THE TEMPORAL NATURE OF ECTOPIC ACTIVITY IN GUINEA PIG VENTRICULAR MYOCARDIUM

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ACADEMIC ABSTRACT

The temporal nature of ectopic activity is important to elucidating the mechanisms that can lead to arrhythmogenesis. However, challenges remain in distinguishing between ectopic and non-ectopic beats. A new methodology was developed and validated to distinguish between beat types. Rapid pacing was used to induce both ectopic and non-ectopic beats. Using an electrocardiogram, the post-pacing recovery beat cycle length (RCL) and QRS were normalized to pre-paced R-R and QRS intervals and analyzed using a K-means clustering algorithm. Control hearts only produced beats with RCL ratios that increased with rapid pacing, suggestive of non-ectopic activity. Hypercalcemia and digoxin both produced significantly earlier beats with wider QRS durations, suggestive of ectopic activity. Increasing pacing further shortened RCL during digoxin + hypothermia, a mechanistic identifier of ectopic activity. When tested against a previously validated analysis, our algorithm performed well. Therefore, this electrocardiogram based algorithm distinguishes between ectopic and non-ectopic beats. In a prospective study, tetrodotoxin increased RCL ratio without changing the QRS duration of excited beats, suggesting neuronal sodium channels play an important role in ectopic beat timing. The next goal was to create a consistent model of ectopic activity. Both sympathetic and parasympathetic stimulation independently potentiate arrhythmogenesis, and we investigated the effects of independent and simultaneous stimulation on the temporal nature of arrhythmogenesis. Isoproterenol (ISO), a sympathetic agonist, transiently produced ectopic activity and increased heart rate. Acetylcholine (ACh), a parasympathetic agonist, did not significantly produce ectopic activity but did slow heart rate. ACh added after ISO also transiently produced ectopic activity, while heart rate remained slowed. Importantly, ISO following ACh <u>persistently</u> increased ectopic activity and heart rate.

Therefore, ISO following ACh is an ideal model for creating sustained ectopic activity. Mature animals exhibited sustained arrhythmogenesis while young animals did not. When ACh was removed and then followed by ISO, ectopic activity and heart rate transiently increased, similar to ISO alone. This suggests that maintained ACh perfusion can sustain ISO sensitivity, in contrast to ISO perfusion alone. The data in this dissertation provide an insight into the mechanisms that affect the ectopic beat timing and arrhythmia propensity.

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GENERAL AUDIENCE ABSTRACT

Cardiovascular disease is the leading cause of death in the United States, and cardiac arrest plays a large role in these deaths. Many of these deaths are due to lethal arrhythmias, or flaws in the heart's electrical activity. Ectopic beats, or beats with abnormal origin and timing, have been linked with the occurrence of these lethal arrhythmias and deaths. The temporal nature of these ectopic beats can reveal the underlying mechanisms that lead to their formation. However, it remains difficult to differentiate between ectopic and non-ectopic beats. One of the goals of this dissertation was to develop a methodology to distinguish between beat types. Using an electrocardiogram, cycle length and QRS duration of beats were normalized and analyzed using a K-means 2-cluster algorithm. Control hearts appeared to only produce non-ectopic beats, as expected. Hypercalcemia and digoxin produced a mixture of ectopic and non-ectopic beats. Importantly, increasing pacing rate decreased the cycle length ratio of the ectopic beat group, as expected of ectopic beats. A neuronal sodium channel inhibitor delayed the formation of these ectopic beats. This suggests that neuronal sodium channel play an important role in ectopic beat timing. The next goal was to create a reproducible model of ectopic activity. Sympathetic stimulation, which is activated during exercise, has been linked with arrhythmogenesis. Similarly, parasympathetic activity, which is activated during sleep, has also been linked with arrhythmogenesis. We investigated how activating sympathetic or parasympathetic stimulation individually and in combination temporally modulates arrhythmia risk. Infusion of a sympathetic agonist transiently produced ectopic activity in the heart, while a parasympathetic agonist produced little arrhythmogenesis. Parasympathetic following sympathetic stimulation reintroduced ectopic activity. Importantly, sympathetic following parasympathetic persistently

produced ectopic activity. Age was an important factor for this sustained response, as young guinea pigs returned transient ectopic activity. Sustained parasympathetic stimulation was also important to the persistent response, and removing parasympathetic stimulation followed by sympathetic stimulation again produced transient ectopic activity. The data in this dissertation provide an insight into the mechanisms that affect the ectopic beat timing and arrhythmia propensity.

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GLOSSARY OF ABBREVIATIONS

Acetylcholine	ACh
β–Adrenergic Receptor	β–AR
Basic Cycle Length	BCL
Delayed Afterdepolarization	DAD
Isoproterenol	ISO
Overdrive Excitation	OE
Overdrive Suppression	OS
Sodium-Calcium Exchanger	NCX
Sodium-Potassium ATPase	NKA
Recovery Beat Cycle Length	RCL
Tetrodotoxin	TTX
Triggered Activity	TA

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Chapter 1: Introduction

On a global scale, cardiovascular disease is the leading cause of death, and sudden cardiac arrest plays a large role in these deaths.[1] In the United States, 300,000 people die from sudden cardiac arrest every year.[2] Sudden cardiac death often occurs as the result of flaws in the electrical activity of the heart, leading to lethal arrhythmias.

Three types of arrhythmias have been linked with sudden cardiac death: abnormal automaticity, triggered activity (TA), and reentry.[3] The aim of this proposal is to study TA. TA can occur within both the atria and ventricles, but the focus of this dissertation will be to study TA observed in the ventricles, or ectopic beats. In order to initiate ventricular fibrillation, a trigger is needed. Ectopic beats are commonly seen in non-pathophysiological hearts and do not necessarily lead to ventricular tachycardia or fibrillation.[4] However, certain arrhythmogenic substrates have increased susceptibility to ectopic beat formation and sudden cardiac death.

Structural heart diseases, such as heart failure,[5] myocardial infarction,[6] and ischemia,[7] and genetic conditions, such as catecholaminergic polymorphic ventricular tachycardia[8] and Andersen-Tawil Syndrome,[9] are vulnerable to ectopic beats and sudden cardiac death. Furthermore, simple everyday changes such as extracellular ion concentrations, rest or alarm, or circadian rhythms have also been linked with ectopic activity and sudden cardiac death. Therefore, we chose to study the temporal nature of ectopic activity in order to elucidate the mechanisms that can increase arrhythmogenesis. To achieve this, two studies were performed. The goal of the first study was to develop and validate an algorithm to distinguish between ectopic beats and non-ectopic beats. Once this was accomplished, we determined that we needed to create a reproducible model of ectopic beats, which was achieved with the second study. During the course of these studies, we have investigated the mechanisms affecting ectopic beat latency and the time course of its incidence.

Cardiac Function Overview

Action potential

The heart acts as a pump moving both oxygenated and deoxygenated blood throughout the body by contracting and relaxing. Electrical activity passes through cardiac myocytes via gap junctions to synchronize the contraction/relaxation process. The sino-atrial node acts as the pacemaker for the heart, setting the heart rate and generating the electrical signal. This signal passes through the atria to the atrioventricular node, where a short pause occurs. This pause allows the atria time to contract and pass blood into the ventricles. After the pause at the AV node, the signal travels through the His bundle into Purkinje fibers that branch into both the left and right ventricle, allowing for the ventricles to contract simultaneously and push blood into the arteries. The ventricles bear the main responsibility for moving the blood to the rest of the body, and thus they are vitally important for keeping an organism alive. Coordinated contraction of the ventricular cells, therefore, is important and this is synchronized by electrical activity.

Electrical activity within the heart takes the form of an action potential, which is a transient change in transmembrane electrical potential. Ventricular cells at rest remain at an intracellular electrically negative potential relative to the extracellular space. Electrical current passing from neighboring cells raises the transmembrane potential, activating the voltage-gated Na^+ channels and allowing for a very rapid influx of positively charged Na^+ ions which quickly depolarize the cell. This is known as phase 0, or the upstroke, of the action potential. Following this upstroke, the transmembrane potential repolarizes as K^+ ions leave by K^+ channels (phase 1). The L-type calcium channels then activate and calcium flows into the cell, creating a depolarized plateau phase (phase 2) for the action potential as calcium flows in and K^+ leaves. In time calcium ceases to flow into the cell as the L-type calcium channels close or inactivate and K^+ channels activate allowing more K^+ to leave the cell, leading to repolarization (phase 3). To maintain the ionic concentration gradients, the Na^+-K^+ ATPase pump removes three Na^+ ions and brings in two K^+ ions, which contributes to the resting negative transmembrane potential. In addition, the Na^+-calcium exchanger (NCX) maintains concentrations gradients as well, although this exchanger

moves three Na^+ ions for every calcium ion and has the ability to move ions in either direction. I_{K1}, a K^+ current, is also responsible for maintaining the resting negative transmembrane potential.

Calcium handling

As stated before, electrical activity propagation through cardiac myocytes allows for synchronized contraction within the cells. This is by a process known as excitation-contraction coupling, where cardiac myocyte excitation also leads to contraction. Following membrane depolarization calcium flows into the cell, and these ions bind to calcium-sensitive ion channels that lie along the sarcoplasmic reticulum, which stores calcium. These calcium-sensitive receptors, known as ryanodine receptors, release calcium into the cytosol, quickly elevating intracellular calcium levels. This is known as calcium-induced calcium-release. Free cytosolic calcium then triggers contraction by exposing the actin protein to the binding of myosin. Calcium is uptaken back into the sarcoplasmic reticulum via a calcium pump known as sarcoplasmic reticulum calcium ATPase (SERCA2a) and is removed out of the cell into the extracellular space by NCX. This allows for calcium to unbind from the contractile machinery and the cell again reaches a relaxed state.

Ventricular Arrhythmias

Three types of mechanisms are known to contribute to the emergence of clinical arrhythmias: automaticity, TA, and reentry. Prior to the 1970s, arrhythmogenesis was attributed to only automaticity and reentry. Some early discoveries in the 1940s,[10] followed by work in 1960s and 70s with glycosides,[11-14] discovered that following the termination of rapid pacing, oscillations were observed in the membrane potential. Oscillatory amplitude increased as a function of pacing rate, while the cycle length of these oscillations, or the interval between the last paced beat and the onset of the oscillation, decreased.[11,12] When the amplitude of these oscillatory afterpotentials was raised to a certain threshold, a beat could be initiated. These oscillatory afterpotentials would later be named delayed afterdepolarizations (DADs), and the beats initiated by these DADs were

called TA, as the initiation was dependent on a rapid pacing protocol or a "trigger".[15] This behavior was in contrast to beats initiated by automaticity as these were not preceded by oscillations and had an opposite relationship with pacing rate where the cycle length progressively lengthened as pacing rate increased.[16,17] However, the question remained whether TA was responsible for ventricular arrhythmias in the clinical setting. Close observation led to the discovery that onset of oscillations coincided with the genesis of ventricular arrhythmias.[13] This suggested that TA could play a role in glycoside-induced ventricular arrhythmias.

Distinguishing arrhythmias

To identify whether TA could be responsible for ventricular arrhythmias in whole heart models, several groups utilized canine intact heart experiments.[17-19] At times DADs may precede triggered beats,[12,20-23] making it simple to identify the mechanism behind beat initiation. In intact or whole heart studies it becomes more difficult as the beat may not necessarily initiate in the field of view (optical mapping or electrode array), or only ECG leads may be available. Without the ability to witness preceding DADs and spontaneous calcium releases it becomes difficult to determine the mechanism behind beat formation. The first step to determining if these arrhythmogenic beats were the result of TA was to see if they behaved as triggered beats do in Purkinje fibers and isolated ventricular myocytes. As a result, studies investigated glycoside-perfused intact heart behavior in the presence of various pacing rates. The objective was to determine if the coupling interval of the recovery beat, or the interval of from the last paced beat to the first recovery beat, could be changed as a result of different pacing rates. It was confirmed that glycoside-perfused hearts exhibited recovery beat coupling intervals that progressively decreased as a function of pacing rate,[18,19] similar behavior to DAD and TA behavior observed in Purkinje fibers and ventricular muscle strips. This is known as overdrive excited behavior, and is associated with electrical activity that initiates prematurely in ectopic sites, or ectopic beats. In contrast, hearts without glycosides, or under non-arrhythmogenic conditions, returned increasing

coupling intervals as a function of pacing rate and manifested overdrive suppressed behavior.[18] In laboratory settings, other factors have also been used to validate the presence of TA as a mechanism, including the acceleration of ventricular tachycardia for a few beats after pacing and specific mechanism-suppressant drugs.[18]

However, the difficulty thereafter lies in differentiating between ectopic and non-ectopic beats, as the same heart in an arrhythmogenic preparation has the ability to respond to rapid pacing with both types of recovery beats. An ectopic beat should have a shorter cycle length than automaticity-induced beat, as they respond differently to rapid pacing.[11,16] Additionally, groups looking to confirm ectopic beats have included recovery beats that have a shorter cycle length than the longest post-paced R-R interval, of ventricular origin, with preceding DADs, or with preceding spontaneous calcium releases.[18,19,24,25] In Chapter 2 we developed and validated a new simple and robust methodology to differentiate between ectopic beats and non-ectopic beats.

Calcium-mediated arrhythmias

Calcium plays a crucial role in arrhythmogenesis. Intracellular calcium rises under certain conditions, leading to spontaneous releases of calcium from the sarcoplasmic reticulum via ryanodine receptors. These transient rises in calcium during diastole, otherwise known as spontaneous calcium releases, are differentiated from the calcium transient during an action potential in that they are not the result of electrical excitation and have much smaller amplitudes. Controversy remains as to what mechanisms lead to the release of calcium via the ryanodine receptor. Increased cytosolic calcium during diastole may trigger calcium release by binding to the ryanodine receptor.[26] Alternatively, calcium build up within the sarcoplasmic reticulum as a result of increased cytosolic calcium may lead to a spontaneous release of calcium by ryanodine receptors.[27-29] The sudden rise in diastolic calcium leads to an oscillatory depolarization of the transmembrane potential, or a delayed afterdepolarization (DAD), presumably via NCX. The DAD

may then initiate TA.[30] Importantly, these calcium-mediated arrhythmias may accompany ventricular tachycardia and sudden cardiac death.[31-34]

Arrhythmogenic conditions

Difficulty remains in creating arrhythmogenic models that will induce ectopic beats and yet survive (i.e. not induce VT/VF) experimental protocols long enough to obtain relevant data. The various models, which have previously been used in ex-vivo Langendorff heart preparations and were used in this research, are described here. As stated above, cardiac glycosides have previously been linked with DADs and TA.[12,21] Increased concentrations of extracellular calcium are associated with augmented amplitude and incidence of calcium events in cardiomyocytes,[35,36] and hypercalcemia has also been linked with spontaneous calcium releases and ectopic beats in ex-vivo guinea pig ventricular preparations.[25] Therefore, both cardiac glycosides and hypercalcemia were used in Chapter 2 to create conditions for ectopic beats to occur. After we developed and validated a model to differentiate between ectopic and non-ectopic beats, we determined that a different intervention was needed to create a reproducible model of ectopic beats, as cardiac glycosides created substrates vulnerable to VT/VF and hypercalcemia seldom produced ectopic beats.

