THE ROLE OF ALTERED CALCIUM-HANDLING IN MECHANICALLY-INDUCED ARRHYTHMIAS DURING ACUTE REGIONAL ISCHEMIA

by

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ABSTRACT

Sudden Cardiac Death, a leading cause of death in Canada, commonly occurs as a result of arrhythmias occurring in the first hour of acute regional ischemia (ARI) after coronary artery occlusion. The mechanisms by which ARI leads to arrhythmias are unclear, with mechanically-induced alterations in electrical activity implicated as a contributing factor. The goal of this thesis was to investigate the role of altered calcium (Ca^{2+})-handling in mechanically-induced arrhythmias during ARI. ARI was induced *via* coronary artery ligation in isolated, mechanically-loaded, Langendorff-perfused rabbit hearts. Four studies were conducted, which investigated: i) ischemia-induced alterations in Ca^{2+} handling (in the absence of mechanical effects); ii) the role of Ca^{2+} as a driver of arrhythmias; and the effects of iii) cytosolic Ca^{2+} buffering, and iv) ryanodine receptor (RyR) stabilization, on arrhythmias. This involved optical mapping (single or dual parametric, of voltage and/or Ca^{2+}) and pharmacological manipulation. It was found that shortening of Ca^{2+} transient duration (CaT) occurred more slowly than action potential duration (APD) during ARI, giving rise to a potential window of vulnerability for Ca^{2+}-driven afterdepolarizations. In contrast, depression in the rate of cellular depolarization and of Ca^{2+} release, as well as the increase in the magnitude of APD and CaT alternans, displayed similar temporal changes. Ca^{2+}-driven arrhythmias were not observed, however application of the Ca^{2+} dye resulted in a near elimination of arrhythmias, thought to be a result of Ca^{2+} buffering. This was confirmed by direct cytosolic Ca^{2+} buffering with BAPTA, which, along with RyR stabilization with the clinically-approved drug Dantrolene, also reduced the incidence of arrhythmias. These findings suggest that during ARI there is a necessary contribution of changes in Ca^{2+} handling to mechanically-dependent arrhythmias.

LIST OF ABBREVIATIONS USED

(AP)	Action Potential	(APD)	Action Potential Duration
(ARI)	Acute Regional Ischemia	(ATP)	Adenosine-Triphosphate
(BAPTA)	1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid	(AM)	Acetoxymethyl
(CaT)	Calcium Transient	(CaT-ALT)	Calcium Transient Alternans
(CaTD)	Calcium Transient Duration	(CaMKII)	Calcium-Calmodulin Protein Kinase 2
(CX-43)	Connexin 43	(cAMP)	Cyclic-Adenosine Monophosphate
(CZ)	Central Ischemic Zone	(CV)	Conduction Velocity
(DADs)	Delayed Afterdepolarizations	(DAG)	Diacylglycerol
(dF_{Ca}/dt_{max})	Rate of Calcium Release	(dF_V/dt_{max})	Rate of Voltage Excitation
(DMSO)	Dimethyl-Sulfoxide	(DPI)	Diphenyleneiodonium
(EADs)	Early Afterdepolarizations	(ERP)	Effective Refractory Period
(ECC)	Excitation-Contraction Coupling	($[K^+]_O$)	Extracellular Potassium
(Em)	Emission	(Ex)	Excitation
(Fps)	Frames Per Second	(I_f)	Funny Current
(G_s)	G-Stimulatory Protein	(HPLC)	High-Performance Liquid Chromatography
(H^+)	Hydrogen Ions	(HCN4)	Hyperpolarization Cyclic-Nucleotide Gated Channels
(IBZ)	Ischemic Border Zone	(KH)	Krebs-Henseleit
(I_{KATP})	ATP-Sensitive Potassium Current	(AVN)	Atrio-Ventricular Node
(I_{Na})	Inward Sodium Current	($K_{ir}2.1$)	Inwardly Rectifying Potassium Channel
(IP_3)	Inositol 1,4,5-Trisphosphate	($[Ca^{2+}]_i$)	Intracellular Calcium
(I_{ti})	Transient Inward Current	(VF)	Ventricular Fibrillation
(LEDs)	Light Emitting Diodes	($I_{Ca(L)}$)	L-Type Calcium Channel
(LV)	Left Ventricle	(LVP)	Left Ventricular Pressure

(MEC)	Mechano-Electric Coupling	(MSC)	Mechano-Sensitive Channels
($Na_v1.5$)	Voltage Gated Sodium Channel	(λ)	Wavelength
(NHE)	Sodium-Hydrogen Exchanger	(Na^+/K^+-ATPase)	Sodium-Potassium ATPase Pump
(NO)	Nitric Oxide	(NOS)	Nitric Oxide Synthase
(NPA)	Non-Perfused Area	(NE)	Norepinephrine
(PCr)	Phosphocreatine	(PLB)	Phospholamban
(PKCα)	Protein Kinase-C Alpha	(PJN)	Purkinje Network
(PRR)	Post-Repolarization Refractoriness	(E_k)	Potassium Equilibrium Potential
(PVEs)	Premature Ventricular Excitations	(PKA)	Protein Kinase-A
(ROS)	Reactive Oxygen Species	(ROIs)	Regions of Interest
(RyR)	Ryanodine Receptor	(SF)	Safety Factor
(SAC_{NS})	Stretch-Activated Cation-Nonspecific Ion Channels	(SCD)	Sudden Cardiac Death
(SAN)	Sino-Atrial Node	(NCX)	Sodium-Calcium Exchanger
(SR)	Sarcoplasmic Reticulum	(SERCA)	Sarcoplasmic-Reticulum ATPase
(V_m)	Membrane Potential	(NOX)	NADPH Oxidase
(VPBs)	Ventricular Premature Beats	(VT)	Ventricular Tachycardia
(α-AR)	Alpha-Adrenergic Receptors	(APD-ALT)	APD Alternans
(β-AR)	Beta-Adrenergic Receptors	($I_{Cl(Ca)}$)	Calcium Activated Chloride Channel

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CHAPTER 1: INTRODUCTION

1.1 Sudden Cardiac Death and Acute Regional Ischemia

The birth of the 20th century heralded the dawn of modern medicine as we know it. Medical advancements such as Alexander Flemming's discovery of penicillin facilitated the transition of mortality from infectious diseases to more burdensome chronic diseases. As epidemiological trends change over the decades, so too must the focus of basic science and clinical research to develop novel, safe and effective treatment strategies. Today, in the developed world, the mortality rate due to heart disease is akin to that of influenza/pneumonia or tuberculosis during the early 1900s.[1] In fact, as of 2013, one of every five Canadians was at risk of dying from cardiac related diseases.[2] A common and particularly elusive cause of mortality is that of *Sudden Cardiac Death* which, as its name may suggest, is defined as either a death occurring within the past 24 hours with no definitive causation, or immediately after (~1 hour) clinical indications.[3]

While Sudden Cardiac Death (SCD) may occur secondary to an array of pathologies,[3] over half of these deaths are the result *ischemic heart disease*.[4] Ischemia occurs in any tissue where the oxygen (O_2) demands exceed that of delivery, profoundly affecting its metabolic status.[5] More specifically, ischemia occurs in the heart as result of coronary artery occlusion reducing blood flow to a region of the myocardium (*regional ischemia)*. Acute regional ischemia (ARI) has an array of effects on cardiac tissue, both acute and chronic, which perturb the electrophysiology of the heart and can facilitate the genesis of abnormal electrical activity (*arrhythmias)*. During ARI, SCD is frequently the sequelae of lethal sustained arrhythmias, such as ventricular fibrillation (VF), which reduce the efficacy

of the heart to pump blood, resulting in rapid syncope and, if not immediately rectified, death.

Despite the large incidence of SCD, investigation into the mechanisms by which lethal arrhythmias occur in humans during ARI has proven difficult. This is in part due to the high mortality rate of cardiac arrest patients (23.8%)[6] before hospital admission, as well as confounding variables such as the size or presence of a preexisting infarction, injury from reperfusion[7] and effects of medical interventions[8]. Fortunately, several mammalian models have been employed to examine the pathophysiological changes promoting arrhythmogenesis during myocardial ischemia.[8] These models indicate that during ARI, non-sustained and sustained arrhythmias occur in two distinct phases. The first, known as Phase I, occurs within the first 0-60 minutes of ischemia and arrhythmias are attributable to reversible electrophysiological changes[9]. The second, known as Phase II, occurs following 90 minutes of ischemia and arrhythmias are the product of cell necrosis and infarction[10]. While both phases are clinically important, Phase I arrhythmias are of particular interest as ~50% of all SCD occur during this reversible period in the absence of tissue necrosis.[9]

Due to the dynamic nature of ischemia, arrhythmias during Phase I have been found to occur in a biphasic time course in a variety of species,[11–13] resulting in further delineation. The first arrhythmic phase, known as Phase IA, occurs over the first ~0-10 minutes, while the second and more lethal[5,11,12], Phase IB, extends from ~20-60 minutes.[5] While the aforementioned time frame of Phase IA and IB ARI-induced arrhythmias is commonly utilized, it is important to note that there exists some temporal variability in the incidence of sustained arrhythmias amongst different species and models.[8]

It is the purpose of this thesis to investigate a recently identified potential mechanism (mechanically-induced[12,14]) of arrhythmias during ARI. It will begin by reviewing the specific conditions required for arrhythmogenesis (Section 1.2), followed by an examination of how ischemia-induced changes during Phase IA (Section 1.3) and Phase IB (Section 1.3) may contribute to these conditions. Understanding how ischemia-induced alterations contribute to arrhythmias during ARI is paramount to the understanding of SCD and the future development of efficacious treatments and therapies.

1.2 Conditions for Arrhythmogenesis

Maintaining the coordinated contraction of the heart's four chambers requires the flawless interaction of electric current generation, conduction, and signal transduction. However, disturbances of the myocardium's rhythmic electrical activity can be the product of a variety of pathologies, both genetic[15–17] and acquired[17]. Should the necessary conditions be met, arrhythmias arising from either genetic or acquired diseases can degenerate into lethal sustained arrhythmias such as VF. As it is an objective of this thesis to discuss the mechanisms of ventricular arrhythmias during ARI, this Section will focus on the conditions necessary for arrhythmogenesis in *acquired pathologies*. Changes in tissue electrophysiology and structure can facilitate arrhythmias via: i) Abnormal Automaticity; ii) Triggered Activity (Afterdepolarizations); and iii) Reentry.

1.2.I Abnormal Automaticity

As a myogenic organ, the heart is able to generate its own electrical activity through a concept known as automaticity.[18] Cells located within the sino-atrial node (SAN), atrio-ventricular node (AVN), and Purkinje network (PJN) are capable of generating a sufficient

depolarizing current to overcome electrotonic-coupling.[19] This concept is known as *diastolic depolarization* and is the product of unstable diastolic membrane potential (V_m) arising from two intertwined mechanisms known as the voltage (membrane) clock and the calcium clock.[20] The former is dependent on the non-selective conductance of sodium and potassium through hyperpolarization cyclic-nucleotide gated channels (HCN4), which provides substantial inward currents upon V_m hyperpolarization.[20,21] The calcium clock on the other hand, contributes to depolarization *via* leak of calcium from the sarcoplasmic reticulum (SR) causing depolarization through the electrogenic sodium-calcium exchanger (NCX).[20]

Diastolic depolarization provides the current required to meet the activation threshold for the L-type calcium channel ($I_{Ca(L)}$, -50mV)[20] in the SAN and AVN, and activation threshold for the voltage gated sodium channel ($Na_v1.5$, -60mV) in the PJN.[18] Despite their shared myogenic ability, there exists slight differences between these groups of cells. For example, the SAN and AVN are characterized by more positive maximum membrane potentials than the PJN or the rest of the myocardium (-50mV, -60mV and -80mV respectively).[18] This is a result of regional differences in expression of the inwardly rectifying potassium channel ($K_{ir}2.1$), whose large conductance to potassium contributes strongly to the maximum membrane potential. As such, expression of $K_{ir}2.1$ is lowest in the SAN and highest in PJN and ventricular cardiomyocytes.[22]

While SAN, AVN and PJN all possess automaticity, the phenomenon of pacemaking in the heart is hierarchical. This implies that overall heart rate is dependent upon the region of the heart which meets two conditions: i) the ability to generate a readily propagating action potential (AP) and ii) the generation of propagating action potentials at a higher

frequency than all other subsidiary sites. During normal physiology the SAN is the dominant pacemaker, however during pathophysiology there can be a shift to lower levels of pacing (AVN and PJN), which depolarize at slower rates resulting in bradycardia.[23] These lower heart rates are potentially pro-arrhythmic, for reasons which will be explained in Section 1.2.III.[24] Conversely, changes in tissue electrophysiology may increase the rate of diastolic depolarization, inducing early activation of these pacemaker cells.

Finally, under certain circumstances cardiomyocytes outside of the conduction pathway may also possess abnormal automaticity. In the instance of disease, automaticity in non-pacemaker cardiomyocytes has been reported to result from heterogeneities in tissue electric potential.[25] This arises as depolarized resting membrane potentials (-70mV) can provide the source of a 'diastolic depolarizing' electrotonic current to coupled cells allowing abnormal excitation.[19,23] For the purpose of this thesis, tissue excitation arising from a local current and independent of preceding propagating APs will be considered abnormal automaticity.

1.2.II Triggered Activity - Afterdepolarizations

While automaticity relies on a depolarizing source current for activation, cardiomyocytes existing external to the pacemaker and conduction system tend to rely upon a preceding wave of excitation to bring them to activation threshold. Therefore, under normal circumstances and in the absence of a diastolic depolarizing inward current, cardiomyocytes remain quiescent until stimulated. However, changes in the properties of a preceding excitation during disease can provide a sufficient trigger for additional excitation to occur. Afterdepolarizations are triggered arrhythmias which are defined by Antzelevitch

& Burashnikov as: “Depolarizations that attend or follow the cardiac action potential and depend on preceding transmembrane activity…”.[25] These events can be divided into two sub-categories, early-afterdepolarizations and delayed-afterdepolarizations as illustrated in Figure 1.1.

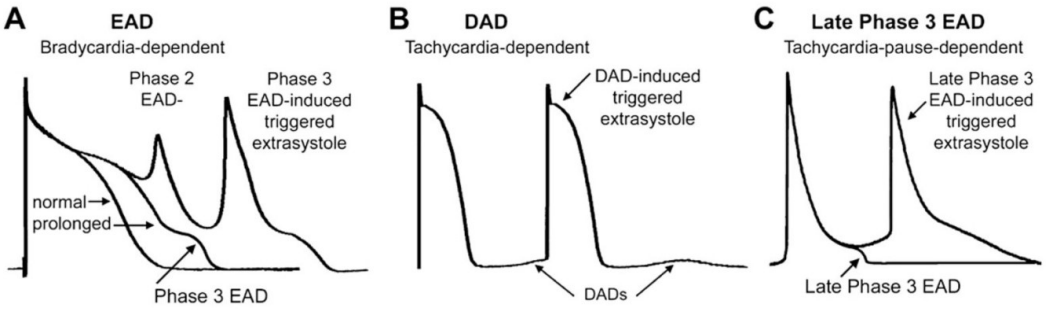

Figure 1.1 Examples of Early and Delayed Afterdepolarizations

Figure illustrates the different types of early afterdepolarizations (EADs) which arise from either prolonged action potential duration (APD) (A) and shortened APD (C) in addition to delayed afterdepolarizations (DADs) (B). Used with permission from: "Overview of Basic Mechanism of Cardiac Arrhythmia" by Antzelevitch and Burashnikov.[25]

Early-afterdepolarizations (EADs) are depolarizations occurring before full repolarization of the cardiac action potential has occurred (phase 4). EADs may occur during either the plateau (phase 2 EADs) or repolarization (phase 3 EADs) portion of the cardiac action potential (Figure 1.1 A). Burashnikov & Antzelevitch define phase 2 and phase 3 EADs as after-depolarizations occurring above or below a V_m of -30 mV, respectively.[27] Phase 2 EADs have been shown to occur most commonly in instances of delayed repolarization and action potential duration (APD) lengthening. These changes may occur as a result of reduced outward currents (carried by potassium) or prolonged/enhanced sodium currents (late sodium current). This extended AP plateau allows adequate time for the reactivation of $I_{Ca(L)}$, which can provide a depolarizing influx of calcium.[18] Opening of these channels during the AP plateau has been shown to provide an ample depolarizing current, subsequently prolonging the AP and delaying time to repolarization.[28,29]

Depolarizations occurring during the repolarization phase (phase 3 EADs) instead have been shown to occur during either augmented or abbreviated APDs. In the instance of delayed repolarization, 'early' phase 3 EADs appear to occur independent of changes in cytosolic calcium, but as the result of electrotonic interactions between regions of varying APD.[29,30] This is unique from the previously described abnormal automaticity (Section 1.2.I) as the current source for phase 3 EADs originates from tissue differences in phase 2 duration, whereas automaticity arises from regional differences in phase 4 electric potential. Contrary to prolonged APDs, 'late' phase 3 EADs may occur in regions of abbreviated APD, where the calcium transient (CaT) is extended beyond repolarization.[31] This 'prolonged' CaT allows intracellular calcium ($[Ca^{2+}]_i$) to remain elevated into phase 4,

whereas under normal circumstances the process of Ca^{2+} extrusion and sequestration do not extend much beyond electrical diastole.[32] Elevated $[Ca^{2+}]_i$ can generate a sufficient transient inward current (I_{ti}) for EAD genesis through the electrogenic extrusion of one Ca^{2+} for three Na^+ through the NCX.[31]

Once resting V_m is achieved, any triggered activity from this point onward is considered a delayed-after-depolarization (DAD). The driving current for this, referred to as the transient inward current (I_{ti}), has a few theorized identities, most of which are related to elevations in $[Ca^{2+}]_i$.[33] Verkerk *et al.* show in isolated ventricular and Purkinje cardiomyocytes, DADs result from the activation of both the calcium activated chloride channel ($I_{Cl(Ca)}$) and the NCX.[34] Increased $[Ca^{2+}]_i$ can occur as a result of a variety of pathologies or sudden changes in depolarizing frequency, both of which dramatically affect calcium handling.[18]

For the purpose of this thesis, all triggered depolarizations occurring as a direct consequence of changes in calcium handling will be referred to as either EADs or DADs. In the instance of depolarizations occurring by which calcium is not a mechanism, arrhythmias will be referred to as either EAD-like or DAD-like.

1.2.III Reentrant Activity

While Section 1.2.I and Section 1.2.II addressed unitary non-reentrant events, lethal arrhythmias occur as the result of reentrant activity.[35] This may occur when a wave of excitation is not extinguished, but re-excites previously activated tissue. This perpetuation of electrical activity may occur following anatomical and/or functional changes to the tissue's characteristics, thus providing the optimal "substrate" for continuous electrical

activity.[36] It is commonly accepted that in order for reentrant activity to occur, the propagating impulse must possess: i) unidirectional conduction block; and ii) an excitable gap.[36,37] Should either of these tenets not be met, the propagating wave may interact with its own wavefront, refractory period, or another wave and consequently become extinguished.

Unidirectional conduction block may occur if a group of activated cells are coupled to regions of disparate excitability. The likelihood of a wave to either propagate or be blocked can be addressed considering the 'safety factor' (SF) for conduction, which reflects the ratio of current a cell is required to generate ("source") in order to excite the cells to which it is coupled ("sink").[18] Should the SF be greater than 1, the source is likely to generate adequate current to excite the cells downstream, and as a result the wave will readily propagate in that direction. Anatomical obstructions, such as blood vessels, connective tissues, or variations in cell-cell junctions may favour conduction in one direction as electrical excitation must propagate around the inexcitable tissue.[18] A practical example of this is the preferential conduction around pre-existing scar tissue *via* multiple paths of viable tissue described as "torturous pathways".[38] During this phenomena, reduced coupling of cells in one direction reduces the input current and may actually increase the SF downstream, promoting stronger conduction in one direction. Functionally, changes in tissue excitability and refractoriness may have profound effects on the size of the electrotonic sink. Should excitation occur in a region that remains refractory or is hyperpolarized, the source current may not be sufficient to overcome the sink and conduction will flow towards regions which are more easily activated.

Regardless of ability to excite tissue in a unidirectional manner, there must be separation between a propagating wavefront and its own refractory period or that of a preceding wave. As such, this 'excitable gap' is essential for the sustenance of an activation wave by preventing wavebreak from occurring.[23] Changes in the length of a propagating wave (λ) has a profound effect on the excitable gap, with shorter wavelengths (under normal circumstances) favoring earlier recovery from refractoriness and greater excitability than longer wavelengths.[37] As wavelength is the product of conduction velocity (CV) and the time to recovery of excitability (ERP); ($\lambda = CV \times ERP$), changes in either CV or ERP will proportionally effect the wavelength of a propagating wave and subsequently the excitable gap. Increasing fibrosis and reductions in gap junctions following scar tissue formation can provide an anatomical mechanism for CV slowing whereas changes in ion channel expression and ion concentrations may alter both CV and ERP.

Although this thesis will not make a distinction between reentrant waves, it would be unwise to neglect the fact that not all reentrant waves are created equal, nor possess similar shape or stability. As such, functional reentrant waves can occur from a variety of forms including: leading circle reentry, anisotropic reentry, figure-eight reentry, reflection, and spiral waves.[23]

1.2.IV Conclusion

Changes in the natural electrical environment of the heart may occur with a variety of diseases, however pathologies that provide both an arrhythmic trigger and substrate are most worrisome. Sustained arrhythmias resulting from the interaction of abnormal automaticity, after-depolarizations and alterations in tissue substrate may provide the

perfect storm for SCD to occur. A variety of alterations in tissue properties can promote this abnormal electrical activity, such as those that occur during ARI. ARI's dynamic characteristics however, cause difficulties in the distinction of arrhythmic mechanisms. The final two Sections of this introduction will address the current literature regarding changes during Phase IA and IB of ARI and how these alterations promote arrhythmogenesis through abnormal automaticity, triggered activity, and reentry.

1.3 Phase IA Ischemia

Immediately upon the cessation of coronary blood supply, a dynamic process of electrophysiological and metabolic changes begins to occur in cardiac tissue. These alterations provide the conditions for both the genesis and sustenance of arrhythmias, however the constantly changing ischemic environment results in phase-dependent mechanisms. Current understanding is that Phase IA arrhythmias are the result of rapid changes in cellular electrophysiology causing ectopic triggers and changes to the substrate for reentry[5,39] secondary to alterations in tissue metabolism and transmembrane ionic concentrations.[40] Specifically, the three principal metabolic/ionic changes that occur during Phase IA of ischemia are: i) elevated extracellular potassium ($[K^+]_o$); ii) hypoxia; and iii) acidosis.[39]

1.3.I Increased Extracellular Potassium

During ARI, $[K^+]_o$ undergoes three time-dependent alterations.[41,42] Within the first ~10 minutes, there is an immediate increase in $[K^+]_o$ (from ~5 mM to ~14.7 mM)[43] resulting from extracellular volume loss (15%)[44] and activation of the ATP-sensitive potassium current (I_{KATP}) (an examination of the activation of I_{KATP} is provided in Section 1.3.II).[5]

Following ~10 minutes, a plateau in $[K^+]_o$ occurs as sodium-potassium ATPase pump (Na^+/K^+-ATPase) function is preserved *via* improved glycolytic capacity stimulated by endogenous catecholamine release.[45] Beyond ~20 minutes of ischemia, accumulation of hydrogen ions (H^+) and production of lactate slows as available energy substrates are consumed, effectively inhibiting anaerobic metabolism. This precipitous plunge in intracellular ATP reduces the efficacy of the Na^+/K^+-ATPase, reducing potassium influx, and inducing a second increase in $[K^+]_o$ during Phase IB.[44] The changes in $[K^+]_o$ during ARI are shown in Figure 1.2. It can be seen that in the central ischemic zone (Figure 1.2 A) there is a rapid rise in $[K^+]_o$ from ~5.4 mM to ~10 mM, then after a brief plateau (10-20 minutes), a second rise occurs. It can also be seen that in the mid-ischemic zone there is a moderate, and slower accumulation of $[K^+]_o$.

