MECHANISM UNDERLYING BRADYCARDIA AND LONG QT 2 RELATED ARRHYTHMIAS: INTERPLAY BETWEEN Ca^{2+} OVERLOAD AND ELECTRICAL DYSFUNCTION

by

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In numerous pathologies, spontaneous Ca\(^{2+}\) release (SCR) emanating from the sarcoplasmic reticulum and occurring during the action potential (AP) plateau can drive voltage instability that initiates arrhythmias, but the direct interplay between SCRs and arrhythmogenesis has not been fully understood in bradycardia and in long QT type 2 (LQT2) models.

Simultaneous optical measurement of intracellular Ca\(^{2+}\) transient (Ca\(_i\)T) and AP were performed in Langendorff-perfused rabbit hearts following AV node ablation. Bradycardia and/or LQT2 was/were induced and the spatial heterogeneity of intracellular Ca\(^{2+}\) handling and its link to voltage dispersion were investigated.

Upon switching from 120 to 50 beats/min, AP duration (APD) increased gradually with increasing occurrence of SCRs during the AP plateau (p<0.01, n=7). SCR was a) regionally heterogeneous, b) spatially correlated with APD prolongation, c) associated with enhanced dispersion of repolarization (DOR), d) reversed by pacing at 120 beats/min and e) suppressed with K201 (1µM) or flecainide (5µM), inhibitors of cardiac ryanodine receptors (RyR2) which reduced APD (p<0.01, n=5) and DOR (p<0.02, n=5). Western blots of Ca\(^{2+}\) channels/transporters revealed intrinsic spatial distributions of Cav1.2α and NCX (but not RyR2, and SERCA2a) that correlate with the distribution of SCR and underlie the molecular mechanism responsible for SCRs.
In LQT2, lability of Ca$_i$, voltage, and ECG signals increased during paced rhythm, before the appearance of early afterdepolarizations (EADs). When EADs appeared, Ca$_i$ occasionally rose before voltage upstrokes at the origins of propagating EADs. Localized, areas of SCRs appeared in LQT2 and corresponded to areas of prolonged Ca$_i$T and APD. Triggered activity appeared after 3-5 min of LQT2 and emanated only at sites with steep membrane potential ($V_m$) gradients ($\Delta V_m$ gradient percentile: 94.9 ± 3.2%, n=6). Pre- or post-treatment with K201 suppressed SCRs and decreased DOR, $\Delta V_m$ and $\Delta$Ca$_i$. The reduction of $\Delta V_m$ suppressed triggered activity (n=8/9 hearts).

The results show that bradycardia and LQT2 elicit spatially discordant SCR, which is tightly correlated with AP instability. The SCR mediated-enhancement of repolarization gradients and AP prolongation can promote arrhythmogenesis. These findings underscore the importance of a detailed understanding of Ca$^{2+}$-dependent arrhythmogenic mechanisms for the development of rational treatment strategies.
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1.0 INTRODUCTION

Coordinated propagation of electrical activity throughout the heart ensures synchronized cardiac contraction generating sufficient forces for blood pumping. However, some pathological conditions can transform an electrically stable heart into one that unstable, which leads to the generation of cardiac arrhythmias. In pathological states, interruption in the spatial organization of cardiac wave propagation as a consequence of functional or anatomical conduction barriers can initiate cardiac arrhythmias such as ventricular tachycardia (VT) or ventricular fibrillation (VF).[1, 2] Experimental studies suggest that dispersion of repolarization (DOR) or spatial heterogeneity of cardiac activity increases in numerous pathologies of the heart and has been proposed as a fundamental mechanism for the initiation and/or maintenance of arrhythmias when the substrate is challenged with premature depolarization (i.e. premature ectopic beats or triggered activity).[3-6] Intrinsic regional differences in electrophysiological properties and pharmacologic responsiveness of ventricular myocardium have been implicated as a factor of DOR. For example, preferential AP prolongation of M-cells in response to antiarrhythmic class III drugs has been linked to mechanisms fundamental to markedly enhanced transmural DOR under LQT conditions.[7] However, our understanding of the augmentation of DOR throughout a heart under various disease conditions still remains rudimentary.

In addition to DOR, it is well established that triggered activity as a consequence of increases in tissue excitability play an important role in cardiac arrhythmogenesis. Although the
emerging role of altered intracellular Ca\(^{2+}\) dynamics in the initiation of arrhythmogenic triggered activity such as early afterdepolarizations (EADs) or delayed afterdepolarizations (DADs) has been extensively reported, the interplay between abnormalities in intracellular Ca\(^{2+}\) handling and arrhythmogenesis is not fully appreciated. In normal conditions, cardiac contraction and relaxation are tightly modulated by membrane potential (V\(_{m}\)) and by Ca\(^{2+}\) uptake and release which are controlled by proteins on the sarcoplasmic reticulum (SR) membrane (V\(_{m}\) \(\rightarrow\) Ca\(^{2+}\) coupling). However, in certain diseases an increase in RyR2 open-probability or interruption in Ca\(^{2+}\) homeostasis due to an imbalance between Ca\(^{2+}\) influx and efflux promotes abnormality in Ca\(^{2+}\) handling, such as ‘non’ voltage gated Ca\(^{2+}\) release. The spontaneous Ca\(^{2+}\) release from sarcoplasmic reticulum (SR), in turn, elicit V\(_{m}\) instability via Ca\(^{2+}\) dependent sarcolemmal transporters (Ca\(^{2+}\) \(\rightarrow\) V\(_{m}\) coupling).[8-11] Specifically, Ca\(_i\) overload in internal Ca\(^{2+}\) stores and altered kinetics of Ca\(_i\) transients have been documented in ischemia-reperfusion arrhythmias, in congenital and drug-induced forms of LQT that produce a characteristic polymorphic ventricular tachycardia, called Torsade de Pointes (TdP), and in heart failure that leads to reentrant arrhythmias.[12-15]

In addition to the impact of spontaneous SR Ca\(^{2+}\) release in the initiation of triggered activity, regional variations in intracellular Ca\(^{2+}\) [2] handling produce long-short-long-short Ca\(_i\) transients that are responsible for spatially discordant V\(_{m}\) instability in heart failure, ischemia, long QT syndrome. Recently, our group reported that spatially inhomogeneous Ca\(^{2+}\) oscillations occur before the onset of ‘electrical’ cardiac arrhythmia during drug-induced LQT2.[16] Such enhanced spatial heterogeneity of Ca\(_i\) handling in pathologies can be explained by multiple factors such as intrinsic ion channel/pump distribution, non-uniform ion channel remodeling, local dispersion of sympathetic nerve ganglion, and regional differences of cellular properties.[5,
Several experimental studies in animal models have shown that the non uniform distribution of cellular properties produces regional differences of Ca\textsubscript{i} handling.\cite{11, 12, 19-21} It is therefore likely that the spatial heterogeneity of Ca\textsubscript{i} handling could be linked to local dispersion of action potential (AP) via Ca\textsuperscript{2+} dependent sarcolemmal transporters.\cite{22} The enhanced AP dispersion could serve either as conduction blocks initiating reentrant arrhythmias or as re-excitant activity along the abnormal voltage gradient. However, evidence that high spatiotemporal heterogeneity of Ca\textsubscript{i} handling is the underlying mechanism of cardiac arrhythmogenesis is lacking.

It is well established that bradycardia increases the likelihood of triggering TdP associated with delayed repolarization.\cite{23-25} However, the mechanism through which bradycardia facilitates TdP remains unknown. In our previous study, we observed a moderate degree of secondary Ca\textsuperscript{2+} elevation with only bradycardia and the bradycardia dependent abnormality in Ca\textsubscript{i} handling was fully reversible by increasing the pacing rate.\cite{16} Despite an extensive body of clinical literature suggesting that tachycardia induced alternations of Ca\textsubscript{i} handling is responsible for electrical instability,\cite{26, 27} the interplay between bradycardia-related abnormality in Ca\textsubscript{i} handling and electrical instability has not been investigated.

Here, we used simultaneous optical measurement of Ca\textsubscript{i} handling and AP dynamics in perfused rabbit hearts, and report for the first time that non-voltage gated secondary Ca\textsuperscript{2+} release (SCR) in drug induced LQT2 or in bradycardia plays a crucial role in the further prolongation of AP. Moreover, the spatial heterogeneity of SCR promotes voltage dispersion across the anterior surface of rabbit hearts. Such Ca\textsuperscript{2+}-dependent augmentation of voltage dispersion promotes lethal cardiac arrhythmias by initiating arrhythmogenic triggered activity along the voltage gradient and/or by providing electrophysiological substrates for reentrant activity.
2.0 SPECIFIC AIMS

The goal of the proposed research is to investigate the interplay between Ca\(^{2+}\) overload and electrical dysfunction in bradycardia and in drug-induced long QT type 2. A complete understanding of the mechanism underlying Ca\(^{2+}\)-dependent electrical dysfunction and enhancement of spatial organization of tissue excitability may provide more reliable anti-arrhythmic therapies. The specific aims of the proposed research are the following:

**Specific Aim #1:** To test the hypothesis that bradycardia can promote an imbalance between Ca\(^{2+}\) influx and Ca\(^{2+}\) efflux, which elicits a secondary Ca\(^{2+}\) release (SCR) from internal Ca\(^{2+}\) stores. SCR can produce an arrhythmogenic substrate if a) it prolongs APDs through a reverse Ca\(_i\)-V\(_m\) coupling during AP plateau and b) SCR is spatially heterogeneous resulting in enhanced DOR. This study will provide a mechanistic explanation for bradycardia-dependent arrhythmias, which is an important clinical problem.

**Specific Aim #2:** To test the hypothesis that secondary Ca\(^{2+}\) oscillations (SCa\(_i\)O) can promote LQT2 related arrhythmias by initiating triggered activity and by promoting dispersion of repolarization. This includes the impact of SCa\(_i\)O on a) voltage instability, b) the initiation of early afterdepolarizations (EADs) or delayed afterdepolarizations (DADs), and c) enhanced DOR leading to triggered activity along lines of voltage gradients. This study will demonstrate the critical role of spatial heterogeneity of Ca\(^{2+}\) abnormalities in the initiation and the maintenance of LQT2-related arrhythmias.
3.0 BACKGROUND

During sinus rhythm, the pacemaker action potential initiates cardiac activation consisting of electrical waves are highly organized from beat-to-beat and control the mechanical contractions associated with each electrical impulse or rhythmic electrical activity. However, during ventricular tachycardia (VT) and ventricular fibrillation (VF), the wave propagation is interrupted by anatomical or functional conduction blocks.[1, 2] The heart loses its rhythmic activity, local muscle contractions become asynchronous with a can-of-worm appearance, and the heart fails to pump blood adequately. The occurrence of arrhythmogenic conduction blocks can be caused by heterogeneities of slowed conduction or of recovery of excitability.[28, 29] More recently, it has been established that disorders in the ion channel kinetics in numerous pathologies produce ionic imbalance between transsarcolemmal membranes. The interruption in ionic homeostasis can also leads to Ca$^{2+}$ overload in internal SR stores, extracellular accumulation of K$^+$ concentration, and intracellular elevation of Na$^+$ concentration, which have been implicated as arrhythmogenic risk factors. In this section, the underlying mechanisms for ionic imbalance and the interplay between the ionic imbalance and arrhythmogenesis will be introduced.
3.1 ELECTROPHYSIOLOGY IN MAMMALIAN HEARTS

Rhythmic excitation and contraction (EC) coupling ensures appropriate blood supply throughout the body. During sinus rhythm, the action potential (AP) of a pacemaker initiates a normal regular cardiac activation, electrical waves are organized, and mechanical contractions associated with the electrical activities are rhythmic (see Figure 1). Such electrical activity, called the cardiac AP, is tightly regulated by changes in membrane conductance and ionic movement across the sarcolemmal membrane. The ventricular AP consists of five phases, number 0 – 4 (see Figure 2).[30] Phase 0 represents membrane depolarization as a consequence of rapid activation of voltage gated Na\(^+\) channels (I\(_{Na}\)). During phase 1 of AP, partial repolarization occurs immediately after the peak of the AP due to the inactivation of Na\(^+\) channels combined with activation of transient of outward K\(^+\) channels (I\(_{to}\)). During phase 2 of the AP, which is the plateau phase that is characteristic of a cardiac AP, Ca\(^{2+}\) channels (I\(_{Ca,L}\)) open and let Ca\(^{2+}\) into the cell while delayed rectifier K\(^+\) channels open letting K\(^+\) come out of the cell. The simultaneous activity of two currents acting in opposite directions causes this plateau phase. Phase 3 represents rapid repolarization of AP as a result of activation of outward K\(^+\) currents. Phase 4 is the final phase of AP, and the membrane potential returns to resting potential.
Figure 1. Heart and conduction system A) Schematic diagram of heart and conduction system.[30] Cardiac rhythms arising in the SA node (SAN) cause atrial contraction (right atria (RA) and left atria (LA)). In parallel, electrical activity travels to the AV node (AVN) via internodal pathways. After a delay, the sinus rhythm is conducted to the left bundle branch (LBB) and the right bundle branch (RBB) then to the purkinje fibers and the endocardium at the apex of the heart, then finally to the ventricular myocardium (right ventricle (RV) and left ventricle (LV)). B) Examples of normal ECG (top) and long QT ECG (bottom). QT prolongation appears in long QT ECG.

Figure 2. Cardiac action potential and ion currents [30] Inward $I_{Na}$ causes a rapid upstroke of cardiac AP (Phase 0). Ito mediates phase 1 notch. $ICaLCa,L$ and delayed rectifying outward $K^+$ currents ($I_{Kr}$, and $I_{Ks}$) sustain AP plateau (Phase 2). Outward $I_{Kr}$ and $I_{Ks}$ mediates rapid AP repolarization (Phase 3) and $I_{K1}$ helps recovery of resting membrane potential (Phase 4).
3.1.1 K⁺ dynamics

K⁺ channels are ubiquitous membrane proteins that contribute to cardiac electrical activity by regulating membrane resting potential and AP repolarization. The resting membrane potential is determined by the K⁺ gradient between the extracellular and cytosolic space. Since activation and the recovery from inactivation of voltage gated I_{Na} and I_{Ca,L} currents are highly dependent on the membrane potential, K⁺ dynamics is a dominant factor in regulating tissue excitability.[31] Alternation of extracellular K⁺ concentration ([K⁺]_{ex}) is also recognized as a major factor of automaticity of both pacemaker cells and working myocytes.[32, 33] For instance, a moderate increase in [K⁺]_{ex} causes the elevation of resting membrane potential in pacemaker cells.

Furthermore, various K⁺ channels contribute to action potential (AP) dynamics. For example, the delayed rectifier current (I_{Kr} and I_{Ks}) elicit AP repolarization. Transient outward current (I_{to}) contributes to the repolarization of phase 1 of AP, and inward rectifier current (I_{K1}) helps recovery to the resting membrane potential by mediating a small hyperpolarizing K⁺ current.[30]

3.1.2 Interventions of K⁺ dynamics and cardiac arrhythmias

Experimental studies have shown that elevations in [K⁺]_{ex}, known as hyperkalemia, can be caused by oxygen depletion due to coronary occlusion and are associated with arrhythmogenesis.[34, 35] For example, ischemia-induced hyperkalemia predisposes the heart to the development of lethal reentrant ventricular arrhythmias by causing sustained membrane depolarization that leads to slowing conduction and altered refractoriness.[36] It is also widely
recognized that potassium K\(^+\) deficiency can be associated with a variety of cardiac arrhythmias. For example, it is well known that reduced K\(^+\) concentration in blood serum, known as hypokalemia, decreases the delayed rectifier current (I\(_{Kr}\)), and results in cardiac arrhythmias by causing AP prolongation.[37]

In addition, due to the primary roles of K\(^+\) currents in AP repolarization, abnormalities in K\(^+\) currents during APs often induce life-threatening tachyarrhythmias. Typically, inhibition of I\(_{Kr}\) is thought to result in arrhythmogenic AP prolongation and promote the occurrence of LQT2-related arrhythmias such as TdP.[38] In addition, abnormal AP shortening reduces refractory periods and provides a favorable substrate for reentrant arrhythmias.[39]

3.1.3 Extracellular K\(^+\) accumulation (EKA) and its role in arrhythmogenesis

Voltage-clamp and K\(^+\) selective electrodes have shown that K\(^+\) accumulation occurs in T-tubules and narrow clefts between cells indicating that K\(^+\) concentration ([K\(^+\)]) in these narrow spaces is not in diffusional equilibrium with the external K\(^+\) concentration (∼4.7 mM).[40, 41] In normal physiology, K\(^+\) efflux from the cell is pumped back via Na/-K pump - and the local external [K\(^+\)] recovers on a beat-to-beat basis.[42] However, at high heart rates or during ischemia, a local EKA rise of 2-5 mM can cause a small but significant depolarization which alters excitability and can change the shape and time course of subsequent APs.[43, 44] Such a spatial and dynamic variation of EKAs can cause variations in tissue excitability, produce local conduction blocks, and has been shown to be sites of premature ectopic beats.[45, 46] High EKAs can occur during stress in physiological conditions such as high heart rates where EKAs may not fully recover from beat to beat resulting in [K\(^+\)]\(_{ex}\) elevation or from local coronary occlusion and the ensuing ischemia and may worsen during VT and VF. [47, 48]
variation of EKAs induces inhomogeneous slowing of conduction velocity, which may alternate
dynamically to cause blocks of wave propagation that can lead to triggered out-of-phase ectopic
beats.[43, 44, 47, 48] The dispersion of EKAs can lead to inhomogeneous tissue excitability, and
alternans, and the spatial excitability variation plays an important role in initiating and
maintaining cardiac arrhythmias.