Sympathetic and parasympathetic stimulation

The autonomic nervous system is composed of two branches: sympathetic and parasympathetic. Both branches play a role in heart rate and contractility. Sympathetic stimulation by β-adrenergic receptors agonists is known to increase both heart rate and contraction. Briefly, sympathetic stimulation activates adenylyl cyclase and therefore increases cAMP production, which then activates Protein Kinase A and leads to the phosphorylation of the calcium handling proteins, thereby increasing intracellular calcium.[37-39] As sympathetic stimulation augments calcium handling, this creates arrhythmogenic conditions, and indeed sympathetic agonists have been linked to ventricular arrhythmias and precursors of ventricular arrhythmias

such as spontaneous calcium releases.[23,39,40] Exercise, which activates the sympathetic system, can increase ectopic beat burden and sudden cardiac death incidence.[41-46]

Parasympathetic stimulation by muscarinic receptor agonists has previously been demonstrated to have opposing effects on calcium handling in the cell.[47-49] However, it has also been associated with increased risk of ventricular arrhythmias. One fascinating study on idiopathic ectopic beats observed that for some patient populations ventricular arrhythmias were preceded by enhanced sympathetic activity, while for other populations parasympathetic activity preceded ectopic beat incidence.[50] Furthermore, studies have shown previously that not only are ectopic beats associated with exercise, but with the recovery phase from exercise as well, which is associated with parasympathetic dominance.[42,51] Indeed, there exist a subgroup of patients who only experience ectopic beats during the recovery phase, and not during exercise.[42] Overall, this suggests that for a certain portion of the population, parasympathetic stimulation can contribute to or facilitate arrhythmogenesis.

The acute effects (seconds) of sympathetic and parasympathetic stimulation on cardiac electrophysiology and calcium handling are well established. Agonization of their respective receptors can lead to not only immediate impacts on the heart, but prolonged exposure (minutes to hours) to these agonists can also impact receptor internalization,[52-54] receptor sensitivity,[55] and protein phosphorylation.[56] Therefore, cardiac electrophysiology responsiveness to these agonists can undergo time-dependent changes. Importantly, these *temporal* effects may impact arrhythmogenesis.

Simultaneous stimulation of sympathetic and parasympathetic activity may also produce time-dependent effects on cardiac electrophysiology and calcium handling. Previous studies have demonstrated that the effect of parasympathetic stimulation can be modulated in the presence of sympathetic stimulation,[57-59] known as accentuated antagonism. This effect, however, is dependent on the length of exposure to sympathetic and parasympathetic stimulation,

and the order in which these branches were activate.[60] The effect of simultaneous sympathetic and parasympathetic stimulation on ventricular arrhythmias remains unclear. A few studies on diving, an example of a natural form of simultaneous autonomic activation, have suggested that simultaneous autonomic activation may increase arrhythmogenic propensity.[61] Furthermore, the time- and order-dependent effects of β adrenergic and muscarinic agonists on the arrhythmogenesis remains unclear. In Chapter 3 we determine how activating one autonomic branch in the absence and presence of the other temporally modulates arrhythmia risk, while also determining the best model to reproducibly create ectopic beats.

Research Objectives

The overarching goal of this work is to investigate the temporal nature of ectopic activity. In order to accomplish this goal, Chapter 2 focuses on differentiating ectopic beats from non-ectopic beats. Therefore, a new methodology is validated to differentiate between overdrive suppressed (i.e. non-ectopic) and overdrive excited (i.e. ectopic) beats. Previous studies have simply used beat cycle length to differentiate between these beat types.[19] This metric may be insufficient as a non-ectopic beat may occur before an ectopic beat at slow pacing rates and be classified as ectopic behavior. Thus, we proposed to use a simple and robust methodology that incorporates both normalized recovery beat cycle length and QRS duration, and we compared the values between the proposed ectopic and non-ectopic beat populations. In a prospective analysis, we validated our algorithm against a QRS complex cross-correlation analysis that has previously been used to distinguish between beat types.[62] Finally, we wanted to demonstrate that ectopic beat cycle length could be pharmacologically manipulated. Previous studies have shown that tetrodotoxin, a sodium channel inhibitor, can blunt calcium handling,[63] decrease arrhythmia incidence,[63] and delay the timing of calcium events.[64] We tested the hypothesis that tetrodotoxin can increase the cycle length of ectopic beats.

In Chapter 3, we investigated how activating sympathetic or parasympathetic stimulation individually and in combination temporally modulates arrhythmia risk. To accomplish this, sympathetic and parasympathetic pathways were activated by direct perfusion of agonists and ectopic beat incidence was evaluated over time. Acetylcholine (ACh) and isoproterenol (ISO) acted as the parasympathetic and sympathetic agonists, respectively. Ectopic activity and heart rate were evaluated for ISO, ACh, ACh following ISO, and ISO following ACh. To test the hypothesis that the concurrent activation of parasympathetic stimulation was needed to produce the persistent response for ISO following ACh, ISO was perfused following ACh removal. Moreover, to address the possibility that pre-paced heart rates may be affecting arrhythmogenesis we also compared the pre-paced R-R intervals for pacing protocols that resulted in ectopic beats versus those which did not. Finally, age has previously been shown to have increase sympathetic-induced arrhythmia burden,[65,66] and we investigated the effect of age on arrhythmogenic risk during simultaneous stimulation.

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Chapter 2: Distinguishing between overdrive excited and suppressed ventricular beats in guinea pig ventricular myocardium

Abstract

Rapid ventricular pacing rates induces two types of beats following pacing cessation: recovery cycle length (RCL) prolongation (overdrive suppression) and RCL shortening (overdrive excitation). The goals of this study were to compare common experimental protocols for studying triggered activity in whole-heart preparations and differentiate between recovery beats using a new methodology. Post-pacing recovery beat cycle length (RCL) and QRS were normalized to pre-paced R-R and QRS intervals and analyzed using a K-means clustering algorithm. Control hearts only produced suppressed beats: RCL ratio increased with rapid pacing (25±4.0%, n=10) without changing QRS duration. Rapid pacing during hypercalcemia + hypothermia (5.5 mM and 34°C) produced significantly earlier excited beats (53±14%, n=5) with wider QRS durations (58±6.3%, n=5) than suppressed beats. Digoxin + hypothermia (0.75 μM) produced the most excited beats with significantly earlier RCL (44±3.2%, n=6) and wider QRS (60±3.1%, n=6) ratios relative to suppressed beats. Increasing pacing further shortened RCL (30±7.8%, n=6). In a prospective study, TTX (100 nM) increased RCL ratio (15±6.0%, n=10) without changing the QRS duration of excited beats. The algorithm was compared to a cross-correlation analysis with 93% sensitivity and 94% specificity. This ECG based algorithm distinguishes between ectopic and non-ectopic beats.

Introduction

The heart electrically excites and contracts in a specific order, as automaticity in sino-atrial nodal cells sets a rhythm faster than other cardiac cells. However, at times electrical activity can originate prematurely at ectopic sites, leading to arrhythmias. Early work in canine purkinje fibers using cardiac glycosides found premature low amplitude membrane depolarizations following rapid pacing.[1-3] These small depolarizations grew in amplitude as the pacing rate was increased, and the interval between the last paced beat and the onset of the oscillation decreased.[1,2] Close observation led to the discovery that the onset of these small depolarizations in Purkinje fibers were concordant with the onset of ventricular arrhythmias.[3] This suggested that small depolarizations may be responsible for cardiac glycoside-induced ventricular arrhythmias. Indeed, these depolarizations, later known as delayed afterdepolarizations (DADs), could initiate an action potential if the amplitude was large enough.[1] These action potentials initiated by DADs later became known as triggered activity (TA), as their initiation was dependent on rapid pacing or "trigger."[4] Finally, the recovery beat cycle length of TA decreases as pacing rate increases, and this is referred to as overdrive excited (OE) behavior and is associated with ectopy.[5-7] In contrast, rapid pacing can also overdrive suppress (OS) the recovery beat by temporarily suppressing normal automaticity,[7-9] presumably by hyperpolarizing the membrane.[10] Therefore, rapid pacing can produce mechanistically different recovery beats: normal (OS) and arrhythmogenic (OE).

Later observations in intact heart models discovered that glycosides decreased recovery beat cycle length following rapid pacing,[7,11] suggesting that triggered recovery beats in intact models with glycoside toxicity were due to TA, as their behavior was consistent with data from Purkinje fibers. More generally speaking, cardiac glycosides and many other interventions such as hypercalcemia,[12] catecholamine perfusion,[13] and myocardial infarction[14] are associated with an increased incidence of DADs and TA .

Importantly, the recovery beat cycle length may be insufficient to distinguish between OS and OE beats. For example, an automatic beat could occur before a triggered rhythm at slow pacing rates and be classified as a triggered rhythm. Therefore, additional criteria are necessary to distinguish between mechanistically different recovery beats. The goals of this study were to 1. Differentiate between ectopic and non-ectopic beats with a new methodology and 2. Demonstrate that ectopic beats can be pharmacologically manipulated.

Materials and Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and has been approved by Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute& State University.

Guinea pig Langendorff preparations

Retired breeder male guinea pigs (n=37) were anesthetized with sodium pentobarbital (325mg/kg) and injected intraperitoneally with heparin to prevent blood clotting. Atria were excised to remove competitive stimulation from atria, and ventricles were perfused in a Langendorff system with oxygenated Tyrode solution (in mM, $CaCl_2$ 1.25, NaCl 140, KCl 4.56, dextrose 5.5, $MgCl_2$ 0.7, HEPES 10; pH to 7.4 with approximately 5.5mL of NaOH) at 37ºC and 50 mmHg. Motion was reduced using 7.5mM 2,3-diacetylmonoxime.

Electrocardiography (ECG) and interventions

A volume-conducted bath electrocardiogram (ECG) similar to lead I was continuously recorded to determine recovery cycle length (RCL) and QRS duration of the QRS complex immediately after the cessation of pacing. Hearts were paced with plunge bipolar electrodes fixed in the basal interventricular septum at pacing rates from 200 to 375 bpm for 15 seconds. Hearts were also perfused with the glycoside digoxin (0.75μM), high extracellular calcium (5.5mM $CaCl_2$), or the sodium channel inhibitor tetrodotoxin (TTX, 100nM). Hearts were maintained at normal

(37ºC) and hypothermic (34ºC) temperatures. ECG measurements were made 10 minutes after the start of perfusion. RCL and QRS ratios were then normalized to the pre-paced beat's cycle length (BCL) and the QRS, called RCL and QRS ratios, respectively. The recovery window was 1500 msec. A k-means 2 cluster analysis, which is an automated clustering algorithm that calculates the distance from each data point to a continuously calculated centroid of each cluster, was used to separate the control, hypercalcemic, digoxin, and hypothermic data into two clusters (Cluster 1 and Cluster 2).

A prospective analysis was performed for the digoxin + tetrodotoxin data set, where the centroids of Clusters 1 and 2 from the original data set (control, hypercalcemia, digoxin, hypothermia) were calculated and recovery beats for digoxin + tetrodotoxin were stratified into two clusters depending on the centroid they were closest to.

To determine sensitivity and specificity of our new algorithm, cluster classification was compared to a previously validated cross-correlation analysis as the gold-standard.[15]. The maximum cross-correlation coefficient was used in this study to analyze QRS morphology similarities between Cluster 1 and 2 recovery beats and their prospective pre-paced beats. True positive was defined as OE beats with a coefficient<0.9, false positive as OE beats with a coefficient≥0.9, true negative as OS beats with a coefficient≥0.9, false negative as OS beats with a coefficient<0.9.

Statistical analysis

Where appropriate, unpaired and paired two-tailed Student's t-tests with equal/unequal variance or the nonparametric Wilcoxon rank-sum test were used to analyze data significance. The F-test for equality of two variances was used to analyze variance significance. A Fisher's exact test was used where appropriate. The Sidak correction for multiple comparisons was used. A $p<0.05$ was considered significant. Values are reported as mean ± standard error. Retrospective power analyses were performed to determine the recommended sample sizes. The

minimum number of samples per intervention was calculated using a power analyses for a 1 sided, two independent sample normal distribution test with alpha 0.05 and a power of 0.8. Minimum number of beats:

Hypercalcemia: 5 OS and 5 OE beats

Hypothermia + hypercalcemia: 5 OS and 5 OE beats

Digoxin: 4 OS and 4 OE beats

Hypothermia + digoxin: 5 OS and 5 OE beats

Digoxin + TTX: 4 OS and 4 OE beats

Results

Overdrive suppression and excitation

Example ECGs from control hearts in Figure 2.1A demonstrate that RCL increases when pacing rate is increased from 200 bpm to 375 bpm. Representative ECGs in Figure 2.1A also demonstrate in hearts perfused with 0.75 μM digoxin that increasing pacing can decrease the recovery beat's RCL. For all experiments, increasing pacing rate in control hearts from 200 bpm to 375 bpm significantly increases RCL, whereas during digoxin, rapid pacing decreases RCL (Figure 2.1B).

The QRS complexes of the recovery beat in the control hearts in Figure 2.1A appear similar, while the digoxin recovery beats at 375 bpm exhibit variable ECG characteristics. Specifically during rapid pacing, digoxin recovery beats can be associated with 1. narrow QRS durations and increased RCL (Figure 2.2, top panel) and with 2. wide QRS durations and decreased RCL (bottom panel) relative to the pre-paced beat. In summary, at 200 bpm, the RCL of digoxin recovery beats was 511±94 ms and the QRS 27±8 ms. At 375 bpm, RCL significantly decreased to 388ms but the standard deviation significantly increased to 154ms. Conversely, QRS increased to 36ms without significantly altering standard deviation. This suggests that recovery beats in the digoxin group (Figure 2.1B) may be composed of beats that could be either

automatic or triggered activity (TA). Therefore, it is important to stratify beat type in order to study mechanistic behaviors.

To stratify recovery beats, we used a k-means 2-cluster analysis on raw RCL and QRS values. Figure 2.3 demonstrates preferential clustering based on RCL values, because the range of RCL values (205 to 1226 ms) is significantly larger than the QRS duration range (18 to 64 ms). More specifically, k-means is based on each data point's distance from a centroid. Therefore the larger range has a larger effect on clustering, and as a result, clustering raw RCL and QRS reduces the influence of QRS duration.

Furthermore, pharmacologic intervention and inter-animal variability can underlie differences in the basic cycle length (BCL) and QRS morphology of native beats, and therefore the recovery beat. For example, any intervention which increased native rhythm rates would likely decrease all recovery beat RCL. This analysis could classify the recovery beat as an OE beat without accounting for the fact that BCL changed. A similar argument can be made for QRS.