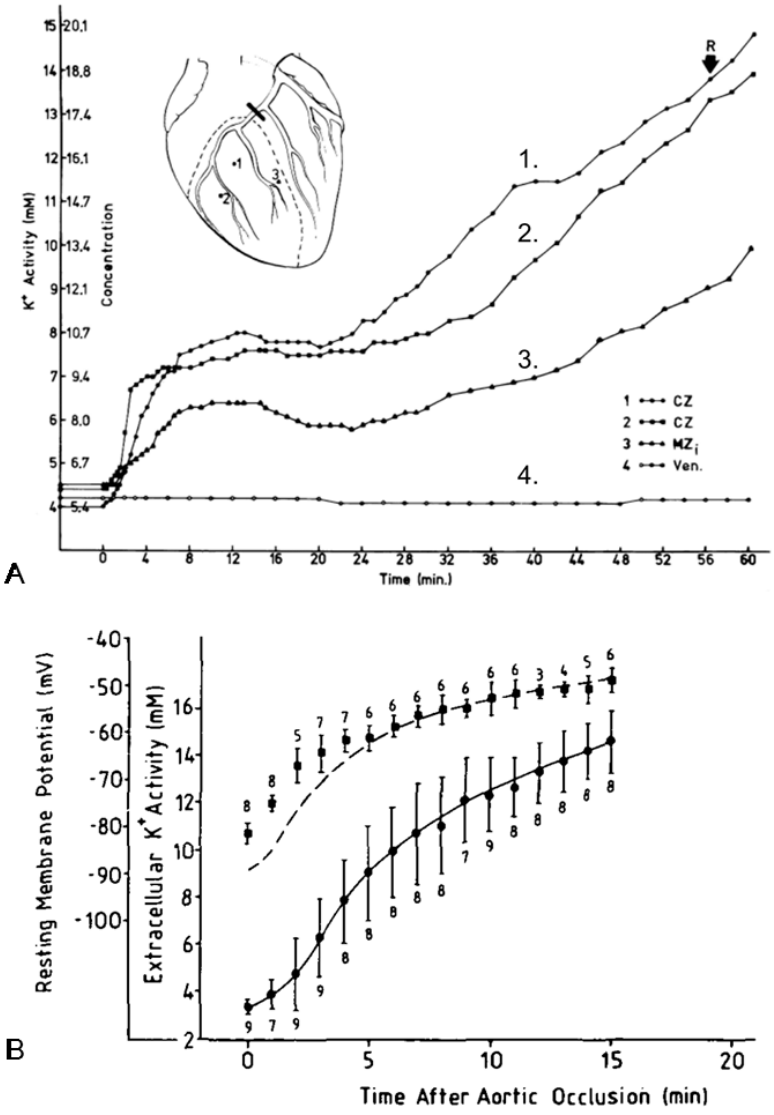

Figure 1.2 Changes in Extracellular Potassium During Acute Regional Ischemia

(A) Changes in K^+ activity (y_1-axis) measured using K^+ -sensitive mini-electrodes and their corresponding K^+ concentrations (y_2-axis) during 56 minutes of regional ischemia. Electrode sites are located in the central ischemic zone (CZ) (1,2), margin of ischemic zone (MZ_i) (3), and coronary vein (4). During ischemia there is a rapid increase in extracellular K^+ followed by a plateau phase and a second increase around 20 minutes. Used with permission from: "Effect of Acute Coronary Artery Occlusion on Local Myocardial Extracellular K^+ Activity in Swine" by Hill and Gettes.[42] (B) Changes in resting membrane potential (y_1-axis) and extracellular K^+ activity (y_2-axis) during 15 minutes of global ischemia. Black circles represent extracellular K^+

activity, black squares represent resting membrane potential. Number of samples shown above each data point ± SEM. Used with permission from: “Resting Membrane Potential, Extracellular Potassium Activity, and Intracellular Sodium Activity During Acute Global Ischemia in Isolated Perfused Guinea Pig Hearts” by Kléber.[43]

As one of the most important cations in the body, changes in trans-sarcolemmal potassium gradients can have profound effects on electrophysiology. In excitable tissue, such as neural or cardiac, potassium plays a significant role contributing to repolarization and resting V_m, through a variety of channels (*e.g.*, $K_{ir}2.1$ (I_{K1}), $K_v11.1$ (I_{Kr}), $K_v7.1$ (I_{Ks}), $K_{ir}3.1$ ($I_{K(Ach)}$)).[46] As mentioned in Section 1.2.I, it is the inwardly rectifying potassium channel ($K_{ir}2.1$), whose conductance is larger than any other channel, which sets the resting V_m.[47] The relationship between potassium's electrochemical gradients and resting V_m is reflected by the similarity of the potassium equilibrium potential (E_k) with that of the resting V_m (-94 mV *vs*. -90 mV, respectively).[43] Thus, during the first ~10 minutes of ischemia, changes in $[K^+]_o$ from ~5 mM to ~10 mM dramatically shifts E_k, and V_m from ~-90mV to ~-60mV, as shown in Figure 1.2 B.[43,44]

Increases in $[K^+]_o$ may contribute to ectopic excitation as elevations in resting V_m may initially cause a period of ectopy and "supernormal conduction".[18] This enhanced excitability occurs in the first ~10 to 20 minutes of ischemia as resting V_m intrudes upon the activation threshold (~-60 mV) of $Na_v1.5$,[18] causing early activation of the inward sodium current (I_{Na}). On the other hand, changes in $Na_v1.5$ availability during ARI can affect the substrate of the tissue by facilitating conduction block. It has been shown that a depolarization of resting V_m to values around -70 mV can inhibit approximately 50% of all sodium channels in the region.[48] This results from downward shift in the steady-state inactivation curve, reflecting a reduction in the probability of channel response to a stimulus. This gives rise to a phenomenon known as "post-repolarization refractoriness (PRR)" which extends the ERP of the tissue by slowing the recovery of the $Na_v1.5$ inactivation gate.[39]

While the described changes occur in the central ischemic region, it is important to consider the junction of ischemic and non-ischemic tissue. In the region where healthy and ischemic tissue meet ('border-zone'), gradients in $[K^+]_o$ can exist. This may cause a gradient in elevations of resting V_m as the more depolarized ischemic region "pulls-up" the resting V_m of cells downstream in the border zone.[49,50] Coronel *et al.* have shown that tissue within the range of -4 mm to +2 mm across the ischemic border requires lower diastolic stimulatory thresholds than that of tissue within the central ischemic zone due to this 'injury current',[51] leading to heterogeneous regions of excitability and repolarization in the border-zone.[18]

1.3.II Hypoxia/Anoxia

The inadequate delivery of oxygen to cardiac tissue during ARI favours a change in the tissue's metabolic status from aerobic to anaerobic metabolism, as electron-accepting oxygen molecules become a limiting factor. Interestingly, during Phase IA of ARI intracellular adenosine-triphosphate (ATP_i) remains relatively stable, as it declines slowly in the first 10-15 minutes.[52,53] This trend can be seen in Figure 1.3 A as a reduction of ~25% in ATP during the first 15 minutes. This ebbing maintenance of ATP_i is the result of additional energy systems, such as the short-lived phosphocreatine (Figure 1.3 B) and self-limiting anaerobic glycolysis system, in addition to reduced metabolic demands of the tissue as contraction is impaired.[52]

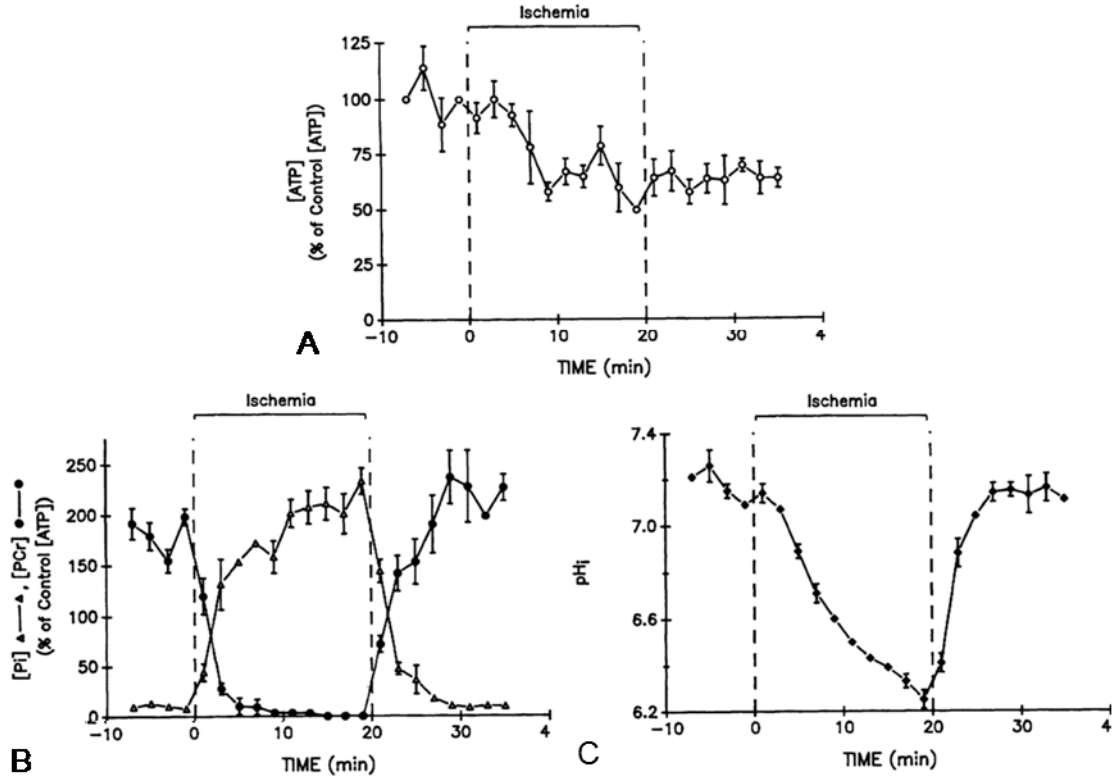

Figure 1.3 Changes in High Energy Substrates and pH During Acute Global Ischemia

Figure illustrates changes in (A) adenosine triphosphate (ATP), (B) phosphocreatine (PCr), and; (C) pH using ^{31}P.NMR techniques. Used with permission from "Quantification of $[Ca^{2+}]_i$ in Perfused Hearts" by Marban *et al.*[52]

Along with declining levels of ATP (which reach ~50% by 20 minutes of ischemia), accumulating levels of its hydrolyzed form, ADP, have been shown to have profound effects on electrophysiology. Weiss *et al.* report that increasing $(ADP)_i$ reduces the inhibitory effect of ATP on I_{KATP} and thus magnifies I_{KATP} activity.[54] As mentioned in Section 1.3.I, activation of I_{KATP} can produce a significant outward potassium current, hastening repolarization and shortening APD. In fact, by use of mathematical modelling, Shaw & Rudy demonstrated that anoxic-activation of I_{KATP} is the most significant contributor to the AP shortening seen with ischemia (much more than any other ischemic mechanism).[39] During ARI, rapid shortening of APD is evident within the first 5 minutes, effects of which can be readily ameliorated using the I_{KATP} specific blocker glibenclamide.[55] Should APD shortening occur in the absence of post-repolarization refractoriness (Section 1.3.I) or calcium transient (CaT) abbreviation, there may be an increased probability of phase 3 EADs to occur given sufficient current.[31] However, an ischemia-induced change in APD in the context of arrhythmogenesis is more commonly viewed as an important contributor to increased dispersion of repolarization and the excitable gap required for reentry, resulting in unidirectional conduction block.[5]

Alterations in O_2 availability can also facilitate adverse alterations in ion channel kinetics. For example, hypoxia has been shown to increase the late (window) sodium current, giving rise to a persistent inward current.[56] This may contribute to increases in intracellular sodium frequently reported during ischemia.[39] Furthermore, hypoxia has been shown to impair the activation kinetics of $I_{Ca(L)}$ (in human-embryonic kidney cells).[57] This response was found in the absence of alterations to inactivation and deactivation kinetics

and has been attributed to the ability of the pore subunit to detect changes in oxygen tension.[58]Acidosis

As mentioned above, during ischemia there is a rapid shift in metabolism towards anaerobic glycolysis.[59] Although this energy system is a quick, albeit inefficient, means by which ATP production can occur, H^+ are rapidly generated as pyruvate is oxidized to lactate and NADH is reduced to NAD^+.[60] As ischemia progresses, intracellular pH decreases linearly from its resting point of 7.4 to values as low as 6.4 within the first 10 minutes.[61,62] Accumulation of H^+ activates exchangers in a futile attempt to maintain homeostatic cellular pH (~7.4). Of these compensatory mechanisms, H^+ is most effectively extruded through the sodium-hydrogen antiporter resulting in an influx of sodium.[63] As a result, additional sodium must then be extruded through the Na^+/K^+-ATPase, sodium pump, or NCX, resulting in a net increase in intracellular calcium.[63] Figure 1.3 C shows the rapid changes in pH during the first 20 minutes of ischemia, which reach a value of ~6.2 by 20 minutes.

Aside from the changes in ionic concentrations required to balance the declining pH during ARI, acidification of the intracellular milieu can have profound effects on the functionality of sarcolemmal, subsarcolemmal, and intercellular ion channels.[39] The sudden decline of pH within the first 10 minutes has been shown to reduce the conductance of I_{Na} by ~25%, leading to a substantial reduction in the maximal rate of depolarization.[64] Acidosis has also been shown to slightly alter I_{Na} steady-state activation and inactivation, facilitating a slight hyperpolarizing shift at a pH of 6.0.[65] Furthermore, conductance of $I_{Ca(L)}$ has been shown to be impaired by ~10-20% by reductions in pH.[66] Reductions in pH to values of 6.3, which may occur within the 10-15 minutes of ischemia, produce a ~70%

reduction in the maximal conductance of $I_{Ca(L)}$.[67] While the predominant inward current carrying channels are affected, outward-repolarizing currents are also altered by acidosis, with an inhibition of I_{K1} contributing to resting V_m depolarization.[5] The combined effects of pH on these three key ion channels causes most notably a reduction in the slope of phase 0 depolarization, in addition to mild shortening of the phase 2 plateau, and moderate phase 4 V_m depolarization.

Electrophysiological effects of intracellular acidification can promote arrhythmogenesis by profoundly affecting the substrate required for reentry. Reduced rate of cellular depolarization (phase 0) by inhibition of I_{Na} can affect conduction velocity in tissue and, when combined with a shortened action potential, reduce excitation wavelength and increase the excitable gap for reentry. Furthermore, conduction slowing may promote wavebreak to occur. As mentioned, these pro-arrhythmic events are linked the excitable gap, however changes in tissue excitability resulting in post repolarization refractoriness may abolish the excitable gap, even despite shortened wavelengths.

Not only can acidosis affect electrophysiological parameters of the heart, but it has also been shown to have profound effects on contractility. For example, acidification has been shown to reduce the sensitivity of Troponin-C for calcium.[68] This, in turn, would reduce the affinity of actin for myosin and impede cross-bridge formation.

1.3.III Conclusion

The metabolic and electrophysiological changes occurring during the first phase of ischemia have been heavily investigated. In their totality, at the cellular level, the changes described above give rise to an elevated resting V_m, reduced upstroke velocity and reduced

APD (Figure 1.4). Sustained arrhythmias at this time are considered to be the result of changes in tissue excitability giving rise to abnormal automaticity and triggered activity, along with alterations in AP morphology providing the conditions for reentry. As a result of the heterogeneous changes in membrane excitability and refractoriness in both ischemic, and border-zone tissue, unidirectional conduction block and the excitable gap are enhanced and ventricular tachycardia is frequently reported.[9] This class of arrhythmia, however, while troublesome is non-lethal, and thus does not generally result in SCD. The more deadly phase of ARI is generally considered to be Phase IB, which will be considered next.

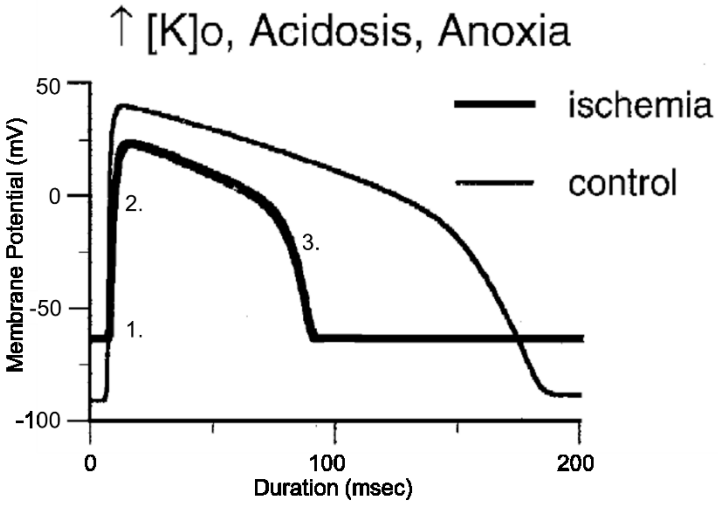

Figure 1.4 Changes in Action Potential Morphology During Ischemia

During ischemia, increases in cytosolic potassium, acidosis, and anoxia lead to an elevation in the resting membrane potential (1.), reduced phase 1 upstroke (upstroke velocity) (2.), and action potential duration shortening (3.) respectively. Used with permission from: "Electrophysiologic effects of acute myocardial ischemia: a theoretical study of altered cell excitability and action potential duration" by Shaw and Rudy.[39]

1.4 Phase IB Ischemia

As ischemia progresses through Phase IA, there is a continual reduction in the excitability of the tissue, secondary to a declining pH, increasing $[K^+]_o$ and reductions in ATP. As such, it may be of little surprise that a period of relative quiescence around 15-20 minutes is frequently reported.[11] The resurgence of not only triggered activity, but of sustained lethal arrhythmias during Phase IB provides an interesting quandary to the relatively few answers that alterations in metabolism and electrophysiology may provide. Currently, Phase IB arrhythmias are considered to be linked to either the individual contribution, or a combination of: i) gap junction uncoupling; ii) endocardial discharge; iii) catecholamine release; and iv) altered mechanics.

1.4.I Gap Junction Uncoupling

As the first phase of arrhythmias during ARI have been reported to be the product of changes in tissue electrophysiology lending to both trigger and substrate, these mechanisms rely on steadfast connectivity of cells.[69] Intercellular coupling of ventricular cardiomyocytes through the medium-conductance hemi-channel connexin-43 (CX-43) is important for the rapid and homogeneous conduction of an action potential throughout the myocardium. However, during ARI, studies have shown that there is a rapid reduction in intercellular coupling and an enhancement in tissue resistance to conduction during Phase IB.[13]

During ARI, acidification, reductions in $(ATP)_i$, and increases in intracellular calcium have been shown to reduce CX-43 phosphorylation and subsequent conduction velocity.[13,62,70,71] Furthermore, these key electrophysiological changes can interact and

complement each other as acidification increases sensitivity of gap junctions to calcium.[13] Smith *et al.* report that during the first 10 minutes of ARI tissue resistivity, inversely proportional to cell-cell coupling, and thus an indicator of uncoupling, modestly increases to a plateau.[72] Then, after a brief hiatus, there is a second substantial rise in tissue resistivity, corresponding to gap-junction uncoupling after ~20 minutes (Figure 1.5).[13,69] Interestingly, this rise has been shown to occur around the same time as the second rise in $[K^+]_o$, however it has been noted that increases in $[K^+]_o$ during this period are quite small.[72] Additionally, changes in intracellular pH by this point during ischemia have plateaued and are unlikely to contribute to further electrophysiological distress.[69]

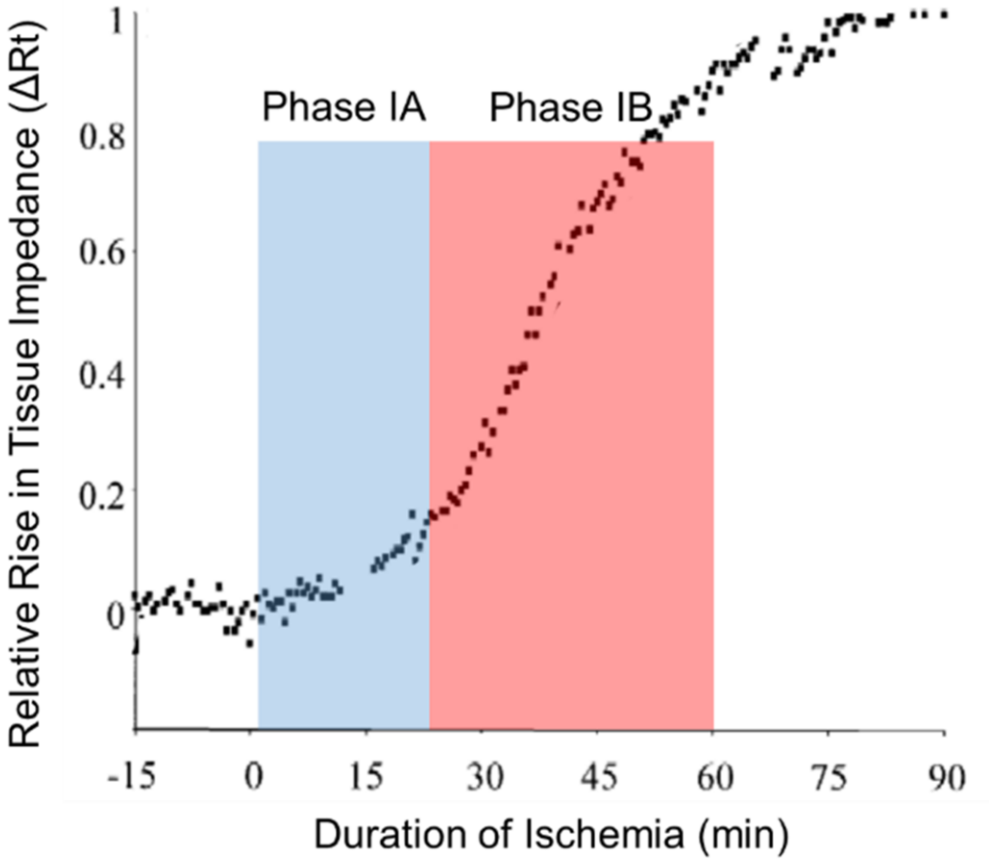

Figure 1.5 Changes in Cell-Cell Coupling During Acute Regional Ischemia

During acute regional ischemia tissue impedance, reflected as resistivity, increases in a sigmoidal fashion. The two phases of acute ischemia have been outlined (Phase IA; blue, Phase IB; red). The latter Phase IB corresponds with a dramatic increase in tissue resistivity. Modified with permission from: "Late ventricular arrhythmias during acute regional ischemia in the isolated blood perfused pig heart: Role of electrical cellular coupling" by de Groot *et al.*[73]

Importantly, the onset of the second increase in tissue resistivity corresponds with an increase in the incidence of VF in regionally ischemic hearts.[72] De Groot and Coronel comment that uncoupling during Phase IB promotes conduction slowing and block, two ideal conditions for reentry.[13] This concept is in agreement with Smith *et al.* who posit that during Phase IB it is the inhomogeneities imposed by uncoupling that promote fibrillation.[72] Contrary to this, it has been reported that uncoupling may reduce the incidence of conduction block by reducing the sink (source-sink) requirements,[74] however this may be more important during Phase IA when moderate cell-cell uncoupling exists. Despite its contribution to the conditions for wave break and reentry, gap junction uncoupling during Phase IB is unlikely to provide the trigger for reentry.[13] As such, this alteration during Phase IB may contribute to the arrhythmic *substrate*, however does not explain the mechanism behind the *triggering* event.

1.4.II Endocardial Discharge

Abnormal activity in the endocardial Purkinje cells has been theorized as a potential mechanism of triggered activity during Phase IB.[75–77] As mentioned above, Purkinje cells possess key characteristics which may contribute to arrhythmogenesis during ARI, such as unstable diastolic membrane potentials and automaticity (Section 1.2.I). In experiments of isolated myocardial (papillary) tissue, the perfusion of solutions mimicking late ischemia has been shown to result in a transient increase in the rate of Purkinje fiber diastolic-depolarization.[78] Furthermore, Huelsing *et al.* show that the coupling of isolated Purkinje fibers to a depolarizing current, analogous to the ischemic injury current, produces an increase in the rate of spontaneous discharge.[79] This work was further extended to display the importance of electrotonic activation of $I_{Ca(L)}$ as the main diastolic depolarizing current,

in addition to the necessity of increased coupling resistance to augment the SF for propagation.[80] While this accounts for automaticity, triggered activity has been shown to occur more readily in ventricular cells than in Purkinje cells, in part due to their higher (more positive) plateau (phase 2) potentials.[81]

Purkinje fibers have not only been implicated as a trigger of ARI arrhythmias, but their specialized conductive highway may provide an optimal route ('substrate') for reentry. Modelling of the junction between Purkinje fibers and ventricular cardiomyocytes has revealed that moderate cellular uncoupling during ischemia promotes unidirectional conduction block by increasing the SF in one direction and forming a reentrant circuit.[82] Furthermore, Purkinje cells have been shown to require significantly less electrotonic input than that of myocardial cells, allowing excitation to occur more readily across a smaller volume of cells.[83] This would increase the likelihood of unidirectional conduction block and reentry to occur in the Purkinje network than in the myocardium. Janse *et al.* have shown that during ischemia, the presence of the endocardium is essential for more complex sustained arrhythmias (VF) to occur.[84] This dependency of sustained arrhythmias on endocardial structures has been recapitulated by Zaglia *et al.* who show that stimulation of the endocardium during ischemia results in sustained arrhythmias more frequently than epicardial stimulation.[83] Both abnormal automaticity and reentry are finite and as ischemia progresses, acidosis, increased $[K^+]_o$, and hypoxia facilitate the uncoupling of Purkinje cells and a reduction in reentry.[85]

Uniquely, these electrophysiological changes (akin to abnormal automaticity and reentry during Phase IA: Section 1.3) are more likely to occur during Phase IB. This may be due to the fact that changes during ischemia in the endocardium lag behind the mid- and

epicardium.[76] Indeed, Wilensky *et al.* report that within the first 12 minutes of ischemia, the most superficial 650 μm of the endocardium possess a resting V_m 20mV lower (polarized), and a higher pH than deeper tissue.[76] This may be due to Purkinje fibers' greater capacity to store glycogen, endowing a greater glycolytic energy reserve, or simply their location adjacent to a cavity filled of oxygen and nutrient rich blood.[75,77,86] Regardless, this indicates that ischemic changes during ARI are substantially delayed in the endocardium, thus providing a later phase of electrophysiological changes.

While abnormal endocardial activity may indeed be a mechanism for sustained Phase IB arrhythmias, it has been shown that during acute ischemia endocardial ablation does not reduce the incidence of ectopic excitation.[84] This would imply that there exists a trigger external to that of the endocardium for ventricular arrhythmias during Phase IB. Furthermore, abnormal automaticity in Purkinje fibers has been implicated as a mechanism for arrhythmias in surviving endocardial tissue post-myocardial infarction, well beyond the acute effects of ischemia.[87]

1.4.III Catecholamine Release

During Phase IB, there is a significant increase in plasma norepinephrine (NE), its precursor dopamine, and epinephrine.[88] While all three increase above baseline values, the former shows the most substantial increase and will be addressed as a potential mechanism for Phase IB arrhythmias. Norepinephrine release during ARI has been shown to occur biphasically, arising from two different mechanisms.[5,89] The initial release of catecholamines is comparatively small to that of the latter release, occurring as a result of sympathetic activation in response to pain or injury, facilitating a rapid exocytotic release

of NE from local postganglionic sympathetic nerve endings within the myocardium.[89] Following ~15 minutes of ARI, reductions in ATP and intracellular sodium accumulation facilitate a reversal of re-uptake carriers in the synapse, favoring massive non-exocytotic (endogenous) NE release.[88,90] This is evident as extension of ischemia beyond 15 minutes sees a further increase in NE up to values 10-100 fold greater than the initial amount.[88] Figure 1.6 A illustrates the progressive increase in plasma catecholamine concentration during the first 60 min of ARI in an isolated Langendorff-perfused rat heart (shown by the values immediately prior to reperfusion). These findings are recapitulated by Lameris *et al. in vivo* (panels B-D) as an increase in intracellular catecholamines, most dramatically norepinephrine (B), occurs over 60 minutes of regional ischemia.