3.1.4 Na⁺ dynamics

The voltage-gated cardiac Na⁺ channel is responsible for the generation of the rapid
membrane depolarization during cardiac AP and thereby plays a crucial role in the excitability of
cardiomyocytes.[49] In addition, since the AP upstroke velocity also determines impulse
conduction velocity in cardiac tissue, this channel also contributes to the conduction of electrical
activity through the heart by facilitating intercellular communication via gap junctions.[50] It is
also well known that Na⁺ dependent transporters such as Na-K pump, Na/Ca exchanger (NCX),
and Na/H exchanger (NHE) [51], play a crucial role in regulation of ionic homeostasis.[52]

Due to the crucial role of Na⁺ channels in tissue excitability and conductivity, its
abnormal activity has been implicated as a major risk factor in various arrhythmogenic
models.[53-55] Inherited mutations in SCN5A that encode the α subunits of Na⁺ channels result
in several different types of arrhythmias. As a consequence, the gain-of-function mutations in the
gene increases Na⁺ influx during APs, then prolongs cardiac AP, and eventually promotes long-
QT type 3 related arrhythmias.[56] Alternatively, deletion or loss-of-function mutations of the
gene have been linked to multiple types of arrhythmias including bradycardia, atroventricular
(AV) conduction delay, and Brugada syndrome.[57-60] Also, pharmacological blocks of cardiac
Na⁺ channels result in slowing conduction and produce marked sudden cardiac death.[61]
In healthy cardiac myocytes, intracellular Na\textsuperscript{+} concentration ([Na\textsuperscript{+}]) is tightly regulated by Na\textsuperscript{+} dependent transporters such as NCX, Na-K ATPase, and NHE. Under some pathological conditions, those regulatory processes can be interrupted and result in [Na\textsuperscript{+}] accumulation.[17, 62] The elevation of [Na\textsuperscript{+}]\textsubscript{i} reverses NCX and promotes sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} overload. Such a cellular Ca\textsuperscript{2+} overload, secondary to Na\textsuperscript{+} overload, can increase the propensity of non-voltage gated SR Ca\textsuperscript{2+} release, which is arrhythmogenic.[63]

3.1.5 Ca\textsuperscript{2+} dynamics

Ca\textsuperscript{2+} is primarily responsible for cardiac contraction. Cardiac AP initiated by Na\textsuperscript{+} entry across the sarcolemma activates voltage-gated L-type Ca\textsuperscript{2+} channels, and Ca\textsuperscript{2+} entry which triggers SR Ca\textsuperscript{2+} release by a phenomenon known as Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR).[64, 65] Transient increases in Ca\textsubscript{i} concentration force Ca\textsuperscript{2+} ions to bind to the myofilament protein troponin C and eventually initiate cardiac contraction (see Figure 3). In addition to its role in cardiac contraction, Ca\textsuperscript{2+} regulates ionic influx and efflux via NCX or calcium-gated activated potassium K\textsuperscript{+} channels (see Figure 3).[66, 67] In Ca\textsuperscript{2+} mediated signaling pathways, Ca\textsuperscript{2+} regulates many different cellular functions.[68, 69]
3.1.6 Abnormality in Ca$^{2+}$ handling and its role in cardiac arrhythmogenesis

Cardiac contraction is initiated by an increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) as a result of SR Ca$^{2+}$ release via RyR2 in response to Ca$^{2+}$ entry through the L-type Ca$^{2+}$ channels, known as the phenomenon of CICR. In general, abnormal Ca$^{2+}$ handling such as SCR has been implicated as a fundamental mechanism that underlies the induction of arrhythmias in numerous cardiac diseases.[11, 16, 70-72] The SCR has been documented in ischemia-reperfusion arrhythmias, congenital and drug-induced forms of long QT that produce polymorphic VT, and in heart failure that leads to reentrant arrhythmias. [8, 14, 70, 73]

In experimental settings, diverse hypotheses for the mechanism underlying SCR have been proposed. For example, in the condition of delayed repolarization or of inhibition of Na-K ATPase by cardiac glycosides, the SR can become overloaded with Ca$^{2+}$. The SR Ca$^{2+}$ overload makes SR more prone to SCR. Such SCR from the SR propagates as a Ca$^{2+}$ wave by CICR.[63] Some of the Ca$^{2+}$ released during a wave is removed from the cell by the electrogenic NCX
resulting in a net depolarization. This is the cellular mechanism underlying delayed afterdepolarizations (DADs), which often precede certain types of fatal cardiac arrhythmias.[74, 75] More recently it has been proposed that arrhythmogenic SCR can also occur due to the increase in RyR2 open probability as a consequence of increase in CSQ or RyR2 sensitivity to luminal free Ca\(^{2+}\) due to CPVT mutations.[76, 77] Redox modification of RyR2 by oxygen free radicals increases RyR2 leakage and facilitates SCR, which triggers ischemia-reperfusion arrhythmias.[71, 78] Protein kinase A (PKA) mediated hyperphosphorylation of RyR2 has been reported in heart failure.[79, 80] If such a SCR occurs before the complete termination of AP repolarization, the SCR elevates cytosolic Ca\(^{2+}\) level and results in arrhythmogenic triggered activity such as EADs by promoting Ca\(^{2+}\) sensitive inward currents (I\(_{\text{NCX}}\)).[81, 82]

### 3.1.7 Regional variation of Ca\(_i\) handling and its link to arrhythmogeneis

Some experimental studies in intact rabbit models have reported that gradients of Ca\(_i\) transients exist on the basal/apical and endocardial/epicardial axes.[19, 20] For example, the endocardium exhibits a slower uptake of intracellular Ca\(^{2+}\) compared with the epicardium, likely as a consequence of the transmural difference of SERCA2a expression. Recently, Sims et al. reported that a higher density of CaV1.2a (L-type Ca\(^{2+}\) channel gene) is expressed more at the base than the apex of the LV, and EADs in LQT2 are more pronounced in the base than the apex of the LV of adult female rabbits.[12] Furthermore, a clinical study has shown that the duration of cardiac contraction, which was measured by an ultrasound technique in LQT patients, is transmurally heterogeneous.[83]
3.2 LONG QT TYPE SYNDROME (LQT2)

Long QT syndrome (LQTS) is an abnormal condition in which prolongation of the APD can lead to sudden cardiac death by inducing polymorphic tachyarrhythmias. Among LQTS, LQT2 is the second most common gene location that is affected in long QT syndrome, making up about 25% of all cases. Typically, two different forms of LQT type 2 (LQT2), congenital LQT2 and acquired LQT2, have been characterized.[84-87] First, the congenital form represents a “pure global repolarization disease”. The congenital form of LQT2 is caused by mutation of human ether-a-go-go gene (HERG), also known as KCNH2 which encodes the alpha subunit of \( K^+ \) channel responsible for \( I_{Kr} \). The loss of function of \( I_{Kr} \) delays repolarization of AP and consequently results in APD prolongation. Second, the acquired LQTS is of critical importance because of its prevalence in the clinical setting. The high binding affinity of the HERG channel to a wide range of drugs due to its very susceptible amino acid residues has been a serious public health problem because cardiac and non-cardiac drugs inadvertently block the \( I_{Kr} \) channel from conducting currents, and impairs repolarization of AP.[88] In addition, several factors (e.g., medications, electrolyte abnormalities, and heart failure) delay ventricular repolarization and may lead to lethal arrhythmias.

3.2.1 EADs and DADs in LQT2

Under LQT2 conditions, the EADs are classically attributed to an inward current carried by L-type Ca\(^{2+}\) channels.[89] In other words, it has been proposed that under conditions conducive to EADs, the sarcolemma spends a longer period of time in the voltage range where both the activation and inactivation gates of L-type Ca\(^{2+}\) channel are partially open. The resulting
enhancement of $I_{\text{Ca,L}}$ during phase 3 of ventricular action potential may be sufficient to cause an 
EAD. Alternatively, secondary Ca$^{2+}$ release from an overloaded SR to the cytoplasm could cause 
EADs by augmentation of the electrogenic $I_{\text{NCX}}$, a mechanism previously shown to be 
responsible for the generation of delayed afterdepolarizations (DADs).[11, 14, 81]

3.2.2 Dispersion of repolarization (DOR)

Conventionally, regional differences in outward K$^+$ currents have been attributed to 
the DOR.[90-93] In mammalian hearts, K$^+$ channels are spatially heterogeneous across the wall 
of the ventricles (epicardium to endocardium) as well along the wall (base to apex). Experimental studies suggest such intrinsic DOR increases in numerous pathologic conditions 
(bradycardia, LQT, ischemia-reperfusion, and heart failure) and has been proposed as a 
mechanism fundamental to the initiation and maintenance of arrhythmias.[94-96] Typically, 
preferential action potential (AP) prolongation of M cells under conditions with reduced $I_{Kr}$ has 
been proposed as a major underlying mechanism responsible for enhanced transmural DOR in 
LQT2.[7, 97] Alternatively, functional differences in levels of $I_{Kr}$ along the surface of a heart that 
can be inhibited by class III anti-arrhythmic agents has been linked to a mechanism underlying 
epicardial DOR in cryoablated LQT2 models.[11]. In LQT2 models, once triggered activity 
occurs, enhanced DOR plays a crucial role in maintaining LQT2 related arrhythmias by 
providing a favorable substrate for unidirectional conduction blocks leading to reentrant 
arrhythmias.[98]
3.2.3 LQT2 related reentrant arrhythmia

If propagation of cardiac electrical activity is disrupted by anatomical or functional conduction blocks,[1, 2] the heart loses its rhythmic activity, cardiac muscle contraction is locally dissociated, and the heart fails to pump an adequate amount of blood. The arrhythmogenic conduction blocks can be caused by heterogeneous slowing of conduction and/or a spatial dispersion of recovery of excitability.[28, 29] In LQT2 conditions, reentrant activity occurs when the wave propagation of premature ectopic beats is interrupted upon encountering a region of refractory tissue but conducts though excitable areas (unidirectional propagation) leading to functional reentry, which results in LQT2 related polymorphic tachyarrhythmias, known as TdP (see Figure 4).[98, 99] An alternative mechanism has been proposed in optical mapping studies of Langendorff perfused rabbit hearts treated with the \( I_{Kr} \) blocker, E4031. In these studies APD prolongation was associated with \( Ca_{i} \) oscillations then became increasingly more severe and elicited EADs where \( Ca_{i} \) preceded the upstroke of EADs. EADs occurred at 2 to 4 sites in the heart and were of sufficient magnitude to propagate and collide with wavefronts emanating from other EADs. TdP was then caused by EADs firing out-of-phase from different sites causing the sinusoidal pattern seen on EKG recordings. Thus, optical images of TdP agreed with the hypothesis of Desertenne that was proposed in 1966.
Figure 4. Typical examples of human ECGs during TdP (top) and ventricular fibrillation (bottom) in LQT conditions [30] QTc prolongation precedes the occurrence of TdP.

Figure 5. ECG recording in lead D2 during T-wave alternans in a LQT patient [100] T-waves alter during 4 consecutive beats (beats I, II, III, IV).

3.2.4 T-wave lability (TWLI)

Electrocardiography (ECG) has been widely used in both clinical and experimental settings for the measurement of electrical activity of a heart. Typically, unusual morphology in ECG represents abnormalities in cardiac rhythms. Abnormal ECG characteristics such as QTc
prolongation and unusual morphology of T wave (alternans and variation) have been reported in LQT patients (see Figure 5).[100-103] Also, in a chronic heart failure model, inter-lead QT dispersion is markedly enhanced and beat to beat variation in repolarization morphology in ECG, known as TWLI, has been thought to be a marker of pro-arrhythmic conditions.[104]

### 3.2.5 Rate and time dependent APD adaptation and its relevance to LQT2 arrhythmias

APD adaptation, also known as QT accommodation, allows a heart to maintain an acceptable ratio between the period of ventricular filling and ventricular contraction.[105] Most studies attribute APD adaptation to changes in cytosolic Ca$^{2+}$ [2] which in turn alters the kinetics of Ca$^{2+}$-dependent inactivation of L-type Ca$^{2+}$ channels[64, 65, 106] but others have implicated the rate-dependence of $I_{NCX}$ [107] and of the late Na$^+$ current, $I_{Na,L}$.[108] Besides ion channel kinetics, changes in ionic concentrations in the cytoplasm (intracellular Na$^+$ and Ca$_i$) or extracellular K$^+$ contribute to APD adaptation.[109, 110] Mathematical simulations of slow heart rate (HR) indicated that the long diastolic intervals result in a complete deactivation of the slow component of the delayed rectifying K$^+$ current ($I_{Ks}$) and complete recovery from inactivation of L-type Ca$^{2+}$ currents ($I_{Ca,L}$) which could theoretically explain APD adaptation in bradycardia.[111-113] Long-term memory, unlike short term memory, is related to pacing induced ion channel remodeling (such as reduction in $I_{Ca,L}$ and $I_{to}$) taking several days to reach to a new steady-state, and plays an important role in long term APD accommodation. [114] The rate and time dependent APD-accommodation occurring within minutes is thought to be spatially heterogeneous due to the intrinsic heterogeneity of ion channel distribution, specifically $I_{Ks}$ distribution.[113, 115] Such a rate dependent APD prolongation is associated with markedly
enhanced dispersion of APD and explains why LQT2 related arrhythmia is more pronounced during bradycardia.

### 3.3 STEEP VOLTAGE GRADIENT AND ARRHYTHMOGENESIS

Experimental studies have suggested that steep voltage gradients can be produced by premature stimuli and/or myocardial infarction.[116, 117] Abnormal voltage gradients as a result of heterogeneous ion accumulation and cellular uncoupling across the ischemic border zone can account for the driving force for the diastolic ‘injury current’ to the normal side of the ischemic border. Such small injury currents can cause a slight depolarization of the normal tissue and may facilitate triggered activity.[116]

### 3.4 MEASUREMENTS OF CARDIAC AP AND INTRACELLULAR Ca\(^{2+}\) DYNAMICS

In the past, although glass pipette microelectrodes have contributed as great deal toward understanding of the ionic basis of the cardiac AP, the application of single cell impalements is not precise for studies requiring simultaneous recording of APs from multiple sites. The spatiotemporal pattern of wave propagation of cardiac electrical activity is an important marker to determine whether cardiac function is rhythmic or arrhythmic. Electrical mapping techniques, by using surface electrodes, have been used to study the spread of cardiac excitation, but the two most basic characteristics, activation and recovery, cannot be identified reliably at the site of recording for conditions other than uniform propagation. In addition, it is not possible to map
while a defibrillating shock is being applied and during slowly changing, low-level depolarization, which is seen in ischemia. Repolarization measured with an electrogram often does not coincide with the actual repolarization at the recording site. Since Salama and Morad for the first time made optical recordings of cardiac AP in 1976, the optical measurements of cardiac APs from the cellular to organ level have made a crucial contribution to cardiac research.[118] The principal theory of optical recordings of cardiac AP is that a membrane binding potentiometric fluorescent dye changes its spectral properties in response to changes in membrane potential. In other words, a membrane binding voltage sensitive dye exhibits wavelength shifts in peak fluorescence and/or absorption due to a change in membrane potential. The major advantages of optical mapping are thought to be 1) the impervious response to electrical defibrillation shock, 2) the fast response to voltage changes, and 3) the high spatiotemporal resolution.[119] In addition to optical recordings of cardiac AP with potentiometric probes, the optical measurements of changes in ion concentration have been widely performed. For example, changes in $\text{Ca}^{2+}$ concentration inside cardiomyocytes have been measured with $\text{Ca}^{2+}$ sensitive fluorescent probes such as $\text{Ca}^{2+}$ indicators Rhod-2 AM and fluo-4.[120, 121] Recently, simultaneous optical measurements of cardiac AP and $\text{Ca}_i$ dynamics at high spatial and temporal resolution have been performed and provide a powerful tool to investigate the role of $\text{Ca}_i$ anomalies in eliciting cardiac arrhythmias (see Figure 6).
Figure 6. Examples of simultaneous measurement of AP (blue) and Ca$_i$ (red) dynamics in a Langendorff perfused rabbit heart before and after inhibition of I$_{Kr}$ LQT2 was modeled by perfusing with Tyrode’s solution containing dofetilide (250 to 500 nM, Pfizer, New York, NY), a selective I$_{Kr}$ blocker and lowering K$^+$ and Mg$^{2+}$ concentrations by 50 %. A) Baseline recording at 500ms cycle length (CL). B) Baseline recording at 1200ms CL. C) 2 minutes after dofetilide infusion at 1200ms CL. D) 5 minutes after dofetilide infusion at 1200ms CL.
3.5 AGENTS TARGETING ABNORMAL Ca\(^{2+}\) HANDLING

Spontaneous SR Ca\(^{2+}\) release under diverse pathological conditions has been linked to initiation of cardiac arrhythmias, and suppression of the SCR has been considered as possible anti-arrhythmic therapies.[122, 123] So far, several pharmacological agents have been introduced as SCR suppressors, though their therapeutic strategies may differ. JTV519, also known as K201 stabilizes RyR2 in the closed state by enhancing the binding affinity of calstabin 2 for RyR2 and preventing the dissociation of calstabin 2 from RyR2.[124] It has been tested in ischemia injury models.[125] Flecainide, which is a well known Na\(^{+}\) channel blocker, also suppresses SCR by inhibiting RyR2 channels by open state block, and has been tested in CPVT mutation.[126] Captopril, which was introduced as an angiotensin converting enzyme inhibitor, acts as a reducing agent and thus reduces SCR by protecting against ischemia-induced oxidation.[127]
4.0 BRADYCARDIA INDUCES NON-UNIFORM SECONDARY Ca\textsuperscript{2+} RELEASE THAT ENHANCES ACTION POTENTIAL DURATION, DISPERSION OF REPOLARIZATION AND ARRHYTHMIA RISK

In heart muscle, the influx and efflux of Ca\textsuperscript{2+} across the plasma membrane are precisely balanced during each phasic contraction as is the coordinated Ca\textsuperscript{2+} release and reuptake across the sarcoplasmic reticulum (SR) network. Any deviation or imbalance between influx and efflux can only be small and transient to maintain cytoplasmic Ca\textsuperscript{2+} homeostasis.[128] A fundamental property of mammalian hearts is the force-frequency relationship or ‘Staircase Effect’ where a change in heart rate causes a transient imbalance between Ca\textsuperscript{2+} influx and efflux until a new steady state is attained where again Ca\textsuperscript{2+} influx equals efflux but at a new state of contractility. The staircase effect is associated with an inverse relationship between action potential (AP) duration (APD) and heart rate (HR) which is critical to achieve a stable state of contractility. The mechanism responsible for APD adaptation as a function of HR remains controversial. Most studies attribute APD adaptation to changes in cytosolic Ca\textsuperscript{2+} [2] which in turn alters the kinetics of Ca\textsuperscript{2+}-dependent inactivation of L-type Ca\textsuperscript{2+} channels[64, 65, 106] but others have implicated the rate-dependence of Na-Ca exchange current, I\textsubscript{NCX}[107] and of the late Na\textsuperscript{+} current, I\textsubscript{Na,L}.[108] Besides ion channel kinetics, changes in ionic concentrations in the cytoplasm (intracellular Na\textsuperscript{+} and Ca\textsubscript{i}) or extracellular K\textsuperscript{+} contribute to APD adaptation.[109, 110] Mathematical simulations of slow HR indicated that the long diastolic intervals result in a complete deactivation of the slow component of the delayed rectifying K\textsuperscript{+} current (I\textsubscript{Ks}) and complete recovery from
inactivation of L-type Ca\(^{2+}\) currents (I\(_{\text{Ca,L}}\)) which could theoretically explain APD adaptation in bradycardia.[111-113]

Of all the proposed explanations for APD adaptation, the role of Ca\(_i\) cannot be over-emphasized since independent of rate, APD is inversely dependent on external [Ca\(^{2+}\)] and Ca\(_i\) by one of the mechanisms that mediate Ca\(_i\) → V\(_m\) coupling. During a transition from slow to fast HRs, Ca\(_i\) rises due to the greater number of APs per unit time and decreased Ca\(^{2+}\) efflux due to shorter diastolic intervals. Higher Ca\(_i\) accelerates the Ca\(^{2+}\)-dependent inactivation of I\(_{\text{Ca,L}}\), reduces Ca\(^{2+}\) influx per AP which decreases APD; an important negative feedback process that limits total Ca\(^{2+}\) influx via I\(_{\text{Ca,L}}\)[129] and enables the system to reach a new steady state. Conversely, a transition from fast to slow HR slows down Ca\(^{2+}\) dependent I\(_{\text{Ca,L}}\) inactivation and the long diastolic intervals allow for the complete recovery of I\(_{\text{Ca,L}}\) from inactivation which prolong APDs.

