labeled with one (p < 0.05, *) or two stars (p < 0.01, **).
Chemicals

Unless noted, all chemicals used for experimentation were from Sigma-Aldrich and prepared fresh daily.
Results

Molecular and Biochemical Phenotype of Transgenic Mice

The shark NCX-c-myc transgene was cloned into the pBS II SK+ bacterial vector containing the α-MHC promoter shown in Figure 13A. To verify expression of this transgene, HL-1 cells, an atrial tumor cell line, was transfected and imaged confocally to visualize expression pattern. Figure 13B demonstrates a field of cells that contains both transfected and non-transfected HL-1 cells. The antibody for c-myc (FITC) is shown in green, while nuclei are stained with DAPI and false colored to red for contrast. The inset of (white box from Figure 13B) is shown in Figure 13C. Expression of the shark NCX-c-myc transgene is shown ubiquitously throughout the cell with a larger portion of that expression shown around the nucleus.
Figure 13: Transgene production and expression in HL-1 cells

A: Rationale for shark NCX-c-myc transgene expression in mammalian system. The tagged protein was ligated in the MCS at SalI and HindIII of the pBS SK(+) vector containing the α-MHC promoter and poly A signal. The transgene was removed from the vector for microinjection by BamHI. B: HL-1 transfected and non-transfected cells shown at magnification of 63x by confocal microscopy. Shark NCX expression is demonstrated by anti-c-myc-FITC (green) and nuclear staining by DAPI (red). C: Inset of panel B (white box) shown at a 4x zoom of the 63x magnification of HL-1 cells expressing the shark NCX-c-myc transgene.
After expression was confirmed in HL-1 cells, the transgene was excised from the bacterial vector and used for microinjection and transgenic mouse production. Three transgenic mice were identified and used to generate three respective transgenic lines, A, B, and D. Offspring of each of these transgenic lines were used to evaluate levels of shark NCX mRNA compared to that of the endogenous mammalian NCX using RT-PCR. Although all three lines were found to have shark NCX mRNA, lines A and D were found to have about 1.4 times more shark NCX than the endogenous mammalian NCX, while line B was found to ¾ less expression of the shark NCX. As a result, only lines A and D were used in experiments.

Western blot analysis of whole heart lysates of WT and TG mice demonstrated no identifiable c-myc bands using two different primary c-myc antibodies. In addition, NCX antibodies π (polyclonal) and R3F1 (monoclonal) demonstrated no discernible difference in amount of NCX present between WT and TG mice. In an effort to look at the cellular level, immunocytochemical approach was used on isolated adult WT and TG cardiomyocytes, isolated from littermates on the same experimental day. Figure 14A shows two cardiomyocytes from WT mice stained with antibodies for c-myc (green) and NCX (red). Both cells demonstrate expression, where NCX is found primarily around the t-tubular membrane and sarcolemma where c-myc expression is found in similar location. Figure 14B is the same staining as in panel A, but for a TG cardiomyocytes. The staining found in WT and TG mice appear to be identical and therefore we were unable to identify the shark NCX-c-myc protein.
Figure 14: Immunocytochemical staining of adult mice WT and TG animals for c-myc and NCX

A: Cardiomyocytes from WT mice where left panels (green) show c-myc expression and right panels (red) show NCX expression. Expression of both proteins is found along the t-tubular membrane. B: Cardiomyocytes from TG mice where left panels (green) show c-myc expression and right panels (red) show NCX expression. Expression of both proteins is found along the t-tubular membrane and is indistinguishable from WT controls.
**Functional Phenotype of Transgenic Mice**

To determine if these transgenic mice manifest any measurable phenotypic changes, we evaluated the cardiac function of paired WT and TG mice both echocardiographically and electrocardiographically. Figure 15A demonstrates a representative pulse Doppler of left ventricular (LV) outflow tract of TG mouse heart from the parasternal long axis view. From this image, one can measure the ejection time (ET – time from aortic valve opening to aortic valve closure) as well as the time to peak (TPk – dT to peak aortic outflow Doppler signal) of aortic outflow before and after 0.1 mg/kg isoproterenol bolus administration. Measurements for WT and TG mice ET and TPk are shown in Table 1. Baseline ET for WT mice was 53.2 ± 2.03 ms and TG was 52.7 ± 1.22 ms, while after isoproterenol the value for WT mice was 38.5 ± 1.31 ms and TG was 42.4 ± 1.47 ms. Baseline TPk values for WT and TG mice were 12.9 ± 1.76 ms and 11.8 ± 1.03 ms, respectively, decreasing as expected after isoproterenol to 6.45 ± 0.41 ms for WT mice and 7.41 ± 0.85 ms for TG mice. In Figure 15B, shows a representative pulse Doppler of the LV inflow-outflow tract using the same parasternal long axis view. From this image, the measured isovolumic contraction and relaxation time (IVCT and IVRT, respectively), are shown in Figures 16C and 16D. IVCT is defined as the period between mitral valve closure and aortic valve opening. The baseline IVCT for WT and TG mice shown in Figure 16C was shortened after isoproterenol application. IVRT, defined as the period of time between aortic valve closure and mitral valve opening, was measured at baseline in WT mice and TG mice in Figure 16D. Following isoproterenol administration, IVRT was also shortened as expected. Neither IVCT nor IVRT values were significantly different in control vs. transgenic mice. Finally, Figure 15C shows a representative m-mode measurement through the anterior and posterior walls of the LV
in the parasternal short axis view. From this view, we can determine posterior and anterior wall thickness at diastole and systole (LVPWd, LVPWs, LVAWd, LVAWs) as well as left ventricular chamber measurements (LVEDd – left ventricular end-diastolic diameter and LVESd – left ventricular end-systolic diameter), summarized in Table 1. Chamber size in WT decreased from $3.36 \pm 0.11$ mm (diastole) and $2.14 \pm 0.18$ mm (systole) to $2.88 \pm 0.13$ mm (diastole) and $1.19 \pm 0.21$ mm (systole) in presence of isoproterenol. In TG mice chamber size decreased from $3.56 \pm 0.18$ mm (diastole) and $2.22 \pm 0.12$ mm (systole) to $2.75 \pm 0.18$ mm (diastole) and $1.02 \pm 0.23$ mm (systole), in presence of isoproterenol. Posterior wall thickness in WT changed from $1.19 \pm 0.09$ mm (diastole) and $1.54 \pm 0.08$ mm (systole) to $1.28 \pm 0.10$ mm (diastole) and $1.93 \pm 0.08$ mm (systole) with isoproterenol administration. In TG mice the same parameters changed from $1.11 \pm 0.11$ mm (diastole) and $1.51 \pm 0.13$ mm (systole) to $1.24 \pm 0.11$ mm (diastole) and $1.70 \pm 0.10$ mm (systole), with isoproterenol. Anterior wall thickness in WT changed from $1.00 \pm 0.09$ mm (diastole) and $1.40 \pm 0.09$ mm (systole) to $0.92 \pm 0.10$ mm (diastole) and $1.60 \pm 0.17$ mm (systole) with isoproterenol administration. In TG the same parameter changed from $0.98 \pm 0.04$ mm (diastole) and $1.41 \pm 0.06$ mm (systole) to $1.01 \pm 0.04$ mm (diastole) and $1.59 \pm 0.09$ mm (systole), with isoproterenol. Additionally, heart rate (Figure 16A) and shortening fraction (a rough estimate of ejection fraction, Figure 16B) can also be calculated and is shown in Figure 16.
Figure 15: Representative m-mode and doppler measurements performed on WT and TG mice

A: Representative measurement taken by pulse Doppler for aortic outflow tract ejection time and time to peak. B: Representative measurement taken by pulse Doppler for LV inflow-outflow IVCT and IVRT. C: Representative measurement taken by m-mode for chamber size, wall thickness, and shortening fraction.
Figure 16: Effect of isoproterenol on heart rate (A), shortening fraction (B), isovolumic contraction time (C), and isovolumic relaxation time (D) in WT and TG mice

A: Comparison of heart rate before and after isoproterenol stimulation in WT and TG mice. B: Comparison of shortening fraction before and after isoproterenol stimulation in WT and TG mice. C: Comparison of IVCT before and after isoproterenol stimulation in WT and TG mice. D: Comparison of IVRT before and after isoproterenol stimulation in WT and TG mice.
Table 1: Comparison of effect of isoproterenol on WT and TG mice cardiac function, chamber size, and myocardial thickness

Measurements were taken for ejection time (ET), time to peak (TPk), left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD), left ventricular posterior wall at diastole (LVPWd), left ventricular posterior wall at systole (LVPWs), left ventricular anterior wall at diastole (LVAWd), and left ventricular anterior wall at systole (LVAWs) in WT and TG animals. Table demonstrates no statistical difference between WT and TG animals.
In addition, protein function was tested in acutely isolated cardiomyocytes from WT and TG mice. Figure 17A shows electrophysiological measurement of membrane capacitance (or cell size). There was no difference between WT and TG mice. Figure 17B shows Ni\textsuperscript{2+} sensitive $I_{\text{NCX}}$ before and after isoproteronol stimulation in order to evaluate the β-adrenergic regulation of NCX. In this set of experiments, we subjected whole-cell patch clamped cells to a ramp-clamp protocol where the cell was clamped from -60mV to +80mV (250ms), and then ramped down to -120mV over 400ms, and step back to -60mV holding potential. This type of recording is able to provide both the inward and outward $I_{\text{NCX}}$. Figure 17B shows that both the inward and outward currents are slightly suppressed in both WT and TG mice, though, not statistically significant. During electrophysiological recordings, we noticed a trend toward larger $I_{\text{NCX}}$ density, only in some TG myocytes. If only such cells with larger currents were evaluated, Figure 18 often noted the typical shark-like NCX cAMP regulation. Figure 18A shows the ramp-clamp protocol and representative traces from control, Ni\textsuperscript{2+} treated, and following washout (all in nifedipine containing solutions) with and without presence of isoproteronol. Figure 18B shows that these cells that have almost double the current density of the WT controls show a significant suppression of the outward current, somewhat similar to the shark bimodal regulation.
Figure 17: Comparison of cell size and Ni$^{2+}$ sensitive $I_{NCX}$ in WT and TG mice before and after isoproterenol

A: Comparison of membrane capacitance in isolated WT and TG cardiomyocytes. B: $I_{NCX}$ in both the inward and outward direction measured before and after isoproterenol application.