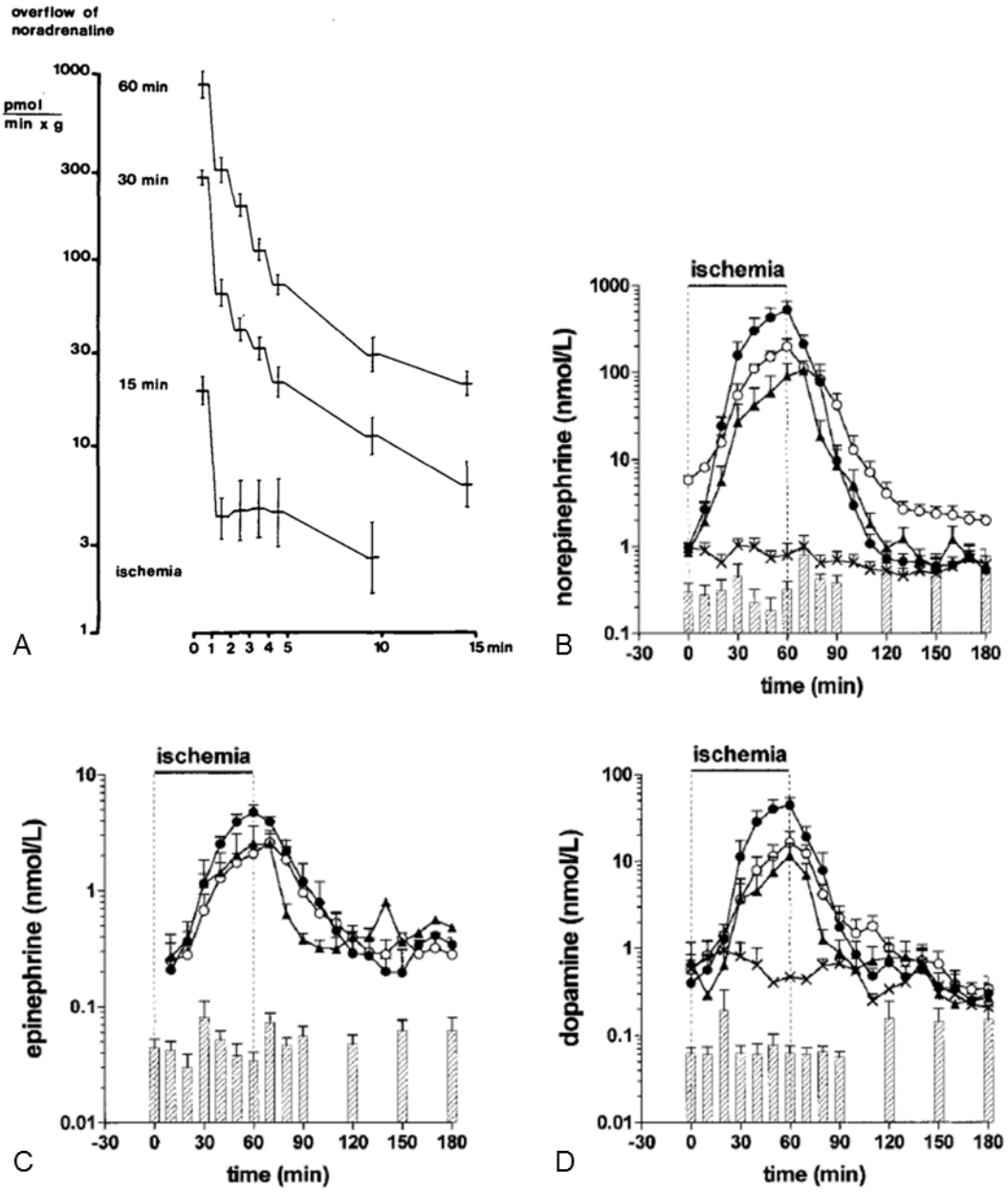

Figure 1.6 Changes in Effluent Catecholamine Concentration During Ischemia

Figure (A) illustrates the concentration of noradrenaline (norepinephrine) using high-performance liquid chromatography (HPLC) during 15 minutes of reperfusion immediately following 15, 30, or 60 minutes of global ischemia. Used with permission from: "Release of endogenous catecholamines in the ischemic myocardium of the rat. Part A: Locally mediated release" by Schomig *et al.*[88] Figure (B-D) illustrates the changes in norepinephrine, epinephrine, and dopamine using micro-dialysis HPLC techniques during 60 minutes of

regional ischemia respectively. Black circles represent central ischemic zone, “X” represents the non ischemic zone, hatched bars represent changes in arterial concentration. Used with permission from: “Time Course and Mechanism of Myocardial Catecholamine Release During Transient Ischemia In Vivo” by Lameris *et al.*[91]

NE is a versatile peptide hormone, acting as an extracellular ligand to two different types of receptors in the heart: i) beta-adrenergic receptors (β-AR_1 specifically) and ii) the alpha-adrenergic receptors (α-AR_1 and α-AR_2), both of which increase membrane translocation during ischemia.[92,93] NE binding to the extracellular domain of β-AR_1 facilitates an activation of a G-stimulatory protein (G_s) coupled receptor which initiates a cyclic-AMP (cAMP) intracellular signaling cascade.[18] The cellular implications of β-AR_1 stimulation are widespread, however through the activation of protein kinase-A (PKA), β-AR_1 stimulation increases the activity of a host of channels.[94] For example, PKA phosphorylation can shift I_{Na} activation and inactivation kinetics (reducing open probability), increase the conductance and open duration of the $I_{Ca(L)}$, increase I_{Ks}, and reduce I_{K1}.[5] In addition to transmembrane protein channels, stimulation of β-AR_1 can facilitate the phosphorylation of the sarcoplasmic-reticulum ATPase (SERCA) inhibitory protein, phospholamban (PLB), thus increasing SR calcium content.[5,94] Furthermore, activation of β-AR_1 also profoundly effects the rate of diastolic depolarization in pacemaker cells (SAN, AVN, PJN), by cAMP amplification of the funny current (I_f).[18]

Alternatively, alpha-adrenergic receptors are G_q protein coupled receptors. The resultant effect of G_q stimulation by NE is the activation of Phospholipase-C, which subsequently hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).[95] IP_3 can facilitate cytosolic calcium release by activating IP_3-receptors on the SR, while DAG activates Protein Kinase-C alpha (PKCα). PKCα has a variety of cellular effects, however in the heart it has been shown to phosphorylate Troponin-I, reducing its affinity for calcium in addition to activating protein-phosphatase-1, which dephosphorylates PLB.[96,97] While activation of α-AR acts to reduce

inotropy (increase lusitropy) and negate the effects of β-AR activation, it is important to note that the relative expression of α-AR in comparison to β-AR is 1:9 respectively.[98]

During ARI, lethal arrhythmias have been linked to increases in cAMP *in situ*,[99] and as such, the blockade of adrenergic receptors has been shown to reduce ventricular arrhythmias in several mammalian models of ischemia.[100–102] This effect has been shown to ameliorated *via* the depletion of endogenous catecholamines in isolated hearts undergoing global low-flow and no-flow ischemia.[103] Recently, Doppalapudi *et al.* have shown that regional perfusion of isoproterenol (β-AR non-selective), in the absence of ischemia, is sufficient for the induction of sustained ventricular arrhythmias.[104] This work was advanced by Myles *et al.* who have shown that local NE injection is sufficient to cause arrhythmias, providing the necessary synchronization of calcium release to induce afterdepolarizations.[105] In the same study, the probability for DADs to occur was enhanced by mild cell-cell uncoupling, akin to that of which occurs during Phase IB. These findings may have important implications during Phase IB, where the second non-exocytotic release of catecholamines coincides with reduced cellular coupling, providing the necessary stimulus for triggered ectopy to occur.

Catecholamine release may also have implications on Purkinje fibers and increases of local cAMP may contribute to abnormal automaticity by augmentation of I_f.[5] Research by Verkerk *et al.* showed that in the presence of NE, moderate uncoupling of Purkinje cells to a simulated ischemic region gives rise to afterdepolarizations.[81]

1.4.IV Altered Mechanics

During ARI, drastic changes in cardiac mechanics occur along with the changes in electrophysiology discussed in Section 1.3. These changes can occur rapidly, with differences in fractional shortening between healthy and ischemic tissues occurring as early as the first 70 seconds.[106] At a physiological level, alterations in contraction occur as metabolic changes occur, namely the reduction in cell pH, high-energy phosphate (ATP) availability, reduced SR calcium release,[107] and reduced affinity of myofilaments for calcium[108]. By the 10th minute of global ischemia, contraction can be entirely inhibited and tissue stiffening begins to occur.[108] In regionally ischemic models, these reductions in contractility within the ischemic zone further contribute to mechanical dysfunction, exposing the border between the ischemic zone and healthy tissue to varying amounts of strain.[109–112]

In addition to reducing the heart's efficacy to contract and generate pressure, mechanics may also play in important role in changes in cardiac electrics. The process by which electrical excitation of the heart is transduced into mechanical activity, known as excitation-contraction coupling (ECC), has been well documented over the decades.[113] Less appreciated however, is a field of physiology referred to as 'Mechano-Electric Coupling (MEC)' which addresses how the mechanical environment alters electrical activity.[114] In the early 1900s, the concept of MEC was taking form as Bainbridge displayed that by increasing venous pressure in a canine model, cardiac acceleration could be observed.[115] Over the years, many mechanisms by which MEC is achieved have been elucidated, including a whole host of stretch-sensitive sarcolemmal channels and cell mediated effects.[114]

Alterations in mechanics can lead to variable changes in electrophysiology, providing both trigger and substrate for sustained arrhythmias to occur.[116] Indeed the distension of the ventricular free-wall which occurs in a variety of pathological conditions has been implicated as a mechanism of SCD.[117] Clinically this effect of mechanics on electrics has been related to regional abnormalities in wall motion enhancing the dispersion of repolarization and subsequent risk for ventricular arrhythmias.[118,119] During ischemia in particular, stretch of ventricular tissue has been shown to enhance tissue repolarization patterns, potentially providing useful insight to arrhythmogenesis through MEC.[120] Coronel *et al.* was one of the first groups to experimentally investigate the mechanical contribution to ventricular arrhythmias during Phase IB ischemia.[12] It was discovered that in regionally ischemic swine hearts the absence of mechanical load significantly reduces the incidence of ventricular arrhythmias.[12] These findings are illustrated in Figure 1.7, which demonstrates that in comparison to *in situ* swine hearts undergoing ARI (A: top), the incidence of arrhythmias is dramatically reduced when hearts are instead isolated and do not pump blood (A: middle). However, the introduction of mechanical load by an intraventricular balloon in the left ventricle (LV) of the heart re-established the incidence of ventricular arrhythmias with ARI (A: bottom), implying that there is a contribution of mechanics to arrhythmias observed during ARI. During the same experiments in loaded hearts, Coronel *et al.* displayed that after a brief pause in pacing, potentiated beats facilitated an increase premature ventricular excitations (PVEs), indicating that strength of contraction correlates with arrhythmic inducibility.[12]

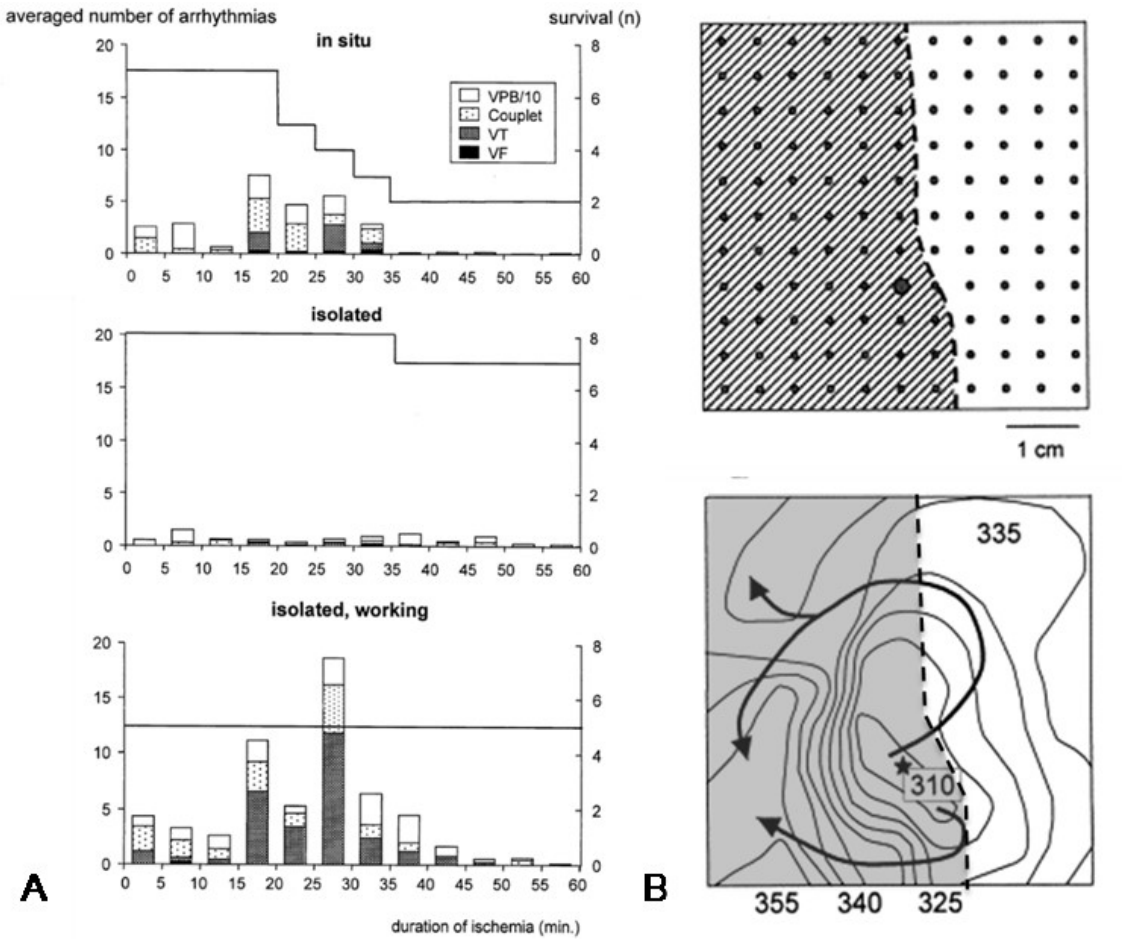

Figure 1.7 The Importance of Ventricular Load in Arrhythmogenesis during Acute Regional Ischemia in Swine

Figure A illustrates the duration of ischemia the x-axis and incidence of arrhythmias (ventricular premature beats (VPBs), white; couplets, dotted; ventricular tachycardia (VT), grey; ventricular fibrillation (VF), black) on the y-axis. Different cohorts of animals had unique mechanical environments ie: normal *in situ* (top), isolated (middle), and isolated, working (bottom). Figure B displays the initiation of an ectopy near the ischemic border zone. Ischemic zone represented by dark grey shading and border zone via interrupted black line. Used with permission from "Origin of Ischemia-Induced Phase 1B Ventricular Arrhythmias in Pig Hearts" by Coronel *et al.*[12]

Furthermore, Coronel *et al.* found that 26% of arrhythmias originated at the zone between healthy and ischemic tissue, shown in Figure 1.7 B, a site known to be under significant stress as ischemic tissue stiffens during Phase IB.[12] By mathematically modelling rabbit hearts, incorporating both electrophysiological and mechanical changes, Jie *et al.* found that arrhythmias occurring during ARI were dependent upon the presence of ion channels that responded to mechanical strain.[121] Most remarkably, the presence of mechanical alterations in the absence of electrophysiological changes was sufficient to substantially alter electrical activity.

Recently, the findings by Coronel *et al.* and Jie *et al.* have been recapitulated by our laboratory in a small animal model (Figure 1.8). Lawen *et al.* displayed in isolated rabbit hearts that the removal of mechanical load (Figure 1.8 B) or the inhibition of myocardial contraction (Figure 1.8 C) significantly reduced the incidence of ectopic triggers for ventricular arrhythmias during ARI.[14]

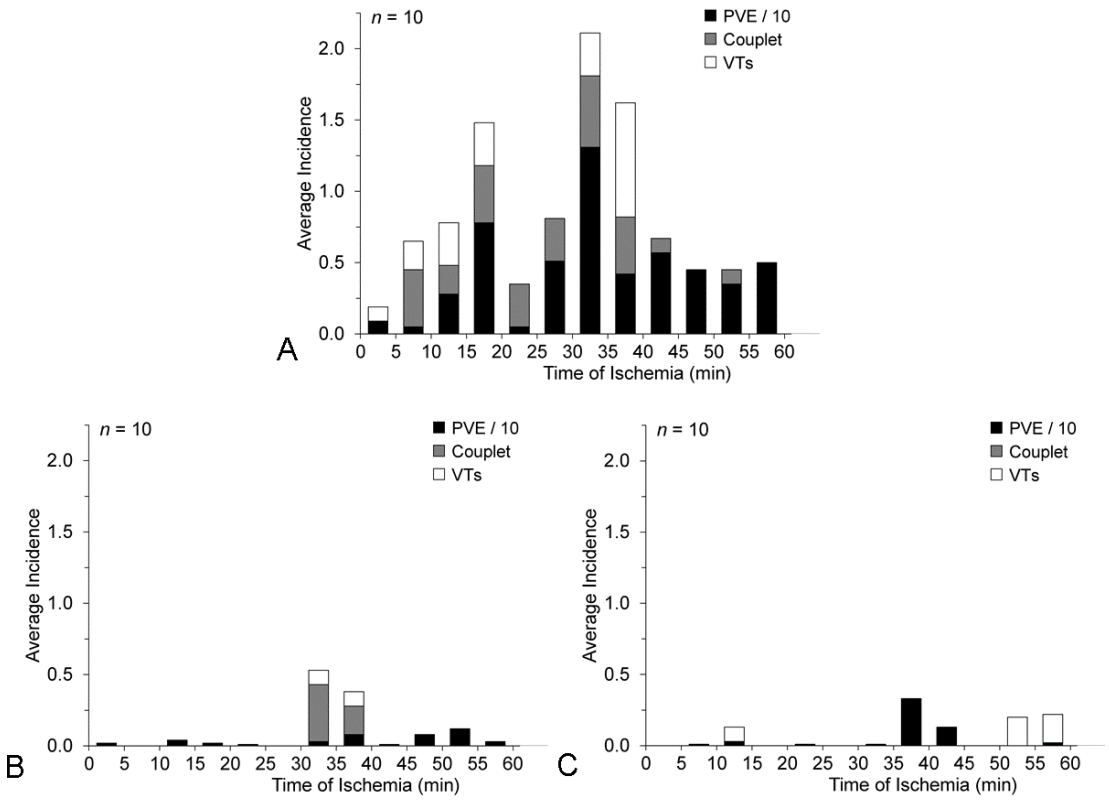

Figure 1.8 Importance of Mechanics during Acute Regional Ischemia in Isolated Rabbit Hearts

Figure (A) illustrates the average incidence of ventricular arrhythmias in isolated rabbit hearts loaded with left ventricular balloon. (B) Isolated contracting hearts in the absence of ventricular loading. (C) Isolated non-contracting hearts, pharmacologically excitation-contraction uncoupled. Black, premature ventricular excitation (PVE); grey, couplets; white, ventricular tachyarrhythmias (VT + VF). Used with permission from "The Contribution of Mechanical Effects to Ventricular Arrhythmias During Acute Regional Ischemia" by Lawen *et al.*[14]

While these findings confirm a mechanical contribution to the genesis of arrhythmias during ARI, the subcellular mechanisms by which mechanics is transduced into arrhythmias in this setting is unknown. MEC may play an important role in arrhythmogenesis during ARI through the acute activation of stretch-sensitive ion channels (stretch-activated and/or mechano-sensitive) and/or mechanically-sensitive calcium handling.

Stretch-activated cation-nonspecific (SAC_{NS}) ion channels open in response to tissue deformation and allow the conductance of cations.[122] Like most channels, the direction of current flow from SAC_{NS} is dependent upon its reversal potential, which has been experimentally determined to be between 0 and -20mV.[123] As such, activation of SAC_{NS} during the plateau phase of the cardiac action potential (phase 2) can hasten repolarization, whereas stretch during late repolarization and diastole can cause depolarization.[123] In the context of mechanical dysfunction during ischemia, activation of SAC_{NS} in conjunction with already shortened APD could potentially provide sufficient inward current for afterdepolarization-like activity to occur. Additionally, activation of SAC_{NS} may affect regions of the heart in different phases of their cardiac action potentials, thus leading to increased dispersion repolarization, favoring conduction block and reentry.[116] However, Barrabes *et al.* have shown that the local perfusion of gadolinium, a non-selective blocker of SAC_{NS}, produced no effect on the incidence of Phase IB arrhythmias in a swine model of ARI.[124] More recently, the same team have reported that the global perfusion of a SAC_{NS}-specific blocker (a peptide isolated from the venom of the *Grammostola spatulata* tarantula, GsMTx-4), caused no significant reduction in ventricular arrhythmias.[125,126] These findings indicate that mechanically-induced arrhythmias during ARI may not involve SAC_{NS}.

In addition to SAC_{NS}, other ion channels found in the heart demonstrate conductance alterations in response to stretch. As these channels' activation is independent of stretch, they will be referred to as mechano-sensitive channels (MSC). A particular channel of interest in the context of regional ischemia is Kir6.2 (I_{KATP}), which has been shown to increase conductance upon mechanical deformation.[127] As the reversal potential of this channel is similar to other potassium-selective channels (~-90 mV), its opening by stretch or reductions in ATP enhances repolarization currents. Thus, while it is unlikely to be involved in triggering arrhythmias, it may contribute to the substrate for reentry by increasing the excitable gap, enhancing dispersion of repolarization, and promoting unidirectional conduction block.[128]

Altering the mechanical environment of cardiac tissue can also have significant arrhythmogenic effects on intracellular calcium handling. It has been shown that in isolated tissues undergoing rapid non-uniform stretch and relaxation there are large surges in cytosolic calcium.[129,130] This effect is the result of increased loading of the myofilaments with calcium (due to a stretch-induced increase in the affinity of troponin-C for calcium[131,132]), which is rapidly released during relaxation. Wakayama *et al.* have shown that in the context of increased calcium, rapid stretch and relaxation can increase the propensity of calcium waves to occur and propagate.[133] This effect has been shown to be independent of calcium conductance through SAC_{NS}, and if sufficient in amplitude, can depolarize the sarcolemma.[134,135] In the context of ARI, these afterdepolarizations may occur with stretch of the ischemic border zone. Stretch can also affect the release of calcium from the SR. Iribe *et al.* have shown that axial stretch of cardiomyocytes leads to an immediate increase in calcium spark-frequency.[136] Interestingly, this occurred in the

absence of extracellular sodium or calcium and during application of SAC_{NS} blocker GSMTx-4, indicating that such an effect was not the result of cation influx through stretch-activated channels. Instead, it was proposed that there exists an anatomical coupling between cytoskeletal microtubules and RyR2 receptors, such that deformation of the cell affects the channel aperture.[136] However, more recent experiments have shown that this increase in calcium spark frequency is in fact the result of stretch-induced reactive oxygen species (ROS) production,[137] and is potentiated by cyclic stretch (as would occur at the ischemic border zone with each heart beat.[138]. Mechanically-dependent alterations in calcium handling may be of particular interest in the context of ischemia where cytosolic calcium overload and increased ROS production both occur.[139,140]

1.4.V Conclusion

Despite indications of Purkinje activation and catecholamine release, research by Coronel *et al.* and Lawen *et al.* provides compelling evidence for the dependence of arrhythmias on cardiac mechanical-dysfunction during ARI.[12,14] While MEC may be an important contributor to ARI arrhythmias, the subcellular mechanisms by which mechanical-dysfunction is translated into electrical-dysfunction in that setting remains unknown. Current literature indicates that the inhibition of stretch-activated ion channels during ARI may not be sufficient to ameliorate the incidence of arrhythmias.[124,126] Furthermore, other mechano-sensitive channels are more likely to contribute to the substrate rather than the trigger. As such, mechanically-dependent alterations to calcium handling may be the "smoking gun" in the triggering of arrhythmias. This thesis will investigate the specific contribution of abnormal calcium handling during mechanically-dependent ARI arrhythmias.

This thesis will investigate mechanisms of mechanically-induced arrhythmias during ARI in the isolated rabbit heart. The principal hypothesis is that there is a contribution of abnormal calcium handling to these arrhythmias. This hypothesis will be addressed by four specific aims:

Aim 1: Characterize mechanically-independent, ischemia-induced changes in calcium handling during ARI;

Aim 2: Investigate calcium's role as a driver of mechanically-induced arrhythmias during ARI;

Aim 3: Determine the effects of cytosolic calcium buffering on the incidence of these arrhythmias; and

Aim 4: Explore the potential of ryanodine receptor stabilization for reduction of these arrhythmias.

CHAPTER 2: METHODS

The methods Section of this thesis will first address the model of ARI used to investigate the specific contribution of altered calcium handling to mechanically-dependent arrhythmias (Section 2.1). Next the reagents used will be described (Section 2.2), followed by technical aspects regarding the use of optical mapping (Section 2.3). Then the protocols and procedures for each specific aim will be outlined (Section 2.4*)*. Finally, the last Section will address the data analysis performed (Section 2.5).

2.1 Rabbit Isolated Heart Model

The rabbit isolated heart is one of the most commonly used models for cardiac electrophysiology research.[141] Rabbit AP morphology is similar to that of humans, possessing a long calcium-dependent plateau phase (phase 2) (distinct from that of rodents)[142], in addition to similarities in repolarization currents.[143] Furthermore, rabbit heart mass in relation to its electrophysiological wave characteristics has been theorized promote wave-patterns and vulnerability to arrhythmias similar to that in humans.[144] Rabbit hearts also share similarities to humans in intracellular calcium handling, namely during diastole ~70% of calcium is re-sequestered by the sarcoplasmic-reticulum ATPase (SERCA) and 30% is extruded by the sodium-calcium exchanger (NCX).[145] Anatomically speaking, the relative lack of collateral coronary artery circulation is advantageous for ischemia studies as it allows a more definitive demarcation of healthy and non-healthy regions.[146,147]

Our experiments were performed in accordance with the ethical guidelines laid forth by the Canadian Council on Animal Care. Female New-Zealand White Rabbits (Charles River

– Montreal, Canada) weighing 1.9-2.3 kg (~9-11 weeks of age)[148] were medically anesthetized *via* injection of pentobarbital (~140 mg/kg) (Cerva Sante Animale) and heparin (~2,000 units/kg) (#SLBL4709V, Sigma-Aldrich) into a branch of the marginal ear vein. Upon absence of pain withdrawal response to toe pinch and presence of pupillary dilation the anterior chest cavity inferior to the xiphoid process was rapidly opened. Hearts were exposed and swiftly excised from the chest cavity and placed in a receptacle containing Krebs-Henseleit (KH) and 0.1 mL heparin. The ascending aorta was located by blunt dissection and cut below the branch of the brachiocephalic artery and firmly secured to a Langendorff-apparatus with a 3-0 wax-coated silk suture.

The heart was perfused with warm (37 °C), oxygenated KH solution through two 11 μm diameter nylon net filters (#NY1102500, Millipore) arranged in parallel and into the ascending aorta in a retrograde fashion at a constant flow of ~20 mL/min (3-roller pump, Watson-Marlow). Effluent KH solution was recycled to the main circulation chamber through an additional nylon net filter, where it was re-perfused throughout all experiments unless otherwise stated. Flow rate was measured using a FLR1000 Series Flowmeter (Omega). Aortic perfusion pressure was measured using a lateral pressure transducer (#MLT0699, ADInstruments) located above the aorta.

Upon perfusion of the heart, superficial coronary artery vasculature was identified. A 3-0 surgical silk suture was placed around the ramus marginalis sinistra, with longitudinal location chosen to obtain a region of non-perfusion of ~40% ventricular mass with ligation, known to confer greatest arrhythmogenicity.[149,150] To prevent build-up of perfusate within the LV with the presence of an intraventricular balloon, a drain was inserted into the apex through a left atrial incision and across the mitral orifice. Cardiac mechanical loading was

controlled by the insertion of a custom polyethylene balloon into the LV by the same route. The balloon was secured to the apex of the LV through the apical drain and mitral orifice *via* a surgical tie. The LV balloon was attached in series to a pressure transducer (#MLT0699, ADInstruments) and passively filled by an adjustable hydrostatic column. A modifiable snare was placed around the balloon-column tubing to adjust LV filling, such that diastolic pressure was ~5 mmHg. Hook electrodes were placed on the right atrial appendage and inferior-lateral right atrium for pacing the heart (at twice the voltage threshold). An image of the instrumented heart can be seen in Figure 2.3.

Hearts were placed in a controlled environment imaging chamber maintained at 37 °C and pH balanced to 7.4 with a carbon dioxide/nitrogen balanced gas mixture (5% CO_2, 95% N_2). Pseudo-ECG was recorded utilizing bath-conducting silver-chloride electrodes, with signals filtered using a 60 Hz notch filter. ECG, LV pressure (LVP), aortic perfusion pressure, aortic perfusion flow rate, chamber temperature, and heart rate were recorded and calculated using LabChart Pro (v8.0.7.) acquisition software.

2.2 Reagents

2.2.I Krebs-Henseleit Solution

KH solution was prepared in advance at eight times concentration and diluted to one times on the day of experiment. All reagents were obtained from Sigma-Aldrich unless otherwise stated. KH solution consisted of NaCl (120 mM), KCl (4.7 mM), NaH_2PO_4 (1.4 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.8 mM), Glucose (10 mM), and $NaHCO_3$ (24 mM). Solution was heated to 37 °C and oxygenated *via* medical grade oxygen/carbon dioxide balanced mixture (95% O_2, 5% CO_2). pH and osmolarity were ~7.4 and ~300 mOsm respectively.

2.2.II Voltage Sensitive Dye: di-4-ANBDQPQ

Epicardial electrical activity was imaged utilizing di-4-ANBDQPQ (di-4-PQ), a styryl based potentiometric fluorescent dye (27.4 mM stock in 100% EtOH; acquired from the University of Connecticut Health Center, Farmington, CT). di-4-PQ rapidly incorporates itself into the phospholipid bilayer of cells, exposing a photosensitive chromophore external to the cell.[151] Alterations in the electrical field of the cell, as which occurs during depolarization, cause a conformational shift in the electron field distribution over the chromophore. These changes are reported as spectral shifts in the emitted fluorescence upon excitation. di-4-PQ can be excited between ~450 and 700 nm, resulting in emission >700 nm, with the percentage change in fluorescence varying with excitation wavelength (in the rabbit isolated heart ~470 nm results in maximal increase, ~640 nm results in maximal decrease, and there is no change in fluorescence with ~540 nm)[152]. di-4-PQ provides several benefits over its commonly used predecessor, di-4-ANNEPS, most notably its red-shifted excitation spectra, which allows imaging over a larger volume (depth) of tissue and greater temporal stability.[151]

2.2.III Calcium Indicator: Rhod-2 AM

Optical mapping of intracellular calcium was accomplished using rhodamine based calcium-indicator Rhod-2 AM (0.89 mM stock in dimethyl-sulfoxide, DMSO;#1209954, Thermofisher Scientific). Rhod-2 is a classified as a high affinity calcium indicator (K_d = 570 nM) which functions by increasing in fluorescence when bound to intra-cellular calcium.[153] Rhod-2 has been shown to be an optimal calcium indicator due to its longer excitation wavelength and 100-fold increase in fluorescence upon calcium binding.[154]

Rhod-2 possesses a BAPTA-based component of its molecular structure, which allows it to capture available cytosolic calcium. The attached acetoxymethyl (AM) group allows its trans-membrane movement, and is cleaved by esterases once inside the cell, making it active and effectively locking it within the cell. To allow this esterase process to occur, we allowed thirty minutes following Rhod-2 loading. Importantly, this esterase period was always performed at physiological conditions (37 ºC) to ensure optimal and rapid cleavage of the AM group.[155] Rhod-2 is most efficiently excited in the green wavelength of the light spectra (~557 nm) and maximal emission is slightly red shifted (~581 nm).[153]

2.2.IV Electro-mechanical Uncoupler: Blebbistatin

When necessary, heart contraction was blocked by global perfusion of the excitation contraction uncoupler (±)blebbistatin (8 mM stock in DMSO #APN14190-1-1, Abcam). Blebbistatin is a myosin II inhibitor which impedes cross-bridge formation of actin-myosin complexes and thus halts contaction.[156] It has been shown that blebbistatin has minimal effects on electrophysiological properties, thus making it an effective tool to measure electrical changes in the absence of motion.[157]

2.2.V Calcium Buffer: BAPTA AM

Buffering of intracellular calcium was accomplished with 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) AM (10 mM stock in DMSO; #APN15002-1-1, Abcam). BAPTA functions by binding to free calcium, making use of four negatively charged carboxylic acid groups.