To account for BCL and QRS variability, we chose to normalize recovery beat RCL and QRS. In Figure 2.4A, an ECG before, during, and after pacing illustrates how the BCL and QRS durations were measured prior to pacing (QRS1), and how the RCL and QRS durations were measured post-pacing (QRS2). We normalized the RCL and QRS duration of the recovery beat to the pre-paced beat's BCL and QRS duration, respectively (Figure 2.4A). The RCL and QRS ratios of all recovery beats from multiple interventions were then plotted and the aforementioned k-means 2-cluster analysis performed. Figure 2.4B demonstrates recovery beat classification, where Cluster 1 (filled circles) is composed predominantly of beats with late relative RCL (≥1) and narrow QRS complexes (~1), and Cluster 2 (open circles) is composed predominantly of beats with early RCL (≤1) and wide QRS complexes (>1). The averages and standard deviations for these clusters are as follows: Cluster 1 RCL 1.31±0.33 and QRS 0.94±0.16 and Cluster 2 RCL

0.64±0.22 and QRS 1.53±0.32. These Cluster 1 beats, therefore, may be overdrive suppressed, while Cluster 2 beats may be overdrive excited.

Control

Keeping the cluster assignment for each recovery beat, we observed that control conditions only produced beats that were within Cluster 1 (Figure 2.5A, solid circles). Consistent with the theory of preferential overdrive suppression (OS) in normal hearts, faster pacing rates (gray circles) prolonged RCL relative to slower rates (black circles). For all experiments, rapid pacing at 375 bpm significantly increased the RCL ratio without affecting QRS duration. Furthermore, mean native and recovery QRS durations from control hearts was 29 ± 4 ms, which is comparable to *in vivo* and *ex vivo* guinea pig measurements with atria intact.(Batey et al., 1997; Stark et al., 1993) Therefore, these data suggest that recovery beats in these experiments with atria removed were all OS, and the pacemaker site for OS beats may be localized to the atrioventricular node or His bundles.

Control + hypothermia

Some studies have investigated spontaneous calcium release dynamics under hypothermic conditions,[12,16] which may impact TA characteristics. Therefore, we assessed recovery beats under physiologic hypothermia (34°C). Consistent with control conditions, hypothermia only produced beats assigned to Cluster 1 (Figure 2.5B). Furthermore, RCL ratio was increased following faster relative to slower pacing (right panel, gray and black bars), while QRS ratio remained unchanged. As in control hearts, this is consistent with OS behavior. Lastly, hypothermia did not affect RCL or QRS ratios relative to control at normothermic conditions.

Hypercalcemia

Hypercalcemia has previously been shown to induce spontaneous calcium releases and TA.[12,16] Hypercalcemia (5.5mM) under normal physiological temperature was investigated, and surprisingly, produced beats preferentially assigned to Cluster 1 (Figure 2.6A, closed circles).

Within Cluster 1, RCL and QRS ratios were unaffected by pacing rate. Due to lack of power, Cluster 2 data is presented but not statistically compared to Cluster 1.

Hypercalcemia + hypothermia

Slower pacing in hypercalcemic + hypothermic hearts resulted in more recovery beats classified in Cluster 1 (Figure 2.6B, black filled circles). During rapid pacing, more hearts exhibited Cluster 2 beats (gray open circles), and the RCL ratio of Cluster 2 beats was significantly shorter and QRS ratio significantly wider than Cluster 1 beats. Thus, overdrive excited (OE) beats were initiated under hypercalcemia during hypothermia. Surprisingly, the RCL ratio of Cluster 1 beats was significantly shorter at faster relative to slower pacing rates (right panel, closed bars). Hypothermia significantly decreased RCL ratio for fast paced Cluster 1 beats relative to normothermia in Figure 2.7A (#).

Digoxin

Digoxin produced beats classified in Clusters 1 (closed circles) and 2 (open circles, Figure 2.7A). Like hypercalcemia, faster pacing did not increase RCL ratio for Cluster 1 beats as evidenced by black and gray filled circle overlap in the left panel and no difference between the average RCL ratios.

At faster pacing rates (Figure 2.7A, gray symbols), RCL ratio was significantly shorter for Cluster 2 relative to Cluster 1 and this was paralleled by a significant increase in the QRS ratio. Cluster 2, therefore, behaved as expected of OE recovery beats.

Digoxin + hypothermia

Digoxin during hypothermia produced beats within Clusters 1 and 2. The RCL ratio for Cluster 1 beats was not significantly affected by rapid pacing, suggesting that Cluster 1 beats were insensitive to overdrive pacing (Figure 2.7B, filled black and gray bars). This is similar to observations in normothermic digoxin conditions.

Regardless of pacing rate, Cluster 2 beats (Figure 2.7B, open bars) manifested decreased RCL and increased QRS ratios relative to the Cluster 1 beats (filled bars). Importantly, increasing pacing rate shortened the RCL ratio of Cluster 2 beats, as evidenced by the comparison of open bars (+, black and gray) without changing QRS ratio. Thus, Cluster 2 beats behaved as expected of OE beats.

With respect to normothermic digoxin in Figure 2.7A, hypothermic digoxin increased RCL ratio for Cluster 1 beats following relatively slower pacing (Figure 2.7B, #). Additionally, hypothermia increased the QRS ratio of Cluster 2 beats relative to normothermic digoxin (#).

Arrhythmia incidence

We also investigated the average incidence of Cluster 2 beats following rapid pacing in order to determine which experimental conditions produced more OE beats. Figure 2.8A demonstrates that temperature (open and filled bars) did not significantly affect average OE beat incidence per animal during digoxin or hypercalcemia (black and gray bars). Further, digoxin produced significantly more OE beats than hypercalcemia. Ventricular tachycardia or fibrillation (VT/VF) incidence was also investigated to determine the susceptibility of interventions. During normothermia, digoxin produced significantly more VT/VF than hypercalcemia (Figure 2.8B).

Mixed population effects

Figure 2.1B suggests that digoxin decreases RCL, but Figure 2.7A suggests that the population of Cluster 2 beats increases at faster pacing rates. Specifically Cluster 2 beats at 200 bpm account for 2 out of 42 beats, while at 375 bpm they account for 33 out of 66 beats. Thus, the decrease observed in Figure 2.1 may be a result of a switch at faster pacing rates from overdrive suppressed to overdrive excited beats. Figure 2.9A summarizes what happens to uncorrected RCL if Cluster 1 and 2 beats (gray and white respectively) are stratified from all recovery beats (black), and Figure 2.9B further demonstrates that the clustering algorithm accentuates the decrease in RCL ratio attributable to Cluster 2 beats. Furthermore, the RCL ratio

demonstrates a similar relationship for stratified Cluster 2 beats, suggesting that the RCL ratio retains any relationship that RCL would have uncovered.

Stratifying between Cluster 1 and Cluster 2 beats also resulted in a much smaller variance for Cluster 2 beats for pacing rates greater than 250 bpm (Figure 2.9C), suggesting that OE beats occurred within a narrow time window. However, these Cluster 2 beats did not manifest any significant changes in variance as a result of pacing rate, suggesting that these beats occurred within a narrow time window regardless of pacing rate.

Digoxin + tetrodotoxin

We sought to prospectively test the performance of this algorithm by utilizing a pharmacologic protocol previously demonstrated to reduce calcium mediated arrhythmia vulnerability.[17,18] Hearts were perfused with digoxin and tetrodotoxin (TTX) and assigned to clusters based on the shortest distance to *a priori* cluster centroids determined from Figure 2.4. Hearts were only paced at 375 bpm to preferentially elicit OE beats. As with digoxin, Cluster 2 beat (Figure 2.10A, open circles) RCL ratios were smaller (0.73±0.04 vs 1.25±0.04) and QRS ratios larger (1.37±0.02 vs 1.02±0.02) relative to Cluster 1 beats. Importantly, while TTX did not change the QRS ratio for Cluster 2 beats, it significantly increased the RCL ratio (gray bars).

A cross-correlation analysis was also performed to determine QRS morphology similarity between recovery beats and pre-paced beats. The maximum cross-correlation coefficient from each analysis was determined for each recovery beat (Figure 2.10C). It was found that Cluster 2 beats had significantly smaller maximum correlation coefficients, indicating that QRS morphologies differed more from pre-paced beats. Stratified beat sensitivity and specificity were calculated using the maximum cross-correlation coefficient as the gold standard. Our clustering algorithm yielded high sensitivity (0.93) and specificity (0.94) relative to the cross-correlation algorithm (Table 1).

Discussion

Rapid pacing is known to induce two types of recovery beats following the cessation of pacing: an interval of quiescence greater than or equal to the native rhythm, or a shortened interval. While we recapitulated previous studies demonstrating that RCL is proportional to pacing rate under control conditions[7,8] and inversely proportional during perfusion with a cardiac glycoside,[7,11] the relationship observed for digoxin in Figure 2.1B may be misleading. More precisely, our beat classification system demonstrates that two distinct mechanistic populations compose recovery beats. The effect of grouping these mixed populations together would therefore significantly underestimate the magnitude of RCL shortening at very fast pacing rates. Therefore, in this study we present a methodology to differentiate between normal overdrive suppressed (OS) and arrhythmogenic excited (OE) rhythms independent of experimental intervention or inter-animal variability.

Previous methods for analyzing triggered activity (TA) in whole hearts have included analyzing recovery beats: 1. of ventricular origin, 2. that have a shorter cycle length than the longest post-paced R-R interval, 3. with preceding delayed afterdepolarizations (DADs), or 4. with preceding spontaneous calcium releases.[7,11,12,19] The use of raw RCL and QRS values resulted in preferentially clustering based on RCL values. Moreover, native rhythms and QRS complexes were altered by certain interventions, thereby affecting the ability to identify normal and arrhythmogenic beats based on raw RCL and QRS values. Therefore, we sought a simple and robust method for differentiating between recovery beats.

Our methodology utilized both normalizing the recovery beat cycle length to the pre-paced beat cycle length and the recovery beat QRS duration to the pre-paced beat QRS duration. The finding that OE beat RCL ratios reduced in response to increased pacing rate is consistent with previous reports of similar measurements using normalized recovery beat cycle length to post-pacing R-R interval as a mechanistic stratifier.[11] Indeed, RCL and RCL ratio for OE beats were

significantly smaller when compared to OS beats, consistent with OE beat behavior. Additionally, the QRS normalization data suggests that OE beats may occur lower in the ventricular conduction system or ventricular tissue than OS beats, as has been previously suggested.[20]

Overdrive –suppression - normothermia

Our results demonstrated that control hearts only exhibited beats classified as OS, consistent with previous studies,[7,9,21] and characteristic of normal automaticity. Other groups have proposed that OS may be caused by rapid pacing hyperpolarizing the membrane in response to activation of the sodium-potassium ATPase (NKA).[10,22] In contrast, the RCL ratio of OS beats during hypercalcemia was insensitive to pacing rate. To our knowledge, this is the first report of the effects of hypercalcemia on OS beats in whole heart preparations. Potential explanations for this finding may be that rapid pacing during hypercalcemia increases diastolic intracellular calcium more than under normocalcemic extracellular concentrations,[23] thus augmenting NCX forward mode calcium extrusion, thereby increasing resting membrane potential,[24] and perhaps making tissue hyperexcitable. Previous studies found that purkinje fibers recover more quickly from overdrive-induced hyperpolarization,[25] or have an increased rate of diastolic depolarization,[26] when placed in high calcium solutions.

Directly inhibiting NKA with digoxin created OS beats insensitive to pacing rates. This is consistent with previous research that demonstrated cardiac glycosides can blunt the prolongation of the recovery interval typically seen under control conditions.[27] Furthermore, others have demonstrated that the glycoside ouabain abolishes overdrive hyperpolarization and shifts the maximum diastolic potential to more positive values in feline purkinje fibers,[28] guinea pig and sheep ventricular muscle, and purkinje fibers.[29] In summary, the increase in RCL could be due to membrane hyperpolarization by NKA and, subsequently, NKA inhibition could blunt hyperpolarization and pacing rate-RCL dependence.

Overdrive –suppression - hypothermia

The expected relationship between OS RCL and pacing rate is maintained for control conditions, but surprisingly increasing pacing rate during hypothermic hypercalcemia decreased OS RCL ratio. Sensitivity and specificity for the ratio analysis was high (0.8 and 0.98 respectively) using a QRS cross-correlation analysis as the gold standard.[15] Furthermore, cross-correlation analysis revealed the same RCL to pacing rate relationship for OS beats. Both analyses suggest the surprising finding that rapid pacing may actually decrease the RCL of hypothermia + hypercalcemia OS recovery beats. As discussed above during normothermia, hypercalcemia may increase NCX activity and depolarize the membrane. Previous studies under severe hypothermia (21-22°C) have suggested that NCX plays a larger role in calcium sequestration,[30] which may augment membrane potential depolarization during rapid pacing, perhaps resulting in automatic recovery beats that return earlier. This hypothesis requires validation, however.

Lastly, OS RCL during hypothermic digoxin was insensitive to pacing rate. Unlike hypercalcemia which directly increases intracellular calcium by increasing the calcium driving force, NKA inhibition increases intracellular sodium prior to calcium. Therefore, NCX may not significantly depolarize the membrane during hypothermic digoxin and rapid pacing because intracellular sodium and calcium rise concurrently.

Overdrive –excitation - normothermia

Once again, control conditions never produced an OE beat in this study. In 6 hearts perfused with hypercalcemia, only 2 hearts produced OE beats and, therefore, conclusions cannot be readily drawn from this protocol. For digoxin, relatively slow pacing rates did not generate OE beats in sufficient hearts for statistical comparison or discussion.

Yet, RCL variance for digoxin OE beats was significantly smaller than for OS beats, suggesting that the mechanisms underlying OE beats may be more temporally deterministic than mechanisms underlying OS beats. Interestingly, RCL variance for OE beats did not change with pacing rate, which may be inconsistent with a previous study that demonstrated that increasing

pacing rate in intact tissue decreases the standard deviation of spontaneous calcium release timing.[16] Furthermore, post-hoc analysis of another study suggests that spontaneous calcium release timing variability in whole hearts decreases as a function of pacing rate as well.[12] This intriguing discrepancy could be a result of differences in spontaneous calcium releases and their subsequent fully propagated TA response.

Overdrive –excitation - hypothermia

In contrast to normothermia, all 5 hearts perfused with hypothermic hypercalcemic solutions exhibited OE beats at the fastest pacing rates, but not at the slowest pacing rate, thus precluding a discussion of mechanisms. However, during hypothermic digoxin, the OE RCL ratio decreased as pacing rate increased, consistent with previous studies demonstrating that rapid pacing decreases RCL of TA.[12,19] The proposed mechanism of OE is due to a rise in diastolic intracellular calcium levels leading to increased probability of a spontaneous calcium release from the sarcoplasmic reticulum causing forward mode calcium extrusion through NCX thereby depolarizing the membrane prematurely.[31,32] As mentioned previously, hypothermia can raise resting membrane potential and make tissue more likely to exhibit a triggered beat. In short, hypothermic digoxin not only produced the most OE beats, but the behavior of these beats was consistent with TA.