Slight suppression of $I_{NCX}$ occurs in both WT and TG with no statistical significance.
Figure 18: Comparison of regulation of Ni$^{2+}$ sensitive $I_{NCX}$ in WT and TG mice when TG cells show up-regulation of $I_{NCX}$

A: Typical ramp-clamp protocol used to measure $I_{NCX}$ along with representative traces from recordings before and after drug administration. B: $I_{NCX}$ in both the inward and outward
Figure 18 legend continued:

direction measured before and after isoproterenol application where cells have up-regulated current. Typical bimodal β-adrenergic suppression of $I_{NCX}$ occurs in TG cardiomyocytes.
Characterization of Shark NCX-YFP Adenovirus Construct

Due to lack of biochemical evidence for shark NCX overexpression in TG mice, we generated another model for NCX overexpression. Figure 19A shows the schematic approach to the generation of a new shark NCX plasmid where eYFP (shown as yellow star) was included in the construct in a domain distal to the XIP region of the intracellular P-loop of the shark NCX. This new construct was then cloned into the viral shuttle vector, DUAL-CCM(+) and used to check its expression in HeLa cells. Figure 19C shows confocal image of the expression of the new plasmid in HeLa cells taken at 63x magnification. Visualization of the YFP indicated direct localization of the shark NCX gene. In addition, the transfected HeLa cells were patch clamped to confirm functional expression and cAMP regulation of the $I_{NCX}$ (Figure 19D). Unlike the cardiomyocytes, HeLa cells were held to a holding potential of 0mV and demonstrated virtually no $I_{NCX}$ when exposed to a low Na$^+$ external solution ($I_{NCX}$ operating in the outward mode). Conversely, transfected cells showed a current density over 1.0pA/pF and demonstrated significant suppression of the outward current with exposure to 8-Br-cAMP. Since the expressed NCX appeared to be functional in our preliminary results, we generated the adenovirus containing the shark NCX-YFP. Figure 19B shows western blot of neonatal cardiomyocytes infected with the adenovirus expressing the shark NCX-YFP. Using GAPDH as a loading control, YFP protein band at the proper size of the tagged NCX was detected in the infected but not control cardiomyocytes confirming protein expression in the adenovirus model.
Figure 19: Generation and functional expression of adenovirus containing shark NCX-YFP in HeLa and neonatal cardiomyocytes

A: Schematic of generation of the adenovirus containing the shark NCX-YFP. B: Western blot of control and infected neonatal cardiomyocytes using YFP and GAPDH primary antibodies. C: Immunofluorescence staining of transfected HeLa cells shows shark NCX distribution in the cells with an emphasis on the membrane. D: Electrophysiological recording in control and transfected HeLa cells show suppression of $I_{NCX}$ with 8-Br-cAMP addition.
cAMP Regulation in Shark-NCX-YFP Infected Neonatal Cardiomyocytes

Neonatal cardiomyocytes were used for adenovirus transduction of the shark NCX-YFP. Spontaneously beating control and infected cardiomyocytes were used to study the functional effects of overexpression of this protein (Figure 20A&B). Endogenous $I_{\text{NCX}}$ was measured in control cells accompanying either their spontaneous Ca$^{2+}$ oscillatory activity or on full release of SR Ca$^{2+}$ pools by rapid application of 3mM caffeine. The average values of caffeine-induced $I_{\text{NCX}}$ in control cells was $0.47 \pm 0.11 \text{ pA/pF}$ and infected cells $1.62 \pm 0.26 \text{ pA/pF}$, Figure 20C. This increase of $I_{\text{NCX}}$ density is also shown in Figure 21 where the I-V relationship of the Cd$^{2+}$ sensitive subtracted current is quantified. Original tracings from three such cells from each group are shown on the left, where the quantification of both the inward (at -100mV) and outward (at 80mV) currents in both control and infected groups are made, right panel. Control cells had an outward $I_{\text{NCX}}$ of $1.12 \pm 0.139 \text{ pA/pF}$ and inward $I_{\text{NCX}}$ of $1.34 \pm 0.228 \text{ pA/pF}$ as compared to infected cells with outward $I_{\text{NCX}}$ of $2.28 \pm 0.169 \text{ pA/pF}$ and inward $I_{\text{NCX}}$ of $2.32 \pm 0.289 \text{ pA/pF}$. This demonstrates about a 2-fold increase in exchanger current in the neonatal cardiomyocytes expressing the shark NCX-YFP. In addition to overexpression of the NCX, we evaluated these cells for regulation by 8-Br-cAMP. Shown in Figure 22 are representative I-V relations before and after 8-Br-cAMP from cells from both control and infected groups. In addition, the distribution of cells and quantification of % suppression by dB-cAMP is shown in Figure 22. While the % suppression by cAMP in control cells is found to be $12.5 \pm 1.66\%$ (outward) and $7.12 \pm 1.64\%$ (inward), the suppression in the shark NCX expressing neonatal cardiomyocytes is significantly larger, $36.0 \pm 3.62\%$ (outward) and $25.5 \pm 3.06\%$ (inward).
Figure 20: Caffeine induced $I_{\text{NCX}}$ in control and infected spontaneously beating neonatal cardiomyocytes

A: Spontaneous beating control neonatal cardiomyocytes before and after rapid caffeine application. B: Spontaneous beating infected neonatal cardiomyocytes before and after rapid
Figure 20 legend continued:

caffeine application. C: Quantification of caffeine-induced $I_{NCX}$ in control and infected neonatal cardiomyocytes.
Figure 21: Subtracted I-V relationship of Cd$^{2+}$ sensitive $I_{\text{NCX}}$ in control and infected neonatal cardiomyocytes

A: Cd$^{2+}$ sensitive subtracted I-V relationships for control (top 3 graphs) and infected (bottom 3 graphs) neonatal cardiomyocytes. B: Quantification of inward and outward $I_{\text{NCX}}$ in control and infected shark NCX neonatal cardiomyocytes using ramp-clamp protocol.
Figure 22: I-V relationship of $I_{NCX}$ and quantification of percent suppression of $I_{NCX}$ in control and infected neonatal cardiomyocytes before and after cAMP application

A: Representative I-V relationships for control (top 3 graphs) and infected (bottom 3 graphs) neonatal cardiomyocytes before and after dB-cAMP exposure. B: Cellular distribution of % suppression of inward and outward $I_{NCX}$ in control neonatal cardiomyocytes. C: Cellular distribution of % suppression of inward and outward $I_{NCX}$ in shark NCX-YFP infected neonatal cardiomyocytes. C&D: Individual cell capacitance is noted between each inward and outward current for each cell. D: Quantification of % suppression of inward and outward $I_{NCX}$ by dB-cAMP in control and shark NCX-YFP infected neonatal cardiomyocytes.
Discussion

Transgenic Mouse Model vs. Adenoviral Overexpression

The major finding of this report is the successful cloning of the shark NCX into mammalian cardiomyocytes through adenoviral and transgenic mouse production. Although the transgenic mouse model suggests expression at the genomic (gDNA) and transcript (mRNA) levels, no evidence for expression of NCX protein was found to confirm the presence of the full-length protein. Using both echocardiographic and electrocardiographic measurements, there was no statistically significant difference in ET, TPk, HR or shortening fraction between the WT and TG mice at baseline levels. Furthermore, isoproterenol administration produced the expected change in heart rate and cardiac contractility as measured by decreases in the R-R interval, ET, TPk, LVEDd and LVESd. An expected increase in fractional shortening was noted as well, indicating a measureable increase in estimated ejection fraction. The values after isoproterenol administration, however, did not significantly differ between the WT and TG mice hearts.

Lastly, functional evidence given by electrophysiology measurements demonstrated a lack of shark NCX expression above that of endogenous mammalian NCX, and similarly a lack of β-adrenergic/cAMP regulation as reported previously (Woo and Morad, 2001; Janowski et al., 2009). In those limited cases where NCX expression was double that of the control WT group cardiomyocytes (where $I_{NCX}$ was normalized to cell capacitance and evaluated for Ni$^{2+}$ sensitive current), cells indeed demonstrated suppression of the outward but not inward current typical of the shark bimodal regulation. Although this was seen in certain cells throughout experimentation, the lack of biochemical evidence for shark NCX protein leads us to believe that something is happening to the protein either during or after translation. It is possible that the C-
terminal c-myc tag has affected this protein, or that this protein is not able to be effectively made in the mammalian system. It is possible that the mammalian systems lack an important protein found in the shark heart, or that the species difference that exist between temperature regulation, heart rate, or osmolarity have limited the expression of this protein. It is also possible that this gene is unable to penetrate into all of the mouse’s cardiomyocytes and hence why in only in a few cells over-expression of $I_{\text{NCX}}$ was detected. If the gene penetrance is limited, it is possible to explain the lack of biochemical evidence (western blotting and immunocytochemistry) of protein expression.

In another effort to confer PKA-mediated regulation of NCX upon mammalian cardiomyocytes, adenovirus overexpression of the shark NCX proved more powerful. Unlike the transgenic construct, we decided to add a visual tag (YFP) in a location where others found little alteration to NCX function, in the intercellular P-loop of the protein (Ottolia et al., 2007). By adding the tag to the non-critical domain of the P-loop, we hoped to avoid any possible concern caused from the C-terminal tagged construct. Under the control of the ubiquitous promoter CMV, the adenovirus transduction proved highly effective in overexpressing the shark NCX. This was done in two ways. The first, spontaneously beating neonatal cardiomyocytes were given a short pulse of 3mM caffeine that causes $I_{\text{NCX}}$ that we can electrophysiologically quantify (caffeine induced $I_{\text{NCX}}$). This caffeine induced $I_{\text{NCX}}$ was 3 times larger in infected cardiomyocytes containing the shark NCX-YFP (1.62pA/pF) as compared to the control (0.47pA/pF). The second electrophysiological approach was to determine $I_{\text{NCX}}$ overexpression using ramp-clamp pulses (as described in results) where we can calculate both the Cd$^{2+}$ sensitive inward and outward current of the exchanger. This method demonstrated a Cd$^{2+}$ sensitive
current of 1.12 pA/pF (outward) and 1.34 pA/pF (inward) for control and 2.28 pA/pF (outward) and 2.32 pA/pF (inward) for transgenic. This is about a 2-fold increase in exchanger current. This level of overexpression is as expected since other mouse models of NCX overexpression show a limited amount of overexpression, due to tight cellular control of the membrane proteins (Adachi-Akahane et al., 1997). Most importantly, shark NCX infected cells demonstrated significantly larger % suppression of $I_{NCX}$ upon cAMP stimulation than controls. This implies that overexpression of the shark NCX into neonatal cardiomyocytes is able to demonstrate typical bimodal regulation as seen in the native shark cardiomyocytes.

Both transgenic and adenoviral overexpression models have their advantages and disadvantages. Transgenic mice provide a way to study the effects of a protein in vivo including cardiac function of the mouse in addition to the cellular level of changes that may occur. It is also possible to create situations that would be considered clinically relevant, such as heart failure or ischemia. One disadvantage is that generally, these mice can compensate to changes that may or may not be the physiological response and that they tightly regulate the amount of protein present in the membrane making large overexpression difficult, especially with the endogenous mammalian NCX still present. Adenoviral overexpression in contrast, is a rapid and easy way to overexpress a protein in primary cells. Although these cells remain in culture, important information can still be obtained. The disadvantage is that this model is limited to transient expression of the protein and will not allow for further studies that could prove whether or not this protein is useful to prevent DADs and EADs.