HR regulates force generation in a physiological range of HRs but fast or slow HRs create severe Ca\(^{2+}\) handling abnormalities that in combination with various pathologies promote arrhythmias.[130] Transitions from slow to fast HR have been extensively studied and shown to create Ca\(^{2+}\) alternans that lead to APD and T-wave alternans and arrhythmias. [26] In the setting of ischemia[22, 131] and heart failure[132], arrhythmogenic Ca\(^{2+}\) and APD alternans occurred at physiological HR. In contrast, few studies have investigated events associated with bradycardia even though bradycardia is known to be an important co-factor required to trigger Torsade de Pointes in long QT types 2 and 3. In drug-induced long QT type 2 (LQT2), slow HR promotes spontaneous SR Ca\(^{2+}\) release that occurs during the AP plateau to produce early afterdepolarizations (EADs) that progress to Torsade de Pointes. [11, 16] At the level of intact hearts, bradycardia prolongs APD but also enhances dispersion of repolarization (DOR),[90,
and the enhanced DOR is thought to be a contributing factor to maintain LQT2-related arrhythmias.[133] In ventricular cells, the longer the cycle length the greater the APD prolongation but in intact hearts, the mechanism linking bradycardia to enhanced DOR and arrhythmia vulnerability remains unexplored.

This project investigates APD adaptation during bradycardia in Langendorff perfused rabbit hearts using dual optical mapping of Ca\textsubscript{i} and V\textsubscript{m} with high resolution CMOS Cameras. The data shows for the first time that bradycardia produces a spatially heterogeneous secondary Ca\textsuperscript{2+} release (SCR) from the SR which modulates APD through Ca\textsubscript{i}→ V\textsubscript{m} coupling and correlates with the enhanced dispersion of APD and DOR.

4.1 METHODS

4.1.1 Heart preparations

New Zealand White rabbits (15 female, 60 to 120 days old) were euthanized with pentobarbital (75 mg/kg intravenously) and anticoagulated with heparin (200 U/kg intravenously). The heart was rapidly dissected and perfused with Tyrode’s solution containing (in mM): 130 NaCl, 24 NaHCO\textsubscript{3}, 1.0 MgCl\textsubscript{2}, 4 KCl, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 50 dextrose, 1.25 CaCl\textsubscript{2}, at pH 7.2-7.4, gassed with 95% O\textsubscript{2} plus 5 % CO\textsubscript{2}. Temperature was maintained at 37.0 °C and perfusion pressure was adjusted to ≈70 mmHg with a peristaltic pump. To minimize motion artifact, blebbistatin (Sigma, St Louis, MO 5-10 μM) was added to the perfusate for 5-10 min. The heart was immobilized in a chamber and stained with a voltage-sensitive dye (PGH1: 200 μl of 1 mg/ml dimethyl sulfoxide (DMSO)) and loaded with a Ca\textsuperscript{2+} indicator (Rhod-2 AM, 200 μl
of 1 mg/ml DMSO). Epicardial bipolar pseudo-EKG was continuously monitored. Epicardial pacing with a unipolar electrode on the right ventricle was performed at a baseline cycle length of 0.5 seconds (baseline heart rate (BHR) of 120 beats/minute) or at a slow HR (SHR) with a cycle length of 1.2 seconds (50 beats/minute; profound bradycardia for rabbit hearts). This investigation conformed to the current Guide for Care and Use of Laboratory Animals published by the National Institutes of Health the Institutional Animal Care and Use Committee of the University of Pittsburgh.

4.1.2 Optical apparatus

An optical apparatus consisting of two CMOS Cameras (SciMedia, Ultima One, 100 by 100 pixels, at 250 frames per second) has been used for simultaneous measurement of intracellular Ca$^{2+}$transients and membrane potential changes, as previously described.[134] The anterior surface of the heart was illuminated with a 520 ± 30 nm excitation beam, and the fluorescence was passed through a dichroic mirror (660 nm) to focus the Rhod-2 and PGH1 fluorescence images on two CMOS Cameras.[134]

4.1.3 Study protocol

The AV node was ablated by cauterization to control heart rate. Hearts were paced at a cycle length of 0.5 s which was taken as a ‘baseline heart rate’ (BHR) then the cycle length was lengthened to 1.2 s to impose a bradycardia or a ‘slow heart rate’ (SHR). APs and Ca$_i$T were continuously recorded for 32 s during transitions from fast to slow or slow to fast HRs while measuring changes in AP duration (APD). Changes in HR lead to gradual changes in APD until
a new steady state was attained. The time-course of ‘APD adaptation’ required continuous recordings of 3-5 minutes to reach a steady state of APDs during transitions from BHR to SHR (bradycardia) and 5-10 minutes in going from SHR to BHR.

Pilot experiments were carried out to select the BHR and the SHR that were used in this study. A slow heart rate of 50 beats/min was chosen because it could be maintained reliably without interruptions by an occasional extra-beat yet was effective to expose spatial heterogeneities of Ca_iT caused by SCR. Similar, but less pronounced effects were observed at 0.9 and 1.0 s cycle lengths. A basic heart rate of 120 beats/min was chosen because a) this HR was well tolerated and did not result in ‘run-down’ of the preparations, b) capture with pacing electrodes was reliable during uninterrupted recordings and c) spatial heterogeneities of AP and Ca_iT were negligible compared to still faster rates.

### 4.1.4 Data analysis

T wave (TW) amplitude relative to QRS complex (TW/QRS) in EKG was used to approximate DOR. [135] Activation time at each site was calculated from (dF/dt)_{max} of the local AP or Ca_iT upstroke. APD and Ca_iT duration (Ca_iTD) at each site was calculated from the interval between (dF/dt)_{max} and the recovery of V_m and Ca_iT traces to 20% of baseline (APD_{80} or Ca_iTD_{80}), respectively. Automatic measurement of APD_{80} and Ca_iTD_{80} from all pixels (100 x 100 pixels) was used to calculate mean APD_{80} and Ca_iTD_{80}. The dispersion of APD_{80} and Ca_iTD_{80} was calculated from the SD of APD and Ca_iTD. The time constant (τ) of mean APD (from all 100x100 pixels) adaptation to heart rate changes was calculated by fitting the transitions from BHR to SHR (τ_{f→s}) and from SHR to BHR (τ_{s→f}) with mono-exponential functions. Briefly, the root mean square of differences between measured and predicted APD
values were used to choose the best curve fit. Rate and time dependent mean APD$_{80}$ and Ca$_{i}$TD$_{80}$ and regional variation in 100 x 100 pixels were statistically evaluated. Bradycardia dependent AP/Ca$_{i}$T prolongation and dispersions before and after K201 (1 µM) or flecainide (5 µM) perfusion were compared. K201 (3-(4-Benzylcyclohexyl)-1-(7-methoxy-2,3-dihydrobenzo[f][1,4]thiazepin-4(5H)-yl)propan-1-one) was synthesized according to the procedure reported by Wehrens et al.[136] The area under the curve [137] of Ca$_{i}$T at each pixel was normalized by setting the minimum value to zero and the maximum AUC to 1. Maps of AUC were generated at steady state BHR and SHR. AUC was used as a measurement of the relative Ca$_{i}$ at each site. Scatter plots of AUC versus APD$_{80}$ were used to correlate these two parameters and calculate a correlation coefficient (r) between AUC and APD$_{80}$ at steady state SHR. All 10,000 recordings (100x100 pixels) were used to generate the scatter plots to correlate spatial heterogeneities of SCR to the dispersion of APD$_{80}$. Regional differences, base of right ventricle (RVB) and apex of left ventricle (LVA) were compared for statistical significance using 2-tailed t-test. Box-whisker diagrams are used to visualize the distribution of the data. The top and bottom whiskers define respectively the maximum and minimum values; the top and bottom of the box define the 75$^{th}$ and 25$^{th}$ percentiles respectively and the line within the box is the median in the data set.

4.1.5 Western blot Analysis

Female New Zealand White rabbits (3 month old) were euthanized, as described above; the hearts were perfused with Tyrode’s solution and ventricular tissue samples (~50 mg) were dissected from the anterior RVB and LVA corresponding to “RVB” and “LVA” regions of the optical mapping studies. Proteins were isolated as previously described,[138] were separated by
SDS-PAGE (50 µg/ sample), transferred to PVDF membranes which were probed by standard techniques. After immunolabeling, band intensities were measured with Image J and normalized with respect to β actin. Differences between the RVB and LVA were analyzed with one-tailed t-test and considered significant at p< 0.05. Antibodies against Cav1.2α, SERCA2, ERG and β actin were obtained from Santa Cruz Biotech (Cat. #: SC-103588, SC53010, 15968 and 81178, respectively); NCX1, and RyR2 were obtained from Thermo Scientific (Cat. #: MA3-926 and MA3-916, respectively) and Nav1.5 was obtained from Alomone (Cat. #: ASC-005)

4.2 RESULTS

APD₈₀ adaptation was reproducible from heart to heart and during repeated cycles (3-4 per heart) of transitions from 120 (BHR) to 50 (SHR) beats/min and back. Figure 7 A illustrates a sequence of two complete cycles of APD adaptation from BHR to SHR. From a steady state heart rate of 120 beats/min (not shown), a transition from BHR to SHR resulted in the expected gradual APD₈₀ and QT prolongation which was fully reversed by shifting back to BHR. The mean ΔAPD₈₀ in the first episode of 5 minutes of sustained SHR increased by 55.2±10.9% which was similar to % increase in the mean ΔAPD₈₀ recorded in second episode of SHR (51.25±8.2%) (p>0.05, n=4). Panels B and C illustrate optical APs and an EKG recording from bipolar surface electrodes, respectively measured at various time points (labeled: a-e in Figure 7A). An AP recorded at the onset SHR (trace a) is markedly shorter than at steady state SHR (trace b). APs measured at the next set of steady state BHR (traces c and e) are shorter than at steady state SHR (trace d). Similarly, T-wave amplitudes were markedly larger at steady state SHR (arrows on traces b and d) than at BHR (arrows on traces c and e) or the initiation of SHR (arrow on trace a).
The increases in T-wave amplitude relative to the QRS amplitude are a measure of enhanced DOR and the ratio of TW/QRS amplitude was statistically greater at steady state SHR than BHR (Figure 7C right panel, p<0.01, n=15 trials from 5 hearts).

Figure 7. APD$_{80}$ adaptation during transitions from BHR and SHR
A) Repeated cycles of pacing from SHR to BHR. Changes in HR (top trace) and the time course of APD$_{80}$ adaptation are shown uninterrupted for 22,000 seconds. B) Optical traces of APs from one of the pixels on the CMOS camera were measured at various time points: a, b, c, d and e, as labeled in panel A (top trace). C) EKG signals were recorded from the epicardium at the same time points: a, b, c, d and e, as depicted in panel A (top trace). The relative amplitude of T-waves measured as the ratio of TW to QRS amplitudes was statistically greater in SHR than BHR which is indicative of an increase in DOR.

APD$_{80}$ adaptation curves showed different time constants in going from BHR to SHR ($\tau_{f\rightarrow s}=48\pm9.2$s) compared to from SHR to BHR ($\tau_{s\rightarrow f}=30.4\pm4.7$s; p<0.05, n=5 hearts) (Figure 8).
Figure 8. Time constants for APD$_{80}$ adaptation: from BHR to SHR and SHR to BHR A) APD$_{80}$ adaptation during transitions from SHR to BHR. B) APD$_{80}$ adaptation during transition from BHR to SHR. In both cases, the time course of APD$_{80}$ adaptation to changes of cycle length were fitted to single exponential curves and their time constant ( ) was calculated based on the best-fit of experimentally measured APD$_{80}$ adaptations.

At BHR, AP and Ca$^2+$ signals exhibited the expected rapid rise and monophasic recovery to baseline. During the transition to SHR, diastolic levels of Ca$_i$ decreased gradually and APD$_{80}$ and Ca$_{iTD~80}$ exhibited the expected time-dependent prolongation (Figure 9A). Most interesting was the gradual prolongation of Ca$_{iTD~80}$ which was associated with an increasingly more pronounced “secondary Ca$^{2+}$ rise” (SCR) during the AP plateau and was associated with APD prolongation (Figure 9A, SCR labeled with an arrow, right traces). With slow pacing, APDs and Ca$_{iTDs}$ increased gradually reached a new steady state. The rate dependent, gradual
increase of SCRs (arrow from BHR to SHR) was associated with changes in the shape and time-course of APs, with a linear relationship between Ca\textsubscript{i}TD\textsubscript{80} and APD\textsubscript{80} (Figure 9A bottom panels). Note the gradual prolongation of Ca\textsubscript{i} (arrow from short to long durations) and voltage (V\textsubscript{m}) traces from BHR, to 16s and 5 min of SHR. Reversal to BHR suppressed SCR gradually, altered the shape of AP repolarization and shortened APD\textsubscript{80} and Ca\textsubscript{i}TD\textsubscript{80} (arrow from long to short duration) (Figure 9B). During APD adaptation, there was a linear relationship between APD\textsubscript{80} and Ca\textsubscript{i}TD\textsubscript{80} during the transition from BHR to SHR (Figure 9A bottom traces) and from SHR to BHR (Figure 9B bottom traces).

The distribution of SCR was heterogeneous and was more pronounced at the bast of the right ventricle (RVB) than the apex of the left ventricle (LVA) as was the distribution of APDs. Figure 10A shows an image of a heart and the area viewed by the CMOS Camera, delineated by the black box. Maps of APD\textsubscript{80} (Figure 10B top panels) and Ca\textsubscript{i}TD (Figure 10B bottom panels) are shown for BHR and 32 s and 5 min into SHR. SHR Caused a marked increase in the dispersion of both APD\textsubscript{80} and Ca\textsubscript{i}TD\textsubscript{80} compared to steady-state BHR (p < 0.01, n = 7 hearts) (Figure 10B and D). The superposition of Ca\textsubscript{i}T measured at BHR, 32 s and 5 min of SHR are shown for three sites (a, b and c) on the anterior surface (Panel A). The traces show that SCR and Ca\textsubscript{i}TD\textsubscript{80} increase during APD adaptation from BHR to SHR in a spatially inhomogeneous, being more pronounced at site a on the RVB than sites b and c that are closer to the LVA (Figure 10C). Similarly, the standard deviations [122] of Ca\textsubscript{i}TD\textsubscript{80} and APD\textsubscript{80} were significantly greater in SHR than BHR (p<0.01, n=7 hearts, Figure 10D).
Figure 9. Time-course of APD and Ca\textsubscript{i} adaptation during first 32 seconds. A) Changes in AP and Ca\textsubscript{i} dynamics during a change in heart rate from BHR to SHR (top traces). APD\textsubscript{80} and Ca\textsubscript{i}TD\textsubscript{80} are plotted as a function of time and APD\textsubscript{80} vs. Ca\textsubscript{i}TD reveals a tight linearly relationship. Inset: superposition of APs and Ca\textsubscript{i}Ts from the same pixel recorded at different times during adaptation to SHR. B) Changes in AP and Ca\textsubscript{i} dynamics during the reversal from SHR to BHR. APD\textsubscript{80} and Ca\textsubscript{i}TD\textsubscript{80} are plotted as a function of time and APD\textsubscript{80} vs. Ca\textsubscript{i}TD are linearly related. Inset: superposition of APs and Ca\textsubscript{i}Ts from the same pixel recorded at different times during adaptation towards BHR.
Figure 10. Dispersion of $\text{Ca}_{\text{TD}}$ and $\text{APD}_{80}$ in BHR and SHR

A) A picture of the heart with a black box identifying the region of the heart viewed by the CMOS cameras. B) Dispersion of $\text{APD}_{80}$ (top panels) and $\text{Ca}_{\text{TD}}$ (bottom panels) at different time-points from steady state BHR (left panels) to SHR after 32 s (middle panels) and at equilibrium 5 min (right panels). C) Optical traces of $\text{Ca}_i$ at site a, b and c, that are identified panel A. D) Standard deviation [122] of $\text{Ca}_{\text{TD}}$ (top) and $\text{APD}_{80}$ (bottom) at steady state BHR and SHR. SDs were calculated from 10,000 recordings (100 x 100 pixels) at 120 beats per minute (bpm) baseline and 5 minute at 50 bpm.
Since the comparison between amplitudes of Ca\textsubscript{i}T and APD is widely used to show the impact of Ca\textsuperscript{2+} abnormality on APD prolongation or shortening, correlation of areas under curves (AUCs) of Ca\textsubscript{i}T, which approximate amplitudes of SCRs, with APD\textsubscript{80} was calculated to assess the interplay between Ca\textsubscript{i} and V\textsubscript{m}. Maps of AUCs and APD\textsubscript{80} were generated and correlation coefficients were calculated from scatter plots of AUC vs. APD\textsubscript{80}. Figure 11A shows the region of a heart viewed by the arrays and Figure 11B and C show the correlation analysis during BHR and SHR, respectively from the same heart. The scatter plot of AUC vs. APD\textsubscript{80} (Figure 11C, rightmost panel) exposes a particularly poor correlation with r = 0.243 during BHR. In contrast, scatter plots during SHR (Figure 11D, rightmost panel) show a tight correlation with a coefficient r = 0.924. Figure 11D illustrates results from a second heart during SHR where AUCs and APD\textsubscript{80} were greater in amplitude at the RVB than the LVA and the correlation coefficient of AUCs vs. APD\textsubscript{80} was 0.987. The hearts in Figure 11A and D were chosen to illustrate the two types of scatter plots that were observed; either a rare bifurcation or non-monotonic AUC vs. APD\textsubscript{80} relationship (n=1/5) (Figure 11D, rightmost panel) or a monotonic relationship (Figure 11C, n=4/5). Most interesting was the enhanced correlation between AUC and APD\textsubscript{80} in SHR (r = 0.93 ± 0.03) compared to that in BHR (r = 0.55±0.29) (p < 0.01, n = 7 hearts) (Figure 11E). The mean APD\textsubscript{80} at BHR was not significantly different between RVB and LVA (p>0.29, n=7) but became statistically different at SHR (p<0.05, n=7) (Figure 11F). In SHR, AUCs were significantly greater at the RVB than the LVA, as shown in AUC maps in Figure 11C and D (p<0.05, n=5).
Figure 11. Regional differences of APD$_{80}$ and AUC of Ca$_i$T in bradycardia