Sodium channel inhibition and excited beats

We also investigated whether inhibition of TTX-sensitive sodium channels could impact the timing of OE beats. OE beat RCL ratio was prolonged in the presence of the sodium channel inhibitor tetrodotoxin (TTX). This is consistent with the finding that non-specific sodium channel blockers can delay the time to spontaneous calcium release.[33] Indeed, previous studies have suggested that sodium channel inhibitors can act as antiarrhythmic agents, decreasing DAD amplitude and preventing OE beats.[17,18] A few mechanisms have been proposed to explain the delay of onset of OE beats, such as decreased membrane excitability secondary to sodium

channel inhibition. However, the native QRS duration and QRS ratio of OE beats remained unaffected by this degree of sodium channel inhibition, suggesting that membrane excitability was not altered. Further, we have shown that this concentration of TTX did not modify membrane excitability in an Andersen-Tawil syndrome guinea pig model of calcium mediated arrhythmias.[17] Another possible mechanism is that TTX prevents intracellular calcium accumulation.[34] While further investigation is needed to understand the mechanisms by which sodium channel inhibition affects OE beats, these results demonstrate that inhibiting sodium channels prolongs the time to the OE.

Algorithmic performance

The maximum coefficient from cross-correlation analysis of QRS complexes has previously been used to identify premature ectopic beats of ventricular origin, and resulted in high specificity and sensitivity values indicating that it is a good predictor for identifying ectopic beats.[15] Therefore, in this study, the lower maximum correlation coefficient found for OE beats suggests that our prospective analysis can distinguish between ectopic and non-ectopic beats. Furthermore, the sensitivity and specificity of our analysis validated against the maximum coefficient was high.

Conclusions

We present a new methodology for distinguishing between automatic and triggered activity. OS beats exhibited automatic behavior supported by data demonstrating that increasing pacing rate increased RCL ratio in control conditions. During hypothermic digoxin conditions OE beats manifested triggered activity characteristics as RCL ratio decreased. Moreover, OE beats manifested overdrive acceleration. These OE beats also exhibited larger QRS ratios and smaller RCL ratios when compared to OS beats within experimental conditions. Hypercalcemia and digoxin were able to create OE beats, whereas control conditions could not, supportive of the hypothesis that calcium overload underlies TA. Hypothermic digoxin produced the most OE beats.

Sodium channel inhibition, which has been shown to ameliorate calcium mediated arrhythmia burden, increased the time to initiation of an OE beat. The prospective analysis performed well, suggesting that our methodology is able to distinguish between OS and OE beats. Our quantitative methodology presents a relatively simple technique for differentiating between automatic and ectopic beats.

Limitations

The electromechanical uncoupler 2,3-diacetylmonoxime (BDM or DAM) was used in this study. This compound alters intracellular calcium concentrations and handling, and therefore may impact ectopic beat formation.[35] Therefore, it may impact the occurrence of ectopic beats. Nevertheless, all experimental conditions were exposed to 2,3-diacetylmonoxime and thus its impact would have been included for all beats.

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Table 1

Truth Table: Sensitivity and Specificity of Digoxin + TTX Stratified Beats

		Cross-Correlation	
		Ectopic Beat	Normal Beat
K-Means	Cluster 2	True Positive: 28	False Positive: 1
	Cluster 1	False Negative: 2	True Negative: 16
		Sensitivity: 0.93	Specificity: 0.94

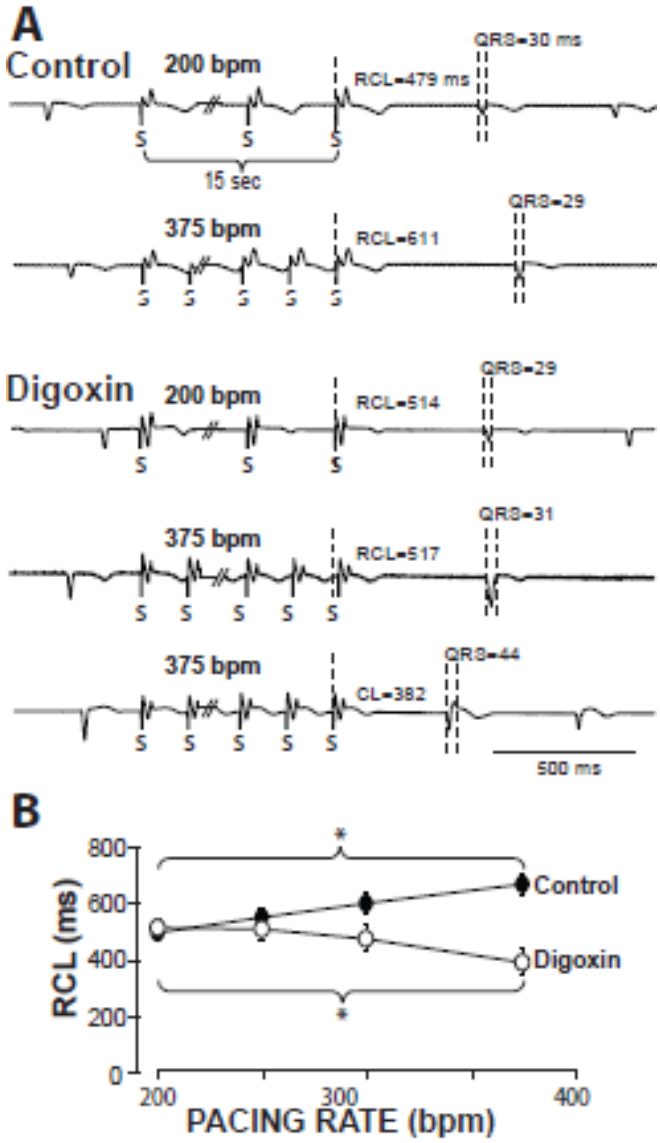

Figure 2.1: Recovery beats characteristics in response to slow and rapid pacing. A) Representative volume-conducted electrocardiograms of control (top) and digoxin (bottom) at pacing rates of 200 and 375 bpm. Paced beats are marked by 'S'. **B)** Summary data of the recovery beat cycle length following different pacing rates. Control data had an increase in RCL (*$p<0.05$, n=11) as pacing rate was increased, while digoxin had a decrease in RCL (*$p<0.05$, n=12).

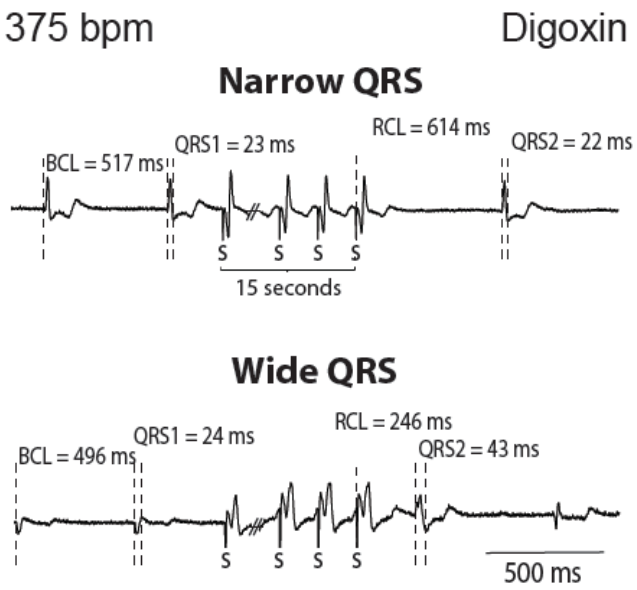

Figure 2.2: Digoxin recovery beats can manifest a late recovery-narrow QRS duration or an early recovery-wide QRS duration. Representative ECGs from digoxin experiments. Recovery beats with narrow and wide QRS complexes are shown in response to 15 seconds of rapid pacing. Figure illustrates representative cycle lengths (CL) and QRS of pre-paced beats, and the cycle length (RCL) and QRS or the recovery beat.

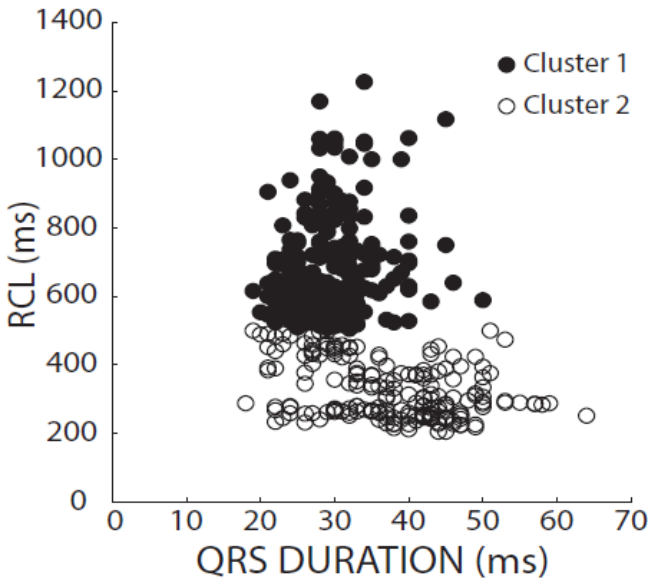

Figure 2.3: K-means separates clusters of raw values by RCL alone. RCL and QRS absolute values from all recovery beats are shown under both normal (37ºC) and hypothermic (34ºC) conditions from control, hypercalcemic, and digoxin conditions for both slow (200 bpm) and fast (375 bpm) pacing rates. Data was stratified into two clusters using a k-means clustering algorithm. As shown, the data preferentially separated in the RCL direction.

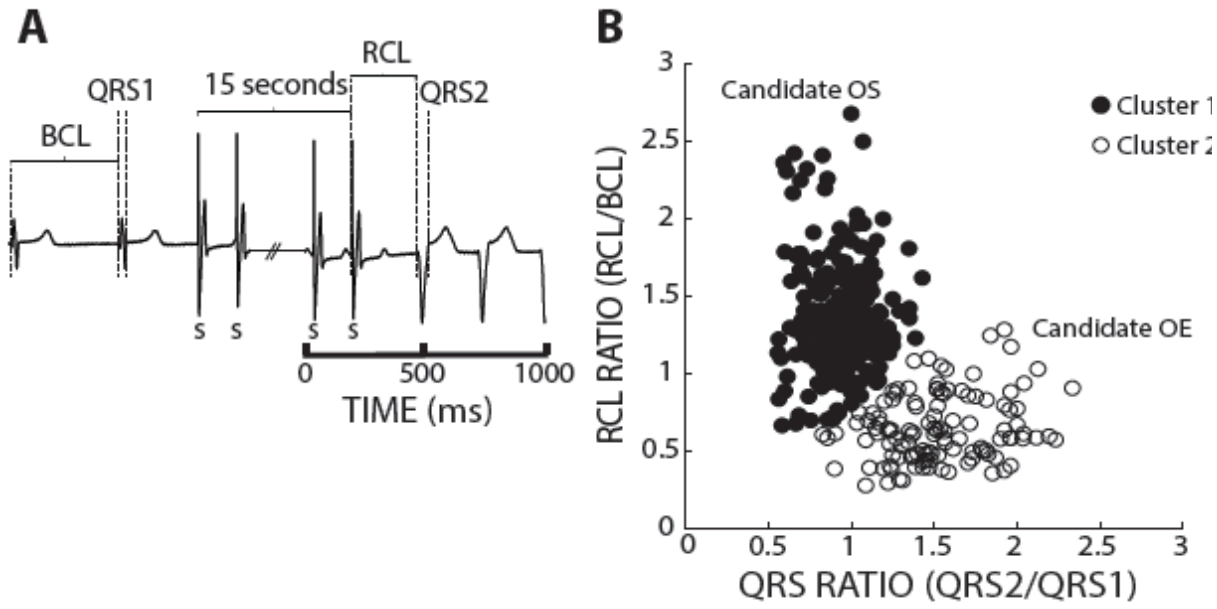

Figure 2.4: K-means separates ratio values by both parameters. A) Representative ECGs showing how RCL and QRS ratios were quantified. The recovery beat's cycle length (RCL) and QRS (QRS2) were normalized to the R-R interval or basic cycle length (BCL) and QRS (QRS1). **B)** Shown are the RCL and QRS ratios of all beats obtained from control, hypercalcemic, and digoxin interventions under both normal (37ºC) and hypothermic (34ºC) conditions for both slow (200 bpm) and fast (375 bpm) pacing rates. A k-means clustering algorithm was used to separate the data into 2 clusters.

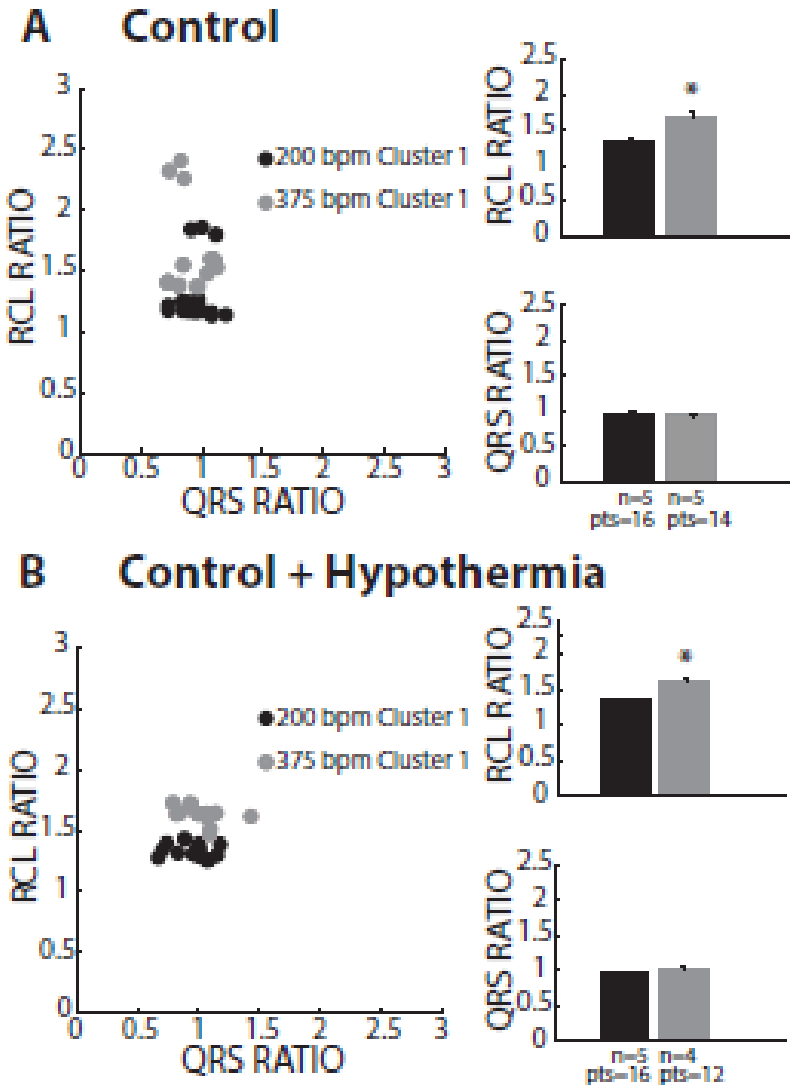

Figure 2.5: Control recovery beats classified as OS. A) All slow (black circles) and fast (gray circles) paced recovery beats fell within Cluster 1 (filled circles). RCL ratio summary data of both slow (n=5) and fast (n=5) paced recovery beat groups. Rapid pacing rates increased the RCL ratio (*p<0.05), while QRS ratio remained unchanged. **Control + Hypothermia B)** All slow (black circles) and fast (gray circles) paced recovery beats fell within Cluster 1. Summary data of RCL ratio from recovery beats shows that rapidly pacing (n=4) increased RCL ratio relative to slow pacing rates (*p< 0.05, n=5), while QRS ratio did not change.