2.2.VI Ryanodine Receptor Stabilizers: JTV-519 and Dantrolene Sodium

JTV-519 has been previously reported to stabilize the RyR2 isoform specifically, reducing their open probability and calcium leak in heart failure models.[158] JTV-519 functions by securing the stabilizing protein FKBP 12.6 with the RyR2 channel.[159] During pilot experiments in contracting hearts, JTV-519 displayed adverse effects on physiology, namely promoting 2:1 block (n = *2*), this finding warranted further investigation into its electrophysiological effects. In order to determine if it had adverse effects on electrophysiology , pilot experiments (n = 2) were performed with optical mapping in isolated non-contracting (by application of blebbistatin) rabbit hearts. JTV-519 was prepared as a 10 mM stock solution in DMSO (#112M4610V, Sigma). Following instrumentation, JTV-519 was diluted to 1 μM and re-circulated for 20 minutes prior to ischemia. An alternative agent for RyR stabilization is dantrolene sodium, a supposedly RyR1-specific agent commonly used to treat malignant hyperthermia (#GR140326-8, Abcam).[163] Recently, there has been evidence that dantrolene may reduce the RyR2 spark frequency in isolated cardiomycytes.[164] Similar to JTV-519, pilot experiments (n = 2) were performed with optical mapping in isolated non-contracting (by application of blebbistatin) rabbit hearts to ensure no adverse effects on electrophysiology. As in previous studies, we utilized dantrolene at a dosage of 1 μM, prepared as a stock of 10 mM dissolved in DMSO.[164,165]

2.3 Optical Mapping

The surface of the LV was imaged during ARI while utilizing a variety of light emitting diodes (LEDs) and a high-resolution 5.5 megapixel sCMOS camera (Andor Zyla 5.5). Image area was 350 x 550 pixels, with a resolution of 167 μm/pixel. Depending upon the

research objective, either mono-parametric or dual-parametric optical mapping was performed.

2.3.I Mono-Parametric

During mono-parametric optical mapping, epicardial electrical activity was measured. This was accomplished by utilizing the potentiometric voltage dye, di-4-PQ, as described in Section 2.2.II. Once loaded into the heart, di-4-PQ was excited (Ex) utilizing LEDs band filtered in the red wavelength of the visible spectra (Ex.2. 640 ± 10 nm; Figure 2.1). Emitted (Em) light was long-pass filtered (Em.2. > 670 nm) and collected utilizing our sCMOS camera, described above.

2.3.II Dual-Parametric

Dual-parametric optical mapping was utilized to quantify both epicardial voltage and intracellular calcium dynamics. Pseudo-simultaneous optical mapping was accomplished by alternating Rhod-2 AM and di-4-PQ excitation LEDs in a 1:1 ratio at a frequency of 520 frames per second (fps). Hardware for this capability was developed in collaboration with Dr. Illija Uzelac and Dr. Flavio Fenton (Department of Physics, Georgia Institute of Technology, GA). di-4-PQ and Rhod-2 AM were excited by red LEDs (Ex.2. 640 nm ± 10 nm) and green LEDs (Ex.1. 550 ± 10 nm), respectively, and emitted light was collected by a sCMOS camera through a multi-bandpass emission filter (Em.2. 800 ± 100 nm; Em.1. 590 ± 20 nm; Figure 2.1).

Important for this method is the fact that as di-4-PQ is a potentiometric voltage indicator, different excitation wavelengths correspond with different emission spectra. Pilot work was performed to ensure that while the excitation spectra of Rhod-2 AM excited di-

4-PQ, this fluorescence did not change with changes in voltage. This was done by utilizing a Rhod-2 AM excitation wavelength (Ex.1.) that did not report a spectral shift in Di-4-PQ upon tissue depolarization, known as the "isosbestic point".[166] Examination of the isosbestic point was performed in isolated non-contracting (blebbistatin) rabbit hearts measuring effects of Rhod-2 AM excitation wavelength (Ex.1. 540 ± 10 nm) in the presence of di-4-PQ, but absence of Rhod-2 AM.

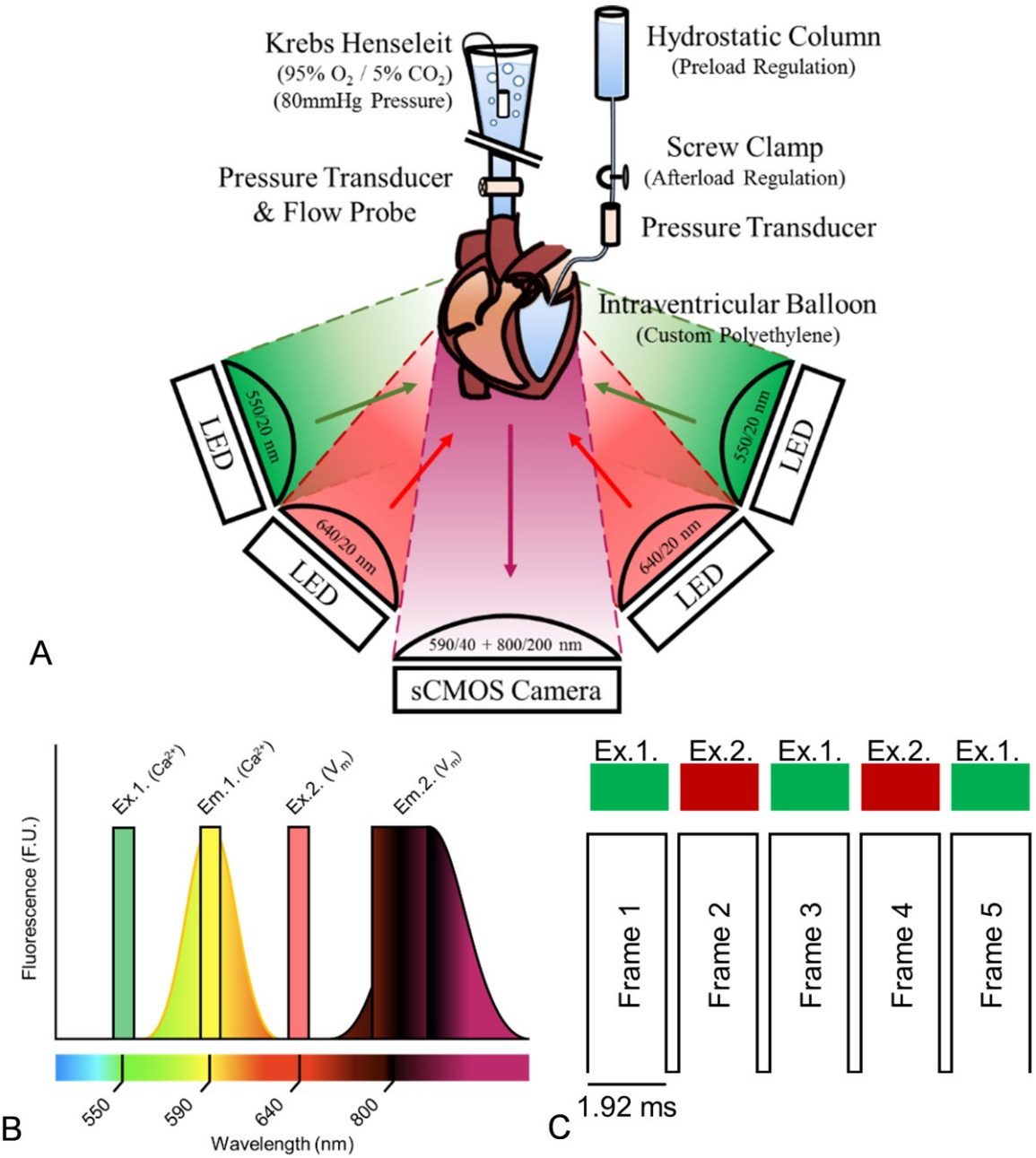

Figure 2.1 Schematic of Dual-Parametric Optical Mapping

(A) Schematic of imaging set-up. Four LEDs excited (Ex) either Rhod-2 (Ex.1. = 550/20 nm) or di-4-PQ (Ex.2. = 640/20 nm) and emitted light (Em) was multi-bandpass filtered (Em.1. = 590/40 nm and Em.2. = 800/200 nm) and collected by a sCMOS camera. (B) Illustration highlighting the excitation and emission spectra for Rhod-2 and di-4-PQ. Boxes represent filtered excitation or emision spectra. (C) Example of alternation of frame acquisition and excitation LEDs.

2.4 Procedures

Figure 2.2. illustrates the experimental design utilized to address the main objectives of this thesis. The first part of this Section will address the protocol used to implement ARI in our animal model. This will provide the "back-bone" for all experiments. Once this has been addressed, the specific protocols used to address each of the specific aims will be described.

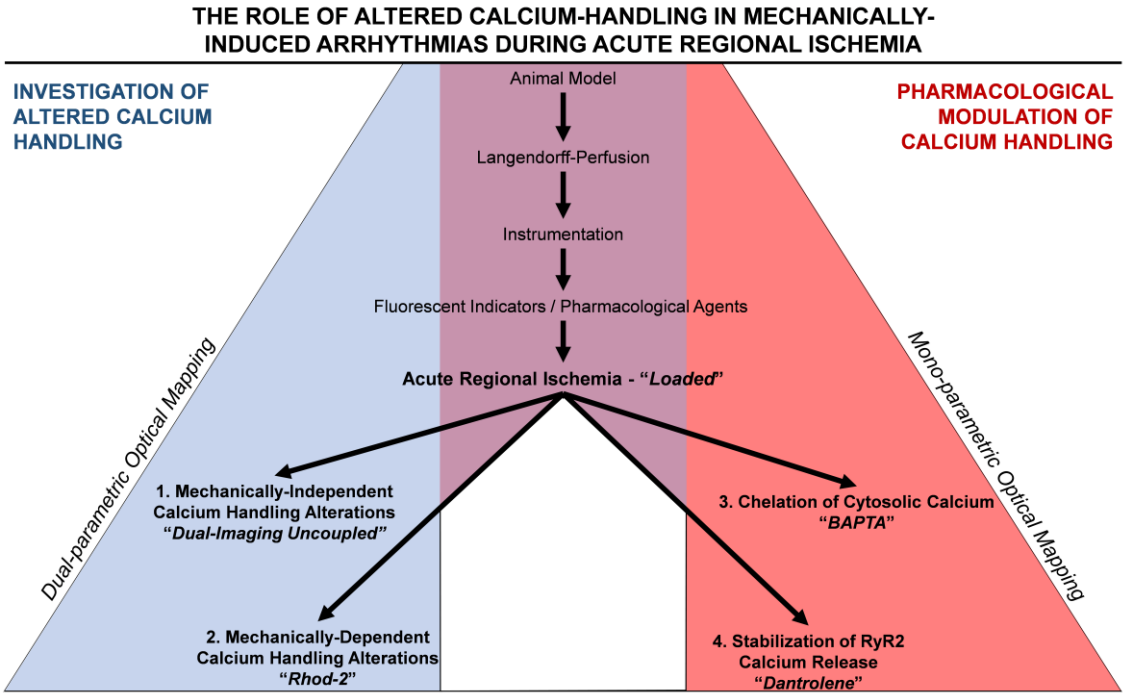

Figure 2.2 Experimental Design

Each experiment utilized the same animal model (rabbit) and was preceded by Langendorff-perfusion and instrumentation. Addition of a variety of fluorescent and/or pharmacological agents was utilized to either investigate the role of, or modulate, altered calcium handling. Each of the objectives are outlined numerically in the order at which they are addressed. For ease of comparison, each group has been given the title shown in quotations.

2.4.I Acute Regional Ischemia

Following Langendorff-perfusion, instrumentation, and addition of fluorescent dyes or pharmacological agents, the Langendorff-apparatus was switched from constant flow to constant pressure (80 mmHg, maintained by an elevated water-jacketed heated chamber). 'Pre-ligation control' optical mapping recordings were taken after a period of stabilization both in sinus rhythm and during atrial pacing (4 Hz, the mid-to-upper end of normal heart rate for rabbit species.[148,167]). Immediately following control recordings, the snare containing the ligation suture was tightened and 60 minutes of ARI was initiated. Throughout the period of ischemia, pacing was continued at 4 Hz and either mono- or dual-parametric optical mapping was performed every minute for a duration of 30 seconds (~15,000 frames). The intermittent recordings were necessary for dye photostability, LED cooling, and saving of data. During all experiments, the ECG in LabChart was monitored for arrhythmias. Following ischemia, 30 minutes of reperfusion was induced by releasing the coronary artery ligation. Immediately following each experiment, the coronary artery was re-ligated and red fluorescent microspheres (Fluoro-Max^{TM} 6 μm diameter, 1.25 mg/mL, Thermo Scientific) were injected through the aortic cannula. Red microspheres were then excited using green LEDs (540 ± 10 nm) and emitted fluorescence was band filtered (620 ± 10 nm) and collected using our sCMOS camera, allowing identification of the perfused and non-perfused area (NPA) (Figure 2.3, right).

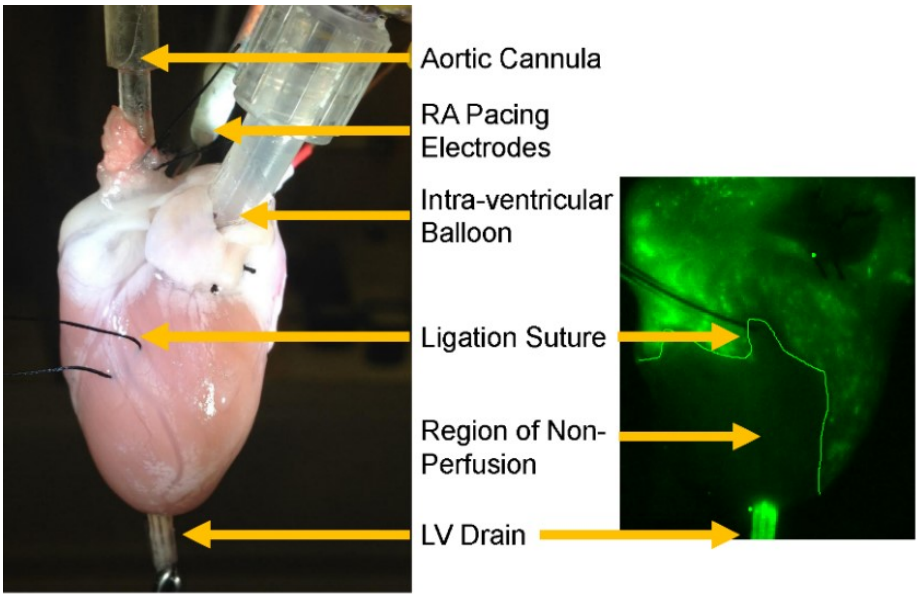

Figure 2.3 Acute Regional Ischemia in a Langendorff-Perfused Rabbit Isolated Heart

Hearts were perfused in retrograde fashion through an aortic cannula. Pacing electrodes were attached to the right atrium (RA) and LV loading was controlled *via* an intra-ventricular balloon (left). A ligation suture was placed around a branch of marginalis sinistra coronary artery. Area of non-perfusion was evaluated using fluorescent microspheres (right).

2.4.II Mechanically-Independent Alterations in Calcium Handling

In order to determine the underlying 'environment' of ischemia-induced changes in calcium handling in the absence of ischemia-induced mechanical alterations we measured changes in both voltage and calcium and their relation to each other in excitation-contraction uncoupled hearts (blebbistatin). This group of experiments will be referred to collectively as "*Dual-Imaging Uncoupled*" throughout this thesis. Dual-parametric optical mapping as described above was utilized for this method.

Pilot experiments ('*Rhod 2-(P)*', $n = 2$) were performed in isolated ECC-uncoupled rabbit hearts (10 μM blebbistatin) to measure the change in fluorescence intensity with increasing amounts of Rhod-2 AM. In brief, this was accomplished by gradually increasing the volume of Rhod-2 injected through the aortic cannula followed by 30 minutes of incubation to allow the acetyl-methyl group to esterase.

Once the optimal amount of Rhod-2 AM was determined, we performed experiments with ARI in rabbit isolated hearts ($n = 10$). Hearts were isolated, perfused, and instrumented as described above (Section 2.1). Hearts were loaded with di-4-PQ voltage dye (20 μL of 27.3 mM stock injected into in the aortic cannula over 2 minutes), Rhod-2 AM (100 μL of 0.89 mM stock injected into in the aortic cannula over 1.5 minutes), and blebbistatin (10 μM in the perfusate), followed by a 30 minute rest period to allow for removal of the AM group from Rhod-2 and for blebbistatin to act. ARI was then induced as described above and dual optical mapping performed by alternating excitation of di-4-PQ and Rhod-2 AM.

2.4.III Mechanically-Dependent Alterations in Calcium Handling

The role of calcium as the driving-event for mechanically-dependent arrhythmias was investigated through the dual-optical mapping of voltage and calcium in a group of isolated contracting rabbit hearts during ARI (n = 10). This group of experiments will be referred to collectively as "*Rhod-2*" throughout this thesis. Similar to that described above, di-4-PQ and Rhod-2 AM were loaded into the heart through an injection port in the aorta prior to ischemia. However, to increase the calcium signal, a larger amount of Rhod-2 AM (310 μL of 0.89 mM stock) was loaded.

2.4.IV Effect of Calcium Buffering on Arrhythmogenesis

Buffering cytosolic calcium may prevent surges in cytosolic calcium and reduce the incidence of calcium-driven afterdepolarizations. This was investigated in isolated contracting rabbit hearts (described above) loaded with di-4-PQ for mono-parametric optical mapping of voltage and BAPTA AM (n = 10). This group of experiments will be referred to collectively as "*BAPTA*" throughout this thesis. The working concentration of BAPTA was determined in pilot experiments ('*BAPTA-(P)*'; n = 2) as the value at which contraction was affected to the same minimal degree as with Rhod-2. For experiments with ARI, BAPTA AM (0.1 μM) was re-circulated for 30 minutes, followed by an additional 30 minute period of BAPTA AM free perfusion. Following BAPTA AM incubation, ARI was induced as described above.

2.4.V Effect of RyR Stabilization on Arrhythmogenesis

Calcium leak from RyR may play an important role in calcium-dependent arrhythmias during ARI. To address the potential role of RyR leak we made use of RyR-stabilizer Dantrolene Sodium in isolated loaded contracting rabbit hearts (n = 10). This group of experiments will be referred to collectively as "*Dantrolene*" throughout this thesis. Hearts were isolated and instrumented as described above. di-4-PQ was incorporated into the hearts for mono-parametric optical mapping. 20 minutes prior to the initiation of ischemia, dantrolene (1 μM) was loaded into the main perfusion apparatus. Following incubation with dantrolene, hearts were subjected to 60 minutes of ARI (described above).

2.5 Data Analysis

2.5.I Non-Perfused Area Analysis

Following fluorescent microsphere injection post ischemia, hearts were removed from the Langendorff-apparatus and ventricles were separated from the atria and frozen. 2 mm cross-sectional slices of the heart were made and transmural distribution of fluorescent microspheres were analyzed by excitation using green LEDs (542 ± 54 nm) and captured using a color CCD camera (The Imaging Source, Charlotte, NC). Non-perfused area was quantified using custom MatLab software to manually select total area and area of non-perfusion (Figure 2.4). Data is reported as a percentage of total ventricular mass. Hearts were excluded if NPA% was < 25%.

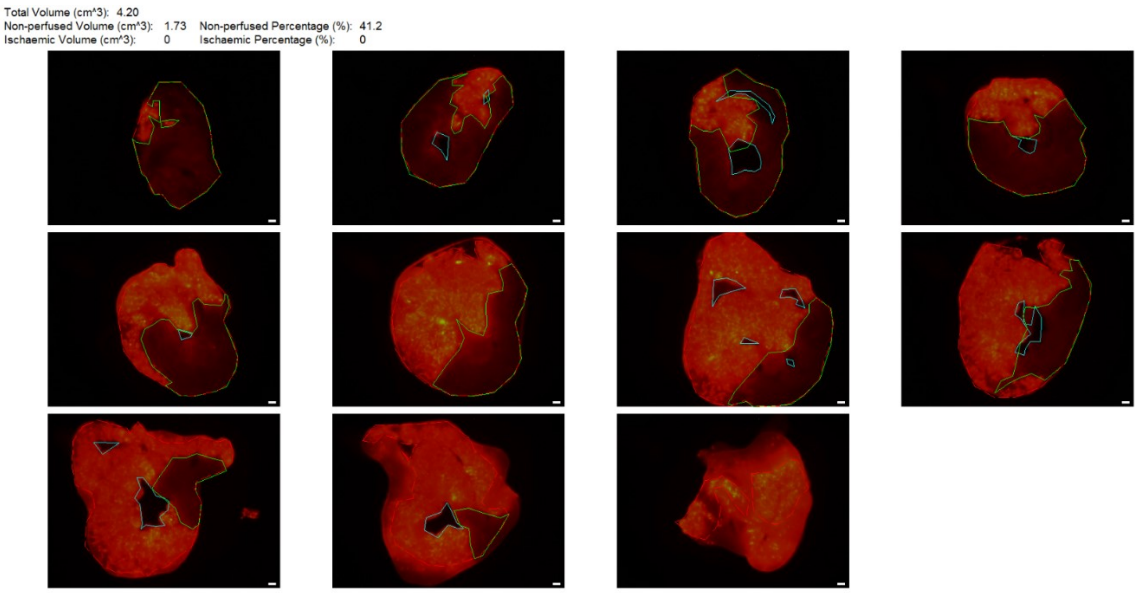

Figure 2.4 Example Quantification of Non-Perfused Area (NPA)

Post-experimental quantification of NPA. 2 mm cross Sectional slices presented in apical-basal order beginning top left. Red fluorescent microspheres delineate region of perfusion.

2.5.II Hemodynamic Analysis

Hemodynamic changes in response to pharmacological interventions, Rhod-2 AM dye, and ARI were analyzed to report their effect on LV function. Data were selected from periods of pacing (4 Hz) to control for rate-dependent effects. Measurements included peak LVP, diastolic LVP, developed pressure (peak LVP – diastolic LVP), maximal rate of developed pressure (dP/dt_{max}), LVP decay constant (τ_{LVP}), and maximal rate of pressure decay (dP/dt_{min}), analyzed using custom Matlab routines. Time points included pre- and post-drug/indicator intervention, in addition to pre- and post-coronary artery ligation. Changes in rate of flow and perfusion pressure in response to coronary artery ligation were also recorded and averaged over 30 seconds. Any experiment that did not have a pre-ligation flow rate of $\geq$ 15 mL/min was excluded, as prior experience associated lower flow rates with adverse effects on hemodynamics.

2.5.III Arrhythmia Analysis

Arrhythmia analysis was performed by examination of LabChart ECG waveforms. Excitation occurring independent of pacing stimulation was deemed an arrhythmia. Arrhythmias were categorized using a classification system similar to Coronel *et al.*[12]. Single ectopic excitations were denoted as 'premature ventricular excitations (PVEs)'. Two PVEs in immediate succession were classified as 'doublets'. Events of three or more PVEs in succession were classified as "tachyarrhythmias", including both ventricular tachycardia and fibrillation. In the instance of sustained ventricular tachyarrhythmias lasting in excess of 5 minutes, data was excluded from the group mean. Examples of each type of arrhythmia is presented in Figure 2.5. Arrhythmias were enumerated and binned into 5 minute periods,

and were grouped as Phase IA (0 – 20 minutes) or Phase IB (20 – 45 minutes). Arrhythmia incidence was compared to previously acquired data from loaded, contracting hearts[14], with significance assessed across groups using one-way ANOVA and Tukey *post hoc* tests, using a threshold of $p < 0.05$.

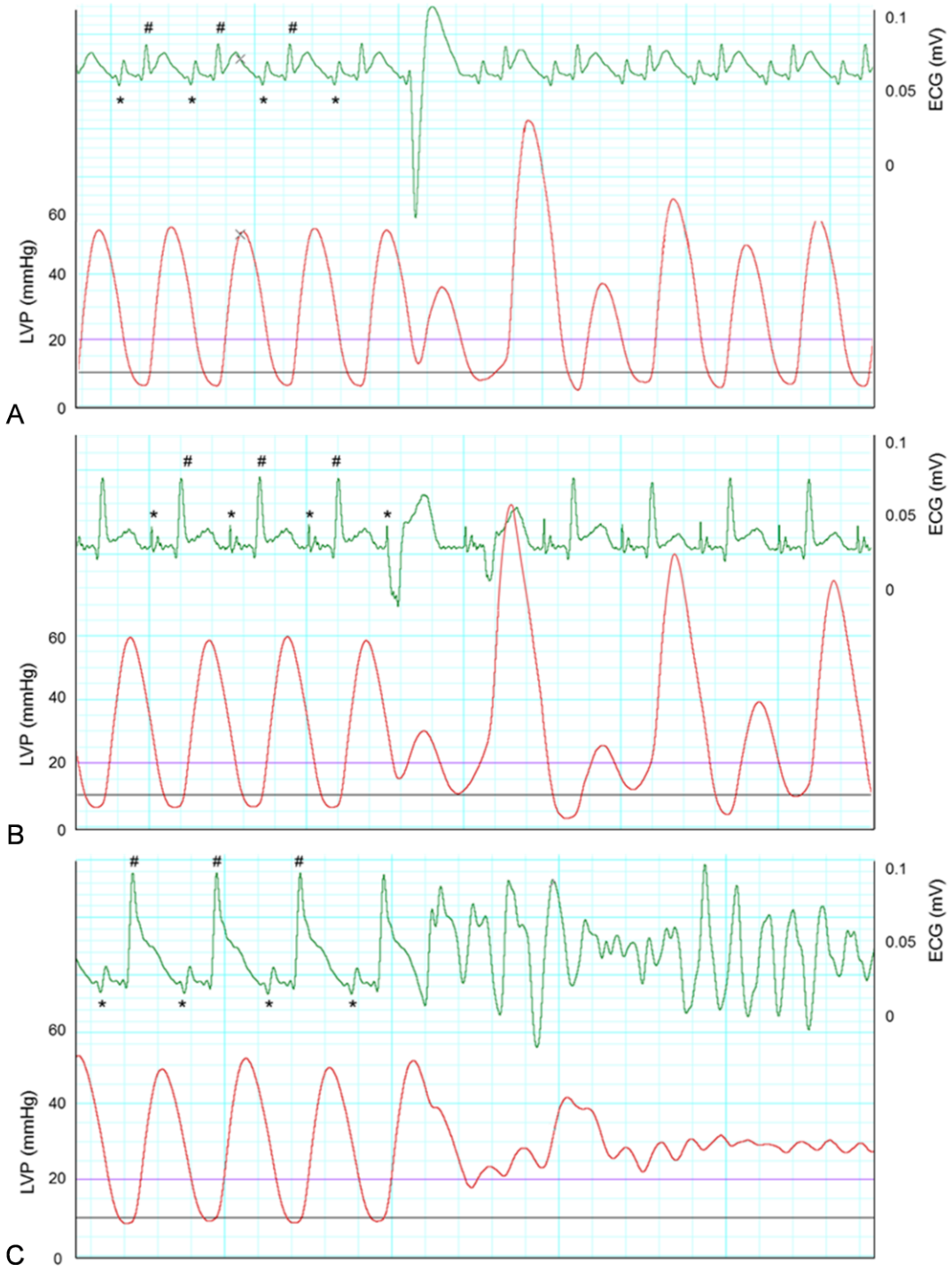

Figure 2.5 Example of Arrhythmia Classification

(A) Premature Ventricular Excitation (PVE); (B) Doublet; (C) Tachyarrhythmia. Top - electrocardiogram (ECG), Bottom - left ventficular pressure (LVP). (*) Denotes atrial pacing spike, (#) denotes QRS complex.