A) Image of heart with a box to delineate the field-of-view of the cameras. B) Maps of AUC (of Ca$_i$T) and of APD$_{80}$ during BHR with scatter plot of AUC vs. APD$_{80}$, in this case the correlation coefficient, $r = 0.243$. C) Same heart as in B but during SHR, maps of AUC and APD$_{80}$ exhibit large regional variations from RVB to LVA, arrows depict gradients of large to small AUC and APD$_{80}$, AUC vs. APD$_{80}$ scatter plot was non-monotonic, with $r = 0.924$. D) As in C but with a different heart. The field-of-view is delineated by the box and maps of AUC and APD$_{80}$ exhibit marked regional variations from RVB to LVA, arrows identify direction of long to short Ca$_i$T and APD$_{80}$. Scatter plot of AUC vs. APD$_{80}$ was monotonic with $r = 0.987$. E) Summary analysis of r values showing a higher correlation between AUC and APD$_{80}$ at SHR than BHR, $p<0.01$ n=7 hearts. F) Statistical analysis of APD$_{80}$ comparison between RVB and LVA during BHR ($p=0.29$, NS) after 5 min of SHR ($p<0.05$ n = 5 hearts); SHR results in statistically significant increase in APD at the base than apex and the percent change in APD, $\Delta$APD$_{80}$ increased by 80% in RVB compared to 40% in LVA as a result of the bradycardia.
To elucidate the interplay between intracellular Ca\(^{2+}\) and voltage, experiments were carried out to assess whether SCR prolonged APDs or prolonged APDs elicited SCRs. In SHR, SCRs are more pronounced and it is reasonable to expect that SCRs might originate from a ‘second’ release of Ca\(^{2+}\) from the SR via cardiac ryanodine receptors (RyR2). Two agents known to stabilize RyR2, K201 (1µM, n=5) and flecainide (5 µM, n=4) were perfused to test their effects on SCR as well as APD and Ca\(_i\)TD adaptation. Ca\(_i\)TD and APD adaptation was measured during transitions from BHR to SHR before and after perfusion with K201 or flecainide. Plots of the percent change of mean ΔAPD\(_{80}\) as a function of time are shown during transitions from BHR to SHR for 24 pixels on the LVA and 24 pixels on the RVB, before and after perfusion with a RyR2 stabilizer. As shown in Figure 12, K201 (panel A) and flecainide (panel B) suppressed SCR and Ca\(_i\)TD during SHR (Ca\(_i\) traces without or with K201 or with flecainide, respectively in Figure 12A and B) and reduced mean APD\(_{80}\) (V\(_m\) traces in Figure 12A and B) at pixels on the RVB (K201: p < 0.01, n=5; flecainide: p < 0.01, n=4) (Figure 12A and B left graphs of ΔAPD\(_{80}\) % vs. time). In contrast to their effect on SCR at the base during SHR, K201 and flecainide did not significantly change AP and Ca\(_i\)T at the apex during SHR (Figure 12). It is important to note that K201 and flecainide did not significantly alter the early phase of APD adaptation, meaning the first 10 s of APD prolongation but both suppressed a second phase of APD prolongation from 10-25 s (plots of ΔAPD\(_{80}\) % vs. time, Figure 12A and B). As shown in Figure 13, the suppression of SCR by K201 (1 µM) or flecainide (5 µM) markedly reduced the dispersion of APD\(_{80}\) at SHR (K201: p < 0.05, n=5; flecainide: p < 0.05, n=4).
Figure 12. Suppression of SCR by K201 and flecainide

A) Percent change of APD<sub>80</sub> as a function of time during adaptation to SHR for sites on the LVA (top graph) and sites on the RVB (bottom graph) before and after perfusion with 1 µM K201; traces of Ca<sub>i</sub> and voltage (V<sub>m</sub>) are superimposed for BHR and SHR without (top traces) and with K201 (bottom traces). Right graph, plots the summary data (n = 5 hearts) of the % change in ΔAPD<sub>80</sub> for hearts without (control) and with K210 (1 µM). B) As for panel A but using flecainide (5 µM) instead of K201.
Figure 13. Suppression of SCR reduces DOR A) Maps of APD$_{80}$ (in ms) before (left panel) and after perfusion with K201 (1μM) (middle panel) at SHR, the standard deviation is used to quantitatively measure the dispersion of APD$_{80}$. Which is significantly greater in controls (ctlr) before K201 than after K201 (right panel) B) Maps of APD$_{80}$ (in ms) before (left panel) and after perfusion with flecainide (5μM) (middle panel) at SHR, the standard deviation is used to quantitatively measure the dispersion of APD$_{80}$. Which is significantly greater in controls (ctlr) before than after flecainide (right panel)
The higher occurrence of SCR at the RVB compared to LVA was possibly due to intrinsic differences in the expression of ionic channels and/or transporters. As shown in Figure 14, the expressions of L-type Ca\(^{2+}\) channel, NCX and voltage-gated Na\(^{+}\) channel, Nav1.5 proteins were significantly higher at RBV than LVA (p<0.05; n=7). In contrast, the levels of SERCA2A, RyR2 and RERG (Rabbit-ERG) were not significantly different (p>0.05; n= 7 hearts). In 5 out of 15 hearts, bradycardia alone was sufficient to produce premature ectopic beats (Figure 15B and D), which were readily eliminated by pacing at BHR (Figure 15C and E). Likewise inhibition of SCR with 1µM of K201 suppressed the onset of ectopic beats at steady state bradycardia (Figure 15F).
Figure 14. Heterogeneities of Ion Channels and Transporters between RVB and LVA
Ventricular tissues were dissected from the epicardium at the base of the RV (RVB) and the apex of the LV (LVA) and processed for Western blots as described in ‘Methods’. Density of peptides was normalized with respect to β-actin to compare levels of channel proteins between RV and LV. A: Illustrates the relative density of RVB versus LVA densities for Cav1.2α, NCX2 (dominant isoform of NCX in heart), SERCA2A, RyR2 and RERG (rabbit ERG). B: Summary of density histograms for each of these channel protein showing a highly significant 2-fold upregulation of Cav1.2α and NCX2 at RVB compared to LVA (p<0.05, n=7 hearts). There were no significant differences between RVB and LVA for SERCA2A, RyR2 and RERG (p>0.05, n=7 hearts).
Figure 15. Bradycardia-dependent ectopic beats A) A pacing protocol consisting of 2-cycles of BHR to SHR with 5 min intervals to achieve steady state followed by treatment with K201 and another cycle to examine the effects of RyR2 stabilization. Red arrows indicate the timing of changes in HR from BHR to SHR, and green arrows indicate changes from SHR to BHR. B & D) Premature ectopic beats were reproducible during the two episodes of bradycardia at 3 min and 13 min of the experiment. C & E) The termination of ectopic beats was consistently obtained during the episode of BHR, shown here at 8 and 18 min time points. F) After pretreatment with K201 (1µM), bradycardia-dependent ectopic beats were suppressed.
4.3 DISCUSSION

Change in heart rate is one of the main mechanisms used by mammals to adjust cardiac output to changing demand. The adaptive role of tachycardia in a “fight-or-flight” situation is well accepted as are the beneficial effects of bradycardia during sleep which diminishes energy consumption by the myocardial tissue. Several parameters of cardiac contraction have to adjust to dynamic HR changes. Most prominent is the change in the duration of mechanical (and electrical) systole to maintain an acceptable balance between the time for ventricular ejection and ventricular filling. At the cellular level, this requires shortening of APD and Ca_{i}T in response to HR increase.

4.3.1 Effects of HR ‘in and out’ of the physiological range

It is not surprising that unusually rapid or slow heart rates contribute to electrical instability and abnormal Ca^{2+} handling by ventricular myocytes has been implicated as a contributor to this instability. For example, an extensive body of clinical literature suggests that microvolt T-wave alternans (mTWA) is a predictor of ventricular arrhythmias in a wide range of conditions.[139-141] mTWA is known to be a tachycardia-related phenomenon, induced by either exercise or atrial pacing for the purposes of clinical testing.[142] At the cellular level, AP alternans underlies mTWA and is preceded by Ca_{i}T alternans,[27] which presumably causes AP alternans by changing the NCX current or similar processes that influences $V_m \rightarrow Ca_i$ coupling as well the reverse $Ca_i \rightarrow V_m$ coupling.[143, 144]

Slow ventricular rate is likewise a critical pro-arrhythmic co-factor known to increase propensity to Torsade de Pointes (TdP), a polymorphic ventricular tachycardia associated with
delayed repolarization.[25, 145] In patients with recurrent bouts of TdP, pacing at relatively fast rates is an effective therapeutic maneuver.[146] Abnormal Ca\textsuperscript{2+} handling and spontaneous SR Ca\textsuperscript{2+} release or I\textsubscript{Ca,L} reactivation are widely accepted as the mechanism of early afterdepolarizations (EADs), the triggered activity which underlies TdP.[14, 81, 147] We have recently reported that in a rabbit model of long QT type-2 related TdP involving bradycardia and I\textsubscript{Kr} block, oscillations of Ca\textsubscript{i}T develop during APD prolongation and precede the appearance of EADs by minutes.[148] The data suggest that the Ca\textsubscript{i} oscillations are caused by spontaneous SR Ca\textsuperscript{2+} release that activates a depolarizing NCX current that serves as a trigger to EAD generation. Although I\textsubscript{Kr} block with dofetilide was required for the appearance of extra Ca\textsubscript{i} upstrokes, we observed that bradycardia alone caused a delay or a “plateau” in the normally smooth downslope of Ca\textsubscript{i}T. This Ca\textsubscript{i}T delay could be eliminated by increasing pacing rate. The experiments reported in this paper were motivated by an effort to elucidate the relationship between bradycardia, Ca\textsuperscript{2+} handling and arrhythmogenesis.

4.3.2 Why is bradycardia arrhythmogenic?

Bradycardia has long been known to prolong APDs and enhance DOR, setting the stage for a more arrhythmogenic substrate with a greater propensity to functional reentry but the mechanisms that enhance DOR are not fully appreciated. Here, we report that bradycardia produces the expected gradual decrease of diastolic Ca\textsubscript{i} but also show for the first time that bradycardia promotes ‘secondary Ca\textsuperscript{2+} release’ (thus labeled SCR) which is non-uniform on the epicardium and contributes to APD adaptation. SCR differs from spontaneous Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} oscillations because the latter can occur at various times along the AP plateau or even diastole which can lead to the initiation of early and delayed afterdepolarizations, respectively.
Bradycardia-dependent SCR occur as extra Ca\(^{2+}\) release 200-300 ms after the AP upstroke, prolong Ca\(_i\)TDs, are more pronounced at the RVB and are associated with a delay of ventricular repolarization. The repolarization delay is most likely caused by SCR in response to a change in HR and correlates closely with the spatial and temporal changes in APD.

To address the question whether Ca\(_i\)T changes drive AP changes or \textit{vice versa}, we performed experiments with K201 and flecainide, agents known to specifically stabilize RyR2. Both agents eliminated SCR and attenuated AP prolongation and DOR during bradycardia. Both agents have off-target effects which must be carefully considered. K201 (1 µM) inhibited \(I_{Na}\), \(I_{Ca,L}\) and \(I_{Kr}\) in addition to its effect on RyR2 but these findings in guinea pig myocytes appear to be species dependent.[149, 150] In rabbit myocytes, K201 was fairly specific for RyR2, at 1 µM; it reduced spontaneous SR Ca\(^{2+}\) release and Ca\(^{2+}\) waves without altering \(I_{Ca,L}\) and SR Ca\(^{2+}\) content. [151] Flecainide at 5 µM targets voltage-gated Na\(^+\) channels which would reduce intracellular Na\(^+\) and Ca\(^{2+}\) load, produce the negative inotropic effect reported for class I anti-arrhythmics[152] which would be expected to prolong not shorten APDs. Flecainide inhibits RyR2 by binding to the open state of the release channel[153] and with respect to APD prolongation; its effect on \(I_{Na}\) normally outweighs[154-156] its inhibitory effect on \(I_{Kr}\). [152] Here, both agents reduce bradycardia-induced APD prolongation which argues that both agents primarily suppress SCR originating from the SR Ca\(^{2+}\) release by stabilizing RyR2 and not by off-targets effects that would prolong APD. Another compelling argument is that both reduce APD in bradycardia but only at the RVB and have no effect at the LVA or during normal HR. A similar argument suggests a role for SCR in bradycardia-induced DOR and the suppression of DOR by K201 and flecainide. It is likely that the dramatic changes in Ca\(_i\)T morphology observed before TdP onset in our LQT2 model represent accentuation of a “normal” Ca\(_i\)T response to profound bradycardia.
We have observed that elimination of SCR by K201 blunts a second slow phase of APD prolongation in response to bradycardia. It thus appears that the dynamics of Ca\(^{2+}\) handling by ventricular myocytes is another candidate mechanism for AP adaptation to HR change, in addition to the mechanisms mentioned in the introduction. Most likely, the Ca\(_i\) handling affects APD by means of Ca\(_i\) → V\(_m\) coupling most likely via NCX. This effect seems to function during bradycardia since the area under the curve of Ca\(_i\)T was tightly correlated to APD\(_{80}\), as both varied as a function of time and location until steady state bradycardia was reached (Figure 11). A major difference between Ca\(_i\)T measured during baseline and slow HR is the appearance of SCR which implies that SCRs influence APDs. The weak correlation between AUCs and APDs in BHR suggests independence between the two parameters and implies that Ca\(_i\) has little effect on the plateau potential and repolarizing K\(^+\) currents. In contrast, SCR in bradycardia augments the forward mode of I\(_{NCX}\), enhances Ca\(_i\) → V\(_m\) coupling and prolongs APDs leading to a high correlation between AUC and APDs. The linear scatter plots suggest that the spatial heterogeneity of AUC is an important determinant of APD\(_{80}\) dispersion. QT adaptation time constants measured in human hearts were comparable in magnitude and exhibited a similar trend.[157]

The enhanced correlation of Ca\(_i\)T and APD\(_{80}\) in bradycardia suggests that SCR raises Ca\(_i\) levels during the AP plateau, which stimulates the forward mode of NCX and its depolarizing current, I\(_{NCX}\) which raises the plateau potential and prolongs APDs. In the absence of adrenergic activity, SCRs were attributed to the re-activation of RyR2 which would be caused by a higher level of SR Ca\(^{2+}\) in a spatially non-uniform manner. The mechanism underlying the regional distribution of SCRs was due to higher levels of expression of Cav1.2α, NCX1 and Nav1.5 (but not RyR2, SERCA2A or RERG) at the RVB than the LVA. In previous reports, we had shown
that in adult female rabbit hearts Cav1.2α and NCX1 were upregulated at the base compared to
the apex of left ventricles. The higher levels of mRNA and proteins corresponded to higher
levels of their respective current densities $I_{Ca,L}$ and $I_{NCX}$, measured by patch-clamping myocytes
isolated from the base and apex.[138, 158] Higher Nav1.5 at the base could contribute to the
higher Ca$^{2+}$ load by increasing intracellular Na$^+$ leading to a stimulation of reverse mode and
inhibition of the forward mode of $I_{NCX}$.

The mechanism through which bradycardia promotes SCR is uncertain at this moment. In
principle, increased diastolic interval during bradycardia should allow more time for Ca$_i$ removal
out of the cell by NCX. However, SR Ca$^{2+}$ pumps can compete effectively with NCX for the
removal of Ca$_i$ during bradycardia. The amount of Ca$_i$ transported to the lumen of the SR by
SERCA during each cardiac cycle may actually be higher during bradycardia due to: a) longer
AP plateau, b) longer duration of Ca$^{2+}$ influx through L-type channels and c) the reduced NCX
driving force during more positive plateau potentials. If there is a significant diffusion limitation
of Ca$^{2+}$ movement between the uptake and release SR compartments, junctional SR may be
replenished after the initial phase of Ca$^{2+}$-induced Ca$^{2+}$-release from the non-junctional
compartment during each heartbeat to a degree which allows spontaneous Ca$^{2+}$ release through
RyR2 in at least some myocytes. This process should be augmented by any intervention which
prolongs plateau duration, such as $I_{Kr}$ blockade.
4.4 STUDY LIMITATIONS

The study focused on the epicardium and did not investigate SCR from different regions of the ventricles. Although previous studies demonstrated higher levels of $I_{Ca,L}$ and $I_{NCX}$ occurred at the base of the rabbit epicardium and not the apex or the endocardium,[138, 158] we cannot exclude the possibility of SCR in other regions of the heart. The study provides evidence that bradycardia increases $Ca^{2+}$ in the lumen of the SR in a non-uniform manner resulting in a SCR at the base, and APD prolongation mediated by $I_{NCX}$. Pilot studies attempted to demonstrate the contribution of NCX to APD prolongation during bradycardia. Unfortunately, the available NCX inhibitors are not sufficiently selective or effective at blocking NCX. Trials with SEA0400 (0.1-2 µM) to block the forward mode of NCX were inconclusive because at these concentrations, inhibition of NCX is partial and at concentrations > 1 µM, $I_{Ca,L}$ is progressively suppressed.[159] An alternative approach of lowering external $Na^+$ has been successfully used to inhibit $I_{NCX}$ in isolated myocytes but cannot be applied fast enough in perfused hearts. Likewise, caffeine can be effectively use to estimate SR $Ca^{2+}$ load in isolated myocytes but not in perfused hearts. Nevertheless, these limitations do not detract from the validity of the study which exemplifies what can be done at the intact heart level to fully appreciate heterogeneities and complexities that cannot be exposed in studies with isolated myocytes.
4.5 CONCLUSION

In summary, the data presented here describe an acute abnormality of myocardial Ca\textsuperscript{2+} handling caused by bradycardia, which may increase propensity to arrhythmia. A recent elegant study has shown the role of remodeling of Ca\textsuperscript{2+} handling processes for arrhythmia in chronic bradycardia.[160] Additional research in this complex field appears warranted. Clinically, it is possible that RyR2 stabilizers and NCX blockers could have a role in acute treatment of bradycardia-induced TdP, at least until pacing therapy can be instituted. On the cellular level, the mechanisms of SCR clearly merit additional investigation.
5.0 Ca\textsuperscript{2+} OSCILLATIONS AND T-WAVE LABILITY PRECEDE VENTRICULAR ARRHYTHMIAS IN ACQUIRED LONG QT TYPE 2

The prolongation of ventricular action potential duration (APD) and QT interval often leads to life-threatening polymorphic VT that show a characteristic electrocardiographic (ECG) appearance known as TdP. Acquired LQT syndrome (LQTS) is of critical importance because of its prevalence in the clinical setting. Several factors (e.g., medications, electrolyte abnormalities, and heart failure) impair ventricular repolarization and may lead to lethal arrhythmias. LQTS is also of conceptual importance because the congenital form represents a “pure global repolarization disease,”\cite{161} which demonstrates a direct link between repolarization delay and sudden cardiac death.\cite{162} Although the molecular defects leading to prolonged APDs in congenital LQTS have been elucidated in remarkable detail,\cite{163} the mechanism by which impaired repolarization causes VT on the tissue level remains less clear. \cite{14, 164, 165}Two hypotheses that are not mutually exclusive have been proposed. First, prolonged APDs result in triggered activity in the form of early afterdepolarizations (EADs).\cite{162} Second, spatially heterogeneous APD prolongation leads to increased dispersion of refractoriness and may form a substrate for functional reentry. \cite{163, 164}Clinically, prolonged APD is reflected on the surface ECG as QT interval prolongation. Several forms of temporal repolarization instability, such as microvolt T-wave alternans (mTWA)\cite{166, 167} and increased QT interval variability,\cite{168-170} have been linked to sudden cardiac death in clinical and experimental settings. Multiple lines of
evidence suggest that Ca\textsubscript{i} alternans leads to APD alternans and mTWA.\cite{27, 61, 144} However, arrhythmias during impaired repolarization are classically associated with bradycardia or pauses, whereas mTWA is usually a tachycardia-induced phenomenon. mTWA is not frequently observed in LQT,\cite{171, 172} and may not be necessary for induction of LQT-related arrhythmias. On the other hand, nonalternans T wave lability (TWL) precedes TdP in LQTS patients\cite{173, 174} as well as in animal models of prolonged repolarization.\cite{162, 175} TWL refers to beat-to-beat changes in T-wave morphology that do not follow an alternans (i.e., ABAB\ldots) pattern. It seems to be a better predictor of arrhythmia than the absolute degree of repolarization delay.