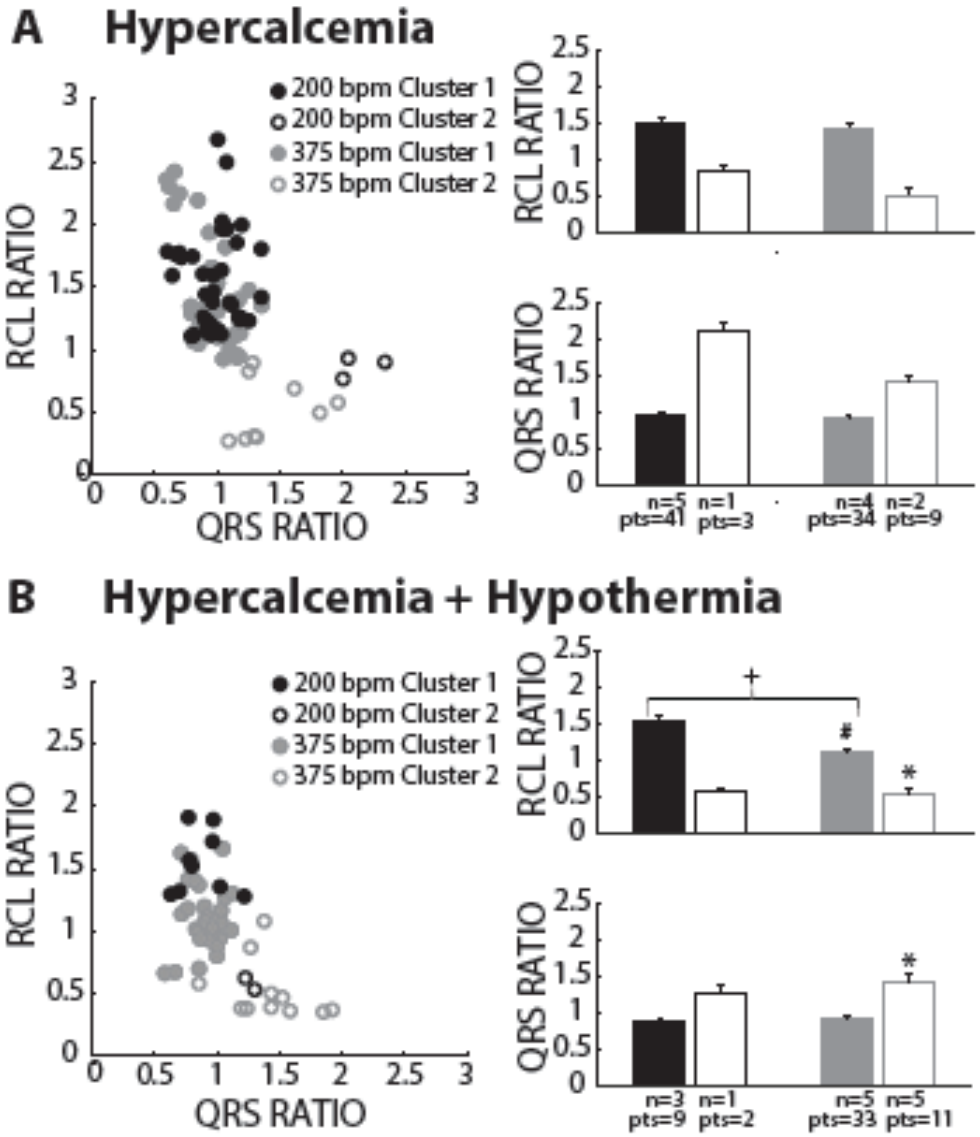

Figure 2.6: Hypercalcemia recovery beats classified as both OS and OE. A) Both slow (black circles) and fast (gray circles) paced beats fell within both Clusters 1 (closed circles) and 2 (open circles). Beats within Cluster 2 for both the slow (n=1) and fast (n=2) paced groups were insufficiently powered for statistical analysis. For Cluster 1 beats, pacing rate did not significantly change RCL or QRS ratio. **Hypercalcemia + Hypothermia B)** Pacing at slow (black circles) and fast (gray circles) rates created beats classified in Cluster 1 (filled circles) and Cluster 2 (open circles). Statistical analyses could not be performed with the fast paced Cluster 1 group due to

lack of power (n=1). Rapidly paced Cluster 2 group (n=5) had a smaller RCL ratio when compared to the rapidly paced Cluster 1 group (n=5,*p<0.05). For Cluster 1, rapid pacing (n=5) decreased the RCL ratio and increased QRS ratio relative to the slow paced group (n=3, +p<0.05). Hypercalcemia and hypothermia (n=5) for the rapidly paced Cluster 1 group (solid gray bar) decreased the RCL ratio when compared to normal hypercalcemia (Fig 5A, n=5, #p<0.05).

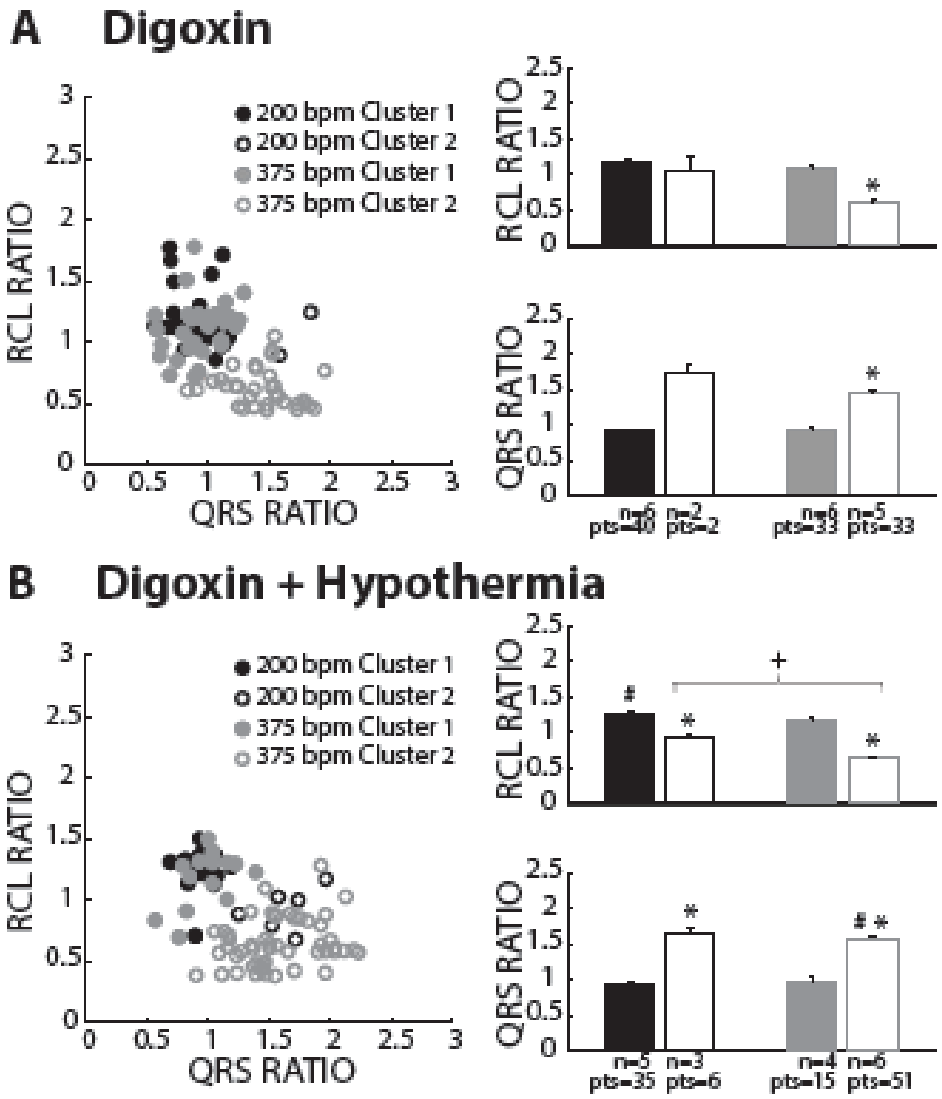

Figure 2.7: Digoxin recovery beats classified as both OS and OE. A) Recovery beats the result of slow (black circles) and fast (gray circles) pacing rates fell within both Clusters 1 (closed circles) and 2 (open circles). Statistical analyses could not be performed with the fast paced Cluster 1 group due to lack of power (n=2). RCL ratio for the rapidly paced recovery beat group in Cluster 2 (n=5) was shortened when compared to Cluster 1 (n=6,*p<0.05). Rapidly paced recovery beats from Cluster 2 had a larger QRS ratio relative to the Cluster 1 group (*p<0.05). **Digoxin + Hypothermia B)** Slow (black circles) and fast (gray circles) paced recovery beats fell within both Cluster 1 (filled circles) and Cluster 2 (open circles). Recovery beats the result of slow

pacing in Cluster 2 (n=3) had a smaller RCL ratio relative Cluster 1 (n=5,*p<0.05). The same occurred for rapidly paced Cluster 2 (n=6) beats relative to Cluster 1 (n=4,*p<0.05). Rapidly pacing the Cluster 2 group (n=6) decreased the RCL ratio relative to the slow paced Cluster 2 group (n=3, +p<0.05). Hypothermia also increased the RCL ratio for the slow paced Cluster 1 group (n=5) relative to normal digoxin slow paced Cluster 1 group (Fig 6A, n=6, #p<0.05). QRS ratio for the slow paced Cluster 2 group increased relative to Cluster 1 (*p<0.05). The same occurred for the fast paced Cluster 2 group relative to Cluster 1 (*p<0.05). Hypothermia increased the QRS ratio for the rapidly paced Cluster 2 group (n=6) relative to normothermia (Figure 2.7A, n=5, #p<0.05).

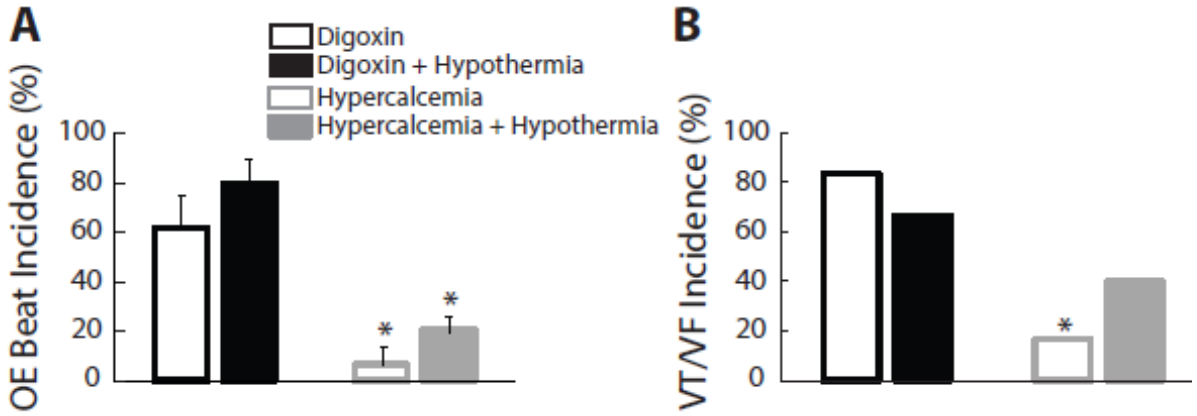

Figure 2.8: Digoxin produces more arrhythmias that hypercalcemia. A) Average OE beat occurrence. Hypothermia did not significantly alter OE beat occurrence under either digoxin or hypercalcemic conditions. OE beats for digoxin (n=7) were significantly more frequent when compared to hypercalcemia (n=6,*$p<0.05$). The same occurred for hypothermic digoxin (n=6) and hypothermic hypercalcemia (n=5). **B)** Ventricular tachycardia or fibrillation (VT/VF) incidence. Incidence was not significantly altered by hypothermia for either digoxin or hypercalcemia. VT/VF was significantly more frequent for digoxin when compared for hypercalcemia. Hypothermic digoxin did not significantly produce more VT/VF than hypothermic hypercalcemia.

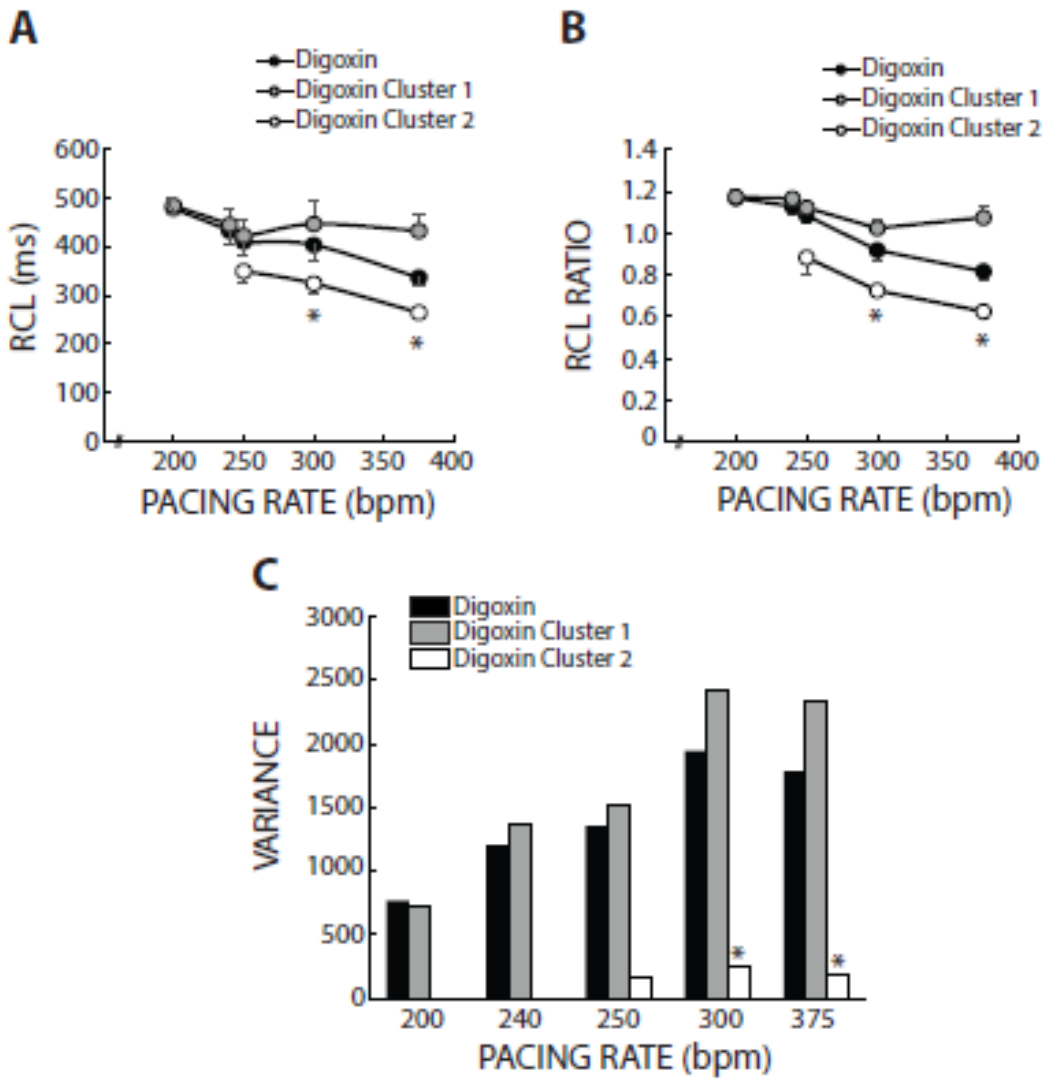

Figure 2.9: Cluster separation reveals distinct OS and OE behaviors during digoxin A) Recovery beat cycle lengths before stratification in black circles. After stratification there are significant difference between Clusters 1 (gray circles) and 2 (white circles) at 300 and 375 bpm. Of note, only data with n≥3 are plotted in B, C, and D. **B)** RCL ratios for the entire dataset (black circles), Cluster 1 (grey circles), and Cluster 2 (white circles). Cluster 2 RCL ratios were significantly smaller when compared to Cluster 1 at 300 and 375 bpm. **C)** Variance of the recovery beat cycle lengths for the entire digoxin data set (black), and Cluster 1(grey) and 2 (white). Cluster 2 variance was significantly smaller than Cluster 1 at 300 and 375 bpm.