2.5.IV Optical Mapping Analysis

Dual-parametric optical mapping was utilized to examine the mechanically-independent, ischemia-induced changes in voltage and calcium in the '*Dual-Imaging Uncoupled'* experiments. Recordings immediately prior to ischemia ("Pre-Ligation Paced") and at 5, 10, 15, 20, 25, 30, 45, and 60 minutes of ischemia were analyzed. From these time periods, the first 2,500 frames of optical mapping data were selected, and voltage and calcium signals were split. The ischemic border zone was superimposed onto the optical mapping data using the post-experiment red fluorescent microspheres image. Images were spatially filtered using a 11 x 11 box filter. 11 regions of interest (ROIs) of 1 × 1 mm (6 × 6 pixels) were placed across the ischemic border zone (from healthy to ischemic tissue) (Figure 2.6). Average signals for each ROI were temporally filtered using a 60 Hz low-pass filter. AP and CaT signals were then analyzed and measurements of upstroke velocity (first derivative of change in fluorescence; dF_V/dt_{max} and dF_{Ca}/dt_{max}, respectively), duration (time from peak upstroke to 50% and 80% recovery; APD_{50}, $CaTD_{50}$, APD_{80}, $CaTD_{80}$), and the period over which calcium was elevated during electrical diastole (difference between CaTD and APD at 50 and 80% recovery, *i.e.*, $CaTD_{50} - APD_{50}$, $CaTD_{80} - APD_{80}$) were averaged over 12 cardiac cycles. CaT decay time constant (τ_{Ca}) was calculated between 50% and 80% recovery. Calcium transient alternans (CaT-ALT) and action potential duration alternans (APD-ALT), characterized by alternation of high and low amplitude calcium release or long and short APD[168], was measured by taking the absolute value of % change between two successive beats[169]. Over the 12 successive cardiac cycles analyzed, any value outside two standard deviations of the mean was eliminated and the average of the remaining measurements was recorded. The averages for

each experiment were combined to obtain a group mean for each distinct time and region of interest (ROI). Two-way ANOVA was used to assess variations *between* factors (time and ROI) and one-way ANOVA with Tukey *post hoc* tests was used to assess variations *within* factors (time or ROI), with a significance level of $p < 0.05$.

Analysis of dual-parametric optical mapping was utilized to examine arrhythmias occurring during *Rhod-2* experiments to determine if calcium-driven depolarizations were occurring. This was accomplished by utilizing custom Matlab software on captured arrhythmias and examining optical data from the arrhythmic event. Should focal epicardial excitation occur, the temporal relationship between calcium and voltage was examined to determine is calcium release had preceded membrane excitation.

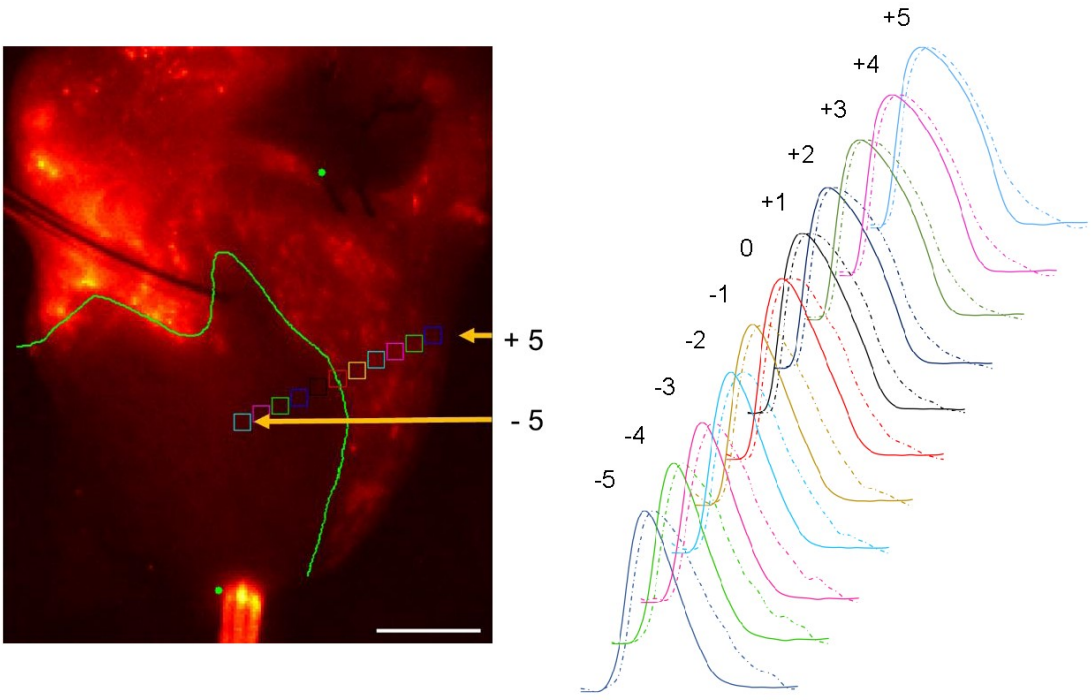

Figure 2.6 Representative Ischemic Zone and Regions of Interest (ROIs)

Selected ROIs (left) and their corresponding averaged signals (right) at 20 minutes of ischemia. +5 (navy blue) = most healthy region, -5 (turquoise) = most ischemic region. Solid lines represent action potentials (APs), dashed lines represent calcium transients (CaTs).

CHAPTER 3: RESULTS

3.1 Mechanically-Independent Alterations in Calcium Handling

3.1.I Effect of Increasing Rhod-2 Volume on Fluorescence

Measuring the effects of ischemia on morphology of the AP and CaT required the absence of motion artifact. As such, this group of experiments was performed in isolated uncoupled (blebbistatin) hearts. In two pilot experiments referred to as '*Rhod-2-(P)'*, we examined the effect of increasing amounts of Rhod-2 on CaT fluorescence. Figure 3.1 A is representative data of CaT signals with increasing amounts of fluorescent indicator. Figure 3.1 B is a quantification of the change in diastolic and systolic fluorescence (primary y-axis; bottom and top of black bar respectively) and the corresponding increase in CaT amplitude (secondary y-axis). It was found that while diastolic fluorescence continually increased with increasing amount of Rhod-2, there was no discernable gain in signal amplitude beyond 100 μL.

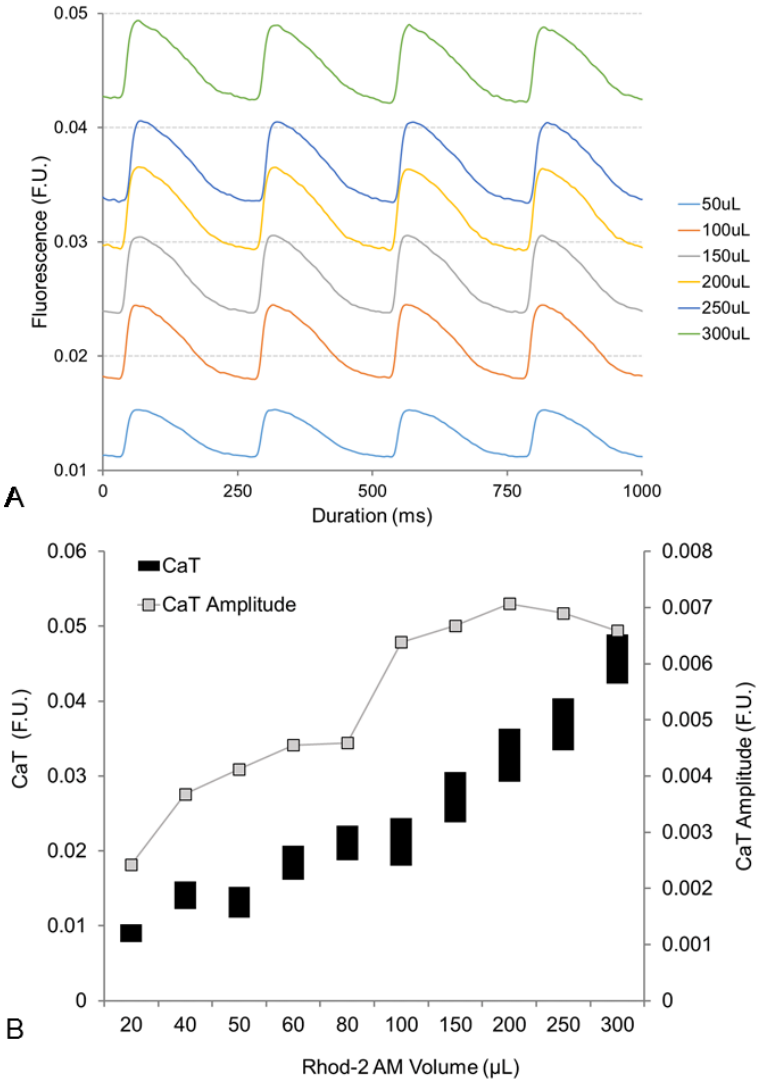

Figure 3.1 Determination of Optimal Rhod-2 Volume

Pilot experiment '*Rhod-2-(P)'* to determine the optimal amount of calcium indicator. (A) Representation of calcium transient (CaT) fluorescence at increasing bolus injections of Rhod-2 AM. Fluorescence is quantified (B) with CaT minimum (diastolic) and maximum (systolic) fluorescence levels indicated on the primary y-axis and amplitude on the secondary y-axis.

3.1.II Ischemia-Induced Alterations in Intracellular Calcium Handling

The ischemia-induced changes in the cytosolic calcium 'environment' and its relation to voltage was investigated by dual-parametric optical mapping of both voltage and calcium in mechanically uncoupled rabbit hearts ('*Dual-Imaging Uncoupled*'; $n = 10$). Figure 3.2 (A) and (B) show the qualitative changes in AP and CaT morphology during the first 30 minutes of ischemia in the ischemic region. These changes are quantified in Figures 3.3 - 3.5.

The rate of depolarization (dF_V/dt_{max}) was significantly reduced by 5 minutes of ischemia within the ischemic region (Figure 3.3 A) compared to pre-ligation values (Figure 3.3 C). Compared to healthy tissue dF_V/dt_{max} was reduced at 15 minutes with slight recovery by 30 minutes (Phase IB) (Figure 3.3 E). The rate of change of calcium (dF_{Ca}/dt_{max}) followed similar dynamics, but showed a smaller overall change (Figure 3.3 B, D, and F). Changes in APD_{50}, APD_{80}, $CaTD_{50}$, and $CaTD_{80}$ had different time courses, and displayed temporal disparities. APD_{50} was significantly shortened by 10 minutes within the ischemic region and ischemic border zone (Figure 3.4 A) compared to pre-ligation values (Figure 3.4 C). Compared to healthy tissue, APD_{50} was reduced at 15 minutes and did not recover in Phase IB (Figure 3.4 E). Reduction of $CaTD_{50}$ (Figure 3.4 B), on the other hand, was delayed until 15 minutes of ischemia, recovered by 45 minutes, and showed a smaller overall change (Figure 3.4 D). Changes in APD_{80} were similar to APD_{50} (Figure 3.5 A, C, and E), but showed a slight recovery in Phase IB. $CaTD_{80}$ for the most part remained unaffected (Figure 3.5 B), only reaching significance by 45 minutes within the ischemic region (-4 mm) (Figure 3.5 D). There also appeared to be a trend towards reductions in $CaTD_{80}$ in Phase IB (Figure 3.5 F).

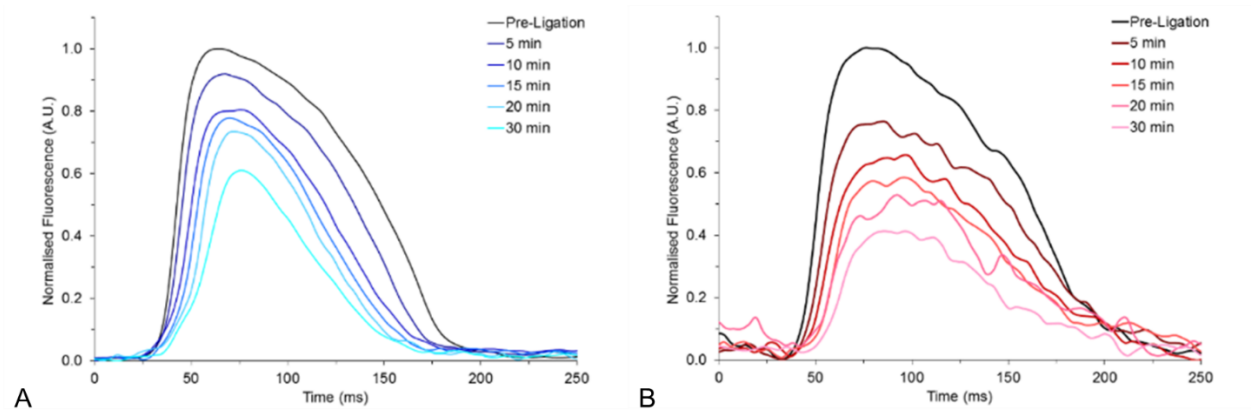

Figure 3.2 Qualitative Changes in Voltage and Calcium Transient Morphologies

Representation of changes in AP (A) and CaT (B) morphology over 30 minutes of ischemia at –5 mm from ischemic border zone (IBZ).

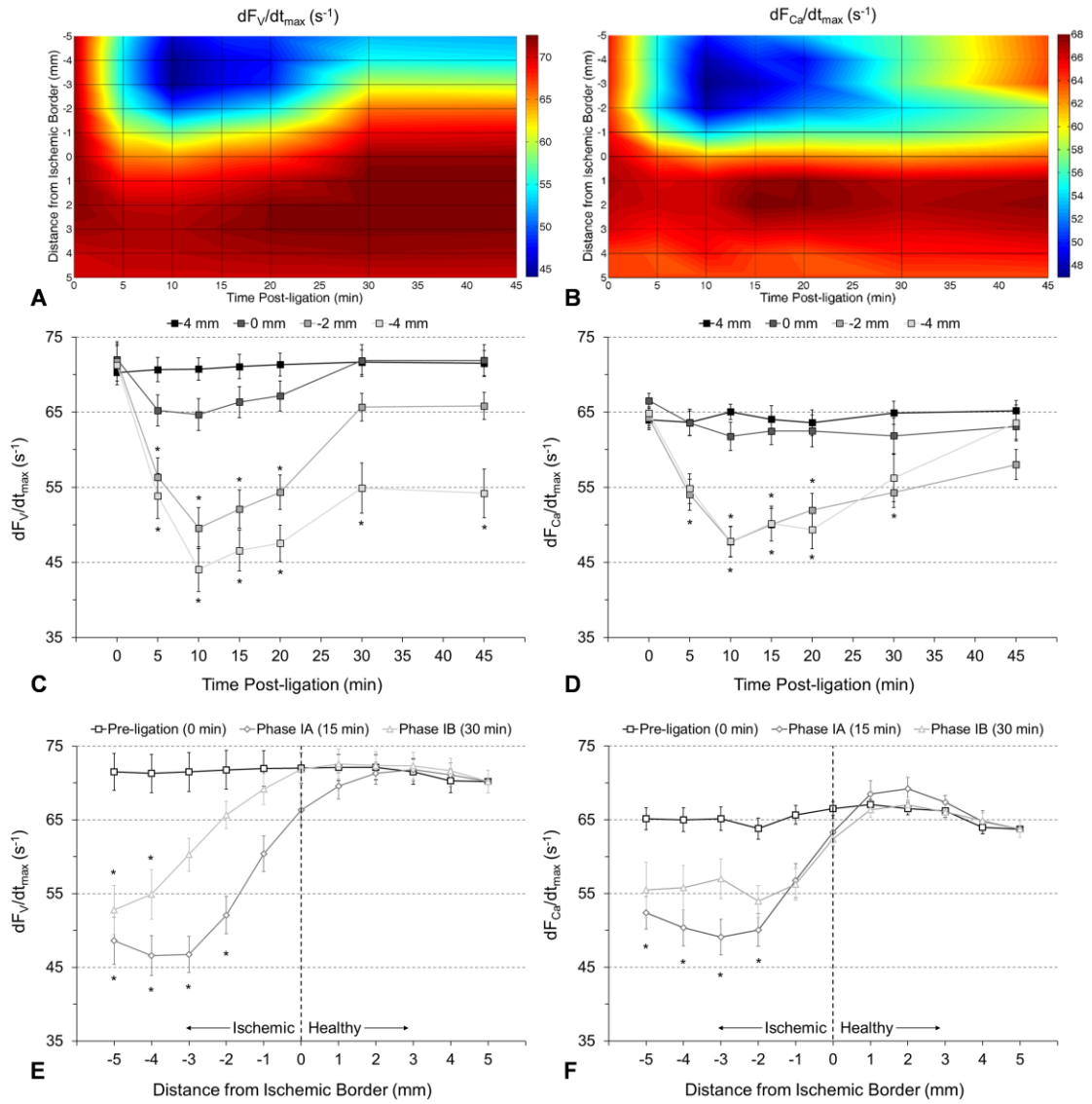

Figure 3.3 Alterations in the dF_V/dt_{max} and dF_{Ca}/dt_{max} during ARI

Changes in voltage (left) and calcium (right) presented as: (A - B) 2-dimensional surface-plots; (C - D) temporal changes at four distinct ROIs (Healthy Zone, 4 mm; IBZ, 0 mm; Mid-Ischemic Zone, -2 mm; Ischemic Zone, -4 mm), and; (E – F) spatial differences across the IBZ during Pre-ligation, Phase IA, and Phase IB. Data represented as means ± SEM (n = 10). (*) For C - D denotes significance compared to 0 min; for E - F denotes significance compared to 5 mm ($p < 0.05$).

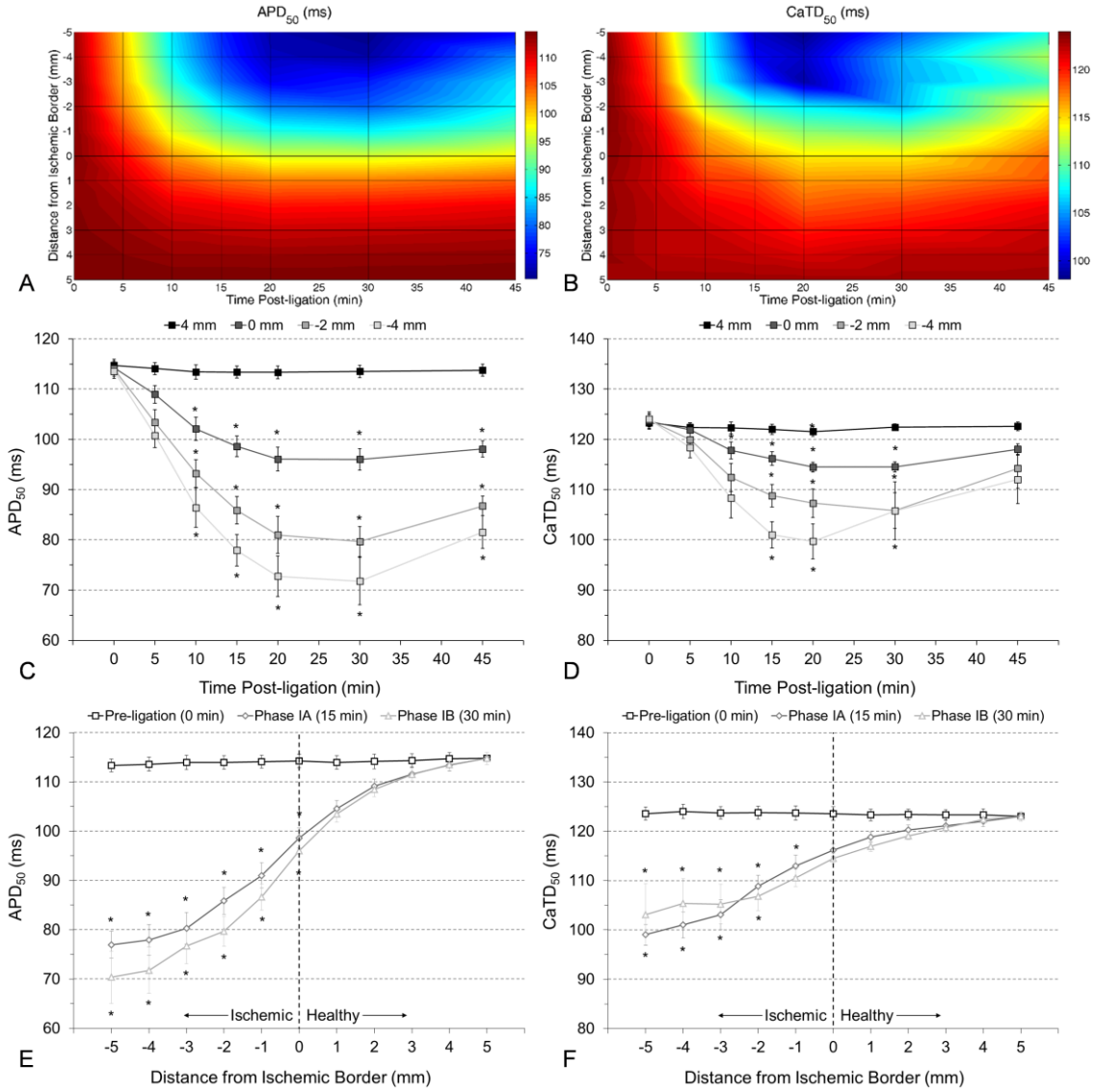

Figure 3.4 Alterations APD_{50} and $CaTD_{50}$ during ARI

Changes in voltage (left) and calcium (right) presented as: (A - B) 2-dimensional surface-plots; (C - D) temporal changes at four distinct ROIs (Healthy Zone, 4 mm; IBZ, 0 mm; Mid-Ischemic Zone, -2 mm; Ischemic Zone, -4 mm), and; (E – F) spatial differences across the IBZ during Pre-ligation, Phase IA, and Phase IB. Data represented as means ± SEM (n = 10). (*) For C - D denotes significance compared to 0 min; for E - F denotes significance compared to 5 mm ($p < 0.05$).

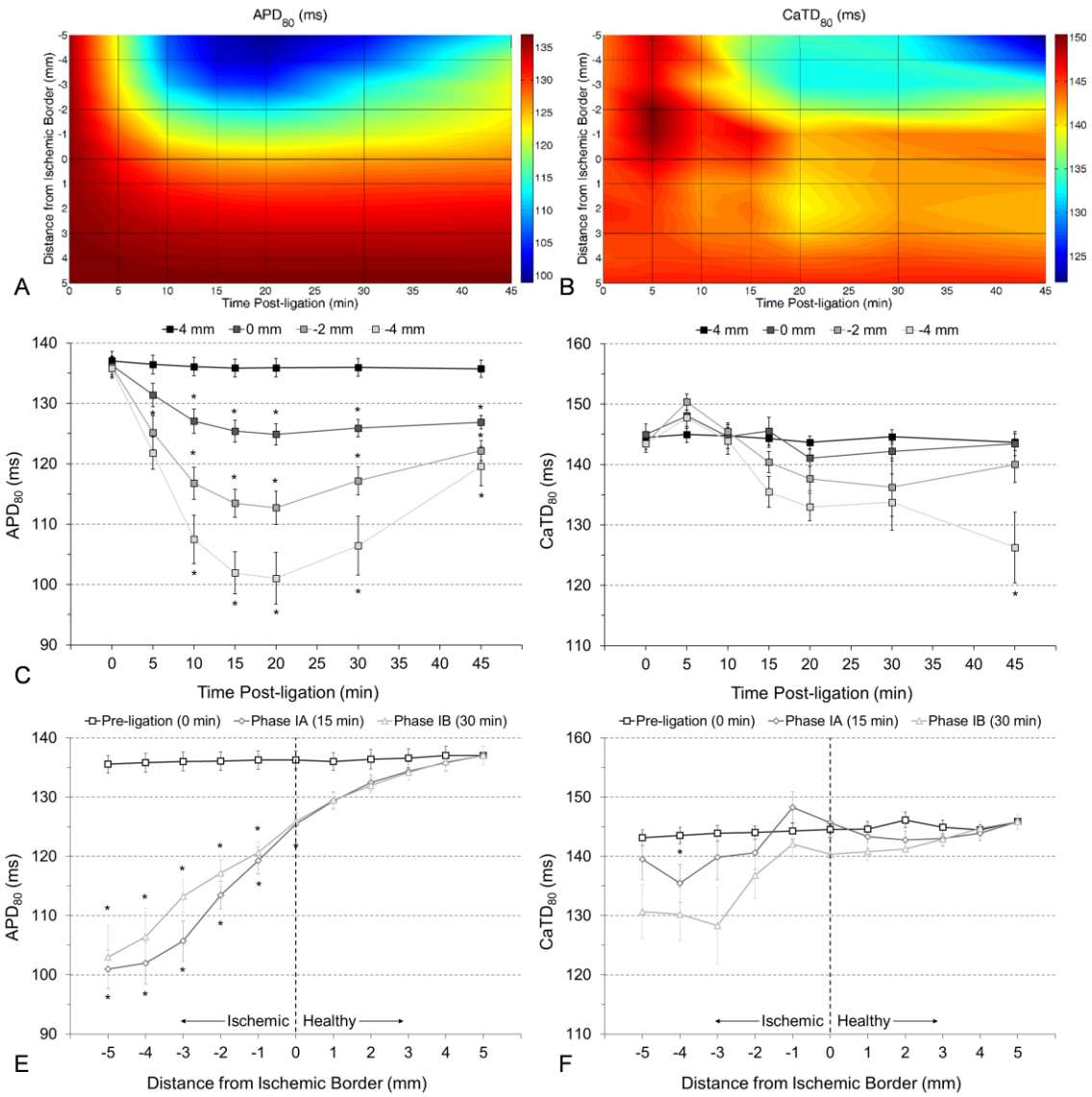

Figure 3.5 Alterations APD_{80} and $CaTD_{80}$ during ARI

Changes in voltage (left) and calcium (right) presented as: (A - B) 2-dimensional surface-plots; (C - D) temporal changes at four distinct ROIs (Healthy Zone, 4 mm; IBZ, 0 mm; Mid-Ischemic Zone, -2 mm; Ischemic Zone, -4 mm), and; (E – F) spatial differences across the IBZ during Pre-ligation, Phase IA, and Phase IB. Data represented as means ± SEM (n = 10). (*) For C - D denotes significance compared to 0 min; for E - F denotes significance compared to 5 mm ($p < 0.05$).

While the changes in both electrical activity and calcium handling may play important independent roles in the arrhythmogeniecity of the heart during ARI, intracellular calcium and voltage have been shown to be inexorably linked.[170] As such, their transient durations in relation to each other may also be important. Figure 3.6 displays representative APs and CaTs at 0, 5, 20, and 30 minutes of ischemia within the ischemic region. Changes are quantified in Figure 3.7. The difference between $CaTD_{50}$ and APD_{50} (Figure 3.7 A) was significantly increased by 10 minutes in the ischemic region, and by 15 minutes at the ischemic border, plateauing after 30 minutes (Figure 3.7 C). The difference between $CaTD_{80}$ and APD_{80} (Figure 3.7 B), on the other hand, was increased by 5 minutes, and returned to normal values by 45 min (Figure 3.7 D). Values of τ_{Ca} are shown in Figure 3.8. τ_{Ca} was increased by 10 minutes in the ischemic region, with slight recovery by 45 minutes (Figure 3.8 B).