The mechanisms underlying nonalternans repolarization lability are a matter of speculation, and the possible role of abnormal Ca\textsuperscript{2+} handling in this phenomenon remains unexplored. It is possible that TWL is caused by the same process that drives EADs, but generates depolarizations of insufficient amplitude to trigger propagating waves.

EADs have been classically attributed to the spontaneous reactivation of the L-type Ca\textsuperscript{2+} current, I\textsubscript{Ca,L}, during the abnormally long APD and slow AP downstroke.\cite{89, 176} Alternatively, SCR from the SR during phase 2 or phase 3 of the AP can further depolarize the plateau potential through the activation of the electrogenic NCX. A similar mechanism has been documented as the trigger of delayed afterdepolarizations (DADs), but its role for EADs remains controversial. In cryoablated rabbit hearts, simultaneous mapping of transmembrane voltage (V\textsubscript{m}) and cytoplasmic free Ca\textsuperscript{2+} in drug-induced LQT2 showed that a rapid increase in Ca\textsubscript{i} precedes the rise of V\textsubscript{m} at the first sites that fire EADs on the epicardium.\cite{11} The dynamic relationship between Ca\textsubscript{i} and V\textsubscript{m} during an EAD supports the notion that SR Ca\textsuperscript{2+} overload and spontaneous SR Ca\textsuperscript{2+} release activate an inward NCX current (I\textsubscript{NCX}), which triggers I\textsubscript{Ca,L} to produce EADs. However, EADs initiated by oxidative stress (with hydrogen peroxide, H\textsubscript{2}O\textsubscript{2} 0.2 to 1 mM) were attributed
to Ca\(^{2+}\)/calmodulin kinase activation, which increased I_{Ca,L}, impaired inactivation of I_{Ca,L} and of voltage-gated Na\(^+\) current \([177]\). Still, SR Ca\(^{2+}\) release should not be excluded as the trigger of EADs because H\(_2\)O\(_2\) acts at numerous targets, including the ryanodine receptor, NCX, and the SR Ca\(^{2+}\) pump.

In this report, we investigated the role of Ca\(_i\) dynamics on TWL in a noncryoablated rabbit model of LQT2 using simultaneous measurements of Ca\(_i\)T, AP, and ECG during paced rhythms and focused on events that precede ventricular ectopy.

5.1 METHODS

5.1.1 Heart preparations

New Zealand White rabbits (female, 60 to 120 days old) were anesthetized with pentobarbital (35 mg/kg intravenously) and anticoagulated with heparin (200 U/kg intravenously). The heart was perfused with Tyrode solution (mM): 130 NaCl, 24 NaHCO\(_3\), 1.0 MgCl\(_2\), 4 KCl, 1.2 NaH\(_2\)PO\(_4\), 50 dextrose, 1.25 CaCl\(_2\), gassed with 95% O\(_2\)–5% CO\(_2\). The AV node was destroyed with electrocautery to control heart rate. To minimize motion artifact, blebbistatin (5 to 10 μM for approximately 15 minutes) was added to the perfusate. The heart was immobilized in a chamber and stained with a voltage-sensitive dye (RH 237: 200 μl of 1 mg/ml dimethyl sulfoxide [DMSO]) and loaded with a Ca\(^{2+}\) indicator (Rhod-2 AM, 200 μl of 1 mg/ml DMSO). Epicardial bipolar pseudo-ECG was continuously monitored. Epicardial pacing with a unipolar electrode on the right ventricle was performed at cycle length 1.2 seconds (50 beats/minute; bradycardia for rabbit hearts). After baseline recordings, LQT2 was modeled by
perfusing with Tyrode solution containing dofetilide (250 to 500 nM, Pfizer, New York, NY), a selective $I_{Kr}$ blocker and lowering $K^+$ and $Mg^{2+}$ concentrations by 50%.[178] This investigation conformed to the current Guide for Care and Use of Laboratory Animals published by the National Institutes of Health.

5.1.2 Optical apparatus

The optical apparatus based on 2 photodiode arrays has been described previously. The anterior surface of the heart was illuminated with a $520 \pm 30$ nm excitation beam, and the fluorescence was passed through a dichroic mirror (660 nm) to focus the Rhod-2 and RH 237 fluorescence images on two $16 \times 16$ photodiode arrays (C4675–103, Hamamatsu Corp, Hamamatsu City, Japan). Outputs from the arrays were amplified, digitized at 1 kHz frequency, and stored in computer memory, along with surface ECG.

5.1.3 Data analysis

Automatic measurement of APD and Ca$_{i}$TD from all pixels was used to calculate APD and Ca$_{i}$TD dispersion, defined as the standard deviation of APD/CTD values. Activation time at each site was calculated from $(dFv/dt)_{max}$ of the local AP upstroke, and APD (Ca$_{i}$TD) at each site was the interval from $(dFv/dt)_{max}$ to the recovery of $V_m$ to 10% of baseline (APD$_{90}$ or Ca$_{i}$TD$_{90}$). Isochronal maps of APD and Ca$_{i}$TD were generated as previously described. [179] In addition, custom software was created in C++ (Microsoft Visual Studio 6.0, Micorsoft Corp., Redmond, WA) for data analysis. Signals were digitally low-pass filtered (3-pole Bessel filter, 20 Hz), and baseline fluctuations were subtracted with a smooth cubic spline. Optical signals were evaluated
from 5 pixels (2-8, 8-2, 8-8, 15-8, 8-15; the numbers stand for the x-y pixel coordinates of the 16 × 16 array). Simultaneous ECG, V_m, and Ca_i signals were displayed, and durations were measured with electronic calipers. During each scan (typically lasting 32 seconds), the interval from pacing stimulus to end of the T-wave (the QT interval equivalent) and the local duration of V_m and Ca_i signals were measured in 2 beats and averaged. The number of Ca_i peaks per AP was determined visually and were averaged over 2 beats from 5 pixels in each of the 32-second scans. Except for the dispersions of APD and Ca_iTD, the average value taken over these 5 pixels was used for subsequent analysis.

The lability of T waves, V_m, and Ca_i signals was calculated as previously reported.[16] Briefly, the ECG, V_m, or Ca_i signals from each beat (unless excluded due to poor signal quality or subsequent PVC) were superimposed using the stimulation artifact, and the root-mean-square of the beat-to-beat differences in signal amplitude measured at corresponding time points of the repolarization segment (150 ms to 900 ms after the stimulus) were calculated. The maximal root-mean-square value (corresponding to the most labile time point) was normalized to the amplitude of the signal-averaged QRS complex (defined as the maximum minus the minimum value, evaluated 10 to 900 ms after stimulation artifact, thus including the QRS complex). The reported T wave, V_m, and Ca_i lability values are taken as a natural logarithm of the normalized root-mean-square values (Table 1).
Table 1. Summary of ECG, $V_m$, and $Ca_i$ parameters before and after dofetilide. The asterix indicates that $Ca_i$ lability was calculated from 2 beats preceding EAD onset, at the site of EAD origin.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Dofetilide, before ectopy</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>APD (ms)</td>
<td>415 ± 118</td>
<td>678 ± 235</td>
<td>.01</td>
</tr>
<tr>
<td>$Ca_i$TD (ms)</td>
<td>474 ± 144</td>
<td>702 ± 227</td>
<td>.005</td>
</tr>
<tr>
<td>QT (ms)</td>
<td>481 ± 105</td>
<td>603 ± 172</td>
<td>.05</td>
</tr>
<tr>
<td>No. of $Ca_i$ peaks</td>
<td>1.332 ± 0.387</td>
<td>2.120 ± 0.469</td>
<td>.002</td>
</tr>
<tr>
<td>T-wave lability</td>
<td>-4.061 ± 0.944</td>
<td>-2.894 ± 0.950</td>
<td>.002</td>
</tr>
<tr>
<td>AP lability</td>
<td>-3.436 ± 0.492</td>
<td>-2.994 ± 0.779</td>
<td>.05</td>
</tr>
<tr>
<td>$Ca_i$ lability</td>
<td>-3.338 ± 0.372</td>
<td>-2.970 ± 0.647</td>
<td>.084</td>
</tr>
<tr>
<td>$Ca_i$ lability*</td>
<td>-4.049 ± 0.685</td>
<td>-3.356 ± 1.033</td>
<td>.05</td>
</tr>
<tr>
<td>$V_m$ x $Ca_i$ correlation</td>
<td>0.9593 ± 0.0231</td>
<td>0.8967 ± 0.0644</td>
<td>.02</td>
</tr>
</tbody>
</table>

The correlation coefficient between $V_m$ and $Ca_i$ signal values during the repolarization segment of the last paced beat not followed by EAD were calculated from 5 pixels as described earlier and averaged. Again, these values were compared to the corresponding values prior to dofetilide perfusion. Unless described otherwise, a 2-tailed paired Student t-test (Excel, Microsoft Corp.) was used to compare signal values obtained from baseline data segments with data segments after dofetilide perfusion, but before the onset of ventricular ectopy. $P$ values of <.05 were considered statistically significant.
5.2 RESULTS

TdP was induced by dofetilide perfusion in all hearts (n = 8) in <10 minutes and was preceded by short-coupled premature ventricular beats. Premature ventricular beats on the ECG corresponded to EADs on optical $V_m$ tracings. In all cases, the onset of EADs was preceded by prolongation of APD (415 ± 118 vs. 678 ± 235 ms; p < .01) and of Ca,TD (474 ± 144 vs. 702 ± 227 ms; p < .005) as compared with controls. The QT interval determined from epicardial ECG also prolonged markedly (481 ± 105 vs. 603 ± 172 ms; p < .02). Occasionally, ectopic ventricular beats with late coupling and $V_m$ tracing suggestive of DADs were observed.

5.2.1 Beat-to-beat lability of repolarization, AP, and CaT

TWL increased in all experiments prior to the onset of arrhythmia (–4.061 ± 0.944 vs. –2.894 ± 0.950; n = 8, p < .002). TWL corresponded to discernible changes of T-wave morphology during regular rhythms preceding ventricular arrhythmias (Figure 16). In contrast, macrovolt T-wave alternans was never observed before the onset of ventricular ectopy (n = 8). Similarly, the beat-to-beat lability of optical $V_m$ signals increased prior to VT onset (–3.436 ± 0.492 vs. –2.994 ± 0.779; p < .05). There was a trend toward an increase in Ca,T lability prior to VT onset that did not reach statistical significance (–3.338 ± 0.372 vs. –2.970 ± 0.647; p = .084). However, at sites of EAD origin, Ca,T lability was significantly higher compared with baseline
for the 2 consecutive beats preceding the onset of EADs ($-4.049 \pm 0.685$ vs. $-3.356 \pm 1.033$; $p < .05$). Table 1 summarizes these ECG, $V_m$, and $Ca_i$ parameters before and after the induction of LQT2 but before the onset of ectopic beats.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before LQT2</th>
<th>After LQT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_m$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Ca_i$</td>
<td></td>
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</tr>
</tbody>
</table>

**Figure 16. Prolonged repolarization induces TWL. Examples of ECG recordings during pacing at 50 beats/minute**

A) Control. B) LQT2. T-wave morphology is constant in A, but changes on a beat-to-beat basis in B. ECG lability (green traces) is plotted before (C) and during LQT2 (D) and is superimposed on signal-averaged ECG (red traces). The Y-axis for lability is expanded 10-fold with respect to signal-averaged ECG. TWL is calculated as the logarithm of maximum ECG lability measured during the repolarization phase and normalized with respect to the amplitude of signal-averaged QRS. TWL is essentially absent in C and highly pronounced in D. Maximum lability occurs at approximately 470 ms after the pacing stimulus in this case.

One might expect that if $Ca_i$ lability causes $V_m$ lability, then maximum $Ca_i$ lability may occur slightly earlier within a cardiac cycle. However, this did not appear to be the case: the average timing of maximum $V_m$ and $Ca_i$ lability before onset of ectopy was $435 \pm 141$ and $580 \pm 95$ ms after pacing spike, respectively ($p=0.044$ by paired t-test). The explanation seems to be that maximum $Ca_i$ lability often occurs towards the end of the AP (late phase 3), when the membrane resistance is lower than during phase 2. Therefore, the effect of $Ca_i$ and $I_{NCX}$ variability on $V_m$ variability may be less than during phase 2. One consistent observation is that
the maximum Caₐ lability occurs later than the second Caₐ peak on signal-averaged CaₐT (580 ± 95 vs. 430 ± 79 ms, p < .002).

5.2.2 CaₐT oscillations

Aside from beat-to-beat lability, CaₐT showed other forms of instability. Normal CaₐTs were monophasic (a single Caₐ peak followed by a recovery to baseline) with Caₐ increasing approximately 10 ms after the rise of Vₘ. Occasionally, a small secondary rise in Caₐ appeared during phase 3 of the AP at a few sites. Perfusion with dofetilide increased the complexity of CaₐT kinetics in all hearts, with the appearance of multiple Caₐ peaks (Figures 17 and 18). The average number of CaₐT peaks occurring during a single AP increased significantly before the onset of EADs (1.332 ± 0.387 vs. 2.120 ± 0.469; P < .002). Interestingly, up to 4 distinct Caₐ peaks could be seen in some experiments, whereas the corresponding local AP remained monophasic, with a smooth downstroke during phase 2 and 3 (Figure 19). Caₐ oscillations (CaₐO) preceded the onset of EADs in all experiments.
Figure 17. Ca\text{\textsubscript{i}}O precede EADs. Simultaneous recordings of V\text{\textsubscript{m}} (blue) and Ca\text{\textsubscript{i}} (red) before (A) and during LQT2 (B, C) A) V\text{\textsubscript{m}} and Ca\text{\textsubscript{i}} are shown at slow (top) and fast (bottom) sweep speeds, and both show monophasic time courses. B) V\text{\textsubscript{m}} and Ca\text{\textsubscript{i}} are shown at slow (top) and fast (bottom) sweep speeds. In the first 2 APs, the LQT2 condition prolonged APD and elicited Ca\text{\textsubscript{i}} oscillations during the paced beats with no V\text{\textsubscript{m}} instabilities. The third AP showed multiple Ca\text{\textsubscript{i}} peaks that were occasionally coincident with EADs. C) An example of TdP onset after the fifth paced beat. The ECG (top trace) and the optical traces of V\text{\textsubscript{m}} and Ca\text{\textsubscript{i}} (bottom traces) were recorded simultaneously. The Ca\text{\textsubscript{i}}O precedes the first EADs at this site.
Figure 18. Time-dependent oscillations of Ca\textsubscript{i} and the evolution of EADs V\textsubscript{m} (blue) and Ca\textsubscript{i} (red) measurements (left) during pacing (A, B) and ventricular escape rhythms (C, D). A) In control, V\textsubscript{m} and Ca\textsubscript{i} are monophasic and similar in shape. B) LQT2 for 3 minutes, a second increase of Ca\textsubscript{i} appears during the AP plateau while V\textsubscript{m} remains free of EADs. C) LQT2 for 6 minutes promotes more complex Ca\textsubscript{i}O that are associated with a single EAD. In this pixel, the Ca\textsubscript{i} upstroke precedes the V\textsubscript{m} upstroke. D) LQT2 for 9 minutes, 2 consecutive EADs follow each AP upstroke. Prominent Ca\textsubscript{i} upstrokes precede EAD upstrokes.
Figure 19. **Spatial and temporal heterogeneity of Ca\(_i\)T and APs during LQT2**

A) Isochronal map of APD\(_{90}\) (A (a), top left) from the anterior surface in LQT2; the field-of-view of the array is shown (inset d), and isochronal lines are 50 ms apart (see color scale). Ca\(_i\) signals recorded at each site are depicted in the symbolic map of the photodiode array (b). Simultaneous \(V_m\) and Ca\(_i\) from a single beat are superimposed for pixels labeled 1, 2, 3 on the maps and are shown at fast sweep speed (c). Marked spatial heterogeneities of Ca\(_i\)O appear, with the highest number of Ca\(_i\)O found at sites with the longest APDs and decrease to sites with shorter APDs. B) ECG, \(V_m\), and Ca\(_i\) recordings from pixel (1) for 30 seconds show a gradual APD prolongation associated with an increasing number of Ca\(_i\)O and a rise of diastolic Ca\(_i\). The right panel shows a shorter segment of the signals (green box on left) with better time resolution.
Figure 19A illustrates a marked spatial heterogeneity of $V_m$ and $Ca_i$ signals during dofetilide perfusion, but before the onset of EADs. An isochronal map of APD$_{90}$ shows a marked APD prolongation (pixel 1) that decreased anisotropically. Before the onset of EADs, the correlation between $V_m$ and $Ca_i$ signals during repolarization decreased in all experiments ($0.9593 \pm 0.0231$ vs. $0.8967 \pm 0.0644$; $p < .02$, 2-tailed sign test), representing an increasing dissociation between the oscillatory $Ca_i$ signal and the monophasic shape and time course of APs. In paced beats immediately preceding the appearance of EADs, the spatial dispersion of $Ca_i$TD$_{90}$ exceeded the dispersion of APD$_{90}$ ($65 \pm 29$ vs. $52 \pm 35$ ms; $p < .02$).