We believe that this adenovirus construct containing YFP in the P-loop of the shark NCX may be a better way to proceed toward making a transgenic model of overexpression. One
advantage is the position and the other, the ability to visually track the gene via fluorescence of YFP. In a future mouse model, we would be able to quickly identify protein expression, location of the protein, and percentage of cells that express the transgene. These advantages would allow for rapid selection for electrophysiological experiments and quick assessment of the translation of the protein. Although western blotting would still need to be used to confirm size of the protein, we would be able to determine potential expression problems quickly. Of note, we would be able to determine if the protein is not only being made to full length, but also if the translocation of the protein to the t-tubular and sarcolemmal membrane is occurring in the adult mouse. We believe that a mouse model would be important to determine if this protein could potentially be anti-arrhythmic for an adult mouse that has been induced into heart failure. Until this model is present, we can only hypothesize that this bimodal regulation could prevent sudden death in these mice.
Physiological Implications of Shark Specific Bimodal Regulation

The cardiac NCX in both shark and frog operate at low temperature, heart rate, and high osmolality in cells where both \( I_{Ca} \) and \( I_{NCX} \) are under β-adrenergic control and cells lack functional release stores of the SR (Morad and Orkand, 1971; Page and Niedergerke, 1972; Nabauer M, 1992). Therefore, these species are highly reliant on \( Ca^{2+} \) influx through both the LTCC and NCX and solely dependent on NCX for the extrusion of that \( Ca^{2+} \). This is in sharp contrast to mammalian cardiomyocytes that beat quickly and rely on CICR from the SR and reuptake and extrusion of that \( Ca^{2+} \) by SERCA and NCX. It is often the inward \( I_{NCX} \) generated during relaxation that triggers EADs and DADs tied to arrhythmias (Sipido et al., 2007) and can increased when it is required to remove even more \( Ca^{2+} \) out of the cell. This could explain why NCX is up regulated and SERCA impaired in cardiac disease such as heart failure (Pogwizd and Bers, 2002).

These findings suggest that bimodal regulation of the shark NCX is an inherent property of this protein and is able to confer its regulation onto the mammalian neonatal cardiomyocyte with adenoviral transduction. Although this modulation is variable, as shown by the distribution of cells in Figure 22, we believe that this cAMP-dependent bimodal regulation is important for shark ventricle when exposed to β-adrenergic stimulation. When this happens, the enhancement of \( Ca^{2+} \) efflux and rapid relaxation occurs as the heart rate increases and the \( Ca^{2+} \) influx mode is suppressed in order to limit its contribution to the \( Ca^{2+} \) flux, so that the phosphorylated LTCC can control the force development of the contraction, possibly similar to that which mammals achieve with smaller \( Ca^{2+} \) flux and CICR (Janowski et al., 2009). We believe that expression of this protein in the mammalian system would provide a potential way to counteract the deleterious
effects of aberrant inward currents generated by the mammalian NCX during times of sickness such as heart failure.
CHAPTER III

Calcium Signaling in iPS Cell-Derived Cardiomyocytes from Normal and Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) Cell Lines
Abstract

Derivation of cardiomyocytes from iPS cells (iPS-CM) allowed us to probe the Ca\(^{2+}\)-signaling parameters of human iPS-CMs from normal and catecholaminergic polymorphic ventricular tachycardia (CPVT1)-afflicted mutant carrying a novel point mutation p.F2483I in ryanodine receptors (RyR2). iPS-CMs were dissociated on day 30-40 of differentiation and patch-clamped within 3-6 days. Calcium currents ($I_{\text{Ca}}$) averaged ~8 pA/pF in control and mutant iPS-CMs. $I_{\text{Ca}}$-induced Ca\(^{2+}\)-transients in control and mutant cells had bell-shaped voltage-dependence similar to that of $I_{\text{Ca}}$, consistent with Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR) mechanism. The ratio of $I_{\text{Ca}}$-activated to caffeine-triggered Ca\(^{2+}\)-transients was ~0.3 in both cell types. Caffeine-induced Ca\(^{2+}\)-transients generated significantly smaller $I_{\text{NCX}}$ in mutant cells, reflecting their smaller Ca\(^{2+}\)-stores. The gain of CICR was voltage-dependent as in adult cardiomyocytes. Adrenergic agonists enhanced $I_{\text{Ca}}$, but differentially altered the CICR gain, diastolic Ca\(^{2+}\), and Ca\(^{2+}\)-sparks in mutant cells. The mutant cells, when Ca\(^{2+}\)-overloaded, showed longer and wandering Ca\(^{2+}\)-sparks that activated adjoining release sites, had larger CICR gain at -30 mV yet smaller Ca\(^{2+}\)-stores. We conclude that control and mutant iPS-CMs express the adult cardiomyocyte Ca\(^{2+}\)-signaling phenotype. RyR2 F2483I mutant myocytes have aberrant unitary Ca\(^{2+}\)-signaling, smaller Ca\(^{2+}\)-stores, higher CICR gains, and sensitized adrenergic regulation, consistent with functionally altered Ca\(^{2+}\)-release profile of CPVT syndrome.
Introduction

Recent breakthroughs in cell biology have made it possible to develop pluripotent stem cells from adult fibroblasts by transfecting them with a set of 4 “stemness” genes (inducible pluripotent stem cells, iPSC, (Takahashi et al., 2007)). This reprogramming allows experimental approaches that drive such cells to acquire cardiac molecular and electrophysiological phenotypes (Zhang et al., 2009; Gupta et al., 2010; Hoekstra et al., 2012), thus creating opportunities for therapy of a host of cardiac pathologies using patient-derived cells. This approach has made it also possible to examine patient-specific mutations in ion channels and Ca$^{2+}$ signaling proteins that might lead to arrhythmia and heart failure in iPS-CM in a laboratory setting, thus devising pharmacological patient-specific paradigms for therapy (Moretti et al., 2010; Fatima et al., 2011; Itzhaki et al., 2011b; Malan et al., 2011; Matsa et al., 2011; Davis et al., 2012; Lahti et al., 2012). In light of such potentials, it is imperative that the electrophysiological and Ca$^{2+}$ signaling properties of iPS-CM as well as their pharmacology are fully identified and quantified.

Ca$^{2+}$-signaling in mammalian hearts is characterized by: 1) $I_{Ca}$-gated Ca$^{2+}$-release (CICR), providing for the characteristic bell-shaped voltage-dependence of Ca$^{2+}$ transients that closely reflect the voltage-dependence of $I_{Ca}$; 2) The gain of CICR is voltage-dependent, not predicted from a strictly Ca$^{2+}$-dependent process (Adachi-Akahane et al., 1996; Bers and Guo, 2005; Wier, 2007); 3) β-adrenergic agonists enhance $I_{Ca}$, Ca$^{2+}$ content of the sarcoplasmic reticulum (SR), Ca$^{2+}$-transients and accelerate their decay kinetics, consistent with PKA-mediated phosphorylation of DHPRs, phospholamban/SERCA2a complex, and the RyR2; 4) Caffeine-triggered Ca$^{2+}$-release activates an inward current ($I_{NCX}$) with time-course and kinetics similar to
rise and fall of cytosolic Ca\textsuperscript{2+}, reflecting the efflux of Ca\textsuperscript{2+} on the electrogenic Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX). Although there are already a number of reports on the electrophysiology of iPS-CM (Dolnikov et al., 2006; Liu et al., 2007; Sedan et al., 2008) there are few detailed reports on their Ca\textsuperscript{2+} signaling pathways and their regulation beyond measurements of Ca\textsuperscript{2+} transients in intact non-voltage clamped cells (Fatima et al., 2011; Itzhaki et al., 2011a).

In this report, we describe the Ca\textsuperscript{2+} signaling properties of iPS-CM by quantifying the activities of Ca\textsuperscript{2+}-signaling proteins that include the density, kinetics, and regulation of Ca\textsuperscript{2+} channels and NCX transporter, the size of SR Ca\textsuperscript{2+}-stores, its regulation by β-adrenergic agonists, the voltage-dependence of $I_{Ca}$ and Ca\textsuperscript{2+}-transients, the gain of CICR, the efficiency of Ca\textsuperscript{2+}-release mechanism, and the properties of the individual dyadic calcium release (sparks). In addition, we have attempted to quantify possible abnormalities in these parameters expressed in cells derived from a catecholaminergic polymorphic ventricular tachycardia (CPVT) mutant, carrying a novel p.F2483I mutation in their ryanodine receptors that were described recently (Fatima et al., 2011). Our data suggests that Ca\textsuperscript{2+}-signaling properties of adult cardiac myocytes are closely replicated in human iPS-CM. That is, $I_{Ca}$-gated SR Ca\textsuperscript{2+}-release is the primary mechanism for the release of Ca\textsuperscript{2+} on depolarization of the cell by the action potential. Relaxation, in a manner similar to mammalian myocardium, is mediated by reuptake of Ca\textsuperscript{2+} into the SR and extrusion of Ca\textsuperscript{2+} by the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger, producing currents often in excess of 2-3 pA/pF. While adrenergic agonists strongly enhanced $I_{Ca}$, and accelerated the rate of decay of the Ca\textsuperscript{2+}-transients, they had insignificant effects on NCX currents, consistent with findings in adult mammalian hearts (He et al., 2003; Ginsburg and Bers, 2004). These findings led us to
conclude that human iPS-CM represent a reliable Ca\(^{2+}\)-signaling model of mammalian cardiomyocytes.

Numbers of recent reports have implicated RyR2-mutations and the resultant abnormal Ca\(^{2+}\) signaling in development of arrhythmia and sudden death associated with intense adrenergic stimulation in patients with CPVT. It has been proposed that such mutation renders the RyRs “leaky” on exposure to β-adrenergic agonists due to hyper-phosphorylation & dissociation of calstabin from RyR2, (Marks et al., 2002; Lehnart et al., 2004) producing localized increases in Ca\(^{2+}\) that is extruded on NCX generating local depolarization (EADs & DADs), triggering at times fatal arrhythmias. Alternatively, overloading of SR Ca\(^{2+}\)-stores by adrenergic agonists has been proposed (Jiang et al., 2005; Priori and Chen, 2011) to lead to increased probability of RyR2 channel openings, resulting in abnormal release of Ca\(^{2+}\) and the resultant membrane-depolarization and arrhythmias. These ideas have been tested in a number of knock-in mice and in-vitro models (Cerrone et al., 2005; Goddard et al., 2008; Fernandez-Velasco et al., 2009; Suetomi et al., 2011), but their validity in the human disease remain still somewhat clouded by both the variability of the RyR2 point mutations producing CPVT, the locus of phosphorylation on RyR2, lack of universal confirmatory results, and absence of clear-cut pharmacology (Priori and Chen, 2011).