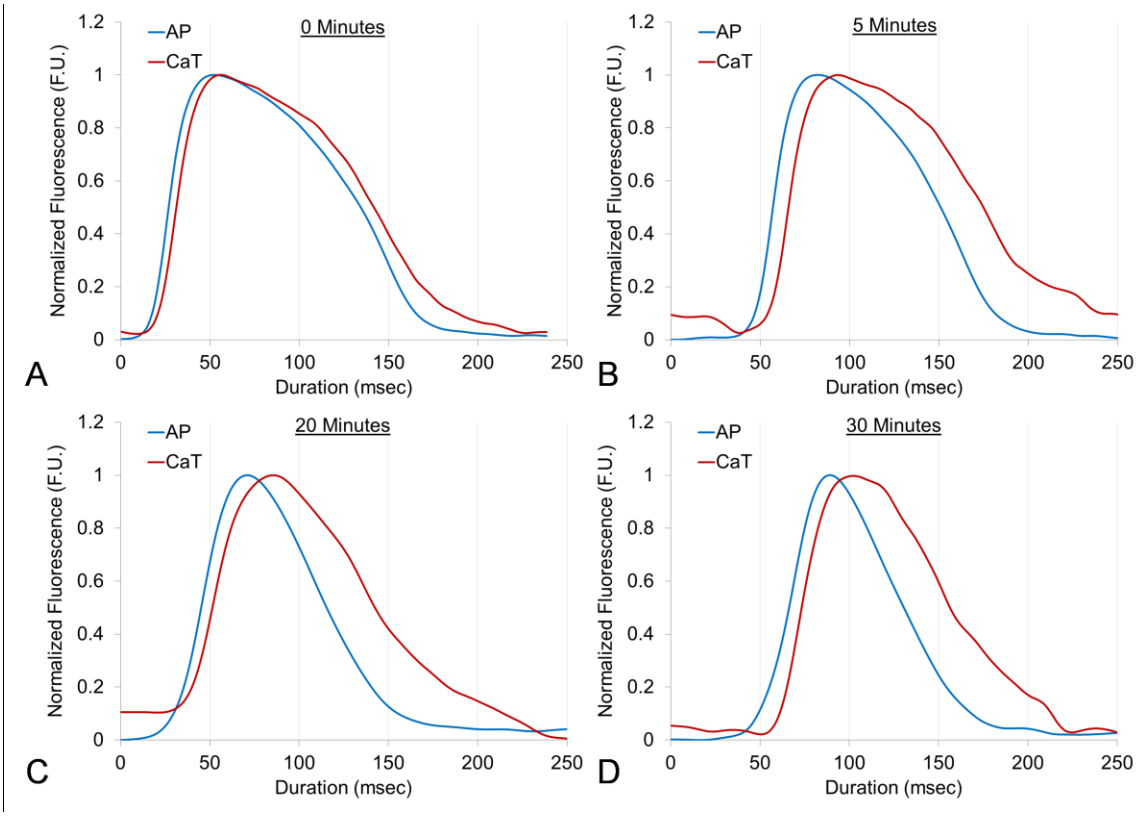

Figure 3.6 Qualitative Temporal Changes in APD and CaTD

AP (blue) and CaT (red) morphology in the most ischemic region (-5 mm) giving rise to a vulnerable window for arrhythmias to occur. Changes at occurring at: (A) 0 minutes; (B) 5 minutes; (C) 20 minutes, and; (D) 30 minutes of ARI.

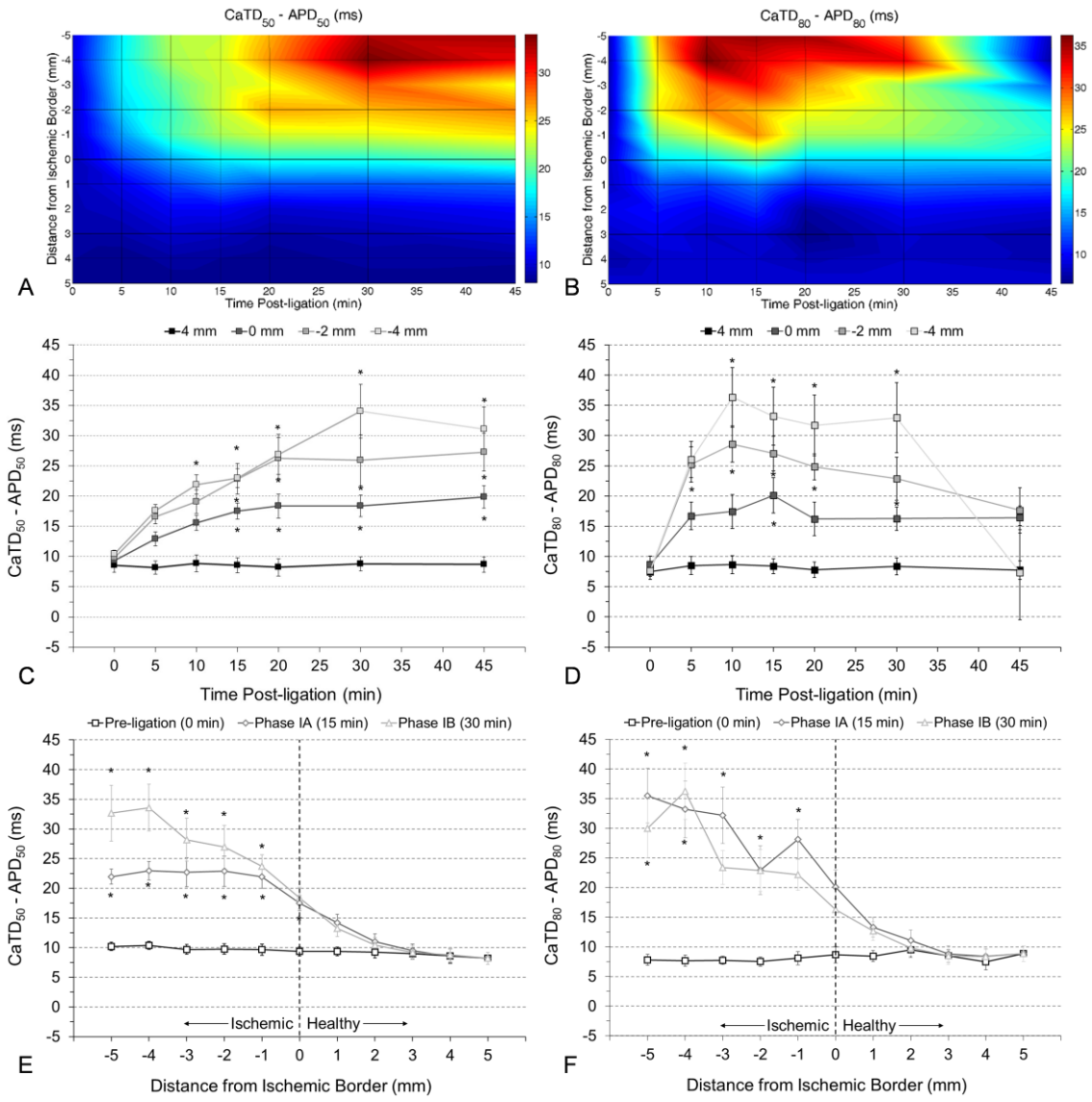

Figure 3.7 Alterations CaTD – APD Difference

Changes in voltage and calcium transient durations to 50% recovery (left) and 80% recovery (right) presented as: (A - B) 2-dimensional surface-plots; (C - D) temporal changes at four distinct ROIs (Healthy Zone, 4 mm; IBZ, 0 mm; Mid-Ischemic Zone, -2 mm; Ischemic Zone, -4 mm), and; (E – F) spatial differences across the IBZ during Pre-ligation, Phase IA, and Phase IB. Data represented as means ± SEM (n = 10). (*) For C - D denotes significance compared to 0 min; for E - F denotes significance compared to 5 mm ($p < 0.05$).

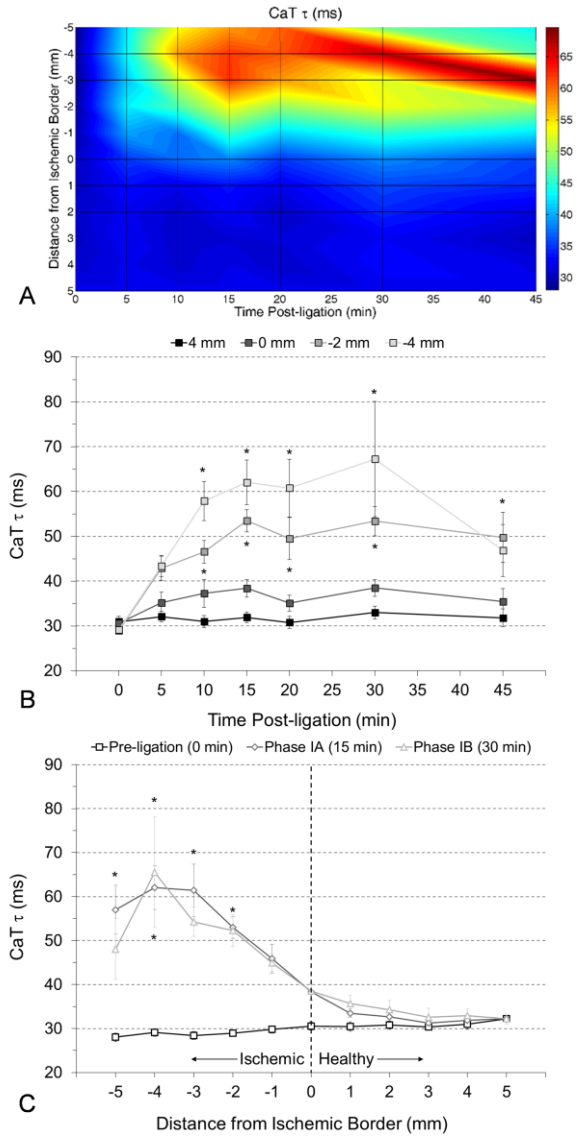

Figure 3.8 Alterations CaT Time Constant to Recovery

Changes in τ between 50% and 80% recovery presented as: (A) 2-dimensional surface-plot; (B) temporal changes at four distinct ROIs (Healthy Zone, 4 mm; IBZ, 0 mm; Mid-Ischemic Zone, -2 mm; Ischemic Zone, -4 mm); (C) spatial differences across the IBZ during Pre-ligation, Phase IA, and Phase IB. Data represented as means ± SEM (n = 10). (*) For C - D denotes significance compared to 0 min; for E - F denotes significance compared to 5 mm ($p < 0.05$).

We also measured the prevalence of both CaT-ALT and APD-ALT, conditions characterized by the alternation of high and low amplitude calcium release or long and short APD, respectively.[168] CaT-ALT has been shown to frequently occur during ARI.[171,172] Changes in amplitude greater than 10% or changes in duration greater than 4 milliseconds between two successive activations were considered CaT alternans and AP alternans, respectively.[169] Figure 3.9 illustrates the absolute difference in APD (left) and % change in CaT amplitude (right) and over successive beats. There is a trend of increasing APD-ALT in the ischemic region (Figure 3.9 A), which surpasses the 4 millisecond guideline (set forth by Pruvot *et al.*[169]) and becomes significant at 45 minutes. (Figure 3.9 C). CaT-ALT on the other hand reached significance by 10 minutes in the ischemic region (Figure 3.9 B, D, and F).

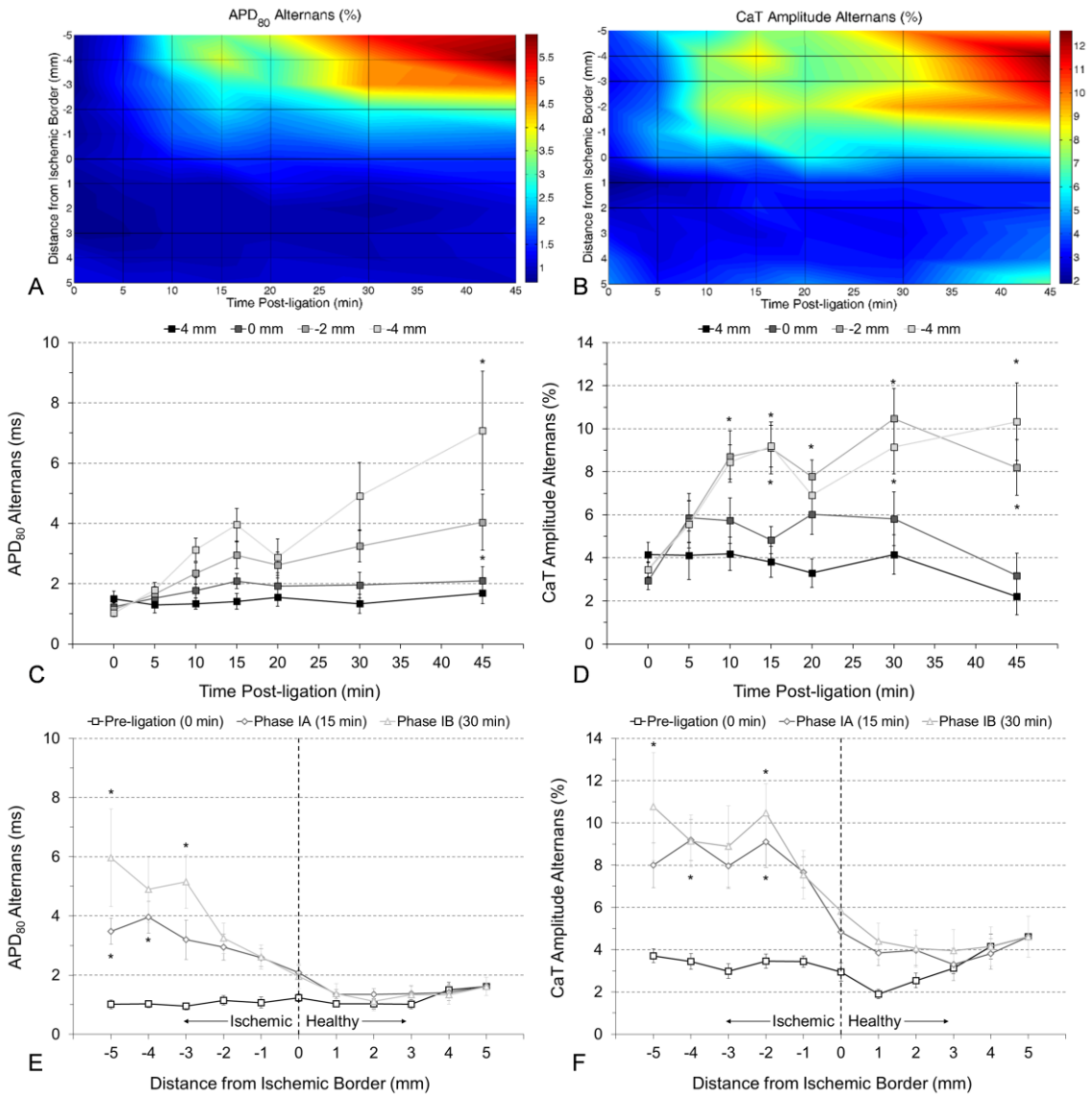

Figure 3.9 Calcium Transient Amplitude and Action Potential Duration Alternans

Absolute difference in action potential duration (left) and absolute percent change of calcium transient amplitude (right) between two successive transients during ARI shown as: (A – B) 2-dimensional surface-plots; (C - D) temporal changes at four distinct ROIs (Healthy Zone, 4 mm; IBZ, 0 mm; Mid-Ischemic Zone, -2 mm; Ischemic Zone, -4 mm), and; (E – F) spatial differences across the IBZ during Pre-ligation, Phase IA, and Phase IB. Data represented as means ± SEM (n = 10). (*) For C - D denotes significance compared to 0 min; for E - F denotes significance compared to 5 mm ($p < 0.05$).

3.1.III Effect of Electro-Mechanical Uncoupling on Arrhythmogenesis

In addition to measuring changes in voltage and calcium during regional ischemia, we also examined the effects of mechanical uncoupling on the incidence of arrhythmias. Previous data from our laboratory has shown that uncoupling with blebbistatin significantly reduces the incidence of arrhythmias during ARI compared to loaded, contracting hearts, an effect that was recapitulated in the current study (Figure 3.10). During these experiments no instances of sustained or non-sustained tachyarrhythmias were experienced.

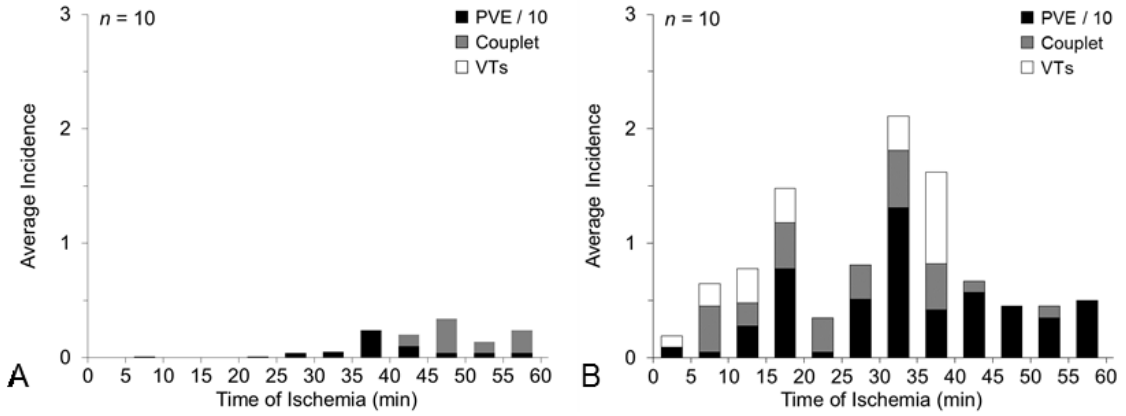

Figure 3.10 Incidence of Arrhythmias in *Uncoupled Dual Imaging* and *Loaded* during ARI

Average incidence of arrhythmias during 60 minutes of acute regional ischemia in (A) *Uncoupled Dual Imaging*, and; (B) *Loaded*, groups. Premature Ventricular Excitation (PVE); Ventricular Tachyarrhythmias (VTs).

3.2 Mechanically Dependent Alterations in Calcium Handling

To assess the potential role of calcium in driving mechanically-dependent arrhythmias during ARI, we optically mapped both voltage and calcium in isolated contracting rabbit hearts - '*Rhod-2'* (n = 10). During these experiments we did not observe any ventricular ectopy being driven by calcium. However, there was a significant reduction in the incidence of PVEs during Phase IB when compared to data from loaded, contracting hearts ('*Loaded'*), suggesting that mechanically-dependent arrhythmias were abolished in this group (Figure 3.11 D). During these experiments, 5 of 10 hearts experienced non-sustained ventricular tachycardia, while 2 of 10 experienced non-sustained ventricular fibrillation. Furthermore, one experiment was excluded due to sustained ventricular fibrillation.

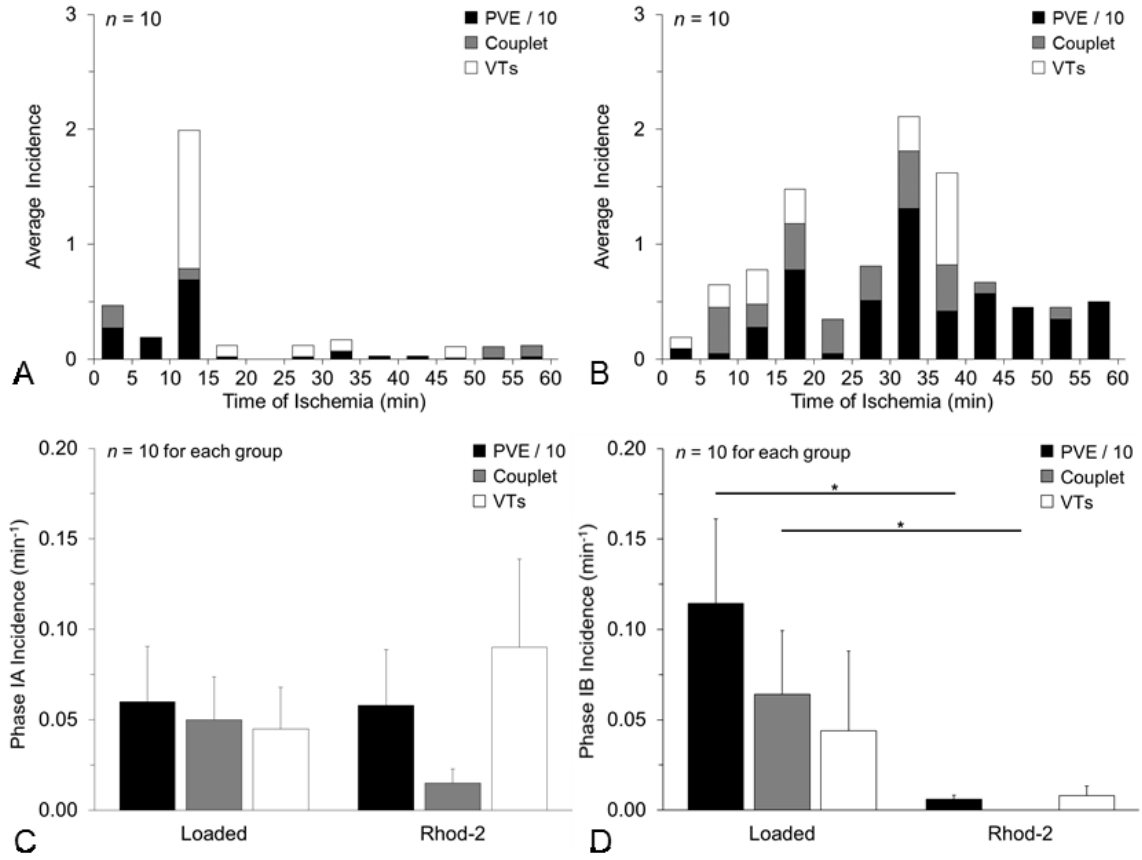

Figure 3.11 Incidence of Arrhythmias During *Rhod-2* and *Loaded* during ARI

Average incidence of arrhythmias during 60 minutes of acute regional ischemia in (A) *Rhod-2*, and; (B) *Loaded*, groups. Incidence of arrhythmias per minute (min^{-1}) during (C) Phase IA (0 – 20 minutes), and; (D) Phase IB (20 – 45 minutes). Premature Ventricular Excitation (PVE); Ventricular Tachyarrhythmias (VTs). (*) Denotes significance from *Loaded*. (Two-Tailed Unpaired T-Test, $p < 0.05$).

3.3 Effect of Calcium Buffering on Arrhythmogenesis

3.3.I The Effect of BAPTA on Arrhythmogenesis

The reduction in the incidence of arrhythmias in hearts loaded with the fluorescent indicator Rhod-2 may be the result of the indicator binding to cytosolic calcium, and thus acting as a buffer.[153] This may be seen as a reasonable conclusion as much of Rhod-2's molecular structure is based around a known calcium chelator (BAPTA). In order to support the idea that the change in the incidence of arrhythmias with loading of Rhod-2 was the result of calcium chelation, we performed experiments in hearts with BAPTA AM ('*BAPTA*'; $n = 10$). First, to determine the concentration of BAPTA that produced a similar degree of calcium buffering to Rhod-2, we performed a pilot study to compare the hemodynamic response with varying concentrations of BAPTA to that with Rhod-2 ('*BAPTA-(P)*'; $n = 2$) as calcium is the main signaling molecule for contraction, contractile force is related to the degree of calcium availability). It was found that concentrations as low as 0.1 μM of BAPTA produced a reduction in developed pressure similar to that of Rhod-2.

Figure 3.12 shows that application of 0.1 μM BAPTA AM resulted in a reduction in the incidence arrhythmias over 60 minutes of ARI compared to our control (*Loaded*) group. During Phase IA, there was a trend towards a reduction in the incidence of single and sustained arrhythmias when compared to *Loaded* (Figure 3.12 C). During Phase IB, BAPTA resulted in a significant reduction in the incidence of PVEs when compared to the *Loaded* group (Figure 3.12 D). In 2 of 10 experiments, non-sustained ventricular tachycardia and fibrillation were observed.

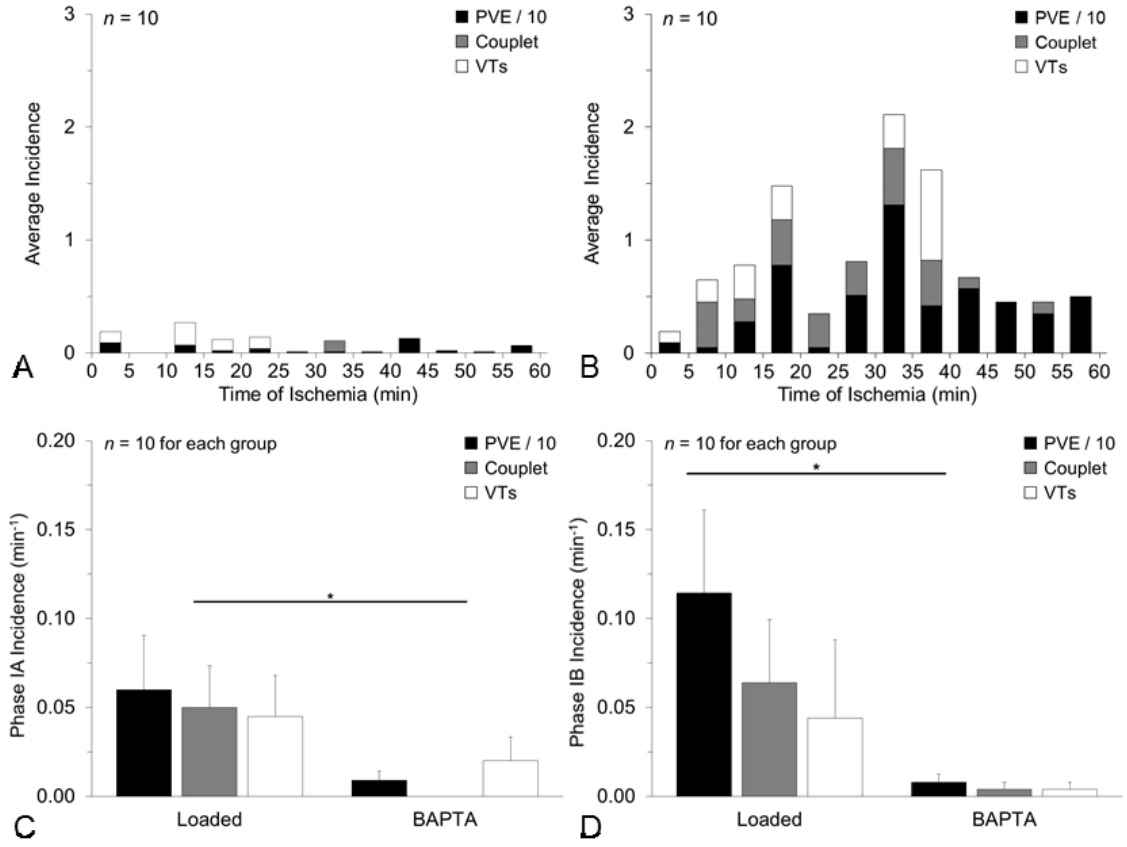

Figure 3.12 Incidence of Arrhythmias During *BAPTA* and *Loaded* during ARI

Average incidence of arrhythmias during 60 minutes of acute regional ischemia in (A) *BAPTA*, and; (B) *Loaded*, groups. Incidence of arrhythmias per minute (min^{-1}) during (C) Phase IA (0 – 20 minutes), and; (D) Phase IB (20 – 45 minutes). Premature Ventricular Excitation (PVE); Ventricular Tachyarrhythmias (VTs). (*) Denotes significance from *Loaded*. (Two-Tailed Unpaired T-Test, $p < 0.05$).