During a normal AP, $Ca_i$Ts are tightly controlled by $V_m$ and AP upstrokes preceded the rise of $Ca_i$ with a delay of $10 \pm 2$ ms. With the appearance of premature ventricular beats, EAD upstrokes often coincided with a secondary $Ca_i$T peak. In 3 of 8 experiments, the first EAD occurred outside of the field of view of the optical maps. In 2 of 5 experiments where the first EAD fell in the field of view of the array, the second rise of $Ca_i$ preceded the second $V_m$ upstroke by 3 to 5 ms, and this temporal relationship reversed gradually as the EAD propagated away from its origin (Figure 20). In 3 of 8 experiments, EAD upstrokes preceded $Ca_i$ upstrokes but with shorter $V_m$–$Ca_i$ delays of 5 to 7 ms compared with delays during normal APs. $Ca_i$O preceding EAD upstrokes (Figures 17B, 18C, and 18D) were routinely observed during complex ectopy, such as bigeminy, trigeminy and VT runs.
Figure 20. Propagation of $V_m$ and $Ca_i$ upstrokes during an EAD A) Isochronal activation map of an ectopic beat (EAD) occurring during an escape rhythm. The origin of the EAD is at site 1. Isochronal lines are 3 ms apart. Note that another independent wavefront emanates from the base of the heart. B) $V_m$ and $Ca_i$ tracings from sites: 1 and 3 in (A). The first AP and $Ca_i$T are monophasic at site 1; during the second AP, there is a distinct second $Ca_i$ peak without an EAD (arrow). On the third beat, an EAD appears with sufficient magnitude to propagate, as in A. C) The temporal relationship between $Ca_i$ and $V_m$ signals are shown at higher resolution at sites 1 to 3 as labeled in A. At the site of EAD origin (1), $Ca_i$ upstroke precedes $V_m$ upstroke (8 ms); at site 2, $V_m$ is coincident with $Ca_i$; and at site 3, remote from the EAD origin, $V_m$ precedes $Ca_i$ (3 ms).

As illustrated in Figure 21, a bigeminy pattern on ECG corresponded to a $Ca_i$O with an EAD during each beat (Figure 21A). Similarly, trigeminy corresponded to beat-to-beat alternations of a large $Ca_i$O with an EAD followed by a smaller $Ca_i$ oscillation without an EAD on the next beat (Figure 21B). Non sustained runs of polymorphic VT corresponded to long $Ca_i$T with multiple $Ca_i$O (Figure 21C).
Figure 21. ECG recordings of complex ectopy: bigeminy and trigeminy correspond to Ca_i O A) ECG recording of paced rhythm with bigeminy. Each paced beat (green arrows) is followed by a ventricular ectopic beat (red arrow). T-waves on ECG signals are indicated by black arrows. Note that neither V_m nor Ca_i recover to baseline before the ectopic beat; in this sense, the paced/ectopic beat can be understood as a single complex AP. B) An episode of trigeminy from the same experiment. Two paced beats are followed by an ectopic beat. The optical tracings indicate that alternans between a short monophasic paced AP and a bigeminal AP underlies the trigeminal pattern. C) Simultaneous ECG and Ca_i tracing with brief runs of polymorphic VT. Pacing rate was 50 beats/min with 2:1 capture. Each run of polymorphic VT corresponds to a single Ca_i T with multiple secondary Ca_i O.
5.2.3 TdP is suppressed by interventions that abolish Ca$_i$O

To corroborate the role of spontaneous SR Ca$^{2+}$ release in eliciting EADs and TdP, several interventions were tested to reduce SR Ca$^{2+}$ load in attempts to suppress Ca$_i$O and the generation of TdP. As shown in Figure 22, nifedipine (5 μM) (Figure 22A) and low external Ca$^{2+}$ (100 μM) (Figure 22B) suppressed Ca$_i$Os, EADs, and TdP (n = 4 for each intervention), although APD remained prolonged. Restoration of original Ca$^{2+}$ resulted in reappearance of Ca$_i$O and TdP. In other experiments (n = 3), the hearts were perfused with Tyrode solution containing ryanodine and thapsigargin (10 μM and 200 nM, respectively) to deplete SR before perfusion with dofetilide. In the presence of ryanodine and thapsigargin, dofetilide prolonged APD, but Ca$_i$O and TdP failed to occur.

![Figure 22. Effect of reduced SR Ca$^{2+}$ load on Ca$_i$O and TdP](image)

TdP was induced with LQT2 solution then nifedipine (5 μM) was added (A) or external Ca$^{2+}$ was lowered (100 μM) (B) in the perfusate. Both interventions attenuated Ca$_i$O and terminated TdP despite continuous dofetilide perfusion and marked APD prolongation.
5.3 DISCUSSION

The main finding of this report is that under LQT2 conditions, Ca\textsubscript{i}T oscillations occur in ventricular myocardium during regular rhythm. They are not caused by oscillations of membrane potential, which they precede by minutes. When EADs do appear, they usually follow Ca\textsubscript{i}T upstroke at the site of EAD origin.

Although Ca\textsubscript{i} oscillations under LQT conditions have been reported before, they were always described in the presence of EADs and often understood as a consequence of I\textsubscript{Ca,L} reactivation with consequent calcium-induced Ca\textsuperscript{2+} release. Because the timing of Ca\textsubscript{i} and V\textsubscript{m} upstrokes is often similar and their relationship may be spatially heterogeneous, this explanation was difficult to disprove. Here, we show that Ca\textsubscript{i}T oscillations consistently occur minutes before the appearance of EADs, at the time when AP downstroke remains smooth and monophasic. Interventions that abolish Ca\textsubscript{i}T oscillations also abolish TdP. These findings indicate that the secondary Ca\textsubscript{i}T peaks are caused by instabilities of intracellular Ca\textsuperscript{2+} handling caused by AP prolongation. These instabilities in turn promote the appearance of EADs, and eventually TdP.

5.3.1 CaT oscillations drive EADs

The driving role of Ca\textsubscript{i} oscillations in EAD generation is supported by the analysis of the Ca\textsubscript{i}T and AP relationship at the site of EAD focus. At the origins of EADs, the rise of Ca\textsubscript{i} preceded the EAD depolarization (n = 2 of 5). At sites remote from EAD foci, V\textsubscript{m} preceded Ca\textsubscript{i}, which is expected if voltage is responsible for EAD propagation. At sites of earliest EAD upstroke, Ca\textsubscript{i} followed the EAD depolarization in 3 of 5 hearts; even then, the short V\textsubscript{m}–Ca\textsubscript{i} delays (<7 ms) suggested that normal voltage-driven SR Ca\textsuperscript{2+} release did not occur. In such cases,
the EAD most likely emanated from deeper in the myocardial wall and propagated to the epicardial surface. In cryoablated rabbit hearts in which a thin layer of epicardium survives, Ca\textsubscript{i} elevation always preceded the EAD depolarization.[11] The cryoablation studies support the interpretation that when EAD depolarizations precede Ca\textsubscript{i} elevation, the site of earliest EAD upstrokes correspond to epicardial breakthrough of a transmural depolarization wavefront. The higher spatial dispersion of Ca\textsubscript{i}TD compared with APD dispersion just before the onset of EADs also supports the notion that Ca\textsubscript{i}Ts are not under the control of APs.

5.3.2 Mechanisms linking Ca\textsubscript{i}T oscillations to EADs

The most likely mechanism linking secondary Ca\textsubscript{i}T upstrokes to membrane depolarization is the NCX current, I\textsubscript{NCX}, consistent with reports that NCX blockers suppress EADs and TdP.[180] We speculate that Ca\textsubscript{i}T oscillations are driven by spontaneous Ca\textsuperscript{2+} release from an overloaded SR. In LQT2, the long AP plateau increases Ca\textsuperscript{2+} influx via I\textsubscript{Ca,L} because the voltage-dependent component of inactivation is incomplete and long APDs also reduce the driving force for Ca\textsuperscript{2+} efflux via NCX. These changes indirectly increase SR Ca\textsuperscript{2+} uptake due to the increase of sarcolemmal Ca\textsuperscript{2+} entry and the suppression of Ca\textsuperscript{2+} efflux mechanisms. Consistent with this view, nifedipine, low external Ca\textsuperscript{2+}, and ryanodine/thapsigargin eliminated Ca\textsubscript{i}T oscillations, EADs, and TdP. Thus, our data indicate that spontaneous Ca\textsuperscript{2+} release from an overloaded SR network is the primary cause of ventricular arrhythmias in LQT2.

In dofetilide-induced LQT2, Ca\textsubscript{o} always appeared before EADs and TdP. Although all Ca\textsubscript{o} are expected to elicit INCX oscillations, not all Ca\textsubscript{o} produced EADs because the ability of I\textsubscript{NCX} (due to a Ca\textsubscript{o}) to sufficiently depolarize the cell membrane and produce an EAD depends on several factors: (1) the magnitude of the Ca\textsubscript{i} elevation, which determines the magnitude of
I_{NCX}; [140] V_m during the plateau phase, a determinant of the magnitude of repolarizing K^+ currents: I_{K1}, residual I_{Kr}, and I_{Ks}; (3) the time point along phase 2 of the AP when I_{NCX} rises, a determinant of the number of L-type Ca^{2+} channels that have recovered from inactivation and can be reactivated; and (4) the activation of the opposing repolarizing K^+ currents (I_{Kr} and I_{Ks}) are also time dependent. The data provide compelling evidence that I_{NCX} is the most reasonable mechanism for the generation of EADs through L-type Ca^{2+} channels reactivation, but it cannot entirely exclude a contribution from spontaneous I_{Ca,L} reactivation.

Nonalternans TWL is caused by the lability of ventricular APs, which is preceded by Ca_{iO} and Ca_{iT} lability. Ca_{iO} are not fully synchronized (Figure 17), and could contribute to AP lability through reverse Ca_{i}–V_m coupling. In contrast to TWL, alternans of Ca_{iT}, AP, or TWA was never observed before the onset of ectopy.

5.3.3 Spontaneous SR Ca^{2+} release and arrhythmias

Abnormal Ca^{2+} handling has been implicated in arrhythmogenesis in numerous pathologies,[130] including digoxin toxicity[181] and catecholaminergic polymorphic ventricular tachycardia.[182, 183] VT is triggered by DADs in both of these conditions. DADs are caused by spontaneous SR Ca^{2+} release during diastole and membrane depolarization by INCX.[9] However, the role of spontaneous systolic Ca^{2+} release from SR in the generation of EADs continues to be debated.[184] Ca_{iO} in the form of sparks and waves have been reported in a wide range of cardiac preparations, ranging from isolated myocytes to intact perfused hearts, and have been implicated as an arrhythmogenic mechanism.[185] In a guinea pig model of ischemia/reperfusion, spontaneous Ca_{iO} appeared to drive ventricular ectopy.[186] Reperfusion arrhythmias thus represent another clinically relevant situation involving spontaneous Ca^{2+}
release from overloaded SR. The link between delayed repolarization, SR overload, Ca\textsubscript{i}T oscillations, and TdP may help the development of diagnostic and treatment strategies in patients with LQTS.

5.4 LIMITATION

Dofetilide was used at a relatively high dose to elicit drug-induced LQT2 because lower doses were less reliable at eliciting TdP. Our LQT2 model shows an extreme degree of impaired repolarization compared to most clinical situations and its clinical relevance must be validated. However, it has the advantage of reproducible TdP induction during an acute study.

5.5 CONCLUSION

In LQT2, APD prolongation promotes Ca\textsubscript{i}T oscillations, which precede the appearance of EADs. The data provide a mechanistic explanation for TWL and enhance our understanding of arrhythmogenesis in LQTS. This may lead to improved clinical management of patients with impaired repolarization.
6.0 REGIONAL HETEROGENEITY OF Ca\textsuperscript{2+} KINETICS PROMOTES MEMBRANE POTENTIAL GRADIENTS AND TRIGGERED ACTIVITY IN DRUG-INDUCED LONG QT TYPE 2

Torsade de pointes (TdP) is a lethal polymorphic ventricular tachycardia associated with delayed repolarization as a consequence of congenital or drug-induced/acquired impairment of the rapid rectifying outward potassium current (I_{Kr}), known as long QT type 2 (LQT2).[84-87, 187] Enhanced spatial heterogeneity of repolarization (i.e. dispersion of repolarization (DOR)) in LQT2 provides a substrate leading to unidirectional conduction blocks, initiating and sustaining reentrant activity and has long been implicated as a driving force for arrhythmogenesis. [93, 96, 163] The prolongation of M cell action potentials (APs) under conditions with reduced I_{Kr} has been proposed as the underlying mechanism responsible for enhanced transmural DOR in LQT2.[7] Alternatively, it has been reported that functional variations in levels of I_{Kr} that can be inhibited by class III anti-arrhythmic agents play a key role in the genesis of apex/base difference in AP repolarization in cryoablated LQT2 models.[11]

More recently, interruption in Ca\textsuperscript{2+} homeostasis (i.e. imbalance between Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+} efflux) caused by a prolonged AP has been shown to promote Ca\textsuperscript{2+} overload in the sarcoplasmic reticulum (SR). [188-190] The Ca\textsuperscript{2+} overloaded SR, in turn, leads to abnormalities in Ca\textsuperscript{2+} handling such as spontaneous release of Ca\textsuperscript{2+} from the SR. [14, 191, 192] If the spontaneous release of Ca\textsuperscript{2+} from the SR occurs during the plateau phase of an AP, the non
voltage gated elevation in cytosolic free Ca$^{2+}$ levels leads to voltage instability via Ca$^{2+}$ dependent sarcolemmal transporters. [11, 16, 81, 105]

We recently reported that the LQT2-induced abnormality in Ca$^{2+}$ handling was pronounced, spatially inhomogeneous, and tightly correlated with the distribution of AP duration even before the onset of TdP. [16] Although experimental and clinical studies have demonstrated that Ca$^{2+}$ abnormality can be promoted and could be spatially discordant in LQT2 animal models or in LQT2 patients, [12, 83] the direct interplay between spatial heterogeneity of intracellular Ca$^{2+}$ handling and voltage dispersion in LQT2 is not fully appreciated.

We optically measured intracellular Ca$^{2+}$ dynamics and membrane potential ($V_m$) in a drug-induced LQT2 rabbit model to demonstrate the impact of enhanced spatial heterogeneity of cytosolic Ca$^{2+}$ handling in the augmentation of voltage dispersion, which initiates and sustains LQT2 related arrhythmias.

6.1 METHODS

6.1.1 Heart preparation

Female New Zealand White rabbits (60 to 120 days old) were anesthetized with pentobarbital (35 mg/kg intravenously) and anticoagulated with heparin (200 U/kg intravenously). The heart was excised and perfused on a Langendorff apparatus with Tyrode solution (mM/L): 130 NaCl, 24 NaHCO$_3$, 1.0 MgCl$_2$, 4 KCl, 1.2 NaH$_2$PO$_4$, 50 dextrose, 1.25 CaCl$_2$, gassed with 95% O$_2$–5 % CO$_2$. The solution temperature was adjusted to 37$^\circ$C with a heater. The atrioventricular node was ablated with electrocautery to control heart rate.
Blebbistatin (Sigma-Aldrich, St.Louis, MO; 5 to 10 μM/L for approximately 15 minutes) was added to the perfusate to minimize motion artifact. The heart was immobilized in a chamber and stained with a voltage-sensitive dye (PGH1: 200 μL of 1 mg/mL dimethyl sulfoxide [DMSO] solution) and loaded with a Ca\(^{2+}\) indicator (Rhod-2 AM, AnaSpec, Fremont, CA; 200 μL of 1 mg/mL DMSO solution). Epicardial bipolar pseudo-ECG was continuously monitored. Epicardial pacing with a unipolar electrode from the lateral left ventricle was performed at cycle length 1.2 s (50 beats/minute; profound bradycardia for a rabbit heart). After baseline recordings, LQT2 was induced by perfusing the heart with Tyrode’s solution containing Dofetilide (500 nM/L, Pfizer, New York, NY) and lowering K\(^+\) and Mg\(^{2+}\) concentrations by 50%.[178] This investigation conformed to the current *Guide for Care and Use of Laboratory Animals* published by the National Institutes of Health.

### 6.1.2 Optical apparatus

The optical apparatus, which is based on two high spatiotemporal resolution CMOS cameras (Ultima Scimedia, Costa Mesa, CA; 100 x 100 pixels, 500-1,000 frames per second), has been used for simultaneous measurement of intracellular Ca\(^{2+}\) transients and membrane potential changes. The anterior surface of the heart with a 1.4 cm x 1.4 cm field of view (140 μm x 140 μm pixel resolutions) was illuminated with a 520 ± 30 nm excitation beam generated by a tungsten lamp with an interference filter. The fluorescence beam was divided by a dichroic mirror (660 nm) to focus the Rhod-2 and PGH1 fluorescence signals on two CMOS cameras. The digitized optical signals were saved on computer HD for off-line analysis.
6.1.3 Data analysis

Optical traces of membrane potential ($V_m$) and intracellular $Ca^{2+}$ transient ($Ca_iT$) at each pixel were normalized and digitally low-pass filtered (60 Hz cutoff, Butterworth). Activation time at each site was calculated from $(dFv/dt)_{max}$ of the local $V_m$ or $Ca_iT$ upstroke, and action potential (AP) duration (APD) and $Ca_iT$ duration (Ca$_i$TD) at each pixel was the interval from $(dFv/dt)_{max}$ to the recovery of $V_m$ [2] to 20% of baseline (APD$_{80}$ or Ca$TD_{80}$). APD$_{80}$/Ca$_{i}TD_{80}$ and APD/Ca$_i$TD dispersion was always calculated during paced rhythm, in the absence of triggered activity. Automatic measurement of APD$_{80}$ and CaTD$_{80}$ from all pixels (100 x 100 pixels) was used to calculate APD$_{80}$ and Ca$_{i}TD_{80}$ dispersion, defined as the standard deviation of APD$_{80}$ (or Ca$_{i}TD_{80}$) values. Amplitudes of secondary $Ca^{2+}$ elevation at each pixel were approximated by calculating ‘area under curve’ [137] of normalized Ca$_iT$ over time-interval from beginning of first secondary $Ca^{2+}$ release (SCR) to end of Ca$_iT$ per AP. A maximal recovery slope (MRS) of normalized Ca$_iT$ at each pixel was calculated from negative maximum value in the first derivative of Ca$_iT$ before the onset of SCRs. A modified phase angle analysis was performed to calculate phase angles of Ca$_i$ and $V_m$ kinetics per AP. Briefly, after local maxima and local minima were defined at a given pixel, individual segments between neighboring local minima and maxima were normalized between 0 to $\pi$ (rising phase) or $-\pi$ to 0 (falling phase). Earliest rising times of SCRs and $V_m$ re-depolarization at each pixel were calculated from first zero phase angles. Numbers of phase singularities in Ca$_iT$ per AP at each pixel were calculated by counting a total numbers of local maxima and minima per AP. The amplitude of the $V_m$ gradient vector is defined as
\[ |\text{grad}(V_m)| = \sqrt{\left( \frac{\partial V_m}{\partial x} \right)^2 + \left( \frac{\partial V_m}{\partial y} \right)^2} \]

The gradients were calculated from discrete data (100x 100 pixels) and we used spatial step of 3 pixel sizes to approximate the partial derivatives in the formula above. Spatial heterogeneity (SH) was calculated as natural logarithm of standard deviation of \( V_m \) (or \( \text{Ca}_i \)) signal amplitude in each 100 x100 pixels, averaged over repolarization time-interval (taken from 100 ms after action potential upstroke to \( \text{APD}_{80} \)). Data analysis and creation of map images and video clips was performed with software created by the first author in MatLab. Statistical comparison between SH of \( \text{Ca}_i \) and \( V_m \) was performed with paired t-test.