Our data here suggests that despite significant quantitative intercellular differences in Ca\(^{2+}\)-signaling parameters of control and RyR2 mutant cells, they both had equivalent and elevated densities of Ca\(^{2+}\) currents and NCX activity, similar bell-shaped voltage-dependence of \(I_{Ca}\)-gated Ca\(^{2+}\)-release, and voltage-dependent CICR gain. Mutant cells, however, were consistently found to have smaller caffeine-triggered Ca\(^{2+}\)-stores higher CICR gain, especially at
-30 mV, consistent with longer, recurrent and often wandering Ca\textsuperscript{2+}-sparks, compared to sporadic and brief sparks of control iPS-CM. Though adrenergic agonists produced equivalent and large enhancements of $I_{Ca}$ in both mutant and control cells, they differentially altered the CICR gain, diastolic Ca\textsuperscript{2+}, and Ca\textsuperscript{2+}-sparks in mutant cells consistent with aberrant Ca\textsuperscript{2+}-release profiles of Ca\textsuperscript{2+}-overloaded CPVT-mutant myocytes, and the higher proclivity for generation of DADs and EADs in mutant hearts.
**Methods and Materials**

**Culture of Undifferentiated iPS Cells**

The iPS cell lines were derived from dermal fibroblasts of a CPVT-afflicted mutant carrying a *de novo* heterozygous autosomal dominant p.F2483I mutation in RYR2 and a control. This generation, cardiac differentiation and characterization of these cell lines were reported recently (Fatima et al., 2011) and conducted in Germany. The iPS cells were maintained on mitomycin C treated murine embryonic fibroblasts (MEF) prepared in DMEM/F12 medium supplemented with Glutamax (Invitrogen), 20% knockout serum replacer (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (βME, Invitrogen), 50 ng/ml FGF-2 (PeproTech). Cells were passaged by manual dissection of cell clusters every 5-6 days.
Cardiac Differentiation

Cardiac differentiation of iPS cells was carried out on the murine visceral endoderm-like cell line, which was provided by C. Mummery (Leiden University Medical Center, The Netherlands). END2 cells were mitotically inactivated for 3 hours with 10 μg/ml mitomycin C (Sigma-Aldrich Chemie GmbH, Munich, Germany) and 1.2×10^6 cells were plated on 6 cm dishes coated with 0.1% gelatin one day before initiation of iPS cell differentiation. To initiate co-cultures, iPS cell colonies were dissociated into clumps by using collagenase IV (Invitrogen, 1 mg/ml in DMEM/F-12 at 37°C for 5-10 minutes). The differentiation was carried out in knockout-DMEM containing 1 mM L-glutamine, 1% NAA, 0.1 mM βME and 1% Penicillin/Streptomycin (100 U/ml and 100 μg/ml, respectively). The co-culture was left undisturbed at 37°C/5% CO₂ for 5 days. First medium change was performed on day 5 and later on days 9, 12 and 15 of differentiation. Spontaneously contracting clusters were dissociated into single cardiomyocytes for experiments.
Preparation of iPS-CM for Patch-Clamp Experiments

Beating areas were micro-dissected mechanically at day 25-35 of differentiation, dissociated with collagenase B, and single iPS-CM then plated on fibronectin (2.5 μg/ml)-coated glass coverslips in 6 well plates. Cells were incubated for 36–72 h before their use in electrophysiological experiments.
Measurements of Cellular Currents and Global Ca$^{2+}$

iPS-CM were voltage-clamped in the whole-cell configuration. L-type Ca$^{2+}$ current ($I_{Ca}$) and $I_{NCX}$ were activated by depolarizing pulses or exposure to caffeine. The voltage-clamped cells were dialyzed with a Cs$^+$-based, moderately Ca$^{2+}$-buffered pipette solution containing (in mM): 110 Cs$^+$-Aspartate, 15 or 5 NaCl, 20 TEACl, 5 Mg-ATP, 0.2 EGTA and 0.1 Fluo-4 pentapotassium, 0.1 CaCl$_2$ ([Ca$^{2+}$]$_i$ ≈ 100 nM), 10 Glucose and 10 HEPES (titrated to pH 7.2 with CsOH; measured osmolarity: 295 mOsm) allowing simultaneous measurements of intracellular Ca$^{2+}$ transients. L-type Ca$^{2+}$ current ($I_{Ca}$) was measured by depolarization to 0 mV from a holding potential of -50 or -40 mV using a Dagan amplifier and pClamp (Clampex 10.2) software. Borosilicate patch pipettes were prepared using a horizontal pipette puller (Model P-87, Sutter Instruments, CA). The pipettes had a resistance of 3–5 MΩ. The extracellular solution used during experiments contained (in mM): 137 NaCl, 5.4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose and 10 HEPES (titrated to pH 7.4 with NaOH). $I_{Ca}$ was normalized relative to the membrane capacitance and plotted in units of pA/pF. Isoproterenol and Bay-K 8644 were dissolved in K$^+$-free external solutions were rapidly applied in close proximity to the cells using an electronically controlled multi-barreled puffing system. Rapid application of 3 mM caffeine was used in the external K$^+$-free solutions to probe the magnitude of SR Ca$^{2+}$ stores. All experiments were carried out at room temperature (22–24°C).

Cellular Ca$^{2+}$ transients in voltage-clamped cells were measured fluorometrically by including 0.1 mM Fluo-4 pentapotassium salt in the dialyzing pipette solution. The dye was excited at 460 nm light generated from a LED-based illuminator (Prismatix, Modiin Ilite, Israel) and Ca$^{2+}$-dependent fluorescent light (>500 nm) was detected with a photomultiplier tube that
placed behind a moveable, adjustable diaphragm, which served to limit the area of detection to the voltage-clamped cell. The cellular fluorescence signals ($\Delta F/F_0$) were normalized by dividing the changes in whole-cell fluorescence ($\Delta F$) with the baseline fluorescence ($F_0$).
TIRF Imaging and Analysis of Ratiometric Images

Single isolated beating cardiomyocytes derived from iPS cells were plated on non-coated WillCo-dish, glass bottom Petri dishes (Ted Pella, Inc. 35x10 mm, 22 mm glass) and allowed to attach for three days before imaging. Intracellular Ca\(^{2+}\) signals were measured with the fluorescent Ca\(^{2+}\)-indicator dye Fluo-4AM (5 µM, Invitrogen) after 30 min incubation at 37°C and 5% CO\(_2\). The cells were imaged using a Leica multicolor total internal reflection fluorescence (TIRF) imaging system (Leica Microsystems, Buffalo Grove, IL) fitted with a 63x oil-immersion objective lens and an Andor iXon3 camera with 512 x 512 pixels. An argon ion laser was used for excitation at 488 nm and fluorescence emission was measured at wavelengths >515 nm. Cells were imaged at 70-100 Hz with a depth of penetration less than 200 nm into the cell, focus on sub-sarcolemmal Ca\(^{2+}\) release in regions where the cell membrane was attached to the underlying glass cover slips. The pixel size in the object plane was 0.25 µm square, or 0.157 µm with 1.6 zoom (Cleemann et al., 1998a; Woo et al., 2005). Images were binned using 2x2 pixel averaging. The external bath solution contained (in mM): 137 NaCl, 4 KCl, 10 HEPES, 10 Glucose, 1 MgCl\(_2\), 2 CaCl\(_2\) with pH 7.4 using NaOH. Sparks were measured in control vs. mutant-iPS-CM in control bath solution and in response to 3 minutes application of 100 µM 8-Br-cAMP.

The obtained image sequences were collected and surveyed with Leica software (LAS AF), but displayed and analyzed in detail using a custom-designed program (Con2i). The method of analysis is illustrated in Figure 23. Initially each frame was typically subjected to 2x2 or 3x3 pixel averaging. This was followed by scaling that equalized the fluorescence intensity throughout the diastolic intervals (Panel A). An average diastolic image (AVE, F\(_0\)(x,y), Panel B)
was then calculated based on a large number of selected diastolic images with little or no
indication of Ca\(^{2+}\) release activity. Such averaged images showed the footprints of the examined
cells that typically had well defined outlines around regions of stable attachment where the
fluorescence intensity varied significantly. This variability may reflect the stationary distribution
of intracellular organelles and some undulation of the cell membrane and extracellular matrix. To
compensate for the stationary variability each frame was divided by the average (#380/AVE,
\(F(x,y,t)/F_0(x,y)\), Panel B). The resulting ratiometric images showed a fairly uniform intensity as a
mottle of dark blue hues which extended throughout the cell except for hotspots of focal Ca\(^{2+}\)
release activity that were displayed in warmer colors of green, yellow, orange and red (arrows).
Panel C shows a sequence of frames (#28 to #43) where a Ca\(^{2+}\) spark first appeared abruptly as a
small bright spot (~1 \(\mu\)m across, frame # 29) that grew in size (#30-33) and flared up at a slightly
different location (#34) before it finally faded and disappeared (#43). The time course of each
Ca\(^{2+}\) sparks could be follow by defining a region of interest based on its first appearance (See
Figures 30, 31 and 34). This approach was used to evaluate the duration of Ca\(^{2+}\) sparks as the
intervals where the intensity exceeded 50\% of the peak value (Figure 30C). To chart the
evolution of Ca\(^{2+}\) sparks and detect shifts from one release site to another, we also used an
algorithm that approximated each image of local Ca\(^{2+}\) release with a Gaussian distribution (Panel
D) and followed changes in location, amplitude, variance and helicity from frame to frame. This
approach allowed the location of Ca\(^{2+}\) release sites to be determined with an accuracy of 0.1 to
0.3 \(\mu\)m depending on the signal-to-noise ratio.
Figure 23: Automated analysis of Ca\(^{2+}\) sparks based on TIRF-imaging

A: Equalization of the diastolic fluorescence intensity. The image (ROI) shows a single frame with superimposed regions of interest that were marked in a semi-transparent manner in red (cell) and blue (surroundings). The fluorescence intensities in these regions were plotted versus time (red and blue curves), and were approximated throughout the diastolic intervals by a black curve and a line that together determined a scale factor that varied with time and was used to compensate for the slow decline in fluorescence that often occurred in the intervals between beats. B: Ratiometric images. The distribution of fluorescence intensity in each frame (e.g. #38) was divided by an average fluorescence intensity calculated based on multiple selected fluorescence images without noticeable Ca\(^{2+}\)-release activity. C: A sequence of partial ratiometric images where the development and decay of local Ca\(^{2+}\) release is shown in bright
Figure 23 legend continued:

colors on a dark blue mottled background representing the resting Ca\textsuperscript{2+} activity. D: Determination of unitary properties of Ca\textsuperscript{2+} release based on a Gaussian approximation. The two sample images (#29 and 34) are shown with superimposed white circles that mark the location and standard deviations of Gaussian approximations. The curves above and to the left of the images show how well horizontal and vertical mid sections through the images were approximated by Gaussian curves. The graph at the bottom of panel D quantifies the horizontal shift in center of the Gaussian approximations as seen also in the curves above the sample images. The shown partial frames correspond to 30x30 pixels or 9.4 x 9.4 \(\mu m^2\).
Confocal Ca\(^{2+}\) Imaging