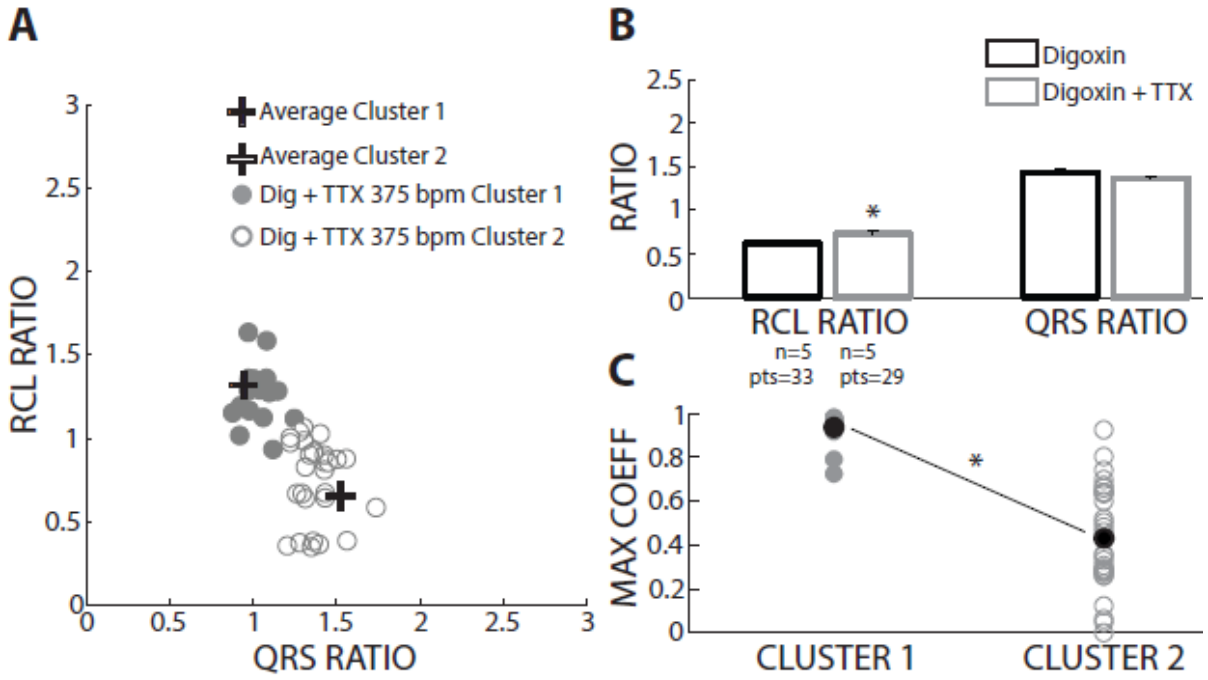

Figure 2.10: TTX prolongs RCL without altering QRS of recovery beats. A) Centroids of Clusters 1 and 2 derived from Figure 4 are plotted as +. Each Digoxin + TTX beat was classified based on least-squares distance from the centroid in either Cluster 1 (gray filled circles) or 2 (gray open circles). **B)** Cluster 2 data from digoxin and digoxin + TTX reveals only RCL ratio increased with TTX (n=5,*p<0.05). **C)** Cross-correlation analysis for QRS morphology reveals that Cluster 2 (n=5 hearts, gray open circles) beat maximum correlation coefficients were significantly lower than Cluster 1 (n=4 hearts, gray filled circles). Averages for each data set are shown as black filled circles.

Chapter 3: Temporal response of ectopic activity in guinea pig ventricular myocardium in response to isoproterenol and acetylcholine

Abstract

Both β adrenergic and muscarinic receptor stimulation independently potentiate arrhythmogenesis. However, the effect of simultaneous stimulation on arrhythmogenesis is not well known. The purpose of this study was to determine the temporal response of arrhythmia risk to individual and combined autonomic agonists. Guinea pig hearts were excised and Langendorff-perfused. The β adrenergic receptor and muscarinic receptor agonists were isoproterenol (ISO, 0.6μM) and acetylcholine (ACh, 10μM) respectively. All measurements with agonists occurred over 21 minutes. ISO induced ectopic activity for the first 8 minutes. ISO also transiently shortened and then prolonged R-R interval over a similar time course. ACh added after ISO transiently induced ectopic activity for 12 minutes, while R-R interval invariantly prolonged. ACh alone produced few ectopic beats, while invariantly prolonging R-R interval. In contrast to ISO alone, ISO following ACh significantly increased ectopic activity and shortened R-R interval for the duration of the experiment. Animals aged 17-19 months exhibited sustained arrhythmogenesis while those aged 11-14 did not. When ACh was removed in older hearts while ISO perfused, a transient increase in ectopic activity and decreased R-R interval was observed, similar to ISO alone. These data suggest that pre-treating with and maintaining ACh perfusion sustains ISO sensitivity, in contrast to ISO perfusion alone.

Introduction

Sympathetic stimulation by β adrenergic receptor (β–AR) agonists, such as isoproterenol or noradrenaline, or by direct stimulation of the stellate ganglion have all been previously used to induce ventricular arrhythmias or precursors to arrhythmias such as spontaneous calcium releases.[1-5] Parasympathetic stimulation via vagus nerves or muscarinic receptor agonists has previously been demonstrated to have opposing effects on calcium handling in myocytes,[6,7] but has also been linked to increased risk of arrhythmias.[8] Both β–AR and muscarinic receptor stimulation can *independently* modify cardiac electrophysiology and calcium handling within seconds. Furthermore, prolonged exposure to agonists (minutes to hours) can also modulate responsiveness.[9-12] Therefore, arrhythmogenic risk due to independent β–AR or muscarinic receptor agonists exhibits multiple time scale dependencies.

On the other hand, there is evidence that *simultaneous* stimulation of sympathetic and parasympathetic pathways, either through receptor agonists or by direct nerve stimulation, can produce time dependent effects on heart rate and contractility. Previous studies have demonstrated that the effect of muscarinic receptor stimulation on heart rate and inotropy can be modulated by β–AR stimulation,[13-15] and this is commonly known as accentuated antagonism. Accentuated antagonism is dependent on the length of exposure to β adrenergic and muscarinic stimulation and the order in which these responses are activated.[16] Nevertheless, the time- and order-dependent effects of β adrenergic and muscarinic agonists on the risk of arrhythmic events is not clear. Furthermore, age is an important determinant in sympathetic-induced responses, as it has previously been shown that the arrhythmogenic propensity during exercise increases with age.[17,18] However, the effect of aging on arrhythmogenic risk during simultaneous stimulation is not well understood either. Therefore, the purposes of this study are to determine how activating one pathway by direct perfusion of an agonist individually and in combination temporally modulates arrhythmia risk, and how age affects this response.

Materials and Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and has been approved by Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute& State University.

Experimental preparations

Retired breeder male guinea pigs (ages 11 – 19 months, weight 800 – 1200 g, n=45) were anesthetized with sodium pentobarbital (325 mg/kg) or isoflurane inhalation. Hearts were rapidly excised, atria removed, and ventricles were Langendorff-perfused with oxygenated modified Tyrode solution (in mM, $CaCl_2$ 1.25, NaCl 140, KCl 4.56, dextrose 5.5, $MgCl_2$ 0.7, HEPES 10; 5.5 mL of NaOH used to pH to 7.4) at 37ºC and 50 mmHg. Experimental preparation time, or the time from start of surgery to Langendorff-perfusion with Tyrode, was approximately 5 minutes. Motion was reduced using 20 μM recirculated blebbistatin (10 min).

Arrhythmia induction protocol

Ectopic beat burden was assessed using a continuously recorded volume-conducted bath electrocardiogram (ECG). A bipolar plunge electrode in the interventricular septum was used to pace the hearts at 300 bpm for 15 seconds. The first recovery beat after rapid pacing was evaluated to determine if it displayed ectopic behavior (Figure 3.1A). A previously established algorithm was used to stratify ectopic beats from other types by normalizing recovery beat latency and QRS width to the corresponding pre-paced intrinsic beat latency and QRS width.[19] Premature beats that occurred during pacing were also considered ectopic beats (Figure 3.1B).

Ectopic beats were counted for 2 minutes prior to autonomic stimulation (Figure 3.1C, Control, open bar), and during the subsequent 21 minute perfusion of the β adrenergic agonist isoproterenol (ISO, 0.6 μM) or muscarinic agonist (ACh, 10 μM, drug A, black filled bar). Similar ISO doses (0.1 – 1 μM) have previously been used with guinea pig myocytes to induce early and

delayed afterdepolarizations.[20,21] ACh physiological concentrations can range from the nanomolar in plasma[22] to the millimolar at the neuromuscular junction,[23] and therefore we chose a concentration that has been associated with augmented calcium handling in guinea pig myocytes.[24] In another set of experiments, the autonomic agonist (drug A) was perfused for 21 minutes, and then was followed by the addition of the other agonist (drug B, grey filled bar, Figure 3.1D). Figure 3.1E displays another set of experiments wherein drug A was perfused for 21 minutes and then followed by the perfusion of just drug B. Vertical dashes below the bars denote when rapid pacing was used to induce ectopic beats. Hearts were rapidly paced every minute for 15 seconds, and then binned into groups of 2 minutes (example in Figure 3.1C). Ectopic beat incidence was calculated by counting the number of pacing protocols that produced ectopic beats and dividing by the total number of pacing protocols performed within the 2 minutes. The following list reports the sample size (number of animals aged 17-19 months) used for each conditions

- Control n=3
- +ISO n=6
- ISO + ACh n=7
- +ACh n=4
- ACh + ISO n=13.
- -ACh + ISO n=5

In a separate set of experiments, animals 11-14 months were studied.

- ACh + ISO n=7. Ages 11-14 Months

Statistical analysis

Data significance was analyzed using paired and unpaired t-tests, Mann-Whitney, and Chi-square where appropriate. A $p<0.05$ was considered significant. Mean ± standard error was reported.

Results

Control

Without agonists, ectopic beats were never observed over the time course of 41 minutes either during or following pacing as illustrated in Figure 3.2A. Figure 3.2B reveals that over the same time period the R-R interval during control conditions was relatively stable but could vary by as much as 29 ms.

+ Isoproterenol

Before β adrenergic stimulation with ISO, or at -2 min, no ectopic beats were observed (Figure 3.3A), consistent with the lack of ectopic beats observed during the entire 41 minute time-control experiment. After ISO perfusion, ectopic beats were observed, reaching a peak incidence within 2-3 minutes. By 8 min, ectopic beats were no longer observed. Therefore, a temporal relationship exists for ISO-induced ectopic beats. Quantification of summary data revealed that ectopic beats were significantly more frequent in the early (0 to 11 minutes) relative to the late (12 to 21 minutes) stage of the experiment (12% vs 0%, grey bar vs white bar, Figure 3.3B), and therefore ISO *transiently* increases arrhythmia risk in the first minutes of stimulation.

Likewise upon administration of ISO, R-R interval significantly decreased within 2 minutes and then slowly recovered to control rates (Figure 3.3C). Summary data revealed that R-R interval in the early stage of the experiment was significantly shorter than in the late stage (286±6 vs 368±8 ms, Figure 3.3D). Furthermore, this change in R-R interval was significantly larger than R-R variation over 41 minutes of control conditions (84±11 vs 29±8), indicating that this change was due to an ISO-induced effect rather than intrinsic changes in Langendorff-perfused guinea pig hearts. Notably, these results suggest that β-agonist responsiveness and arrhythmia risk acutely increases within a few minutes and then decreases in *ex vivo* preparations.

Isoproterenol + Acetylcholine

In a separate set of experiments where ISO was perfused for 21 minutes and then ACh added to the perfusate, ectopic activity before ACh (time -2 min, Figure 3.4A) remained at 0% consistent with the last 10 minutes of the ISO experiments above. Surprisingly, the addition of ACh reinitiated ectopic beats for 12 minutes. However, the response was *transient*. This is confirmed by the summary data, which revealed more ectopic activity in the early (15%) stage of ISO + ACh than the late stage (3%) as illustrated in Figure 3.4B.

Data in Figure 3.4C reveals that unlike ISO alone, ISO + ACh increased the R-R interval immediately upon perfusion and maintained the increased R-R interval for the duration of the experiment. This is supported by summary data in Figure 3.4D (465±11 early vs 485±14 ms late).

+ Acetylcholine

The muscarinic agonist ACh was not significantly arrhythmogenic. More specifically, only two hearts produced ectopic beats (1 per heart) with ACh between 18 and 21 minutes (Figure 3.5A). As a result, ectopic incidence was not significantly different between the early (0%, Figure 3.5B) and late (5%) measurement stages. ACh increased R-R interval (Figure 3.5C), and R-R remained prolonged throughout the remainder of the experiment (Figure 3.5D).

Acetylcholine + Isoproterenol

In another set of experiments where ACh was perfused for 21 minutes before addition of ISO, few ectopic beats were produced (5%, time -2, Figure 3.6A). The addition of ISO significantly increased the incidence of ectopic beats. Moreover, ectopic beats were *persistently* produced throughout the ACh + ISO perfusion period. Comparisons between the early and late stages revealed that ectopic incidence remained relatively high and unchanged over 21 minutes (32% vs 33%, Figure 3.6B). Importantly, these data demonstrate that pre-treatment with ACh followed by simultaneous ACh + ISO produces a sustained β-adrenergic arrhythmic responsiveness.