6.2 RESULTS

6.2.1 Increase in spatiotemporal heterogeneity of intracellular \text{Ca}^{2+} handling in long QT type 2

At a baseline heart rate of 1.2 s cycle length (CL), moderate bradycardia dependent SCRs, which were fully reversible at 500ms CL, occurred during the plateau phase of the AP. After induction of LQT2 but before the onset of triggered activity, the episodes of SCRs per AP, defined with numbers of local minima and maxima, were more pronounced compared to those at baseline (\( p<0.01, n=5 \)) (Figure 23A). Intriguingly, dynamic alternations in the spatial distribution of cytosolic \text{Ca}^{2+} levels (or spatial heterogeneity of amplitudes in \( \text{Ca}_i \)T) were consistently observed in LQT2 as a result of enhanced spatial heterogeneity of \( \text{Ca}_i \) handling (i.e. kinetics of
Ca\textsuperscript{2+} recovery, onset times of SCR, and frequencies of SCR (Figure 23B&C). Occasionally, sub-millimeter scale spatial heterogeneities of cytosolic Ca\textsuperscript{2+} levels with small ‘islands’, which were isolated from neighboring pixels, were formed (Figure 24).

**Figure 23. Abnormality in Ca\textsubscript{i} handling in LQT2** A) Augmentation of SCR in LQT2. Left three panels show superimposed traces of Ca\textsubscript{i}T and phase angles in different conditions. A heart was paced at 1.2s cycle length before induction of LQT2 (baseline) and during LQT2. A right panel shows statistical comparison of numbers of phase singularities (local maxima and minima) during plateau per AP between baseline and LQT2. B) Dynamics alternation of spatial distribution of Ca\textsubscript{i}T amplitude. A left panel shows superimposed traces of Ca\textsubscript{i}T in three different sites (a, b, and c in C). A middle panel shows variation of standard deviations of normalized Ca\textsubscript{i} amplitude in 100 x 100 pixels with a time dependent manner. A right panel shows the statistical comparison of calculated spatial heterogeneity between baseline and LQT2. C) Snapshots of Ca\textsubscript{i} amplitude maps at given times (240ms, 360ms, 480ms, 600ms, and 720ms in B). Square dots indicate site a, b and c in B. D) Spatial heterogeneity of Ca\textsubscript{i} handling. A left panel shows a map of maximum recovery slopes (MRS) of Ca\textsubscript{i}Ts before the onset of SCR. A middle panel shows a map of onset times of SCR. A right panel shows a map of numbers of local maxima and minima during plateau phases of APs. Square dots indicated site a, b, and c.
Regional differences in the delayed recovery of Ca\textsubscript{i}T due to the spatial heterogeneity of SCRs eventually led to dispersion of area under curve [137] of Ca\textsubscript{i}T per AP and Ca\textsubscript{i}TD (Figure 25A). Spatial distribution of calculated AUC was correlated with the dispersion of Ca\textsubscript{i}TD\textsubscript{80} in LQT2 (r=0.932±0.065, n=5). The AUC distribution in LQT2 reflected one of baseline (r=0.88±0.054, n=5), but was more pronounced than in baseline (p<0.02, n=5) (Figure 25B).

Figure 24. Sub-millimeter scale heterogeneity of Ca\textsubscript{i} handling A) A picture of a Langendorff perfused heart. A black square (1.4 cm x 1.4 cm) indicates a field of view of optical mapping. B) Superimposed optical traces of normalized Ca\textsubscript{i} in site a, b and c in C. Vertical dot lines labeled with 1, 2 and 3 indicate time lines of Ca\textsubscript{i} mapping in C. C) Maps of normalized Ca\textsubscript{i} amplitudes at a given time. 1, 2, and 3 indicate the corresponding time lines in B. square dots indicate site a, b, and c with 400 µm distances.
Figure 25. Spatial heterogeneity of AUC and dispersion of Ca\textsubscript{T}D\textsubscript{80} A) A picture of a Langendorff perfused heart. A black square indicates a field of view of optical mapping. a, and b in the square indicate site a and b in the middle panel. A middle panel shows superimposed optical traces of normalized Ca\textsubscript{i} in site a and b in A during baseline (top) and LQT2 (bottom). A right panel shows a time course of variation of SD of normalized Ca\textsubscript{i} amplitudes in 100x100 pixels during baseline (top) and LQT2 (bottom). B) Spatial heterogeneity of AUC (of Ca\textsubscript{T}). Left two panels show spatial distribution of AUC during baseline (left) and LQT2 (right). A right panel shows statistical comparison of SD in AUC between baseline and LQT2. C) Spatial heterogeneity of Ca\textsubscript{i}TD\textsubscript{80}. Left two panels show spatial distribution of Ca\textsubscript{i}TD\textsubscript{80} during baseline (left) and LQT2 (right). A right panel shows statistical comparison of SD in Ca\textsubscript{i}TD\textsubscript{80} between baseline and LQT2.
6.2.2 Spatiotemporal interplay between SCRs and $V_m$ re-depolarization in LQT2

In LQT2, the calculated spatial heterogeneity of $Ca_{i,T}$ amplitudes was more striking than calculated regional differences in $V_m$ (p<0.01, n=6) (Figure 26A). The spatial organization of cytosolic $Ca^{2+}$ levels was closely correlated to dispersion of $V_m$ during the plateau phase of APs at a given time (Figure 26B&C). To investigate whether spatial organization of SCRs promotes $V_m$ dispersion or regional differences in $V_m$ instability elicits spatial heterogeneity of SCRs, we compared the rise times of SCRs with those of phase 2 EADs. Highly selective suppression of SCRs with RyR2 stabilizer, 1μM of K201, was also performed to further elucidate the interplay between SCR and $V_m$. SCRs during the plateau phase of APs in LQT2 always preceded phase 2 EADs (time delay= 9.2±5.2ms, n=50,000) (Figure 26D). This time delay between SCRs and phase 2 EADs caused a loss in the spatial correlation between $Ca_{i}$ and $V_m$ because the spatial heterogeneity of $Ca_{i}$ occurred before $V_m$ dispersion (Figure 26E). Suppression of SCRs eliminated phase 2 EADs (Figure 26E). Furthermore, more oscillatory events were observed at regions with longer APD$_{80}$/Ca$_{TD_{80}}$ (Figure 27 B). The delayed recovery of APs due to phase 2 EADs corresponding to SCRs in LQT2 ($r=0.9546±0.0319$, n=5) promoted further prolongation of AP duration, and regional differences in the delayed recovery of APs resulted in augmented dispersion of repolarization (Figure 27B). Suppression of SCRs with K201 significantly reduced dispersion of repolarization in LQT2 (p<0.01. n=5). These findings suggest that the spatial heterogeneity of SCRs in LQT2 is a driving force of voltage dispersion and/or DOR.
Figure 26. Spatiotemporal coupling between Ca\textsubscript{i} and V\textsubscript{m} A) statistical comparison between calculated spatial heterogeneity (SH) of Ca\textsubscript{i} and V\textsubscript{m}. B) Maps of normalized Ca\textsubscript{i} and V\textsubscript{m} amplitudes at a given time. Square dots indicate site a, b, and c for C. C) Spatial correlation between Ca\textsubscript{i} and V\textsubscript{m} amplitudes. A left panel shows superimposed optical traces of normalized Ca\textsubscript{i} (top) and V\textsubscript{m} (bottom). A vertical dot line indicates a timeline of Ca\textsubscript{i} and V\textsubscript{m} mapping in B. A right panel shows a scatter plot of Ca\textsubscript{i} vs. V\textsubscript{m}. r represents correlation coefficient between Ca\textsubscript{i} and V\textsubscript{m} amplitudes. D) Maps of times of SCRs/Ca\textsubscript{i} oscillations (top) and EADs/V\textsubscript{m} oscillations (bottom). The rise of SCR precedes the rise of EADs in all 100 x 100 pixels. E) Continuous spatial correlation during a single AP in LQT2 (top) and in LQT2 with 1µM of K201 perfusion (bottom) at the same pixel. r represents a spatial correlation coefficient between Ca\textsubscript{i} and V\textsubscript{m}. Higher r values show that either Ca\textsubscript{i} or V\textsubscript{m} causes the other.
Figure 27. Correlation between spatial heterogeneity of $\text{Ca}_i \text{TD}_{80}$ and dispersion of $\text{APD}_{80}$
A) A picture of a Langendorff perfused heart. A black square indicates a field of view of optical mapping. a, and b in the square indicate site a and b in B. B) Tight linear relationship between dispersion of $\text{Ca}_i \text{TD}_{80}$ and $\text{APD}_{80}$. A left panel shows superimposed optical traces of normalized $\text{Ca}_i$ and $V_m$ in site a (top) and b (bottom) in A. A middle panel shows maps of $\text{Ca}_i \text{TD}_{80}$ and $\text{APD}_{80}$. A right panel shows a scatter plot of $\text{Ca}_i \text{TD}_{80}$ vs. $\text{APD}_{80}$. C) Decreases in dispersion of $\text{Ca}_i \text{TD}_{80}$ and $\text{APD}_{80}$ after suppression of SCRs with 1µM of K201. A left panel shows superimposed optical traces of normalized $\text{Ca}_i$ and $V_m$ in site a (top) and b (bottom) in A. A right panel shows maps of $\text{Ca}_i \text{TD}_{80}$ and $\text{APD}_{80}$. D) Statistical comparison of dispersion of $\text{APD}_{80}$ (top) and $\text{Ca}_i \text{TD}_{80}$ (bottom) in LQT2 before and after K201 infusion.
6.2.3 The impact of enhanced \(V_m\) dispersion during phase 3 of APs in the initiation of arrhythmogenic triggered activity

Abnormally high \(V_m\) dispersion, which was correlated with the spatial heterogeneity of cytosolic \(Ca^{2+}\) levels, was observed during phase 3 of APs before the onset of triggered activity in LQT2 (Figure 28A). To test the role of enhanced voltage dispersion during phase 3 of APs in the initiation of triggered activity, \(V_m\) gradients immediately before the onset of triggered activity was calculated. Intriguingly, such \(V_m\) gradients preceding the onset of triggered activity were spatially lined up with the origins of triggered activity (Figure 28B&C). The triggered activity was more pronounced at regions with higher \(V_m\) gradients (>94 percentile) (Figure 28D). The triggered activity was completely suppressed by reducing \(V_m\) gradients as a result of suppression/prevention of SCRs with K201 in 8 out of 9 hearts (pre-perfusion of 1\(\mu\)M of K201 in 4 hearts and post-perfusion of 1\(\mu\)M of K201 in 5 hearts) (Figure 28E). These findings suggest that abnormally enhanced \(V_m\) gradients during phase 3 of APs in LQT2 are \(Ca^{2+}\) dependent and can play a crucial role in the initiation of triggered activity.
Figure 28. $V_m$ gradients and triggered activity

A) An example of $V_m$ gradients and triggered activity in LQT2. A left panel shows superimposed optical traces of normalized $C_{ai}$ (top) and $V_m$ (bottom) in site a, b and c. A middle panel shows maps of normalized $C_{ai}$ and $V_m$ amplitude 10ms before the onset of triggered activity. A right panel shows a map of activation of triggered activity. B & C) Spatial correlations between $V_m$ gradients and the origins of triggered activity in two different hearts. D) Percentile ranks of $V_m$ gradients at the origins of triggered activities in six different hearts. E) Elimination of triggered activity after reduction of $V_m$ gradients with K201 perfusion. A left panel shows superimposed optical traces of normalized $C_{ai}$ and $V_m$ during LQT2 arrhythmia. A right panel shows superimposed optical traces of normalized $C_{ai}$ and $V_m$ after suppression of LQT2 arrhythmia with K201 perfusion in the same heart. VG: $V_m$ gradient, and AT: activation time.
6.2.4 The role of SCEs in the initiation of local early afterdepolarizations (EADs) and propagating delayed afterdepolarizations (DADs)

Previously, we reported that secondary Ca\(^{2+}\) oscillations, which precede \(V_m\) instability, are capable of eliciting reactivation of the L-type calcium channel via Na-Ca exchangers leading to triggered activity such as EADs and DADs.[16] We further investigated the direct interplay between SCRs and triggered activity without mediation of \(V_m\) gradients. In addition to triggered activity along the \(V_m\) gradients, numerous EADs and DADs were observed in this LQT2 models (Figure 29A). At the origination points of EADs and DADs (or the earliest sites of occurrence), a rise of \(Ca_i\) always preceded the rise of \(V_m\) re-depolarizations. The majority of early phase EADs were localized without propagation and were spatially discordant. Occasionally, local propagation of EADs occurred and was driven by voltage (Figure 29B). On the other hand, once DADs were initiated with consistently preceding SCRs to \(V_m\) re-depolarization during phase 4 of APs, global propagation of DADs was persistent and driven by voltage (\(Ca_i Ts follow APs\)) (Figure 29C). These observations are consistent with our previous studies.
Figure 29. EADs and DADs in LQT2  
A) Superimposed optical traces of normalized Ca\textsubscript{i} and V\textsubscript{m} during LQT2 arrhythmia. Green arrows indicate a diastolic elevation of Ca\textsubscript{i} and V\textsubscript{m}. Black arrows labeled with 1, 2, and 3 indicate DADs. Red arrows labeled with a, b, and c indicate EADs occurring immediate after DADs.  
B) Zero phase maps of Ca\textsubscript{i} (left) and V\textsubscript{m} (right) during DADs indicated in A. At the origins of DADs, rising of Ca\textsubscript{i}T precedes rising of V\textsubscript{m}. In the regions of DAD propagation, rising of V\textsubscript{m} precedes to rising of Ca\textsubscript{i}.  
C) Zero phase maps of Ca\textsubscript{i} and V\textsubscript{m} during EADs indicated in A. Most of early phase EADs were localized without propagation, but once propagation of EADs occurred, rising of V\textsubscript{m} precedes rising of Ca\textsubscript{i}. 
6.3 DISCUSSION

LQT2 is known to increase the spatial heterogeneity of AP repolarization (i.e. dispersion of repolarization (DOR)) and has been widely implicated as an arrhythmogenic factor. However, mechanisms fundamental to LQT2 related augmentation of DOR and its impact of arrhythmogenesis remain unclear. Here, we report for the first time that spatially discordant abnormality in $\text{Ca}^{2+}$ handling promotes $V_m$ gradients, which is responsible for triggered activity in LQT2. This $\text{Ca}^{2+}$ dependent dispersion of repolarization offers an alternative to the M-cell hypothesis to explain increase in repolarization gradient in LQT2. Consideration of the mechanisms of these interesting findings requires further discussion.

6.3.1 Transmural DOR vs. epicardial DOR

Because $I_{Ks}$ current density in midmyocardial cells (M cells) is relatively weak, M cells are more sensitive to many APD-prolonging conditions than epicardial and endocardial cells.[193] The preferential AP prolongation of M-cells has been linked to underlying mechanisms of markedly enhanced ‘transmural’ DOR in LQT2.[7, 97] Conversely, experimental studies have shown that heterogeneous AP prolongation occurs along the surface of a heart in various species in LQT2.[11, 93, 96, 194] It is controversial whether or not electrotonic influence of subepicardial M-cells contributes to the large epicardial dispersion of repolarization (Base $\rightarrow$ Apex). However, it is unlikely that M-cells promote epicardial DOR since 1) M cells reside throughout the deep subepicardial layers without any base-apex preference[98, 195] and 2) cryoablation of septum and conduction system except for a 1 mm thick layer of epicardium shows marked base to apex gradients of repolarization in LQT2. Thus, other factors rather than
M-cells may account for epicardial repolarization gradients.[11, 196] Alternatively, it has been proposed that there is a functional difference in the level of $I_{Kr}$ along the epicardium that can be blocked by class III antiarrhythmic drugs.[11] However, experimental evidence supporting this hypothesis is lacking.

### 6.3.2 Possible effects of Ca$^{2+}$ abnormality on AP prolongation

Intriguingly, AP prolongation in LQT2 is often associated with oscillatory events and modulated with agents which increase $I_{Ca,L}$.[7] The role of Ca$\text{_{i}}$ handling in further AP prolongation in LQT2 is not fully demonstrated, but it is possible that cytosolic Ca$^{2+}$ elevation during AP plateau due to spontaneous SR Ca$^{2+}$ release could produce membrane potential instability (i.e. EADs) and eventually result in further repolarization delay in LQT2. Previously, we reported that oscillations in Ca$\text{_{i}}$T occurred on the surface of the heart in a rabbit LQT2 model before the onset of TdP and were correlated with further AP prolongation.[16, 197] It is not surprising that spontaneous elevation (oscillation) in Ca$\text{_{i}}$ level, which is not voltage gated, delays AP repolarization as a consequence of positive Ca$\text{_{i}}$-V$_m$ coupling via Ca$^{2+}$ dependent sarcolemmal transporters namely as NCX, but the interplay between Ca$\text{_{i}}$ handling and AP prolongation in LQT2 has not been fully understood.

### 6.3.3 Markedly enhanced Ca$^{2+}$ heterogeneity in LQT2

Ca$^{2+}$ homeostasis (i.e. balance between transmembrane Ca$^{2+}$ influx and Ca$^{2+}$ efflux) in cardiac myocytes is tightly modulated by membrane potential and by the Ca$^{2+}$ uptake and release controlled by proteins on the SR. Experimental studies have shown that these processes are
altered in numerous pathologies and result in changes in systolic and diastolic Ca\textsuperscript{2+}.\cite{70, 71, 78, 80, 198} Specifically, conditions with delayed repolarization alter the amount of Ca\textsubscript{i} uptake in the lumen of the SR by SERCA during each cardiac cycle due to the delayed voltage dependent inactivation of L-type Ca\textsuperscript{2+} channels (increase in Ca\textsuperscript{2+} influx) and the reduced NCX driving force (decrease in Ca\textsuperscript{2+} efflux). Thus, beat to beat imbalance between Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+} efflux results in gradual SR Ca\textsuperscript{2+} overload leading to spontaneous SR Ca\textsuperscript{2+} release. The next question is whether or not this phenomenon can be spatially heterogeneous. Experimental results have suggested that intercellular Ca\textsuperscript{2+} diffusion through gap junctions may facilitate synchronous spontaneous Ca\textsuperscript{2+} releases in neighboring cells.\cite{197, 199} On the other hand, it was reported that spontaneous SR Ca\textsuperscript{2+} release can be persistent and even more pronounced when gap junctions are inhibited.\cite{143} This observation suggests that intracellular oscillatory events in a single cell can result from its own intrinsic properties rather than intercellular synchronization.