Voltage-clamped iPS-CM dialyzed with fluorescent and non-fluorescent Ca\(^{2+}\) buffers (0.2 mM Fluo-4, 0.5 mM EGTA, with 0.5 mM Ca\(^{2+}\)) were imaged at a frame rate of 240 Hz on a Noran Odyssey confocal microscope as previously described (Cleemann et al., 1998a). The focal plane was adjusted to the midsection of the cell intersecting its nuclei.
**Immunocytochemistry of RyR2 Distributions**

Immuno-labeled RyR2 in fixed iPS-CMs were imaged on a Leica confocal microscope following published procedures (Tufan et al., 2012), but using a primary antibody to RyR2 (1:100 anti RyR Mab by Thermo Scientific). Briefly, immunocytochemical approaches were used to detect RyR2 in cultures of control, IMRC8 iPSC-CM. Media was removed and cells were rinsed with phosphate-buffered saline (PBS), and then fixed with -20°C methanol for 10 min at 4°C. After washing the cells with PBS, the cells were blocked in 1% bovine serum albumin and 0.1% Triton in PBS. Then the blocking solution was removed, and primary antibody (1:100 anti -RyR Mab by Thermo Scientific) was applied overnight at 4°C in block. After washing out the cells 3 times with PBS, anti-mouse FITC (1:100) in block was applied for 2 h at room temperature. The secondary antibody dilution was removed, washed, and the cover slip was mounted on a slide using a drop of ProLong Gold with DAPI and allowed to cure for 24 h at room temperature and stored at – 20 °C. Imaging was performed on a Leica TCS SPS AOBS confocal microscope system using a 488 nm argon ion laser to examine FITC expression of RyR2 and a 405 diode laser to examine nuclear DAPI expression using a 63x oil objective with zoom function.
Statistical Analysis

Average values are presented in histograms and in the text as the mean ± the standard error of the mean for “n” cells. The distribution of data in individual cells is shown in separate panels. T-test was used to determine statistical significance. Significant findings are labeled with one (p < 0.05, *) or two stars (p < 0.01, **).
Chemicals

All chemicals used in experiments were from Sigma Aldrich unless otherwise stated and prepared fresh daily.
Results

Calcium Current in iPS-CM from Control and CPVT Cell Lines

Cardiomyocytes from two control iPS cell lines (clones 5 (C5) and 8 (C8)) and one CPTV iPS cell line (clone 1, NP0014-C1)) were used in the comparative electrophysiological and Ca\(^{2+}\)-signaling experiments. To approximate as closely as possible, the internal media of intact contracting cells, \(I_{Ca}\) was measured in cells dialyzed with low Ca\(^{2+}\)-buffered solutions (in mM: 0.2 EGTA, 0.1 Fluo-4, 0.1 Ca\(^{2+}\), see methods), containing either 5 or 15 mM Na\(^{+}\). Figure 24D, shows that \(I_{Ca}\) averaged about 8 pA/pF in control and mutant cells, in bathing solutions containing 2 mM Ca\(^{2+}\), with significant distribution in \(I_{Ca}\) density between individual cells of three cell lines (Figure 24A). The variability in the current density could not be attributed solely to the age of cells in culture nor to cell size (cell capacitance, indicated at the bottom of each column) or intracellular Na\(^{+}\) concentrations (panels, B & D).

The time course of inactivation of \(I_{Ca}\) in low Ca\(^{2+}\)-buffered dialyzing solutions was best fit with two exponentials (\(\tau_{fast} = \sim 10\) ms, and \(\tau_{slow} = \sim 60\) ms, Figure 24C). Increasing the intracellular Na\(^{+}\) from 5 to 15 mM had little effect on the magnitude or kinetics of \(I_{Ca}\). Mostly, spontaneously beating single cells were selected for Ca\(^{2+}\) signaling experimentations.
Figure 24: Distribution of calcium currents ($I_{Ca}$) density and average time constant (tau) in control (IMR-C8) and mutant (NP0014-C1) iPS-CM

Cells were cultured for 3-6 days. $I_{Ca}$ was recorded from a holding potential -40mV with step depolarization to 0 mV. The number at the bottom of each bar indicates the capacitance of individual cells. Panels A and B: Distribution of $I_{Ca}$ density in control and mutant iPS-CM recorded with $[Na^+]_i$ at 5 or 15 mM $Na^+_i$. C: Average fast (tau f) and slow (tau s) time constants of inactivation of $I_{Ca}$ in control and mutant iPS-CM recorded with 5 mM $Na^+$ in the pipette solution. D: Average $I_{Ca}$ density of control and mutant iPS-CM and representative $I_{Ca}$ traces from each group. The average membrane capacitance values were: Panel A, IMR-C8, $32.7\pm 2.3$ pF, NP0014, $43.6\pm3.7$ pF, and panel B, NP0014, $45.8\pm4.2$ pF. Various colors in the distribution panels represent different days of experimentation.
**\( I_{\text{Ca}} \)-gated \( \text{Ca}^{2+} \) Release**

Figure 25 A & B compares the voltage-dependence of \( I_{\text{Ca}} \) and the accompanying \( \text{Ca}_i \)-transients in representative control and mutant cells. In a manner similar to that observed in mammalian myocardium for developed tension and \( \text{Ca}_i \)-transients (Morad and Goldman, 1973; Cleemann and Morad, 1991; Bers, 2008), a bell-shaped voltage-dependence was found for both parameters that activated at -30 mV, peaked at \( \sim 0 \) - 10 mV and decreased back to baseline levels between +60 to +80 mV (middle panels in Figure 25A & B). \( I_{\text{Ca}} \) reactivated on repolarizations from positive potentials (+60 to +80) also triggered \( \text{Ca}^{2+} \)-release (inset traces, panels A & B) as was first described for the rat ventricular myocytes (Cleemann and Morad, 1991). This strict dependence of \( \text{Ca}^{2+} \)-transients on \( I_{\text{Ca}} \) and not on the direction of voltage change, is considered as phenotypic cardiac EC-coupling characteristic, distinguishing cardiac from skeletal muscle that is primarily regulated by voltage-induced \( \text{Ca}^{2+} \)-release (VICR) mechanism (Morad and Goldman, 1973; Cleemann and Morad, 1991; Bers, 2008). The findings of Figure 25A & B suggest that human iPS-CMs express only the cardiac-type EC-coupling. Although there was considerable variability as to the degree of development of the maintained components of \( \text{Ca}_i \)-transients in both mutant and control cell lines, \( \text{Ca}_i \)-transients always decreased at positive potentials and activated \( \text{Ca}_i \)-transients on repolarizations from these potentials (60 to 80mV, inset traces, Figure 25A & B). Comparison of control and mutant cells did not reveal significant differences in the rate of activation and decay or the voltage dependence of \( I_{\text{Ca}} \)-gated \( \text{Ca}_i \)-transients, suggesting no apparent global EC-coupling defects resulting from p.F2483I RyR2 point mutation under baseline conditions.
Confocal spatial imaging of $I_{\text{Ca}}$-triggered $\text{Ca}^{2+}$ release of Figure 25C-E shows that $\text{Ca}^{2+}$ release within the first 25ms of depolarization occurred primarily near the cell surface membrane before rising in the interior cytoplasmic space. Figure 25C shows the typical representation of the rounded appearance of a bi-nucleated (n, n) voltage-clamped Fluo-4 dialyzed iPS-CM, where step-depolarization produced regional $\text{Ca}^{2+}$ signals that developed more quickly around the perimeter of the cell (red trace in Figure 25C representing the red mask of Figure 25D) than in its deeper layers (orange, green) or nuclei (blue). The release of $\text{Ca}^{2+}$ from superficial SR $\text{Ca}^{2+}$ stores is consistent with Figure 25F, showing an immunofluorescence distribution of RyRs in a cluster of iPS-CMs suggesting punctate clustering of RyR2s near the surface membrane. These distributions are reminiscent of adult atrial or immature ventricular cardiomyocytes (Woo et al., 2003a), but differ from those of adult ventricular cells where synchronous activation is supported by a t-tubular network (Cleemann et al., 1998a) as occasionally observed in a small subset of iPS-CM (Figure 26).
**Figure 25: Voltage-dependence and subcellular distribution of $I_{Ca}$-activated Ca$^{2+}$-transients**

Representative current–voltage (I–V) relations normalized relative to the membrane capacitance and the corresponding fluorescence (Fluo-4) Ca$^{2+}$ signal recorded from control (C5, panel A) and mutant (NP0014, panel B) iPS-CM. Currents were recorded with a 250 ms step from holding potential of -50mV in 10 mV steps to +60 mV. The middle panel shows the I-V curves for $I_{Ca}$ and the corresponding Ca$^{2+}$ Fluo-4 signal. The internal solution contained 5mM Na$^+$, and was Ca$^{2+}$-buffered with 0.1mM Fluo-4, 0.2 mM EGTA, and 0.1mM Ca$^{2+}$. The right panel shows the $I_{Ca}$ and fluorescence traces at +60 or +70 mV activating rises in Ca$^{2+}$ on repolarization. C: Confocal image of baseline fluorescence (Fluo-4, $F_0$). D: Color-coded regions of interest (ROI) corresponding to nuclei (n), patch electrode (e) and cytoplasmic regions with increasing distances from the cell membrane (red, orange, green). E: Differences in the time course of the normalized Ca$^{2+}$-dependent fluorescence ($\Delta F/F_0$). The color coded traces corresponds to the ROI in panel D. The 2-D confocal fluorescence images were recorded at 120 Hz using a focal plane intersecting the nuclei. The initial response is shown on an expanded time scale. The voltage-clamp pulse (from -60 to -30 mV) and the resulting (Na$^+$ and Ca$^{2+}$) membrane currents are shown at the top. F: Confocal image of immunofluorescence labeled RyR2 (green) in a small cluster of control iPS-CM with DAPI-labeled nuclei (blue).
Figure 26: Sarcomeric Ca$^{2+}$ release in voltage-clamped elongated iPS-CM

A subset of iPS-CM were elongated and showed some indication of sarcomeric striping as found in adult ventricular cardiomyocytes. A: Membrane currents measured with depolarizing pulses from -60 mV to -30 mV (black) and 0 mV (red). B: Cellular transients of Ca$^{2+}$-dependent Fluo-4 fluorescence ($\Delta F/F_0$). C: Frequency of Ca$^{2+}$-sparks binned per 3 frames of 4.17 ms. D: Average fluorescence intensity ($F_0$). E & F Sample frames showing Ca$^{2+}$ sparks 4 (E) and 12 ms (F) after depolarization from -60 to 0 mV (arrows). G: Distribution of Ca$^{2+}$ sparks. The dashed lines suggest the presence of a faint sarcomeric pattern. Confocal measurement at 240 Hz.
SR Ca$^{2+}$ Stores