3.4 Effect of RyR Stabilization on Arrhythmogenesis

3.4.I Effect of RyR Stabilizers on Tissue Electrophysiology

In consideration of side-effects experienced while utilizing JTV-519 in loaded contracting hearts (2:1 block), the effects of RyR2 (JTV-519) and RyR1 (dantrolene) stabilizers on tissue electrophysiology was investigated during pilot studies. The effects of JTV-519 (1 μM) and dantrolene (1 μM) on APD_{80} and dF_V/dt_{max} were assessed in isolated non-contracting (blebbistatin) hearts (‘*JTV-519-(P)’*, $n = 2$; ‘*Dantrolene-(P)’*, $n = 4$). Figure 3.14 illustrates the change in APD_{80} and dF_V/dt_{max} after 30 minutes of perfusion with dantrolene or JTV-519. 1 μM JTV-519 caused a significant reduction in dF_V/dt_{max} and a trend towards increased APD_{80}. After 30 minutes, the ability to pace at 4 Hz was lost in one heart, resulting in a 2:1 rhythm. In contrast, with dantrolene there was no change in APD_{80}, and dF_V/dt_{max} was reduced to a lesser extent than with JTV-519 (Figure 3.14 (black box)). Furthermore, during ‘*Dantrolene-(P)”* experiments there was no loss of pace capture. As such, dantrolene was selected as the pharmacological agent for stabilization of RyR channels in our principal experiments.

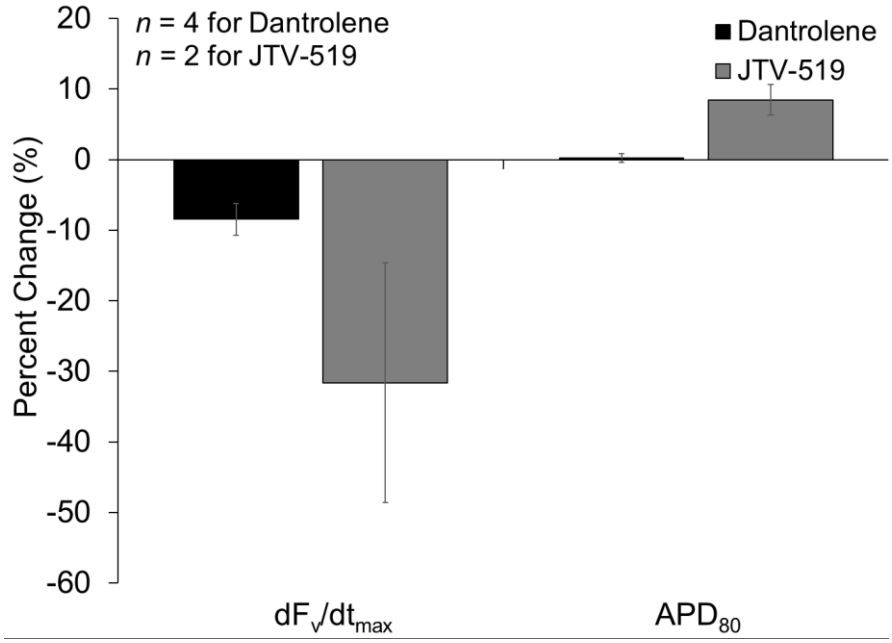

Figure 3.13 Effect of RyR Stabilizers JTV-519 and Dantrolene on Electrophysiology in Isolated Uncoupled Hearts.

Change in maximal rate of change in fluorescence (dF_V/dt_{max}) and action potential duration to 80% repolarization (APD_{80}) in *Dantrolene-(P)* (black) and *JTV-519-(P)* (grey) experiments.

3.4.II Effect of RyR Stabilization on Arrhythmogenesis

The effect of RyR stabilization on the incidence of arrhythmias was examined in *Dantrolene* (n = 13). Data presented in Figure 3.14, shows the incidence of arrhythmias during 60 minutes of ARI in (A) *Dantrolene* and (B) *Loaded* hearts. During Phase IA there was no significant reduction in the incidence of single or sustained arrhythmias when compared to *Loaded* **(**Figure 3.14 C). During Phase IB however, Dantrolene, resulted in a significant reduction in the incidence of PVEs during Phase IB when compared to *Loaded* data (Figure 3.14 D). During dantrolene experiments, non-sustained ventricular tachycardia was experienced in 4 of 13 hearts, while non-sustained ventricular fibrillation was observed in 1 of 13. One experiment was excluded due to sustained ventricular fibrillation.

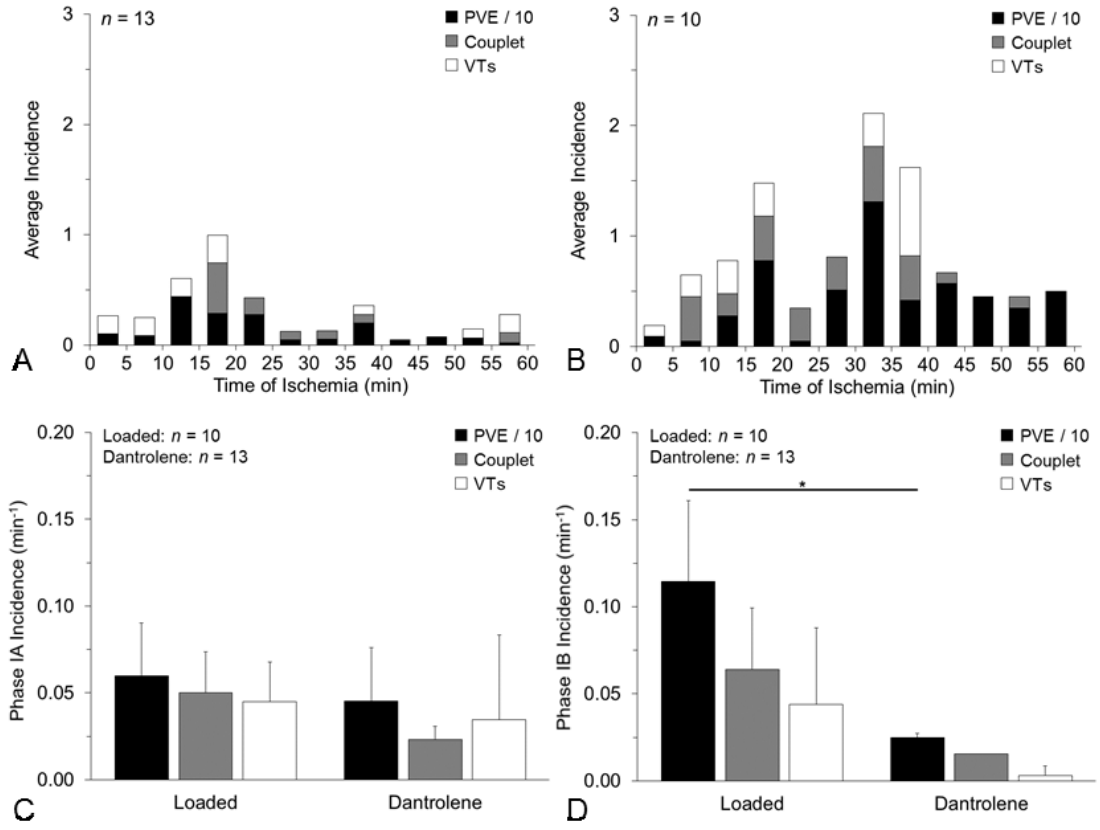

Figure 3.14 Incidence of Arrhythmias During *Dantrolene* and *Loaded* during ARI

Average incidence of arrhythmias during 60 minutes of acute regional ischemia in (A) *Dantrolene*, and; (B) *Loaded*, groups. Incidence of arrhythmias per minute (min^{-1}) during (C) Phase IA (0 – 20 minutes), and; (D) Phase IB (20 – 45 minutes). Premature Ventricular Excitation (PVE); Ventricular Tachyarrhythmias (VTs). (*) Denotes significance from *Loaded*. (Two-Tailed Unpaired T-Test, $p < 0.05$).

3.5 Phase Analysis of Previously Reported Arrhythmia Incidence during ARI

The effect of calcium chelation and RyR stabilization on the incidence of arrhythmias revealed changes specific to Phase IB. We examined data previously collected by our laboratory examining the effects of mechanics on ARI arrhythmias,[14] segregating it into Phase IA and IB. Reduced ventricular load (*Unloaded*) and pharmacological uncoupling (Blebbistatin - *Non-Contracting*) reduced the incidence of both Phase IA and Phase IB PVEs when compared to *Loaded* (Figure 3.15 A and B). In addition, reducing ventricular load or inhibiting mechanics resulted in a significant reduction in the incidence of doublets compared to *Loaded* during Phase IA (Figure 3.15 A). During *Loaded* experiments, non-sustained ventricular tachycardia was observed in 5 of 10 experiments, however there were no instances of non-sustained ventricular fibrillation.

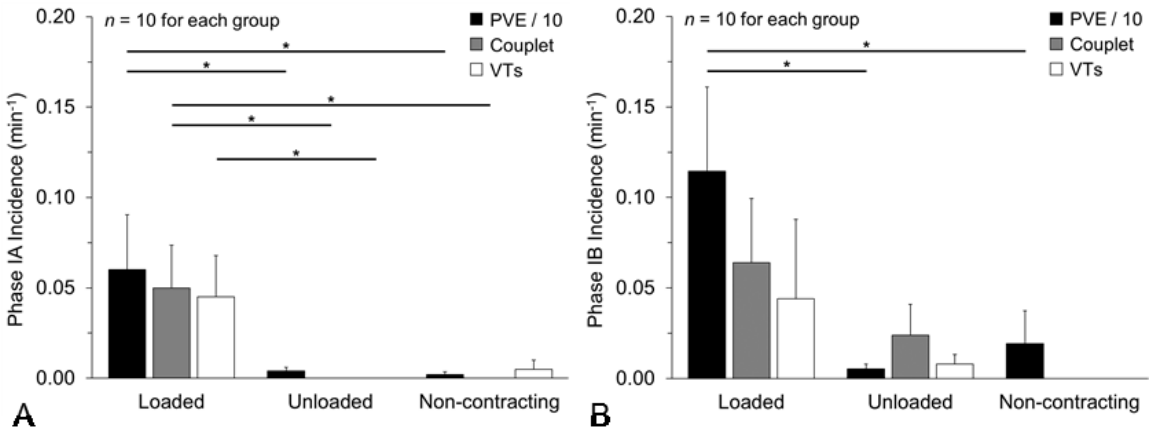

Figure 3.15 Incidence of Arrhythmias during Altered Mechanics during ARI

Average incidence of arrhythmias in *Loaded*, *Unloaded*, and *Non-contracting* groups during ARI. Incidence of arrhythmias per minute (min^{-1}) during (A) Phase IA (0 – 20 minutes), and; (B) Phase IB (20 – 45 minutes). (*) Denotes significance from *Loaded.* (One-Way ANOVA, $p < 0.05$).

3.6 Non-Perfused Area and Hemodynamic Group Comparisons

The size of the ischemic zone is an important factor in arrhythmogenesis.[150] In order to affirm similar sizes of non-perfusion in all experimental models, the quantification of the region of non-perfusion induced by coronary artery ligation was assessed utilizing fluorescent microspheres (example shown in Figure 2.3.). Figure 3.16 presents the percentage area of non-perfusion and the related percentage reduction in coronary artery flow with artery ligation. One-way ANOVA showed no significant difference across the groups.

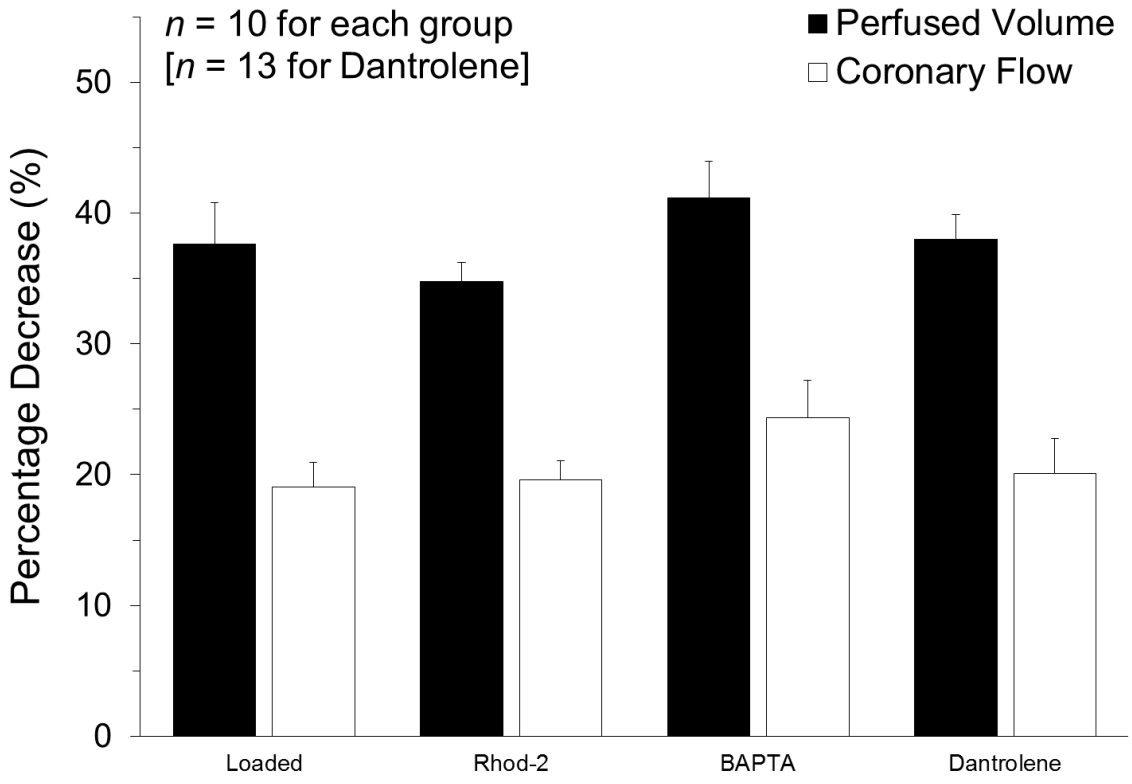

Figure 3.16 Group % Non-Perfusion (NPA) and % Reduction in Coronary Flow Upon Ligation

Data presented as mean ± SEM.

Changes in LV function in response to loading of Rhod-2, BAPTA, and dantrolene were analyzed and are reported in Figure 3.17. All three agents resulted in a decrease in developed pressure and the maximum rate of pressure decay (dP/dt_{min}) compared to baseline. When compared to each other, dP/dt_{max} differed between dantrolene and BAPTA, developed pressure differed between dantrolene and BAPTA / Rhod-2, dP/dt_{min} differed between all groups, and there was no significant variation in τ_{LVP}.

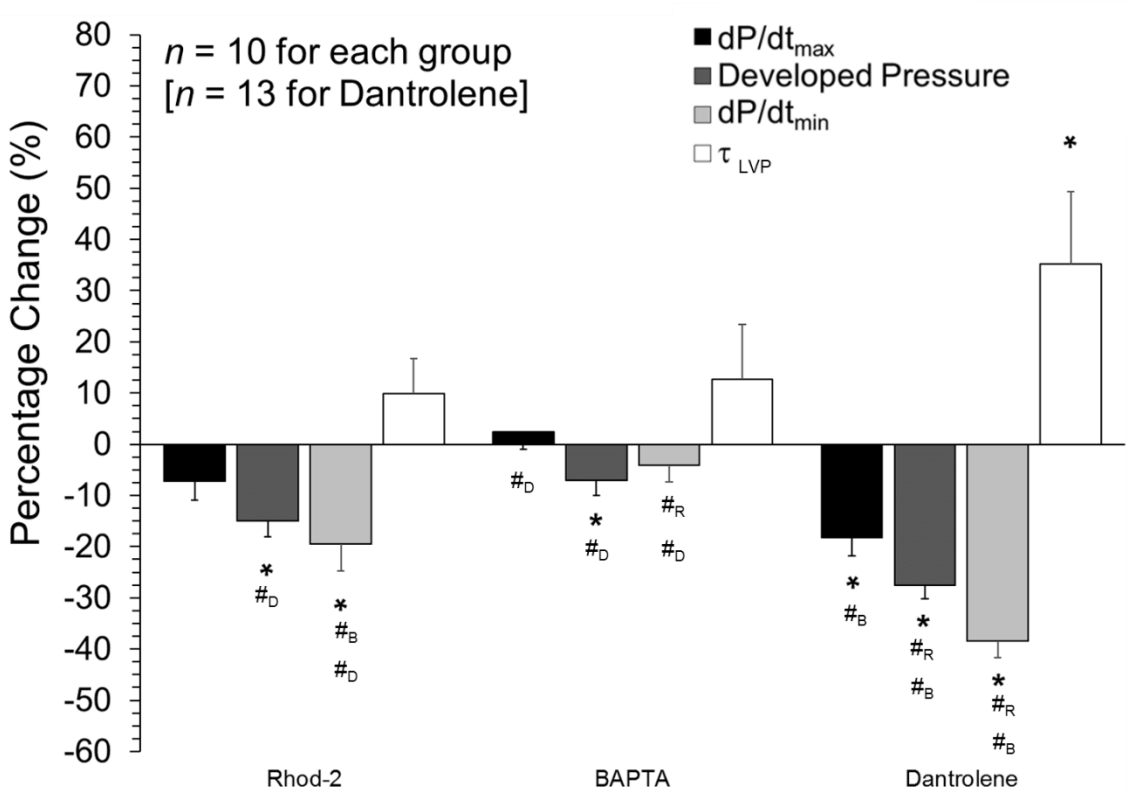

Figure 3.17 Hemodynamic Changes in Response to Intervention

Changes in hemodynamics for each experimental group as a percent change from baseline (pre-intervention) values. Black boxes represent dP/dt_{max}; dark grey boxes represent developed pressure; light grey boxes represent dP/dt_{min}, and; white boxes represent τ_{LVP}. Data presented as mean ± SEM. (n = 10 for *Rhod-2* and *BAPTA*; n = 13 for *Dantrolene*). (*) Denotes significance from baseline, ($\#_R$, $\#_B$, $\#_D$) denotes significance from Rhod-2, BAPTA, or Dantrolene, respectively. (One-Way ANOVA, $p < 0.05$).

In light of reductions in developed pressure with application of Rhod-2 and pharmacological agents, it was essential to ensure that contraction was not significantly reduced compared to our control group (*Loaded*). Assessment of developed and end diastolic pressure both with and without pacing immediately prior to induction of ARI is displayed in Figure 3.18 for all contracting groups. This shows that values for developed pressure were mostly similar across the groups, with *BAPTA* having lower values than *Rhod*-2 / *Dantrolene* without pacing and *Loaded* having lower values than *Rhod-2* / *BAPTA* with pacing. The fact that *BAPTA* and *Rhod-2* hearts displayed a slightly higher developed pressure prior to the initiation of ARI than *Loaded* Supports the notion that difference in arrhythmia incidence were not due to direct effects on cardiac mechanical function. There was no difference in end diastolic pressure across the groups.

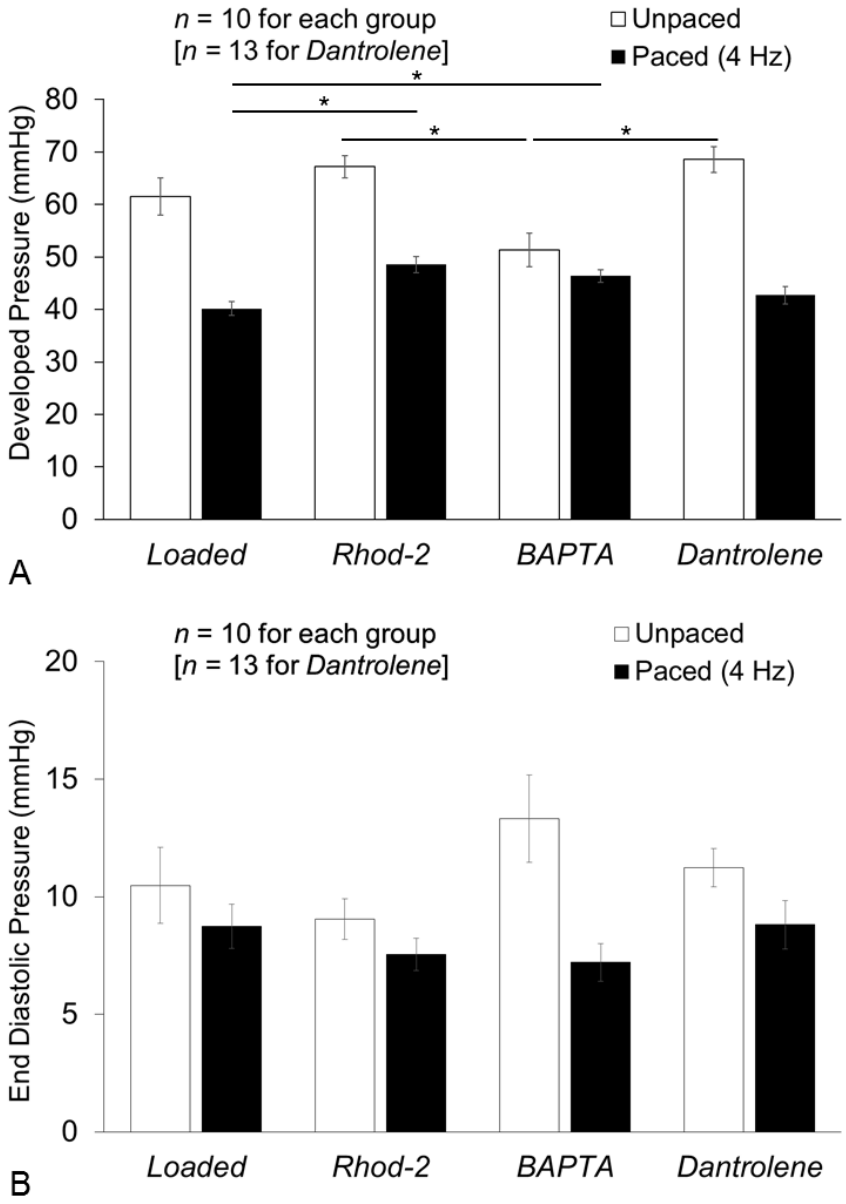

Figure 3.18 Left Ventricular Hemodynamics Prior to the Induction of ARI

Changes in (A) developed pressure and (B) end diastolic pressure in each experimental group immediately prior to the induction of regional ischemia. Unfilled and filled boxes represent values during normal sinus rhythm and pacing (4 Hz) respectively. Data presented as mean ± SEM. (*) Denotes significance between groups. (One-Way ANOVA, $p < 0.05$).

CHAPTER 4: DISCUSSION

Sudden Cardiac Death is a major cause of death in the developed world, including here in Canada. A major cause of SCD is the development of coronary artery stenosis, artery occlusion, and subsequent ischemia. During acute ischemic events sustained arrhythmias can reduce the heart's efficacy to pump blood and result in rapid syncope and death. Interestingly, arrhythmias may occur early during ischemia in the absence of cell-death and tissue necrosis. Arrhythmias arising within the first hour of ischemia occur biphasically, with the first and well documented Phase IA being attributed to changes in tissue electrophysiology. However, current literature does not fully address physiological changes occurring beyond 20 minutes of ischemia, during which the more lethal sustained arrhythmias such as VF predominantly occur. One of the suggested mechanisms for Phase IB arrhythmias is the feedback of changes in the mechanical function of the heart to its electrical activity, which has been supported by recent data from our laboratory, and may act through mechanically-induced alterations in intracellular calcium handling. As such, it was the main goal of this thesis to investigate the contribution of abnormal calcium handling to mechanically-dependent arrhythmias during ARI in the isolated rabbit heart.

4.1 Mechanically-independent, Ischemia-induced Changes in Calcium Handling

As the principal purpose of this thesis was to investigate the potential contribution of altered calcium handling to mechanically-induced arrhythmias during ARI, it was pertinent to first investigate mechanically-independent, ischemia-induced CaT changes in our model and how they relate to changes in the AP. This was accomplished by performing dual-parametric optical mapping of voltage and calcium in electromechanically uncoupled isolated hearts ('*Dual Imaging Uncoupled*').

Our findings (Section 3.1) illustrate that during ARI, there are rapid changes in electrophysiology, in agreement with previous investigations. Most notably, we observed a swift depression in dF_V/dt_{max}, APD_{80}, and AP amplitude, similar to that shown by Janse *et al.*[77] The mechanisms of these changes have been addressed in detail in Section 1.2 and are mainly the result of alterations in extracellular potassium, acidification, and anoxia.[39] Here we have also shown a similar rapid decline in dF_{Ca}/dt_{max} and CaT amplitude within the first 5 minutes of ischemia. These findings recapitulate prior literature, which has shown that between 2 and 5 minutes of ischemia both CaT amplitude and rate of SR calcium release are significantly impaired.[173] The rapid effects of ischemia on the rate of calcium release may reflect a relative desensitization of the RyR channels to calcium-activation due to the accumulation of hydrogen ions.[174]

As ischemia progressed into Phase IB, CaT amplitude continued to decrease, while dF_{Ca}/dt_{max} partly recovered. These changes have been shown by others to be accompanied by an increase in the diastolic level of calcium in the cytosol, which plateaus at around 3 - 4.5 times that of initial values between 10 and 30 minutes.[67,175] The mechanism of increased cytosolic calcium is thought to be related to the activation of NCX in reverse-mode, to extrude accumulating intracellular sodium as reductions in intracellular pH activate the sodium-hydrogen exchanger (NHE) to maintain homeostasis.[176,177] During Phase 1B, however, NHE is progressively inhibited by acidosis and its activity may be greatly reduced, partly accounting for the plateau in cytosolic calcium.[63] Interestingly, rises in diastolic fluorescence in similar magnitudes to that of prior literature were not observed in our study, however this may be explained as moderately acidic environments ($pH < 6.6$) reduce the affinity of Rhod-2 for calcium in a linear manner.[175] The recovery of dF_{Ca}/dt_{max}

during Phase IB may be attributed to endogenous catecholamine release[88] facilitating PKA-dependent RyR phosphorylation and increased sensitization to calcium-induced calcium release[178]. Furthermore, these catecholamines may increase conductance through $I_{Ca(L)}$, which has shown increased sensitivity to β-AR stimulation during hypoxia.[179,180] This increased conductance would facilitate an increase in the coupling gain seen within the dyad. Increased ROS has also been shown to occur rapidly during ischemia in both isolated cells[140,181] and intact hearts,[182] which may amplify $I_{Ca(L)}$ current density[183] or RyR2 sensitivity,[184] *via* redox modification.[185,186] As ischemic contracture begins to occur, ATP sparing may also allow partial recovery of both excitability and calcium release.