Likewise, ACh + ISO decreased R-R interval acutely, but R-R intervals did not return to pre-ISO rates, as happened with ISO alone (compare Figure 3.6C to Figure 3.3C). Interestingly,

summary data does reveal that early stage R-R intervals (417±10 ms) were significantly shorter than late stage R-R intervals (442±8 ms, Figure 3.6D). However, while this change was significant, the absolute change in R-R interval was not significantly different to changes observed during control conditions (26±16 vs 29±11 ms). Furthermore, this change was significantly smaller relative to changes during +ISO (26±16 vs 84±11 ms), suggesting that pre-treating with ACh followed by simultaneous ACh + ISO blunts β-adrenergic desensitization.

Acetylcholine + Isoproterenol – young guinea pigs

Previous experiments presented in Figures 1 – 6 were completed with mature guinea pigs ages 17 – 19 months. In order to account for the effects of age on ectopic incidence, ACh + ISO experiments were performed in guinea pigs ages 11 – 14 months. Similar to the mature animals, ACh alone produced one ectopic beat. Upon ISO perfusion, ectopic beats were *transiently* produced, with only one ectopic beat observed after 8 minutes (Figure 3.7A). The early period ectopic incidence was 7% (Figure 3.7B), while the late period was 1%. Importantly, young guinea pigs no longer demonstrated persistent arrhythmogenesis.

Transient behavior was observed in changes in the R-R interval as well. As expected, ISO decreased the R-R interval, but this was followed by an increase in R-R interval over time (Figure 3.7C). This was evident in the summary data as well (496±8 early vs 568±9 ms late, Figure 3.7D). As would be expected, the change in R-R induced by Ach + ISO was significantly larger compared to control measurements without intervention (72±21 vs 29±11).

Heart rate and age effects on ectopic appearance

Figure 3.8A summarizes the age distribution of the guinea pigs for differential ectopic incidences with ACh + ISO. Importantly, the mature guinea pigs (17 – 19 months) manifested greater ectopic incidences relative to the young guinea pigs (31% vs 5%). Total ectopic incidence was defined as the incidence over 21 minutes of ISO perfusion. This would suggest that age is an important factor in ectopic beat manifestation.

Rapid intrinsic heart rates may also contribute to the trigger of ectopic beats. In order to account for any effects that intrinsic heart rate may have on the formation of ectopic beats, the preceding intrinsic heart rate is compared for the pacing protocol that did or did not produce ectopic beats during ACh + ISO perfusion. There was no significant difference in intrinsic R-R interval between these two groups (Mann-Whitney Test, Figure 3.8B), suggesting that intrinsic heart rate was not a factor in ectopic beat manifestation.

-Acetylcholine + Isoproterenol

The previous experiments only explored the concurrent activation of autonomic pathways following chronic activation of the other pathway. However, during normal physiology, parasympathetic tone is usually withdrawn upon activation of sympathetic stimulation. Therefore, we sought to determine whether the persistent behavior observed with ACh + ISO would also be displayed with ACh washout + ISO. As with the initial experiments (Figures 3.1 – 3.6), ACh washout + ISO was performed in mature guinea pigs. Once again, before changing the solutions, 2 ectopic beats were observed from one heart during ACh perfusion (time -2 min, Figure 3.9A). At time 0, the perfusate with no ACh but with ISO reached the heart. Ectopic beat incidence was elevated for a short time before falling again to a 0% incidence at 6 min. Therefore, withdrawal of ACh and addition of ISO produced a similar *transient* response as ISO alone (compare Figure 3.9A to Figure 3.3A). Summary data revealed the same (13% early vs 2% late, Figure 3.9B).

Changes in the R-R interval with washout of ACh and wash-in of ISO produced *transient* behavior as well. Specifically, ISO initially shortened R-R interval, but then R-R interval gradually prolonged (Figure 3.9C). Early and late stage comparisons revealed that the R-R interval was shorter during the early stage (313±13 vs 400±15 ms, Figure 3.9D). Importantly, this change in R-R interval was significantly larger than the variations observed under control conditions (88±27 vs 29±11 ms).

Discussion

In this study, we demonstrated that continuous pre-perfusion of acetylcholine (ACh) followed by isoproterenol (ISO) creates a persistent arrhythmogenic substrate in mature guinea pigs. ISO and ISO followed by ACh, on the other hand, reveal transient arrhythmogenic substrates. Furthermore, when ACh was washed out during ISO perfusion, the arrhythmogenic substrate became transient again. Therefore, time course and order of β adrenergic and muscarinic receptor stimulation can impact the development of arrhythmias.

β adrenergic receptor stimulation

ISO perfusion in this study initially increased ectopic activity and heart rate, but this was followed by a gradual decrease in both parameters, suggesting that sympathetic stimulation itself decreases β adrenergic receptor (β-AR) responsiveness. These results are consistent with previous manuscripts that have demonstrated a similar temporal response of heart rate[25] and left ventricular diastolic pressure[26] (post-hoc test) during minutes of β-AR stimulation. Consistent with other studies, β-AR stimulation triggers ectopic activity in the whole-heart.[1,27] β-ARs are coupled to the stimulatory G-proteins (G_s), which activate adenylyl cyclase and cAMP production, thereby augmenting calcium handling and inducing ectopic activity.[1] To our knowledge, however, this is the first study to demonstrate that ectopic activity can decrease within 8 minutes during ISO perfusion.

The proposed mechanism for this transient response may be attributed to the hypothesis that sympathetic stimulation has a time-dependent, negative feedback mechanism. Specifically, β agonization leads to internalization of β receptors,[9] desensitization of β2 by switching from coupling with the stimulatory G-protein to the inhibitory G-protein[11] (G_i), and desensitization of β1 and β2 through activation of phosphodiesterases which degrade cyclic AMP.[28] Additionally, β3 is also coupled to the G_i protein. Therefore, during β1 and β2 internalization, stimulation of β3

continues to activate G_i, further inhibiting global β-AR responsiveness.[29] As a result of all of these mechanisms, β agonists induce transient and biphasic responses in chronotropy, inotropy, and, as we demonstrate, ectopic activity.

Muscarinic receptor stimulation

Stimulating the muscarinic receptors with ACh led to a sustained decrease in heart rate and few arrhythmias, consistent with previous studies in canine[30] and mice.[31] Indeed, the predominant muscarinic receptor subtype in the heart[32] is coupled to the inhibitory G-protein (Gi), which inhibits adenylyl cyclase and the production of cAMP. Therefore, ACh prevents ion channel phosphorylation and decreases calcium handling,[33] both of which can lead to decreased heart rate and arrhythmia propensity. Of note, parasympathetic stimulation has been associated with arrhythmogenesis in conditions such as long QT syndrome[34,35] and Brugada syndrome.[36] Generally, however, muscarinic receptor stimulation alone is not arrhythmogenic,[37] and therefore our results are consistent with previous work.

Muscarinic following β adrenergic receptor stimulation

Surprisingly, ACh following ISO induced a transient increase in ectopic activity. This is in seeming contradiction to a different study in isolated guinea pig myocytes,[24] as Song et al. found that ACh decreased the amplitude of ISO-induced delayed after-depolarizations, and therefore seemingly displayed anti-arrhythmic behavior. These studies, however, may not be directly comparable because the Song et al. study evaluated the effect of ACh remodeling after only ~5 minutes of ISO perfusion, and with a significantly smaller ISO concentration (20 nM vs 600 nM). We chose to use 600nM ISO, because it was previously demonstrated to induce phosphorylation of proteins that desensitize β-ARs.[38]

β adrenergic following muscarinic receptor stimulation washout

The effects of ISO following ACh washout have been extensively studied. When ACh is washed out in the presence of ISO, a so-called rebound effect is observed. Specifically,

contractility[15,39] and calcium current[40] increase and triggered activity is induced[24] upon ACh washout in the presence of β-AR stimulation. It has previously been proposed that this may be due to activation of the pertussis toxin insensitive G protein (G_q) pathway,[41] which has been linked to augmented chronotropy[42] and inotropy.[43] Specifically, certain muscarinic receptor subtypes are coupled G_q which activates phospholipase C. Phospholipase C then cleaves phospholipids to form inositol 1,4,5-triphosphate and diacyl glycerol. Calcium handling can then be altered by two mechanisms. The inositol phosphate can then bind to the IP_3 receptor located on the sarcoplasmic reticulum to trigger a release of calcium into the cytosol (for review see Kockskamper, J et al. 2008).[44] Also, diacyl glycerol facilitates translocation of protein kinase C to the cell membrane and its activation. Activation of protein kinase C has been linked to the phosphorylation of a number of ion channels, thereby modulating calcium flux (for review, see Ferreira, JC et al. 2012).[45] Importantly, the Gq pathway effects on chronotropy[42] and inotropy[43] is transient (over minutes), just as reported here.

β adrenergic receptor following muscarinic receptor stimulation

It has been demonstrated that sympathetic stimulation within seconds can elevate heart rate even in the presence of chronic parasympathetic stimulation,[16] consistent with this study. To our knowledge, this is the first demonstration that ISO following ACh *persistently* elevates heart rate and ectopic beat incidence. Importantly, the persistent elevation of ectopic beat incidence reveals that ISO perfusion in the presence of chronic ACh perfusion in mature animals is a more robust model for producing sustained ectopic activity.

Mechanisms of enhanced arrhythmic risk

If ISO exposure reduces β-AR responsiveness, it is not apparent why the order of ACh and ISO perfusion produces temporally different responses. While there is no direct explanation for the persistent elevation of heart rate and ectopic activity only when ISO follows and is concurrently perfused with ACh, previous studies suggest that this may not be an entirely

unexpected result. Specifically, β-AR desensitization can occur via Protein Kinase A activation of phosphodiesterases or Protein Kinase A phosphorylation of the β2 receptor, and/or internalization of β-AR. One possible mechanism is that pre and persistent ACh treatment may preserve β-AR sensitivity by inhibiting cyclic AMP production(see Harvey and Belevych),[33] thereby reducing Protein Kinase A activation, and preventing β2 phosphorylation and therefore desensitization. Due to the lack of β2 receptors in guinea pig ventricular myocardium,[46,47] however, it seems unlikely that β2 receptors are playing a role in parasympathetic-induced preserved β-AR sensitivity.

It is important to note ACh can also activate PKC, which enhances G-protein coupled receptor kinase phosphorylation[16] and thereby can increase β internalization.[48] By increasing internalization, ACh may blunt the β-AR negative feedback mechanism. However, this seems unlikely because if receptors were internalized, then washing out ACh and adding ISO should have had similar effects as ACh and ISO, as the time course of 40-50% β-AR receptor recovery post-internalization has been estimated to be about 20 minutes in rodent.[49,50] Despite these intriguing hypotheses, further studies are necessary to determine the mechanisms by which ACh modulates β internalization.

Downstream factors may also be playing a role in sustained arrhythmogenesis. Phosphodiesterases, which degrade cAMP, are activated by Protein Kinase A and associated with agonist-induced β adrenergic desensitization. As parasympathetic stimulation prevents Protein Kinase A phosphorylation, this may also decrease phosphodiesterase activation and therefore cAMP degradation. By this mechanism, isoproterenol may therefore have a sustained effect on the heart in the presence of acetylcholine. Further investigation is needed to determine if this mechanism plays a role, however.

Age-related effects on β adrenergic receptor following muscarinic receptor stimulation

Ventricular arrhythmia prevalence has previously been shown to increase with age, consistent with our results. In particular, age is an important factor in exercise-induced arrhythmias or during sympathetic stimulation.[17,18] Structural heart changes[51] or remodeling of calcium handling proteins[52] may account for these changes. However, Molina et al. demonstrated phosphodiesterase activity decreases with age.[53] Therefore, with the decreased phosphodiesterase activity this may allow ACh to sustain arrhythmogenesis with ISO. This requires further investigation though.

Conclusions

In summary, we present evidence that muscarinic receptor stimulation produces ectopic beats in the presence of β adrenergic receptor (β-AR) stimulation in mature animals. Importantly, the order in which muscarinic and β-AR stimulation is introduced has important transient and persistent effects on ectopic beats and heart rate, and these data support the hypothesis that muscarinic receptor stimulation may be impacting ectopic beat formation by modulating β-AR desensitization. Therefore, future studies may consider chronic ACh perfusion before β-AR agonists in order to elicit more ectopic beats. Furthermore, while beta-blocker therapy may be highly efficacious for preventing sudden cardiac death, these findings suggest that preventing ACh induced preservation of β-adrenergic responsiveness may be a new target for preventing sudden death.

Limitations

The concentrations of ISO and ACh were chosen to elicit measurable responses in the heart. However, as only one concentration for each autonomic agonist was used in this study, it should be noted that other concentrations may elicit different responses. Finally, it is important to note that this study utilized autonomic agonist perfusion into the heart rather than directly stimulating nerves, and direct nervous stimulation might produce different responses than what has been found here.

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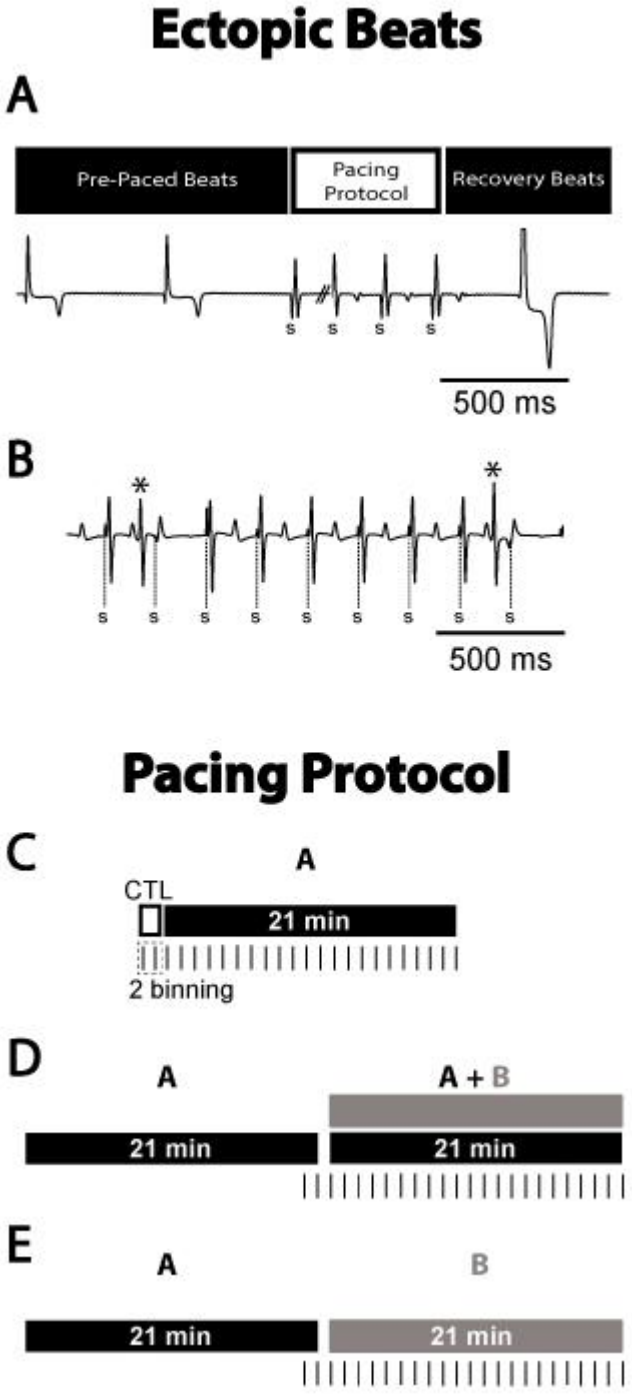

Figure 3.1: Ectopic beats during and after pacing. A) Representative ECG from ACh + ISO experiment. The first recovery beat following 15 seconds of rapid pacing and the preceding pre-paced beats are shown. Paced beats are denoted by 'S'. Latency and QRS width of the first

recovery beat were normalized to the R-R interval and QRS width of the pre-paced beats to determine which beats were ectopic. Displayed is an example of an ectopic recovery beat. **B)** Representative ECG from ACh + ISO experiment. The rapid pacing drive train is shown with premature beats (*), marked as ectopic beats. Arrhythmia induction protocol. **C)** Control conditions without autonomic agonists, were observed for two minutes. ISO or ACh was then perfused for 21 minutes. Hearts underwent rapid pacing for 15 seconds every minute and data was binned into 2 minute intervals. **D)** ISO or ACh was perfused for 21 minutes, and hearts were paced for the last two minutes. The other autonomic agonist was then added to the perfusate for an additional 21 minutes. **E)** ISO or ACh was perfused for 21 minutes with hearts paced for the last two minutes. The solution was then switched to the other autonomic agonist for 21 minutes.