1. Fractional Ca$^{2+}$-release:

Fractional Ca$^{2+}$ release, in the context of this study, is defined as the ratio of $I_{Ca^{-}}$ to caffeine-triggered Ca$_{i}$-transients. This ratio is generally close to unity (0.8-0.9) in adult mammalian ventricular cells and 0.3-0.4 in atrial cells (Belmonte and Morad, 2008). Since Ca$^{2+}$ release in cardiac myocytes depends on the magnitudes of both $I_{Ca}$ and Ca$^{2+}$ stores, the ratio of Ca$^{2+}$ released by $I_{Ca}$ and caffeine (representing near-full release) may be taken as the effectiveness of Ca$^{2+}$ channel-gated release. Figure 27 quantifies the fractional Ca$^{2+}$ release in control and mutant myocytes dialyzed with 5 or 15 mM Na$^{+}$ (Figure 27C). The higher intracellular concentrations of Na$^{+}$ used in the low Ca$^{2+}$-buffered pipette solutions are thought to increase the myocyte Ca$^{2+}$-load (Bers, 2008). The fractional release averaged ~0.3, in control and mutant cells (Figure 27C), with some cells showing ratios as high as 0.6 and 0.9 (Figure 27A & B). The fractional release was not significantly altered in 15 mM Na$^{+}$ dialyzed cells in either control or mutant cells (Panel C). Figure 27D shows representative traces of Ca$_{i}$-transients activated by $I_{Ca}$ or triggered by caffeine recorded in control and RyR2-mutant myocytes, (upper and lower traces, respectively).
Figure 27: Fractional Ca\textsuperscript{2+} release in control and mutant iPS-CM

Fractional release was calculated by dividing the $I_{\text{Ca}}$-triggered Ca\textsubscript{i}-transient by that generated by application of caffeine ($\Delta F_{(I_{\text{Ca}})}/\Delta F_{(\text{Caff})}$). Cells were voltage-clamped from $-50$ to $0$ mV or were held at $-50$ mV while subjecting them to $0.5$ s long $3$ mM caffeine pulses. A and B: Distribution of the value of efficiency in control (A) and mutant iPS-CM (B). The numbers at the bottom of each bar indicate the membrane capacitance of each cell. C: Average efficiency of Ca\textsuperscript{2+} release with 5 and 15 mM Na\textsuperscript{+} in the pipette solution. D: Representative Ca\textsuperscript{2+} signals triggered by $I_{\text{Ca}}$ or caffeine in control (a) and mutant (b) iPS-CM.
2. SR Calcium Load:

To quantify the magnitude of SR Ca\(^{2+}\) stores iPS-CMs were subjected to rapid puffs of caffeine containing solutions. In whole-cell patch-clamped iPS-CMs caffeine applications produced large transient rises in the global Ca\(^{2+}\) accompanied by activation of large inward \(I_{\text{NCX}}\). Figure 28A & B represent tracings of the time course of caffeine-triggered Ca\(_i\)-transients and \(I_{\text{NCX}}\) in control and CPVT-mutant cells. Although there was significant variability in the magnitude of caffeine-triggered Ca\(^{2+}\) release between individual cells within control (panel C) and mutant (panel D) cell lines, on average, there was markedly smaller caffeine-triggered Ca\(^{2+}\) release in mutant compared to control cells (panel, E), suggesting significantly smaller SR Ca\(^{2+}\) stores in the mutant cells. Consistent with this idea, the Na\(^+\)-Ca\(^{2+}\) exchanger current (\(I_{\text{NCX}}\)) representing the electrogenic extrusion of Ca\(^{2+}\), as cytosolic Ca\(^{2+}\) rises by caffeine-triggered Ca\(^{2+}\) release, was also significantly larger (~2.2 pA/pF) in control compared to the mutant cells (~1.5 pA/pF) suggesting smaller caffeine-triggered Ca\(^{2+}\)-stores in mutant cells. The time course of Ca\(^{2+}\) rise and fall was also quite variable in different cells, but there was no consistent change in the duration of the release or its relaxation rate between control and mutant cells.

Figure 28F quantifies the rise in cytosolic Ca\(^{2+}\) as measured by the integral of \(I_{\text{NCX}}\) activated by caffeine-triggered Ca\(^{2+}\) release in control and mutant cells as the cellular Na\(^+\) load was increased from 5 to 15 mM. This procedure enhances the entry of Ca\(^{2+}\) on NCX leading to increased Ca\(^{2+}\) load of the SR. Note that increasing Na\(^+\) from 5 to 15mM enhanced only slightly the Ca\(^{2+}\) load of the SR in control cells. In sharp contrast, however, the elevation of [Na\(^-\)]\(_i\) in mutant myocytes increased significantly the Ca\(^{2+}\) load of the SR. This proclivity to Ca\(^{2+}\) loading in mutant myocytes may contribute to leakiness of SR and arrhythmogenesis under the in vivo
conditions where mutant RyR2 would become more susceptible in causing localized releases of \( \text{Ca}^{2+} \) and activation of early and delayed after-depolarization (EADs and DADs).

3. Gain of \( I_{\text{Ca}} \)-gated \( \text{Ca}^{2+} \)-release:

The degree to which \( I_{\text{Ca}} \) triggers \( \text{Ca}^{2+} \) release normalized for the \( \text{Ca}^{2+} \) content of the SR store is defined as the gain of CICR (Wier, 2007). The gain has been used as a parameter to evaluate the effectiveness of CICR under different physiological and pharmacological conditions in cardiac myocytes (Adachi-Akahane et al., 1996; Bers and Guo, 2005). Surprisingly, the gain of \( I_{\text{Ca}} \)-induced \( \text{Ca}^{2+} \) release in mammalian myocytes is voltage-dependent such that more \( \text{Ca}^{2+} \) is released per Coulomb of \( \text{Ca}^{2+} \) influx at voltages negative to zero (high gain) than at positive voltages (0 to +40 mV, lower gain). Irrespective of the mechanisms suggested by different investigators (Wier, 2007), the voltage-dependence of CICR is unique to cardiac EC-coupling, as \( I_{\text{Ca}} \)-gated \( \text{Ca}^{2+} \)-release in neurons, for instance, shows no voltage-dependence (Solovyova et al., 2002). To test the voltage-dependence of the gain factor in the iPS-CMs, we quantified \( \text{Ca}^{2+} \) release triggered by \( I_{\text{Ca}} \) as a function of SR \( \text{Ca}^{2+} \) store at two potentials: -30 mV, where \( I_{\text{Ca}} \) is marginally activated, and at 0 mV, where \( I_{\text{Ca}} \) is near its maximal value. Figure 28G illustrates the quantification of the gain factor at both -30 and 0 mV, in 7 control and 15 mutant myocytes. In a manner similar to adult cardiomyocytes the gain was significantly higher at -30 than at 0 mV, in both control and mutant myocytes (Figure 28G). The CICR gain though equivalent at 0 mV was significantly larger at -30 mV in mutant compared to control myocytes. Considering the smaller \( \text{Ca}^{2+} \) content of the SR in mutant myocytes (Figure 28), this finding suggests a more sensitive \( \text{Ca}^{2+} \) release mechanism in mutant cells for equivalent \( I_{\text{Ca}} \) densities (Figure 24), especially under conditions that would increase the SR load.
The gain factor was calculated using the following equation: \( \text{Gain} = 100 \times \frac{\Delta F_{I_{Ca}}}{(\Delta F_{I_{caff}} \times I_{Ca})} \), (Where \( \Delta F_{I_{Ca}} \) and \( \Delta F_{I_{caff}} \) are the Ca-signals produced, respectively, by activation of \( I_{Ca} \) and exposure to caffeine) averaged ~20 in CPVT mutant compared to ~12 in control iPS-CM at -30 mV (Figure 28G). The higher gain of mutant cells at the threshold of activation of \( I_{Ca} \) may contribute to the instability of release mechanism at voltages near resting potentials, especially under \( \text{Ca}^{2+} \)-overload conditions.
Figure 28: Caffeine-releasable Ca\(^{2+}\) stores

A-E: Simultaneous measurements of caffeine-induced $I_{\text{NCX}}$ current and Ca\(^{2+}\)-dependent fluorescence ($\Delta F/F_0$) in control (IMR-C8) and mutant (NP0014) iPS-CM dialyzed with 5 mM Na\(^{+}\) and superfused with standard Tyrode’s solution. Cells were voltage-clamped to -50mV and exposed rapidly to 3mM caffeine for 500 ms. A and B: Representative caffeine-induced NCX
Figure 28 legend continued:
currents and corresponding fluorescence Ca$^{2+}$ signal from control (a) and mutant iPS-CM (b). C and D: Distribution of $I_{NCX}$ values in control and mutant iPS-CM. E: Average values of caffeine-activated Ca$^{2+}$ signals (top) $I_{NCX}$ currents (bottom) in each group. Stars indicate significance levels (*p<0.05, **p<0.01). F and G: Effect of intracellular Na$^+$ on the amount of Ca$^{2+}$ released from SR in response to application of caffeine. Average values of Ca$^{2+}$ release from SR calculated from the integral of the caffeine-induced $I_{NCX}$ in control and mutant iPS-CM with 5 or 15 mM Na$^+$ in the internal solution. G: Average values of gain factor and $I_{Ca}$ density at -30 and 0 mV. The gain factor is plotted in units corresponding to the fraction in % of the caffeine-induced Ca$^{2+}$ release that is release by a Ca$^{2+}$ current with a density of 1 pA/pF.
4. Modulation of SR-stores by isoproterenol:

In this set of experiments, we quantified the effect of isoproterenol on the magnitude of caffeine-triggered Ca\(^{2+}\) stores and found significant enhancement in the magnitude of the store in the presence of isoproterenol (Figure 29). Interestingly, although the caffeine-triggered Ca\(^{2+}\) releases (\(\Delta F/F_0\)) were significantly enhanced by isoproterenol (Panel E, upper bar-graphs), the accompanying \(I_{\text{NCX}}\) were significantly reduced (lower bar-graphs) as may be expected by isoproterenol-induced enhancement of recirculation fraction (\(R_f\)) of released Ca\(^{2+}\) back into the SR (Morad and Goldman, 1973).