$CaTD_{50}$ displayed significant shortening within the first 10 minutes of ARI in our experiments (followed by partial recovery), while $CaTD_{80}$ was not reduced until 45 minutes. The lack of change in $CaTD_{80}$ may be explained by the increase in τ during ischemia, which by 10 minutes was two times greater within the ischemic region (Figure 3.8). This is in agreement with Venkataraman *et al.* who showed that after 15 minutes of regional ischemia in isolated murine hearts there is a dramatic increase in τ, while transient duration remains unaltered.[187] This change in τ may relate to the slowed extrusion of calcium by NCX, as it begins to function in reverse-mode, as well as impairment of SR calcium uptake by the sarcoplasmic-reticulum ATPase pump (SERCA2a). While expression of SERCA2a at 30 minutes of ischemia has been shown to be unchanged, reduced $[ATP_i]$ and dephosphorylation of the inhibitory protein phospholamban (PLB) by calcineurin activated protein phosphatase-1 will result in impaired SERCA2a activity and reduced calcium re-sequesteration.[188] Chen *et al.* have shown that over 30 minutes of ischemia there is no significant change in the content of the SR, despite a slight trend

upwards, suggesting equivocal impairment to both release and reuptake mechanisms.[67] These findings are not unanimous, however, as it has been shown in isolated wedge preparations that low-flow and no-flow ischemia depresses CaTD to a similar extent as APD by 15 minutes.[189] Discrepancies may relate to differences between regionally and globally ischemic tissue, where in addition to increased heterogeneity there exists coupling of ischemic and healthy tissue. In addition, while *low flow* ischemia simulates some effects of ARI (hypoxia, acidosis), there remains the potential for the remaining perfusion to prevent extracellular buildup of ions and metabolites (such as potassium). The recovery seen in APD_{50} and APD_{80} may be the result of improved metabolic status as contraction is reduced. Additionally, catecholamine release may stimulate glycogenolysis and increase the availability of glucose for anaerobic catabolism and ATP production. Combined, these effects would alter the ADP:ATP ratio, potentially reducing the activity of I_{KATP}. Furthermore, increases in $[Ca^{2+}]_i$ have been shown to reduce the activity of I_{KATP} in skeletal myocytes,[190] which may have interesting consequences in our model where calcium overload is commonly reported. The genesis of a persistent late-sodium current has been shown to occur during ischemia, potentially providing an additional means by which prolongation of the APD occurs.[191]

As voltage and calcium are coupled systems, temporally distinct changes may contribute to arrhythmogenicity. As such, we analysed the difference between CaTD and APD during ARI, which reflects the period during which intracellular calcium remains elevated after membrane repolarization, creating a 'vulnerable window' for calcium-driven after-depolarizations (due to inward currents through NCX).[192] Figure 3.5 B illustrates that within the first 5 minutes of ischemia, changes in $CaTD_{80}$ - APD_{80} were greater than in

$CaTD_{50}$ - APD_{50}, due to the previously discussed temporal difference in changes of $CaTD_{50}$ and $CaTD_{80}$. This window of vulnerability continuously increased into Phase IB, with $CaTD_{50}$ - APD_{50} and $CaTD_{80}$ - APD_{80} becoming equal by 30 minutes. After 30 minutes of ischemia $CaTD_{50}$ – APD_{50} remained high, however as $CaTD_{80}$ began to fall, $CaTD_{80}$ - APD_{80} returned to pre-ischemic levels.

Recently, a vulnerable window for calcium-driven arrhythmias has been implicated with β-AR remodeling in heart failure[192] and pharmacological APD abbreviation with I_{KATP} opener pinacidil[31]. In those studies, it was found that relatively greater shortening of the AP than the CaT set the *foundation* for afterdepolarizations to occur. However, despite this increased vulnerability, a sudden increase in cytosolic calcium (*via* β-AR stimulation or rapid-pacing) was required for arrhythmias to occur.[31,192] Thus, the absence of ectopy during our '*Dual Imaging Uncoupled*' experiments may indicate that despite the environment for calcium-driven events, mechanical effects are needed to trigger arrhythmogenic surges of intracellular calcium (discussed below). The idea that mechanically-induced effects may act on top of an ischemia-induced arrhythmogenic background during ARI is supported by the observation that the increase and subsequent decreases in $CaTD_{80}$ - APD_{80} in our experiments aligned with the increase and decrease in arrhythmia incidence in *Loaded* hearts, potentially accounting for the arrhythmias seen in that group during this period.

Alterations in calcium handling during ischemia have also been shown to give rise to beat-to-beat variations in the magnitude of calcium release, typically at shorter cycle lengths.[193] Known as calcium transient alternans (CaT-ALT), this typically occurs when upon excitation the preceding CaT has not completely recovered, resulting in a reduced SR

load and calcium release, followed by a compensatory increase. Greater alternation of calcium release amplitude may be arrhythmogenic if beats with augmented calcium release are sufficient to initiate calcium-driven afterdepolarizations. Furthermore, if accompanied by spatial dispersion of calcium release, CaT-ALT may result in increased heterogeneity of contraction, which could have important implications for mechanically induced arrhythmias.

Ischemia is also associated with beat-to-beat variations in APD (APD alternans, APD-ALT).[194] This cycle length-dependent alternation can be the result of the incomplete recovery of potassium currents or reduced sodium currents,[195] or alternatively may be linked to changes in sarcolemmal calcium fluxes.[196] Calcium modulates cellular electrophysiology through inhibition of $I_{Ca(L)}$ and activation of I_{NCX} in forward mode, and as such, preferential modification of those currents by a large amplitude CaT will facilitate asynchronous (APD shortening) or synchronous (APD lengthening) APD-ALT.[168]

Using a method validated by Purvot *et al.*, we measured both CaT-ALT and APD-ALT by calculating the absolute percent change in amplitude (calcium) or action potential duration (voltage) between two successive transients.[169] Figure 3.9 shows that during ischemia there is a time-dependent increase in CaT-ALT and APD-ALT in the mid-ischemic (-2) and central-ischemic (-4) zones. While our findings suggest that CaT-ALT and APD-ALT may be occurring, alternans has been shown by others to occur more rapidly and to a greater magnitude during ischemia (for instance, our values of CaT-ALT fall just shy of the cutoff for alternans laid out by Pruvot *et al.* ($\geq$ 10%)[169]).[172] However, this may not be a surprise, as both CaT-ALT and APD-ALT are highly cycle-length dependent[196] and at no point during our experiments did the duration of the calcium transient approach,

or exceed, that of the pacing cycle length of 250 milliseconds. Additionally, CaT-ALT may not have been observed on the epicardium, as there exists both transmural and apical-basal heterogeneity in calcium handling.[197,198] Namely, subendocardial tissue expresses significantly less SERCA2a than the mid- or sub-epicardium, and has been shown to possess slower calcium uptake kinetics and earlier CaT-ALT during rapid-pacing.[197] The increase in APD-ALT, which appears to follow that of CaT-ALT, may imply that altered calcium release may be affecting the electrical activity. Interestingly, APD-ALT has been shown to be pressure development (load) dependent[199], which may explain why only modest alternations in APD were witnessed in our electromechanically uncoupled hearts. The most worrisome effect of CaT-ALT aside from triggered activity is its ability to facilitate APD-ALT, which can increase dispersion of refractoriness and conduction slowing across the heart, promoting block and reentry.[200]

Despite the potentially pro-arrhythmic ischemia-induced alterations in intracellular calcium handling, our data suggest that these are not enough for the initiation of ventricular arrhythmias. As we saw virtually no arrhythmias in our uncoupled hearts, yet a relatively high incidence in loaded, mechanically contracting hearts, it would appear that the triggering event is mechanically dependent. This further corroborates the findings of Coronel *et al.* and our own laboratory who stress the importance of cardiac mechanics in the genesis of arrhythmias.[12,14] Thus, the findings of this objective suggest that while ischemia-induced changes in the calcium environment provide a background for arrhythmias, mechanical effects are necessary to provide the additional trigger for arrhythmias to occur.

4.2 Mechanical Effects on Calcium Handling

Following the investigation of the mechanically-independent, ischemia-induced changes in the calcium environment, we investigated the role of mechanical-effects in facilitating abnormal calcium handling in the context of ARI. Alterations in cardiac mechanics occur rapidly as ischemic tissue stops generating force (0 – 10 minutes) and begins to stiffen (10 – 20 minutes).[108] These changes expose tissue within the central ischemic region and border zone to systolic stretch, which may contribute to alterations in intracellular calcium handling.

Our specific aim was to investigate the role of calcium as a driver of mechanically-induced arrhythmias during ARI, using dual-parametric optical mapping in loaded, contracting hearts (*'Rhod-2'*). However, there was a significant reduction in PVEs compared to *Loaded* hearts in these experiments (Figure 3.11), which suggests that mechanically-induced arrhythmias were no longer present in these hearts. We believe this reduction in PVEs with the use of Rhod-2 is due to buffering of calcium by the calcium indicator. Rhod-2 functions by binding to cytosolic calcium to cause an increase in fluorescence, thus acting as a calcium sink. The prevention of calcium-driven events by buffering of calcium dyes has been demonstrated previously in sinoatrial node cells, where the use of Fluo-4 AM prevented calcium sparks from causing calcium waves[201] (an effect that was ameliorated by the use of lower concentrations of dye[202]).

Interestingly, by re-analyzing data previously collected by our laboratory, we showed that the reduction in PVEs and couplets with Rhod-2 application were restricted to Phase IB (Figure 3.11 C), while reductions in unloaded or non-contracting hearts occurred in both

phases (Figure 3.15). This suggests that mechanically-induced arrhythmias in Phase IA and IB occur by different mechanisms, with Phase IB arrhythmias being calcium-driven.

To examine if Rhod-2 was indeed buffering cytosolic calcium we retroactively investigated its effects on LV function. We found a slight reduction in developed pressure with application of the calcium indicator, suggesting that calcium availability may have become slightly impaired (Figure 3.17). In light of this finding, we wanted to ensure that the reduction in arrhythmias was not a mechanical effect, by an impaired ability to generate pressure. This appears not to be an issue, as developed pressure immediately prior to the induction of ARI in the *Rhod-2* group was in fact slightly *higher* than in the *Loaded* group (Figure 3.18). This suggests that the reduction in the incidence of Phase IB arrhythmias with Rhod-2 was not due to a reduction in mechanical activity, but rather was a result of the pharmacological properties of the dye.

We attempted to reduce the applied volume of Rhod-2 in our experiments to reduce the degree of calcium buffering, however this caused unacceptable reductions in signal quality, impairing our ability to detect the CaT upstroke. While this is a clear limitation of our technique, higher sensitivity cameras with an improved signal:noise ratio may help overcome this problem. Additionally, lower affinity calcium indicators could be used to reduce cytosolic buffering. This was not possible in our experiments, however, as the excitation/emission spectra of available lower affinity calcium dyes are not compatible with the single camera, isosbestic point technique of dual voltage-calcium imaging.

The few arrhythmias that did occur (and which we captured) in the Rhod-2 group did not show calcium-driven activity, however these may not have been mechanically-induced events (as they were presumably prevented by calcium buffering). Moreover, our ability to

measure activity only at the epicardial surface may be a potential limitation. Current practices utilized to image both endocardial and epicardial surfaces make use of ventricular wedge[189] or cut-open LV preparations[31], which are not practical for our use, as they severely compromise normal cardiac contraction. As mentioned above, the endocardium may be of particular interest for calcium-driven arrhythmias, as its lower expression of SERCA2a and attenuated CaT recovery may enhance arrhythmogenicity compared to the epicardium.[197] In fact, slow calcium recovery in the endocardium has been implicated in spontaneous calcium releases and triggered activity in the context of pacing-induced calcium overload in isolated wedge perparations.[203] As a result, calcium-driven ectopic activation may in fact occur within the endocardium, but once propagated to the epicardium, will be voltage-driven (and at best appear only as a shortening in the delay between voltage and calcium signals [32,204,205]).

The mechanism by which stretch may lead to surges in calcium sufficient for excitation is multi-factorial. Iribe *et al.* have shown that axial stretch of isolated cardiomyocytes results in an increase in calcium sparks, attributed to an anatomical coupling of cytoskeletal myofilaments and RyR channels.[136] Since, it has been proposed that this stretch-induced increase in SR calcium release occurs instead through the generation of reactive oxygen species (ROS), such as hydroxyl radicals or hydrogen peroxide.[137] ROS may be generated endogenously by ongoing mitochondrial oxidation, sarcolemmal nicotinamide adenine dinucleotide phosphate oxidases (NOX),[184] and the uncoupling of nitric oxide synthase (NOS) enzymes, such that they promote the synthesis of ROS instead of nitric oxide (NO)[206]. ROS have been shown to alter the oxidative status of RyR and lead to enhanced calcium-sensitivity and leak.[184] Pacing induced tachycardia has been shown to enhance

NOX activity and ROS-dependent RyR *S*-glutathionylation, increasing the open probability during systole.[207] These findings have been extended by Prosser *et al.*, who demonstrated that both static and sustained cyclic stretch increases ROS production and subsequent spark frequency in isolated rat cardiomyocytes,[137,138] an effect that was inhibited by the use of both NOX non-specific (diphenyleneiodonium) and NOX2 specific antagonists (Gp91ds-tat). Additionally, stretch has been shown to act through NOS pathways. Petroff *et al.* have shown that sustained stretch increases calcium spark frequency through eNOS-dependent NO *S*-nitrosylation of the RyR channel.[208] Furthermore, it has been indicated that nNOS activation may be a mediator of spark frequency in the context of increased afterload.[209]

Another way in which stretch can lead to transient increases in intracellular calcium come from work by Ter Keurs *et al.*, which revealed in isolated rat trabeculae that non-uniform contraction and relaxation can lead to calcium wave formation.[132] This finding was attributed to a stretch dependent increase in calcium-myofilament loading, which upon relaxation is suddenly released. Interestingly, this effect has also been linked to ROS production and RyR sensitization, increasing the likelihood for calcium sparks and waves to occur.[138,210]

The above describe surges in calcium, if sufficient in amplitude, may contribute to afterdepolarizations though the transient inward current (I_{ti}) carried by I_{NCX} (predominantly), the calcium-activated chloride channel ($I_{Ca(Cl)}$), and calcium-activated nonselective current (I_{Ca}).[211]

In a different vein, as ischemia transitions into Phase IB there is a massive endogenous (i.e., independent of extra-cardiac neural stimulation) release of catecholamines (especially NE).[89] NE released at this time may play a significant role in the induction of sustained

arrhythmias by heterogeneously shortening ERP, providing a substrate for reentry,[104] and it has been shown that depletion of endogenous NE stores reduces ischemia-induced arrhythmias.[103] There is also evidence that localised increases in NE can induce calcium-driven afterdepolarizations.[105] This may be enhanced by mechanical effects during ARI, as Moreau et al. have shown that increasing preload in an isolated working heart during ARI proportionally increases NE release.[212] This finding could have important implications for mechanically-dependent arrhythmias during ARI where mechanical dysfunction may trigger localized NE release.

4.3 Confirmation of the Effects of Cytosolic Calcium Buffering on Arrhythmia Incidence

During our *Rhod-2* experiments a significant reduction in the incidence of PVEs during Phase IB was observed. This was attributed to the buffering of cytosolic calcium by Rhod-2, which prevented calcium-driven ectopy from occurring. In order to substantiate this claim we investigated the effects of a well-documented calcium buffer on the incidence of arrhythmias in mechanically loaded isolated hearts ('*BAPTA*'). The chelating agent BAPTA AM was selected as it shares a molecular backbone with Rhod-2, conferring similarities in its ability to associate with available calcium ions.

Some of the early uses of BAPTA in cardiac research were by Billman *et al.* who during the early 1990s investigated the effects of calcium buffering on sustained arrhythmias.[213] In their studies, BAPTA was reported to ameliorate calcium overload following an ischemic stress-test in a canine model and subsequently reduce the incidence of VF.[213] More recently, Tang *et al.* have shown in a model of calcium overload (pacing-induced) and APD abbreviation (with pinacidil), that pretreatment with BAPTA reduces the

incidence of late phase 3 EADs.[31] This effect was attributed to a reduction in the amplitude of calcium release following rapid pacing and reduced calcium-dependent AP modulation. Furthermore, Wu *et al.* show that ischemic augmentation of CaTD is ameliorated by BAPTA, reducing the period over which the calcium transient intrudes into electrical diastole, contributing to a reduction in endocardial late phase 3 EADs.[214] In these examples, BAPTA reduced the incidence of triggered afterdepolarizations by reducing the calcium available for the activation of the I_{ti}.

While BAPTA appears to be able to confer a significant anti-arrhythmic effect, it has also been shown to significantly reduce cardiac hemodynamics. Calcium chelation reduces the calcium available to bind Troponin-C and activate cross-bridge formation. In fact, in their studies reporting that pre-treatment with BAPTA reduces the incidence of VF, Billman *et al.* also report that 1 mg/kg of BAPTA significantly attenuates the rate of developed pressure prior to ischemia in addition to reducing systolic and diastolic pressures immediately following coronary artery occlusion in vivo.[213] To date, several studies have utilized BAPTA in *ex vivo* hearts at concentrations of 20 μM to ameliorate calcium handling dysfunction,[31,214] however due to its efficiency and rapidity at chelating cytosolic calcium, concentrations as low as 2.2 μM have been shown to reduce developed pressure (60%).[215] As such, a fine line of what constitutes 'optimal' calcium buffering exists, with improved calcium dysfunction and reduced contractile function on opposing sides. We titrated BAPTA to a concentration that had a similar effect on hemodynamics as that of Rhod-2. During pilot experiments ('*BAPTA-(P)', n* = 2) we titrated [BAPTA] based on developed pressure. It was found that 0.1 μM of BAPTA corresponded to ~10% reduction in developed pressure, similar to that seen during our *Rhod-2* experiments.

Our investigation into the effects of low dosage BAPTA in mechanically loaded hearts (‘*BAPTA’*), revealed a significant reduction in the incidence Phase IA couplets and, similar to that of our *Rhod-2* experiments, Phase IB PVEs (Figure 3.12). This finding is intriguing considering the dosage of BAPTA was 1:200th of that previously used by researchers to suppress calcium driven events.[31,214] Importantly, with this in mind, the amount of BAPTA utilized in our experiments may not have significantly buffered the increase in diastolic calcium that occurs during ischemia, nor would it have been expected to abbreviate the CaTD, thus reducing the CaTD - APD vulnerable window. Should regular calcium handling have been significantly altered then substantial effects on developed pressure would have been expected. Pre-treatment with BAPTA in our experiments caused only a small reduction in developed pressure (~7 %) in comparison to that of Rhod-2 (~15 %). This would suggest that there was less calcium buffering in our *BAPTA vs. Rhod-2* experiments.

Our findings regarding the effects of BAPTA support the idea that mechanically-dependent arrhythmias occurring during Phase IB are linked to calcium handling dysfunction. We believe that BAPTA and Rhod-2 may have played a role in buffering sudden increases in cytosolic calcium, which may occur during non-uniform contraction or relaxation. Interestingly, despite the rapid changes in calcium-handling occurring during ischemia, BAPTA and Rhod-2 significantly affected PVEs during Phase IB only. From this we can posit that while changes in cardiac mechanics contribute to both Phase IA and Phase IB arrhythmias,[12,14] the former may be linked to mechanisms independent of calcium-driven afterdepolarizations. As ischemia progresses, mechanically-induced calcium release occurs with an increasing efficacy to depolarize surrounding tissue. As mentioned in

Section 1.4, cell-cell uncoupling begins to occur during Phase IB as the accumulation of calcium and reduction in ATP facilitate CX43 closure.[13,69] These changes in tissue connectivity, namely increased tissue resistance and reduced source-sink interactions, may augment the propensity for calcium-driven events to evolve into propagating activations. Indeed, Myles *et al.* have shown that mild uncoupling of tissue increases the area of tissue experiencing shortened voltage – calcium activation delays post-NE injection, suggesting greater calcium release synchronization.[205]

4.4 The Effects of RyR Stabilization on the Incidence of Arrhythmias

While the utilization of BAPTA provided useful insight into whether increases in cytosolic calcium contribute to ventricular arrhythmias, calcium is located in a variety of cellular and subcellular organelles. As a high affinity calcium chelator, BAPTA rapidly and non-discriminately binds to available cytosolic calcium. Therefore, using this method we were unable to determine the origin of these mechanically-dependent cytosolic calcium surges.

As mentioned previously, rapid and non-uniform relaxation causes a large release of calcium from the myofilaments. In order to investigate the contribution of calcium-myofilament offloading, we would need a method for attenuating calcium release from Troponin-C. This effect would be difficult to attain without altering excitation contraction coupling of the myocardium. As such, we decided a more prudent approach would be to ameliorate stretch-induced SR RyR leak (sparks), which has been shown to occur in isolated cardiomyocytes through cytoskeletal, NOS, or NOX dependent pathways.[136,138,208] In pilot experiments (results not shown), we tested the use of diphenyleneiodonium (DPI), which has been used in cell experiments to block NADPH-oxidase (NOX) ROS

production.[140] Interestingly, even at a low dosage (3 μM), DPI significantly and progressively reduced developed pressure (n = 2). This suggests that either ROS production is essential for force generation or DPI is a non-specific agent to NOX. It has been shown in macrophages that DPI may antagonize nitric oxide synthase (NOS) activity,[216] that of which appears to be important for fractional shortening in isolated cardiomyocytes[209]. As contraction is essential to our experiments, it was decided to avoid agents, either NOS or NOX antagonists, which may reduce the contractility of the heart and instead target the RyR channel and its accessory proteins.

The RyR2 channel, the isoform found most abundantly in the heart, is regulated by many accessory proteins including cytosolic calmodulin, FK506-binding protein (FKBP) 12.6, PKA, and protein phosphatases, as well as luminal triadin, junction, and calsequestrin.[113] FKBP 12.6 is of particular interest as it regulates the open probability of the RyR2 channel. As such, phosphorylation of FKBP 12.6 by PKA or dephosphorylation by phosphatase activity augment or attenuate RyR2 release, respectively.[217]

The first pharmacological agent utilized in our studies was 1,4-benzothiazepine derivative, JTV-519 (also known as K201), which has been shown to increase the association of FKBP 12.6 with RyR2[218] and ameliorate spontaneous calcium release[219,220]. During pilot experiments (n = 2) we investigated the effects of this agent on electrophysiological parameters and a reduction in excitability (dF_V/dt_{max}) and 2:1 atrioventricular block was quickly discovered (Section 3.4). This finding recapitulated prior literature regarding the significant off-target effects of JTV-519, namely antagonism of I_{Kr},[160] I_{Na},[161] and activation of mitochondrial $I_{KATP.}$[162] Furthermore, similar to our findings, JTV-519 was shown by Ito *et al.* to cause 2:1 atrioventricular block.[162]

Since the late 1970s, a therapeutic known as 'dantrolene sodium' (commonly used to treat malignant hyperthermia, a condition caused by the delayed closure of RyR1 channels in response to general anesthetics)[221] was noted for its potential to act as an anti-arrhythmic. This observation came as the infusion of dantrolene was shown to reduce arrhythmias, a common sequelae of malignant hyperthermia.[222] Despite its proposed skeletal muscle specificity, early cardiac electrophysiological studies of dantrolene have found prolonged refractory periods,[222] extended APD,[223,224] and reduced AP plateau and peak calcium release (similar to that of verapamil)[225]. This was theorized to be a potential anti-arrhythmic agent by creating wave break and reducing the frequency of sustained arrhythmias.[226] Recently, dantrolene sodium is experiencing a renaissance of sorts, gaining recognition as an agent to improve the stability of the RyR2 channel. Dantrolene is thought to achieve this by preventing subunit "domain unzipping"[165] and has been shown to reduce calcium sparks in cardiomyocytes isolated from failing hearts[164,165] and in the context of catecholaminergic polymorphic ventricular tachycardia[163,227]. Furthermore, there is evidence supporting the use of dantrolene *in vivo* to improve survival outcomes post VF.[228] These results are attributed to reduced diastolic SR calcium release and improved CaT-ALT threshold, ameliorating both afterdepolarizations and heterogeneity of repolarization. However, dantrolene is not without its side-effects and at high concentrations (10 μM) has been shown to prolong APD and slow conduction velocity.[226] In pilot experiments ('*Dantrolene-(P)*') we investigated the effects of a commonly used low dose of dantrolene (1 μM)[164,229] on cardiac electrophysiology (n = 4). While there were no apparent effects on APD, there was a slight reduction in the Phase 0 upstroke velocity after 30 minutes (Figure 3.12). This may imply that there is a slight reduction in the availability of I_{Na}, however there is non-conclusive evidence as to whether or not dantrolene alters the AP upstroke.[224,225]

Our investigation into the effects of dantrolene in loaded regionally ischemic hearts ('*Dantrolene*') revealed a significant reduction in the incidence of PVEs during Phase IB (Figure 3.14). This finding was similar to that of calcium buffering addressed in the last section and suggests that mechanically-dependent arrhythmias during Phase IB are the result of aberrant SR RyR2 channel openings, which have been shown to contribute to intracellular calcium overload during ischemia.[230] Should these openings be the result of cytoskeletal-RyR2 anatomical coupling as suggested by Iribe *et al.*, it is unlikely that the pharmacological stabilization of RyR2 subunits would have been effective. It may in fact be more conceivable that post-translational modifications to the RyR2 channel are responsible for the increased open probability. These modifications may be the result of NO or ROS production and subsequent RyR2 *S*-nitrosylation[231,232] or *S*-glutathionylation[207]. NOX activation may play a more dominant role during ischemia as its cytosolic and nuclear expression has been shown to increase in an *in vitro* model of ischemia (*via* metabolic inhibition).[233] Furthermore, NOX expression has also been found to be increased in human hearts following acute myocardial infarction.[234] This implies that ischemia-induced ROS production, in addition to mechanically-induced ROS production resulting from non-uniform contraction, may be sufficient in augmenting the open probability of RyR2 during late ischemia. If calcium sparks initiate calcium waves and afterdepolarizations, they may be the source of calcium driven events. As mentioned above, this mechanical-effect on SR leak may be arrhythmogenic during the latter Phase IB, as cell-cell uncoupling reduces source-sink interactions. Furthermore, altered cardiac mechanics at the ischemic border zone may in fact provide a means for synchronization of calcium release from the RyR2 channels.

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

This thesis sought to investigate the mechanisms of mechanically-induced arrhythmias during ARI in the isolated rabbit heart. It was hypothesized that there is a contribution of abnormal calcium handling to these arrhythmias.

We have shown that during ARI, profound alterations to intracellular calcium handling occur in the absence of cardiac mechanics. These changes, namely: i) genesis of a vulnerable window for calcium-driven afterdepolarizations (CaTD – APD); ii) impaired calcium release and removal, and; ii) CaT-ALT and APD-ALT, occur with increasing magnitude into Phase IB and may promote an optimal environment for calcium driven events to occur. Despite these profound changes, the presence of cardiac mechanical activity was shown to be essential for the genesis of arrhythmias. Dual-parametric optical mapping of voltage and calcium in mechanically-coupled hearts did not reveal calcium driven events in the face of significantly reduced PVEs, which was attributed to the buffering of calcium by Rhod-2, and validated using BAPTA. Calcium buffering experiments revealed that calcium-dependent ectopy occurred specifically during Phase IB, with buffering potentially playing a role in attenuating sudden calcium surges, and preventing afterdepolarizations. Through the stabilization of the RyR2 using dantrolene, we were able to attenuate the incidence of Phase IB arrhythmias. Thus, overall our results suggest that mechanically-induced arrhythmias during Phase IB, are linked to alterations in calcium handling. Alterations in mechanics, namely non-uniform contraction and relaxation at the ischemic border may synchronize cytosolic calcium release from RyR2 channels and, in light of cell-cell uncoupling, overcome source-sink interactions to facilitate triggered activity.

The investigation of the importance of mechanics in the genesis of arrhythmias during ARI has more-or-less taken a 'top-down' approach in the hierarchy of Systems Biology.[235] Kaplinsky *et al.* first illustrated the biphasic response of arrhythmias in an *in vivo* canine model of coronary artery occlusion.[11] This finding, in conjunction with literature indicating that stretch of cardiac tissue alters electrophysiology, led Coronel *et al*. to the discovery of the importance of ventricular load both *in vivo* and *ex vivo*.[12] Research from our laboratory has since complemented this work in an *ex vivo* small animal model,[14] illustrating the importance of altered calcium handling. However, to continue this reductionist approach we must move from organ to tissue and tissue to cell to better define the effects of mechanics during ischemia on intracellular calcium handling. Future work should focus on an in-depth examination of the changes in calcium handling occurring at the cellular level during simulated ischemia and in the context of cyclic stretch. Single cell studies will allow us to test whether altered mechanics (cyclic stretch) during simulated ischemia is facilitating RyR calcium leak and afterdepolarizations to occur.

Ultimately, our findings may provide useful insight for the development of novel treatments for ARI arrhythmias. As Phase IB arrhythmias are documented to be more lethal than Phase IA, with a greater occurrence of VF, we hope that our findings on the importance of abnormal calcium handling will open the doors to a more targeted approach for treating SCD. As a clinically approved pharmaceutical, dantrolene may be an interesting agent for the treatment of ARI arrhythmias. However, current literature has shown increasing dosages of dantrolene can have substantial, potentially pro-arrhythmic off-target effects. Further investigation is required to affirm the optimal and safe dosage of dantrolene in humans. It is our hope that the continuation of basic science research in this field will

contribute to translational research in medicine to improve the quality of life of people around the world.

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