Here, after induction of LQT2, the spatial and temporal complexity of cytosolic Ca\textsuperscript{2+} levels was significantly enhanced as a consequence of spatiotemporal changes in Ca\textsubscript{i} kinetics. This is consistent with clinical observations showing that the relaxation of cardiac contraction in LQT2 patients was spatially inhomogeneous.\cite{83} The underlying mechanisms of mechanical dispersion in LQT2 and its influence to voltage dispersion remain elusive. It is possible that this spatial heterogeneity of Ca\textsubscript{i} handling in LQT2 reflect repolarization gradients. However, since Ca\textsuperscript{2+} uptake in SR by SERCA and spontaneous Ca\textsuperscript{2+} release are not fully voltage dependent, regional variations in intrinsic properties of Ca\textsubscript{i} dynamics are likely to cause the Ca\textsuperscript{2+} heterogeneity. Experimental studies have shown that in mammalian hearts, Ca\textsubscript{i} handling molecules such as Cav1.2\textalpha, NCX1, and SERCA2a are differentially expressed across the ventricular wall as well as along the epicardial surface and this regional difference is pronounced
more in female than in male.[12, 20, 21, 200] As a result, intrinsic inhomogeneous distribution of Ca\(^{2+}\) handling molecules could explain markedly enhanced Ca\(^{2+}\) heterogeneity in LQT2.

6.3.4 Spatial similarity of Ca\(^{2+}\) abnormality between bradycardia and LQT2

In a previous chapter, it was shown that bradycardia alone elicits spontaneous Ca\(^{2+}\) releases, which are spatially inhomogeneous and tightly correlated with the regional expression of Ca\(^{2+}\) handling molecules. The similarity in spatial distribution of Ca\(_i\) handling between bradycardia baseline and bradycardia-LQT2 (Figure 25B) illustrates that complex spatial patterns of Ca\(_i\) handling in LQT2 may reflect intrinsic heterogeneity of Ca\(_i\) handling, which was already accentuated in bradycardia alone. Even if this conjecture is accepted, it is unclear why the submillimeter-scale spatial heterogeneity of Ca\(_i\) should only appear after LQT2 induction. In addition to complex heterogeneity in Ca\(_i\) dynamics, Ca\(_i\) signaling mechanisms are likely to be activated and to complicate the system through positive and negative feedback processes. Additional experiments are required to fully elucidate the answer to this problem.

6.3.5 Correlation of Ca\(^{2+}\) and V\(_m\) gradients

In numerous pathologies, spatially discordant alternations in cytosolic Ca\(^{2+}\) handling are responsible for the dispersion of V\(_m\) instability/alternans, presumably due to positive Ca\(_i\)-V\(_m\) coupling via electrogenic Ca\(^{2+}\) dependent Na-Ca exchangers.[10, 26, 102] The most striking observation in this report is that spatiotemporal alternations in Ca\(_i\) handling in LQT2 were tightly correlated with dynamic changes in V\(_m\) dispersion. The notions 1) that SCRs, whose regional variation is responsible for the spatial heterogeneity of Ca\(_i\), always precede early phase
membrane re-depolarization, 2) that spatial heterogeneity of Ca\textsubscript{i} and Ca\textsubscript{TD} are consistently bigger than spatial heterogeneity of V\textsubscript{m} and APD respectively, and 3) that perfusion with 1µM K201, which acts exclusively on ryanodine receptors and specifically affects Ca\textsubscript{i} handling\cite{151}, abolished the spatial heterogeneity shows that the regional variation of Ca\textsubscript{i} handling can promote voltage dispersion.

### 6.3.6 V\textsubscript{m} gradients initiate triggered activity

Repolarization gradients in LQT2 have been shown to initiate unidirectional conduction blocks and have been separated from that of spontaneous SR Ca\textsuperscript{2+} release which initiates triggered activity such as early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs). In this model, triggered activity was initiated in the steepest region of voltage gradients (3.12±0.64 mV/mm\textsuperscript{2}, n=6). A voltage gradient along the direction of propagation of electrical activity is normal and facilitates synchronized contraction throughout the heart. However, in experimental studies, steep voltage gradients in pathologies can generate arrhythmogenic triggered activity\cite{116, 201}. For example, in myocardial infarct models, a voltage gradient across the ischemic border zone can lead to a diastolic injury current at the normal side of the ischemic border. Such small injury currents can slightly depolarize the normal tissue and facilitate the occurrence of triggered activity. \cite{116} Although it is well established that gradients of diastolic Ca\textsuperscript{2+} levels promote repolarization gradients in numerous pathologies\cite{20, 22, 26, 102} the direct interplay between Ca\textsuperscript{2+} dependent voltage gradients and triggered activity has not been established.

Here, we observed that LQT2 mediated triggered activity is also initiated by V\textsubscript{m} gradients during phase 3 of APs. Despite high spatial correlation between origins of triggered activity and
voltage gradients in this study, further investigation of clear mechanistic explanation on the
impact of Ca\(^{2+}\) mediated voltage gradients in the initiation of triggered activity is required.
Theoretically, regional differences in cytosolic Ca\(^{2+}\) levels can generate voltage dispersion via
electrogenic NCX during the plateau phases of APs. If electrotonic currents from ‘donor’ cells are sufficient to partially depolarize ‘recipient’ cells, triggered reactivation of L-type channels can initiate triggered activity.

In cryoablated rabbit LQT2 models, Maruyama et al. recently reported similar observations. According to the report, enhanced voltage gradients during phase 3 of APs can promote phase 3 EADs that lead to triggered activity. Intriguingly, they argued that the late phase voltage dispersion in acquired LQT syndrome was independent of regional variations in Ca\(^{2+}\) handling because the phase 3 EADs were not suppressed with BAPTA infusion.[196] However, the efficacy of BAPTA in chelating Ca\(^{2+}\) in intact animal models is controversial.[10] In contrast, we observed here that highly specific inhibition of secondary Ca\(^{2+}\) release with 1µM of K201, a dose at which it selectively decreases the RyR2 open probability, consistently eliminated triggered activity. Previous interventions into cytosolic Ca\(^{2+}\) handling with partial inhibition of L-type Ca\(^{2+}\) channels with nifedipine or by reducing [Ca\(^{2+}\)]_o suppressed triggered activity.[16] Such experimental evidence supports that triggered activity is Ca\(^{2+}\)-dependent.

Since we tested the role of voltage gradients in the initiation of triggered activity on the surface of hearts, further investigation is required to elucidate whether a steep voltage gradient on the surface of hearts account for the triggered activity or the triggered activity is initiated in deeper regions and propagates to the surface along the voltage gradient. However, experimental evidence has shown that triggered activity was observed in cryoablated rabbit hearts under similar LQT2 conditions,[11, 196] which suggests that TA may not originate intramurally. In the
canine wedge model of LQT2, breakthrough sites of triggered activity occurred consistently along the epicardial voltage gradient and not deep inside the ventricular walls.[202] If the triggered activity originates intramurally independent of voltage gradient, the breakthrough pattern of TA could not follow epicardial voltage gradient but rather propagates to earlier recovery areas.

6.3.7 Non voltage-gradient mediated triggered activity such as EADs and DADs in LQT2

In a way, the observed requirement for steep $V_m$ gradient in triggering propagated EADs is not surprising: secondary Ca$_i$ oscillations occurring during the AP plateau in the absence of local $V_m$ gradient would be expected to cause a “local” EAD, but no propagation because the EADs may not be sufficient to initiate propagating triggered activity – this is a phenomenon we have repeatedly observed (Figure 7). On the other hand, Ca$_i$ oscillations that occur even in the absence of a local $V_m$ gradient in excitable tissue can certainly cause propagating ectopic beats, also known as DADs, which has been consistently observed by our group and others (Figure 29).[11, 16, 196, 203]
6.4 LIMITATIONS

Optical recordings of membrane potential and Ca\(^{2+}\) dynamics on the anterior surface of rabbit hearts revealed a complex spatial heterogeneity of Ca\(_i\) handling which was responsible for voltage dispersion leading to triggered activity in LQT2. The 2D nature of optical mapping does not provide LQT2 related enhancement of transmural differences in Ca\(_i\) handling and its link to triggered activity which originates from deeper layers of the ventricles. In addition, positive coupling of Ca\(_i\)-V\(_m\) via I\(_{\text{NCX}}\) most likely accounted for Ca\(_i\) dependent V\(_m\) dispersion in LQT2, but due to the lack of a reliable NCX inhibitor, further assessment of the NCX mediated interplay between Ca\(_i\) heterogeneity and V\(_m\) dispersion was not fully investigated.

6.5 CONCLUSION

In summary, we report that a marked increase of Ca\(_i\)T heterogeneity develops under LQT2 conditions, with the formation of unexpectedly small, irregularly shaped regions of elevated Ca\(_i\). We propose that the increase in Ca\(_i\)T heterogeneity contributes to augmentation of V\(_m\) gradients and DOR and is highly arrhythmogenic by generating triggered activity or by initiating reentrant circuits. The suppression of TdP by a RyR2 selective K201 concentration underscores the importance of a detailed understanding of arrhythmogenic mechanisms for the development of rational treatment strategies.
APPENDIX A

INTRACELLULAR Ca\textsuperscript{2+} OSCILLATIONS AND T-WAVE LABILITY (TWL) PRECEDE TORSADE DE POINTES (TDP) IN A RABBIT MODEL OF LONG QT TYPE 2 (LQT2)

Authors: Jan Nemec, Jong Kim, Bethanny Gabris, Guy Salama, University of Pittsburgh, Pittsburgh, PA ; J. Am. Coll.Cardiol.2010;55;A2.E16 doi:10.1016/S0735-1097(10)60017-4

Background: Action potential (AP) prolongation results in TdP in clinical and experimental LQT2, but the exact mechanism remains uncertain. T-Wave alternans has been proposed as a marker of arrhythmic risk and is elicited by APD and Ca\textsuperscript{2+} alternans. However, non-alternans TWL has been associated with TdP in LQT syndrome. We hypothesized that TWL at constant heart rate precedes TdP in a LQT2 model and that it is caused by abnormal Cai dynamics.

Methods: Female rabbit hearts were perfused, AV node was ablated and ventricles paced at 50 beats per minute. Epicardial ECG was recorded during optical mapping (RH 237 and Rhod-2 AM) of AP and Cai. LQT2 was mimicked by Dofetilide (D; 500 nM/L) and decrease in [K\textsuperscript{+}]\textsubscript{o} and [Mg\textsuperscript{2+}]\textsubscript{o}. TWL was calculated as logarithm of root-mean-square of differences between T wave amplitude from subsequent beats.

Results: D prolonged AP durations and induced TdP (n=8/8). Baseline Ca transient (CaT) was usually monophasic. Occasionally, a small secondary peak was observed. The number of CaT peaks per AP during paced rhythm increased upon D addition and prior to VT onset compared to
baseline in all cases (Number of CaT per AP: 1.33±0.39 vs 2.32±0.47, p<0.002). This was accompanied by increased TWL (-4.06±0.94 vs -2.89±0.95, p<0.002).

**Conclusions:** TWL precedes TdP in rabbit LQT2 model. Complex Ca dynamics (>1 CaT peak per AP) occurs during prolonged AP, promoting TWL and TdP. Possible mechanisms include secondary Ca2+ release from an overloaded sarcoplasmic reticulum and enhanced Na/Ca exchange current.
APPENDIX B

ACQUIRED LQT2 LEADS TO MARKED SPATIAL HETEROGENEITY (SH) OF Ca$^{2+}$ TRANSIENT

Authors: Jong J. Kim, Jan Nemec, Guy Salama, University of Pittsburgh, Pittsburgh, PA; J. Am. Coll. Cardiol. doi:10.1016/S0735-1097(11)60003-X 2011;57;E3.

Background: Enhanced dispersion of repolarization (DOR) in LQT2 and abnormalities of intracellular Ca$^{2+}$ [2] have been proposed as arrhythmogenic mechanism of Torsade de Pointes (TdP). However, the interplay and relative roles of DOR and SH of Cai is not understood.

Methods: Simultaneous optical maps of membrane voltage (Vm) and Cai were recorded from the anterior surface of Langendorff rabbit hearts (n=7) paced at 50 bpm after AV node ablation. After perfusion with normal Tyrode (baseline; B), LQT2 was induced by adding dofetilide (0.5 μM) and reducing K+ (2 mM). Standard deviation of Vm and Cai signal amplitude was calculated in each 100x100 pixel frame and averaged over repolarization time-interval (from 100 ms after action potential upstroke to APD80). SH was calculated from paced beats as natural logarithm of this average at B and during LQT2.

Results: Irregular regions of elevated Cai (~2x3 mm) appeared during phase 2 and 3 of action potential. During LQT2, SH of Cai exceeded SH of Vm in all hearts (-3.28±0.45 vs -3.55±0.41; p<0.01), although high Cai correlated with high Vm. At B, SH of Vm and Cai were similar and
significantly lower than during LQT2.

**Conclusions:** SH of Cai transient during LQT2 exceeds SH of Vm. Regions of elevated Cai exhibit complex oscillations on a mm scale. SH of Cai may reflect intrinsic regional differences of Cai handling mechanisms accentuated by repolarization delay of LQT2 rather than follow SH of Vm. Instead, SH of Cai appears to enhance SH of Vm, elevate DOR and facilitate TdP.
APPENDIX C

BRADYCARDIA ELICITS A SECONDARY Ca\textsuperscript{2+} ELEVATION DURING THE ACTION POTENTIAL (AP) PLATEAU WHICH IS SPATIALLY HETEROGENEOUS, PROLONGS AP DURATIONS (APD) FURTHER, ENHANCES DISPERSION OF REPOLARIZATION (DOR) AND MAY EXPLAIN THE ARRHYTHMOGENIC PROPERTIES OF BRADYCARDIA

Authors: Jong J. Kim, Jan Nemec, Rita Papp, and Guy Salama, University of Pittsburgh, Pittsburgh, PA; AHA 2011 Scientific Session APS.403.03a

Introduction: Bradycardia is known to prolong APD and DOR and is a factor that promotes arrhythmia in long QT type 2. In numerous conditions, spontaneous Ca\textsuperscript{2+} release from sarcoplasmic reticulum has been shown to trigger early afterdepolarizations, ectopic activity that initiate arrhythmias, but the interplay between secondary Ca\textsuperscript{2+} elevation (SCE) and membrane excitability has not been demonstrated in bradycardia.

Objectives: To correlate voltage-depolarization during the AP plateau to SCE during bradycardia.

Methods: Dual optical mapping of intracellular calcium transient (CaT) and AP was performed in Langendorff perfused rabbit hearts. After AV node ablation, CaT and AP dynamics were investigated at physiological (120 beats per minute (bpm)) and slow heart rate (50 bpm).
**Results:** Upon changing HR from 120 to 50 bpm, APD gradually increased with a time-constant of $53.8\pm8.9$ s, consistent with clinical QT measurements. The shift from 120 to 50 bpm elicited SCE during the AP plateau that was a) regionally heterogeneous, b) associated with enhanced depolarization of the AP plateau and was reversed by pacing at 120 bpm. Regional differences of SCE at 50 bpm were significantly increased ($P<0.01$, $n=7$) and were correlated with dispersion of APD ($r=0.9277\pm0.03$, $n=7$). SCE and APD prolongation were more pronounced at the base of right ventricles than the apex of left ventricles ($P<0.01$, $n=7$). Suppression of SCE with K201 (1 µM/L) (to stabilize RyR2) reduced APD ($P<0.01$, $n=5$) and DOR ($P<0.02$, $n=5$). The molecular basis of the spatial distribution of SCE is currently being correlated to the intrinsic distributions of Ca$^{2+}$ handling channels and transporters (Cav1.2α, RyR2, NCX and SERCA2a).

**Conclusion:** These data show for the first time that bradycardia elicits SCE which contributes to AP prolongation and its spatial heterogeneity increases DOR. These changes explain why bradycardia is a critical factor to trigger Torsade de Pointes in LQT2.
APPENDIX D

THE ROLE OF SPATIAL HETEROGENEITIES OF INTRACELLULAR Ca\(^{2+}\) OSCILLATIONS (Ca\(i\)O) IN LONG QT TYPE 2 (LQT2)-RELATED ARRHYTHMIAS

Authors: Jong J. Kim, Jan Nemec, and Guy Salama, University of Pittsburgh, Pittsburgh, PA; Heart Rhythm, Vol 8, No. 5, PO6-113, May Supplement 2011

Introduction: Spontaneous Ca\(^{2+}\) release from internal stores, Ca\(i\)O and dispersion of refractoriness (DOR) have been implicated as underlying mechanisms of Torsade de Pointes (TdP) in LQT2. DOR remains a fundamental arrhythmogenic mechanism because it generates conduction blocks and the steep voltage (\(V_m\)) gradients that sustain reentry. Enhanced DOR is partly due to intrinsic heterogeneities of ionic currents but may also depend on spatial and dynamic heterogeneities of Ca\(i\)O.

Methods: Simultaneous optical maps of membrane voltage (Vm) and Cai were recorded from the anterior surface of Langendorff rabbit hearts (n=7) paced at 50 bpm after AV node ablation. After perfusion with normal Tyrode’s, LQT2 was induced with dofetilide (0.5 µM) and low K\(^+\) (2 mM) and Mg\(^{2+}\) (0.5mM). Time delays between Ca\(i\)O and Vm was quantitatively analyzed using phase maps. Spatial correlation between Ca\(i\)O amplitudes and DOR/Vm dispersion in LQT2 but before the onset of triggered activity (TA) was investigated. Cai and Vm heterogeneities were mapped and correlated to the origins of TA.
**Results:** Spontaneous Ca\textsubscript{i}O preceded phase 2 and 3 depolarization by 9.1503±5.1874ms. Local CTD\textsubscript{80} was dependent on the amplitude of Ca\textsubscript{i}O and was spatially correlated to APD\textsubscript{80} (COE = 0.9546±0.0319, n=5). The spatial heterogeneity of preceding Ca\textsubscript{i}O was correlated to Vm dispersion (COE=0.8172±0.093, n=5). TA occurred primarily at the boundaries of high and low Vm.

**Conclusions:** Spatial heterogeneities of Ca\textsubscript{i}O can alter local V\textsubscript{m} dynamics resulting in enhanced DOR or large Vm gradient that can initiate TA and provide the substrate of local conduction blocks needed to sustain TdP.
BIBLIOGRAPHY