Figure 29 also shows that isoproterenol was more effective in increasing the frequency of generation of spontaneous Ca\(^{2+}\) releases when Ca\(^{2+}\) load of the cells was increased by dialyzing them with higher Na\(^+\) (panel G, and original tracings panel F). Similarly isoproterenol increased consistently the diastolic Ca\(^{2+}\) levels irrespective of intracellular Na\(^+\) load (panel H). These findings are consistent with idea that Ca\(^{2+}\) overload of the myocyte is a critical in manifestations of CPVT syndrome.
Figure 29: Effects of isoproterenol

A-E: Caffeine-induced $I_{\text{NCX}}$ current in control iPS-CM (IMR-C5) recorded with 5 mM Na$^{+}$ before and after exposure to isoproterenol. A and B: Representative $I_{\text{NCX}}$ currents and corresponding fluorescence Ca$^{2+}$ signal before (A) and after (B) exposure to 75 nM isoproterenol. C and D: Distribution of Ca$^{2+}$-transients ($\Delta F/F_0$) and $I_{\text{NCX}}$ values before (C) and
Figure 29 legend continued:

after (D) exposure to isoproterenol. E: Comparison of average values of ΔF/F₀ and I_{NCX} in each group (*p<0.05, **p<0.01). F-G: Effects of isoproterenol on spontaneous Ca^{2+} release activity in regularly depolarized (0.2 Hz) mutant iPS-CM (NP0014-C1) dialyzed with pipette solutions containing 5 or 15 mM Na^+. F: Changes in Ca^{2+} transients (Fluo-4), I_{Ca} and I_{NCX} at the onset of exposure to 100 nM isoproterenol in cells dialyzed with 5 (top) or 15 mM Na^+ (bottom). G: Fraction (in %) of mutant cells with spontaneous Ca^{2+} releases (intervening between the I_{Ca}-triggered transients) before and after exposure to isoproterenol. H: Effects of isoproterenol on baseline Ca^{2+} (F₀).
**Focal Ca\(^{2+}\) Releases and Ca\(^{2+}\) Sparks**

In intact spontaneously beating cells from control and CPVT-mutant incubated in Fluo-4AM containing solutions, we measured the properties of focal Ca\(^{2+}\)-release using two-dimensional confocal imaging with moderate success. Much clearer and more distinct Ca\(^{2+}\)-sparks and focal releases were, however, observed when we used a TIRF imaging system Leica, Inc.) with higher spatial resolution (0.1 µm, Figure 23). Measurements of sparks in control and mutant cells, Figure 30C, shows a histogram of spark-duration suggesting that control sparks rarely lasted longer than 20 ms, while mutant sparks frequently reached durations of 100 ms or more, consistent with longer mean open time of RyR2s.

Our method of analysis made it possible to determine the location of Ca\(^{2+}\) sparks in the focal plane with an accuracy of ~0.1 µm (See Methods and Figure 23). Considering that the imaging resolution in the vertical direction, determined by the evanescent field of illumination was also in the order of 0.1 µm, we achieved an overall resolution that revealed distinct sites with properties that were often preserved from one Ca\(^{2+}\) release event to the next. Figure 30D & E suggests that the Ca\(^{2+}\) release sites may be classified in different categories. One type of Ca\(^{2+}\) release site produced rare and brief Ca\(^{2+}\) releases that with imaging speed of 70-100 Hz produced strong, highly localized fluorescence hot-spots in only a single frame (Panel D, Control, #116). These sparks faded and spread in 1-2 frames, but remained centered at the same location. At a second type of Ca\(^{2+}\) release site, the events were equally brief, but recurred every few hundred milliseconds (D: Control #56; E: mutant #31). While the latter sites were found in both control and mutant cells, sites with much longer lasting release events were found predominantly in the mutant cells. In some cases the epicenter of the release site remained stationary in several
frames (E: Mutant #21 and 103), yet the Ca\textsuperscript{2+} release often appeared to terminate abruptly rather than gradually (Figure 31D). In other cases it leaped from site to site (E: Mutant # 109, Figure 31E). The long-lasting wandering Ca\textsuperscript{2+} release events in the mutant cells may generate inward $I_{NCX}$ that in turn may predispose for EADs and DADs. We consistently found that different Ca\textsuperscript{2+} release sites had distinct properties that were repeatable at individual sites, but varied from site to sites. It may be significant to note that Ca\textsuperscript{2+} release sites in mutant cells had a broad spectrum of Ca\textsuperscript{2+} release times that included the normal brief sparks possibly supporting the idea that the mutation does not by itself cause prolonged wandering Ca\textsuperscript{2+} releases but does so only when other factors come into play.
Figure 30: Ca\textsuperscript{2+} sparks in control (A & D) and mutant (B & E) iPS-CM

A & B: From left to right the panels show: 1) Images of average diastolic fluorescence distributions, 2) Color coded regions of interests corresponding to locations of Ca\textsuperscript{2+} sparks, and 3) The time course of the normalized fluorescence intensity at these locations. The histogram in
Figure 30 legend continued:

panel C shows the distributions of the duration of Ca$^{2+}$ sparks measured at half peak amplitude for control cells (black, average = 40.4±3.5 ms, n=76 ) and mutant cells (red, average = 89 ±7.5 ms, n=50 ). D & E: Image sequences showing the evolution of Ca$^{2+}$ sparks. The colored labels correspond to the traces in panels A & B.
Figure 31: Unitary properties of Ca\textsuperscript{2+} sparks

From top to bottom each panel illustrates the time course of local Ca\textsuperscript{2+} releases in terms of their amplitude (F\textsubscript{1}/F\textsubscript{0}), full-width-at-half-amplitude (FWHA), “elliptical elongation” (d\textsuperscript{2}), and shifts in location (\Delta x\textsubscript{0} and \Delta y\textsubscript{0}). A: Distribution of measurements. The individual traces are interpreted as follows: B: Three ordinary Ca\textsuperscript{2+} sparks (red, blue, and green) with a well-defined center of release within the evanescent field of illumination. C: Diffuse Ca\textsuperscript{2+} releases suggesting Ca\textsuperscript{2+} release outside the evanescent field or asymmetric spread. D: Abrupt termination of long-lasting Ca\textsuperscript{2+} sparks. E: Local Ca\textsuperscript{2+} releases with rapidly shifting centers.
**Pharmacology of iPS-CM**

Pharmacological agents known to activate adrenergic cascade or serve as agonists of Ca\(^{2+}\) channel enhance \(I_{Ca}\) and potentiate Ca\(_i\)-transients in cardiac muscle. Figure 32 shows the effectiveness of isoproterenol in enhancing \(I_{Ca}\) and \(I_{Ca}\)-triggered Ca\(_i\)-transients in control and CPVT-mutant myocytes. Generally we found that isoproterenol while doubling \(I_{Ca}\), only slightly (\(~20\%) enhanced the Ca\(^{2+}\) transients (Figure 32C & D). Similarly, Bay K 8644 also potentiated \(I_{Ca}\) strongly, but only moderately enhanced the Ca\(_i\)-transients (Figure 33). There appeared to be no significant differential potentiating effects of these agents in control vs. the mutant cells.

We also analyzed the extent to which isoproterenol altered the gain of CICR in control and mutant myocytes. Figure 32E quantifies the gain factor in control and mutant myocytes at -30 mV where the gain is high and at 0 mV where the gain is lower, as observed in adult ventricular myocytes and confirmed in Figure 28G in iPS-CMs. Interestingly, isoproterenol appeared to lower the gain at both voltages in control, but not in CPVT-mutant myocytes. Such differential effect of isoproterenol in control and CPVT-mutant myocytes may contribute to higher sensitivity of mutant RyRs to be activated in presence of adrenergic agonists as compared to control cells.
Figure 32: Effects of adrenergic stimulation on $I_{\text{Ca}}$, Ca$^{2+}$-transients, and gain factor in control and mutant iPS-CM

A and B: $I_{\text{Ca}}$ traces and the corresponding Ca$^{2+}$ fluorescence in control and mutant iPS-CM before (black) and after (red) treatment with isoproterenol. Cells were depolarized from -40 mV to 0 mV. C and D: Average values of peak $I_{\text{Ca}}$ and $\Delta F/F_0$ in each group before and after exposure to isoproterenol. D & E: Effects of isoproterenol on the gain factor at -30 and 0 mV. The average
Figure 32 legend continued:

values of $I_{Ca}$ and $\Delta F/F_0$ in panels C and D are based on the distributions illustrated for individual cells in Figure 33. Similarly the average gain factor and $I_{Ca}$ in panel E correspond to a different set of experiments.
Figure 33: Effects of the Ca$^{2+}$ channel agonist Bay K8644 on $I_{Ca}$ and Ca$^{2+}$-transients in control and mutant iPS-CM

A and B: Representative $I_{Ca}$ traces and the corresponding Ca$^{2+}$ fluorescence in control (a and b) and mutant (c and d) iPS-CM before and after exposure to Bay K. The voltage-clamped cells were depolarized to 0 mV from a holding potential of -40 mV. C and D: Average values of peak $I_{Ca}$ and fluorescence in each group before and after Bay K.
Effect of dB-cAMP on Ca\textsuperscript{2+} Signals in Intact Mutant iPS-CMs

Using TIRF microscopy we found that adrenergic stimulation of mutant iPS-CMs often caused Ca\textsuperscript{2+}-overload. In other cells, that tolerated exposure to 8-Br-cAMP, or dB-cAMP for 1-3 minutes, we generally observed increase in the frequency of spontaneous beating (Figure 34A vs. E), and occasionally periods of increased diastolic Ca\textsuperscript{2+} release activity in the form of bursts of Ca\textsuperscript{2+} sparks and low level Ca\textsuperscript{2+} waves (B vs. F). The location of Ca\textsuperscript{2+} sparks were obtained by computerized analysis and are shown as maps superimposed on sample images showing Ca\textsuperscript{2+} sparks (C vs. G) or the onset of synchronous activation of Ca\textsuperscript{2+} release (D vs. H). These maps suggest that isoproterenol may increase the tendency of focal Ca\textsuperscript{2+} releases to wander or jump (white lines) from one frame to the next. The sites of the mapped Ca\textsuperscript{2+} sparks (white triangles) showed little correlation with the highly reproducible regions of synchronous Ca\textsuperscript{2+} release (D & H) suggesting that the spontaneous diastolic Ca\textsuperscript{2+} sparks may occur at sites that are not fully integrated in $I_{Ca}$-induced Ca\textsuperscript{2+} release mechanism.
Figure 34: TIRF imaging of Ca$^{2+}$ sparks in mutant iPS-CM before (A-D) and after (E-H) 3 minute exposure to 100 µM dBcAMP

From top to bottom the matched panels show: The time course of cellular Ca$^{2+}$ transients (A & E), Ca$^{2+}$ sparks at selected color coded sites (during the diastolic interval showed in gray above, B & F), and maps of the locations of Ca$^{2+}$ sparks superimposed on sample ratiometric images of Ca$^{2+}$ sparks (C & G) or the onset of Ca$^{2+}$ release (D & H at the times indicated by *s in A & E). Connecting lines show movements of the center of release from one frame to the next.
Discussion

The major finding of this study is that spontaneously beating cells derived from human skin fibroblasts show similar Ca\(^{2+}\) signaling properties as those of adult mammalian hearts, *i.e.* they express a robust \(I_{\text{Ca}}\)-gated CICR signaling pathway modulated by adrenergic and Ca\(^{2+}\) channel agonists, shown in Table 2. Nevertheless, there were significant quantitative but not qualitative differences in the density of \(I_{\text{Ca}}\), the SR Ca\(^{2+}\) load, and the response to adrenergic and Ca\(^{2+}\) channel agonists among the control as well as mutant cells, perhaps as a consequence of developmental stage of the myocytes. The most prominent change in the Ca\(^{2+}\) signaling profile of p.F2843I RyR2-mutant cells was their consistently smaller caffeine-triggered Ca\(^{2+}\) stores and higher CICR gain, resulting most likely from higher frequency of recurrent longer and spatially wandering sparks. Although isoproterenol was equally effective in enhancing \(I_{\text{Ca}}\), Ca\(^{2+}\)-transients, and caffeine-triggered Ca\(^{2+}\)-stores in control and mutant cells (Figure 29 & 32), it differentially increased the diastolic Ca\(^{2+}\) levels, and the frequency of spontaneous Ca\(^{2+}\) releases in mutant cells (Figure 29F). Given the altered Ca\(^{2+}\) signaling profile of CPVT-mutant cells, it is likely that such cells when exposed to conditions that increase the SR Ca\(^{2+}\) load, such as adrenergic stimulation, higher beating frequency, and increased cytosolic Na\(^{+}\) load, would render the mutant myocytes in intact heart more susceptible to triggering of DADs or EADs, and arrhythmogenesis.
Table 2: Comparison of baseline Ca\textsuperscript{2+} signaling parameters and the effects of adrenergic stimulation in control and mutant iPS-CM
**Ca\textsuperscript{2+} Stores and Their Regulation: Control vs. Mutant**

In cardiac-type EC-coupling there are three critical elements that contribute to the effectiveness of CICR: the density of $I_{Ca}$, the activation-state of RyRs, and magnitude of the Ca\textsuperscript{2+} stores. This has been amply demonstrated by many investigators and is confirmed here by the bell-shaped voltage-dependence of Ca\textsuperscript{2+}-transients reflecting the voltage-dependence of $I_{Ca}$ (Figure 25). This characteristic appears to be fully intact in CPVT-mutant myocytes. Interestingly, the fractional Ca\textsuperscript{2+} release seems to be higher in mutant cells, but the effect was not statistically significant (Figure 27C).

A novel and consistent finding of our studies, also reported for P2328S iPSC-CMs (Kujala et al., 2012) and in R4496C knock-in mice model (Fernandez-Velasco et al., 2009), was that the caffeine-triggered Ca\textsuperscript{2+}-stores were significantly smaller in mutant cells (Figure 28E). The lower Ca\textsuperscript{2+} store content is likely to be caused by enhanced mean open time of RyRs (leakiness) reported in almost every animal model of CPVT, irrespective of the alternative mechanisms proposed. It is clear that a lower Ca\textsuperscript{2+}-content of SR in hearts with hyperactive RyR2s would serve as a protective compensatory mechanism to reduce irregularities in rate and rhythm in whole animal models. Our finding of similar decreases in the SR Ca\textsuperscript{2+}-content in human cellular model of CPVT, suggests that such compensatory decreases are likely to result from the molecular cross-talk between the cellular Ca\textsuperscript{2+} signaling proteins.

The Ca\textsuperscript{2+}-content of the SR was measured either by using Fluo-4, the Ca\textsuperscript{2+}-sensing dye, or by the integral of NCX current generated in response to rise of intracellular Ca\textsuperscript{2+} by caffeine. The total charge carried by $I_{NCX}$ (Figure 28F) provides a more accurate estimate of Ca\textsuperscript{2+} extruded from the cytosol following application of caffeine, as this measurement is free of complications.
resulting from dye-saturation or quantification of Ca\(^{2+}\) using a non-ratio-metric fluorescent dyes. Figure 28F & G confirms the findings of Figure 28E that the mutant Ca\(^{2+}\) stores are significantly smaller than those of control myocytes, but in addition shows that cellular conditions that increase the cytosolic load of Ca\(^{2+}\), for instance, higher intracellular Na\(^{+}\), can strongly enhance the Ca\(^{2+}\) content of the stores in mutant cells. A similar loading of Ca\(^{2+}\) store seems also to occur on exposure of cells to isoproterenol (Figure 29E). In the latter case, even though the caffeine-triggered Ca\(^{2+}\)-release (Fluo-4 signal) is strongly enhanced by isoproterenol, the accompanying \(I_{NCX}\) is suppressed significantly as maybe predicted from the PKA-induced enhancement of SERCA2a/PLB activity and the increased re-uptake of Ca\(^{2+}\) into the SR, \textit{i.e.} the enhancement of recirculating fraction (\(R_f\)) of Ca\(^{2+}\) (Morad and Goldman, 1973).
Gain of CICR

In a purely CICR-gated signaling system, only the numbers of coulombs of Ca\(^{2+}\) that enter the cell and interact with RyRs determine the magnitude of released Ca\(^{2+}\). Such a strictly Ca\(^{2+}\), but not voltage-dependent property of CICR has been observed in neurons (Solovyova et al., 2002). In cardiac myocytes, however; CICR gain shows significant voltage-dependence, such that the gain increases exponentially at voltages negative to 0 mV (Adachi-Akahane et al., 1999). It has been proposed that the larger unitary Ca\(^{2+}\) currents at negative voltages are responsible for the voltage-dependence of CICR gain, but this idea has been dismissed by a number of other investigators (Wier, 2007) and the issue remains unresolved. Interestingly, in atrial myocytes, only the surface, but not the centrally located RyRs appear to show the cardiac phenotypic voltage-dependent gain. Woo et al. (Woo et al., 2003b) by introducing various fragments of carboxyl-tail of Ca\(^{2+}\)-channel into atrial myocytes probed for possible interactions between specific domains of carboxyl tail and RyR2 in mediating the voltage-dependent gain of CICR. In support of direct interaction of DHPR with RyRs it was found that only the CaMKII/CaM binding fragment (LA) of the carboxylic tail of the DHPR (Soldatov et al., 1997) rendered voltage-dependence to the centrally located “naked” RyRs when the fragment was introduced into the atrial myocyte (Woo et al., 2003b). Irrespective of the underlying mechanism for the voltage-dependence of CICR, it is clear that hiPS-CMs show not only voltage-dependent gain of CICR, but also comparatively enhanced gain at negative potentials in mutant cells (Figure 28G), making the CPVT-mutant myocyte Ca\(^{2+}\)-signaling mechanisms poised for release.
CPVT Ca\(^{2+}\) Signaling Models: Of Mice and Men

The CPVT cellular model presented here was made possible by the finding that the p.F2483I mutation could be expressed in the iPS cell-derived cardiomyocytes from the fibroblasts of a CPVT mutant (Fatima et al., 2011), making it possible to study the pathophysiology of human disease in the laboratory setting. Considering the large number (>80) of point mutations in RyRs that bring about CPVT, it is difficult to generalize that this point mutation would have the same Ca\(^{2+}\)-signaling profile as those reported for other point mutations either in transgenic mice: R2474S (Lehnart et al., 2008); S2246L (Suetomi et al., 2011); P2328 in (Goddard et al., 2008); and R4496C (Liu et al., 2006; Fernandez-Velasco et al., 2009) or in iPS-CM: M4109R (Itzhaki et al., 2012); S406L (Jung et al., 2012); and P2328S (Kujala et al., 2012). It is more likely that there will be subtle differences in Ca\(^{2+}\) signaling profiles of the various models, because the proposed mechanisms responsible for enhancement of diastolic Ca\(^{2+}\) release in CPVT1 vary from: 1) altered sensitivity of mutant RyR2, 2) activation by luminal Ca\(^{2+}\), 3) decreased FKBP12.6 binding to mutant RYR2, and 4) to abnormal local intermolecular RYR2 domain interactions. Our findings, summarized in Table 2, suggest that p.F2483I mutation produces unitary Ca\(^{2+}\) release events that are longer in duration, more wandering and recurrent in nature, and therefore are likely to lead to activation of Ca\(^{2+}\)-waves and generation of EADs and DADs. Although in some mutant cells we observed higher frequencies of spark occurrence, we believe that such measurements in intact cells are less reliable as the frequency of sparks, even in control cells, may vary greatly depending on physiological state of the myocyte. Cells that showed higher spark frequencies are likely to be more Ca\(^{2+}\)-overloaded. Interestingly, we found consistently that exposure of myocytes to isoproterenol increased the proclivity of
sparks to migrate/wander and generate diastolic rises of $\text{Ca}^{2+}$ as the frequency of spontaneous beating increased (Figures 29 & 34). In 15 mM Na$^+$ dialyzed mutant cells, held at -40 mVs, we often found higher frequencies of spontaneous releases of $\text{Ca}^{2+}$ (accompanied by activation of equivalent $I_{\text{NCX}}$) than in control cells, or mutant cells dialyzed with 5mM Na$^+$, consistent with the idea that $\text{Ca}^{2+}$-overloading conditions increase the proclivity to aberrant $\text{Ca}^{2+}$ releases in mutant cells.

The consistent decrease in the $\text{Ca}^{2+}$-content of the SR (Figure 28) in the mutant myocytes observed in our study and in recently reported CPVT-iPS-CM carrying P2328S mutation in RYR2 (Kujala et al., 2012) might be expected considering the generally agreed upon finding of increased mean open time of mutant RyRs, irrespective of underlying mechanisms. Nevertheless, in mice knock-in CPVT model with S2246L mutation in RYR2 (Suetomi et al., 2011) $\text{Ca}^{2+}$-content of the SR appears not to have changed significantly even though spark frequency was markedly increased. On the other hand, the Gomez group (Fernandez-Velasco et al., 2009) found that R4496C mutation decreased the $\text{Ca}^{2+}$ content of the SR, while increasing the spark frequency, consistent with our findings of smaller $\text{Ca}^{2+}$-content of the SR. It is of course possible that the transgenic mice models with heart rates exceeding 500 beats/minutes do not adequately represent the pathology of human CPVT where the heart is working at ~60 beats per minute. We posit that the lower $\text{Ca}^{2+}$-content of the SR in human CPVT may serve as a compensatory protective mechanism to dampen the higher gain of CICR, and the longer and more recurrent spark activity of the CPVT myocytes. The findings that both higher cellular Na$^+$ load and exposure to isoproterenol strongly increase the SR $\text{Ca}^{2+}$ load in our model leads us to
suggest that such conditions would overcome the protective compensatory decreases in the SR Ca\(^{2+}\) content, that leads to aberrant Ca\(^{2+}\)-release and arrhythmias.


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