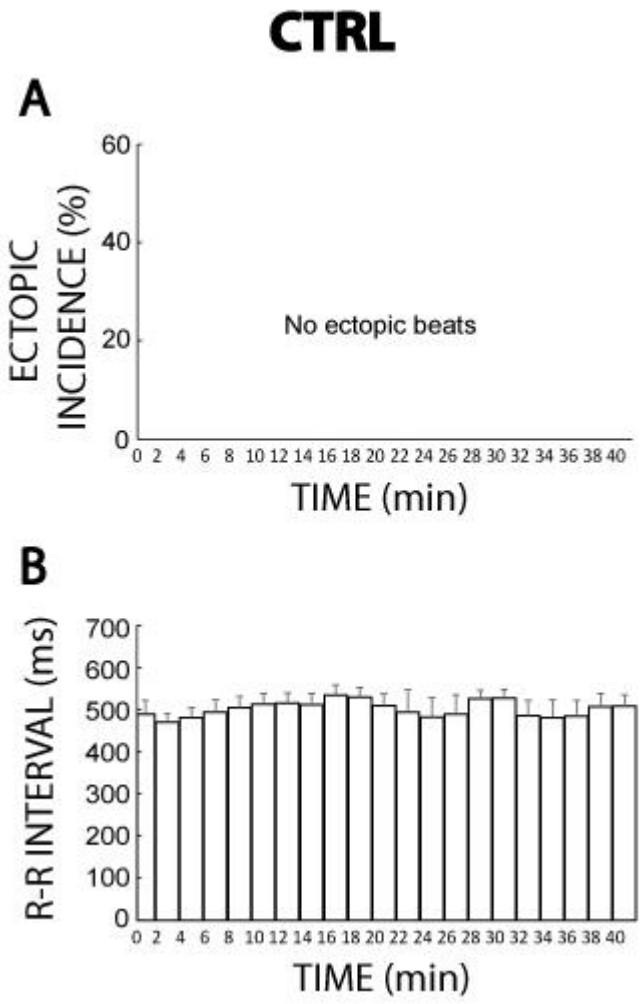

Figure 3.2: Control ectopic incidence and R-R interval time course. A) No ectopic beats were observed during 41 minutes of control conditions (n=3). **B)** R-R interval changed by as much as 29ms over 41 minutes without a reproducible temporal pattern.

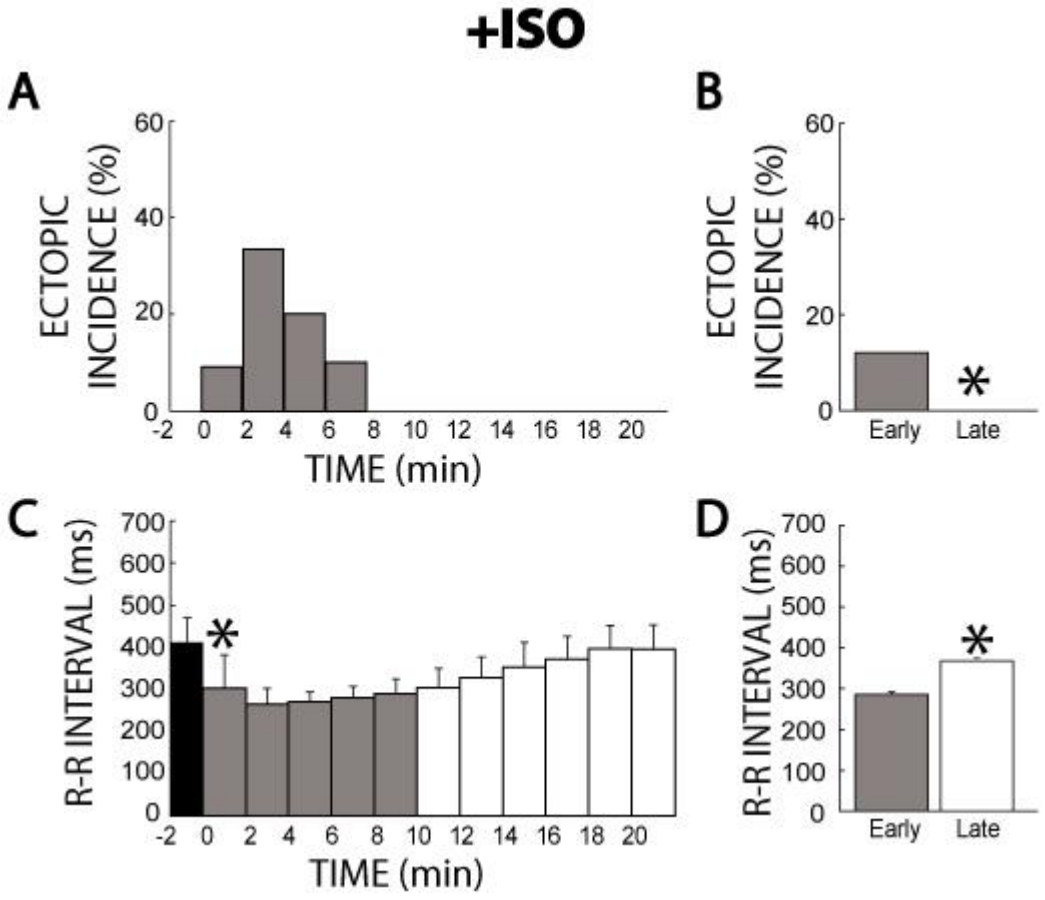

Figure 3.3: +ISO ectopic incidence and R-R interval time course. A) Without ISO (time -2) no ectopic beats were elicited. With the addition of ISO, ectopic beats were produced for 8 minutes, reaching the peak incidence by 2 minutes, before reaching 0% incidence at 8 minutes. Black: without ISO, Gray: early stage with ISO (0 – 11 min), and White: late stage with ISO (12 – 21 min), respectively. **B)** Significantly more ectopic activity (*) was produced in the early stage (0-11 min, grey bar, 12%, n=6) relative to the late stage (12-21 min, white bar, 0%). **C)** ISO significantly shortened R-R interval within 2 minutes (*) relative to time points before ISO perfusion (Black). Over time, R-R interval gradually returned to control cycle lengths. **D)** The R-R interval for the early stage was significantly shorter relative to the late stage (286±6 vs 368±8 ms, *).

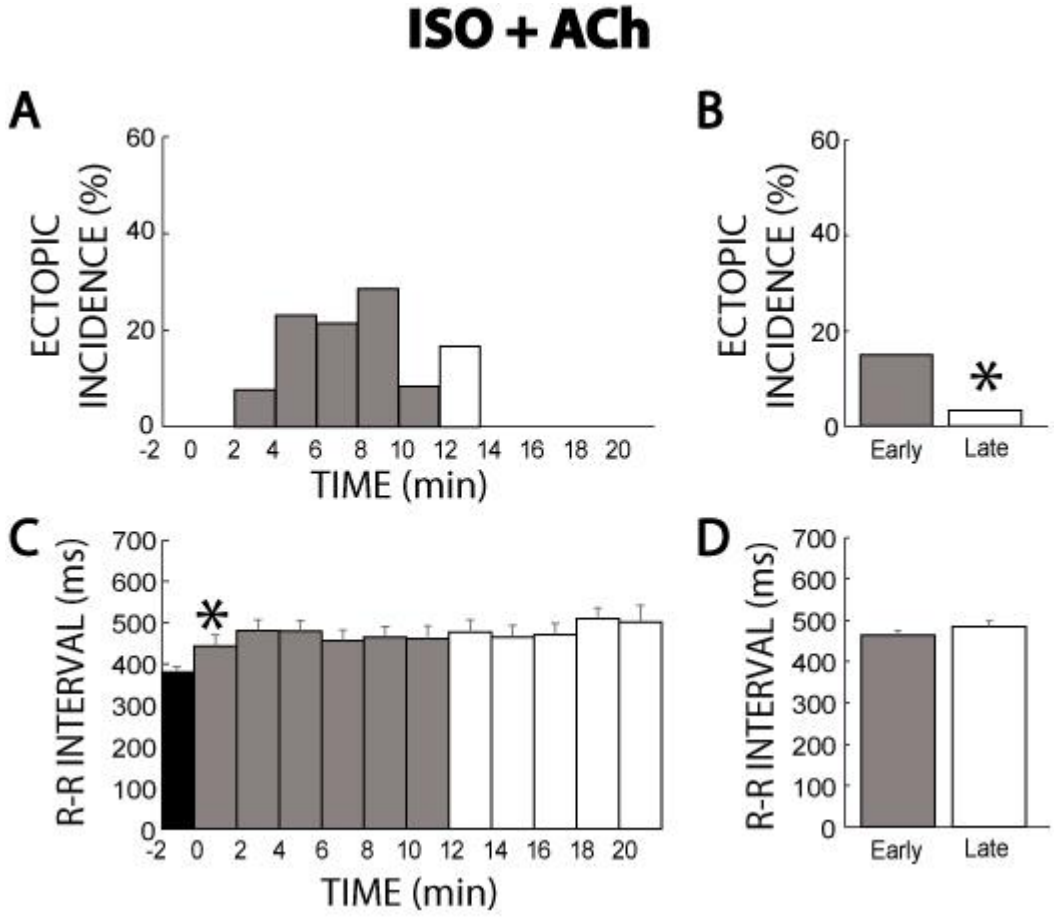

Figure 3.4: ISO + ACh ectopic incidence and R-R interval time course. A) After 21 minutes of ISO perfusion (time -2) no ectopic beats were produced. The addition of ACh re-initiates ectopic beats starting at time 2 minutes and lasts for 12 minutes before reaching 0% incidence at time 14 minutes. Black: without ACh, Gray: early stage with ACh (0 – 11 min), and White: late stage with ACh (12 – 21 min), respectively. **B)** Ectopic beat incidence was significantly larger for early (15%, n=7) relative to the late stage (*, 3%), demonstrating a transient temporal relationship. **C)** ACh prolonged R-R interval within 2 minutes (*), and cycle length remained elevated for 21 minutes. **D)** No significant R-R interval differences were found between the early and late stages (465±11 vs 485±14 ms).

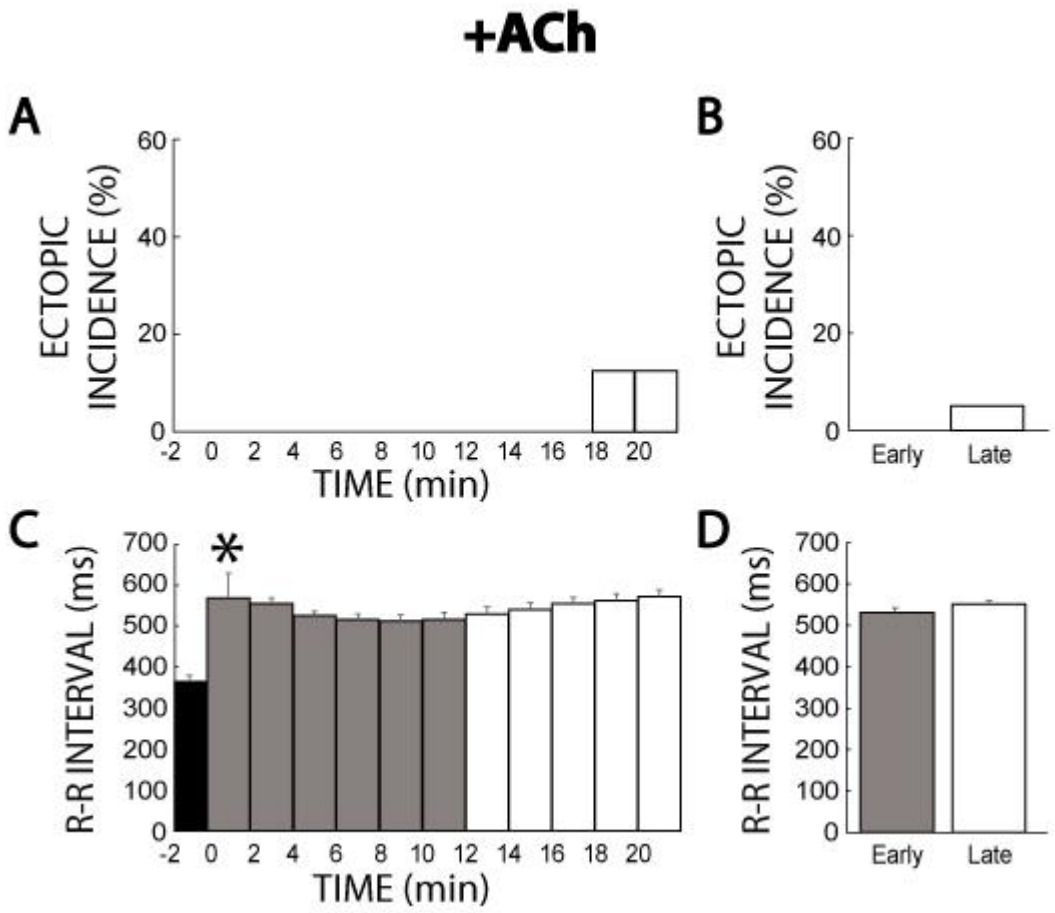

Figure 3.5: +ACh ectopic incidence and R-R interval time course. A) Ectopic beats were not produced without ACh (time -2). ACh perfusion produced few ectopic beats. **B)** No significant difference of ectopic activity was found between early (0%, n=4) and late (white bar, 5%) stages. **C)** ACh increased R-R interval within 2 minutes (*). **D)** R-R interval remained prolonged for both the early and late stages (531±10 vs 551±7 ms).

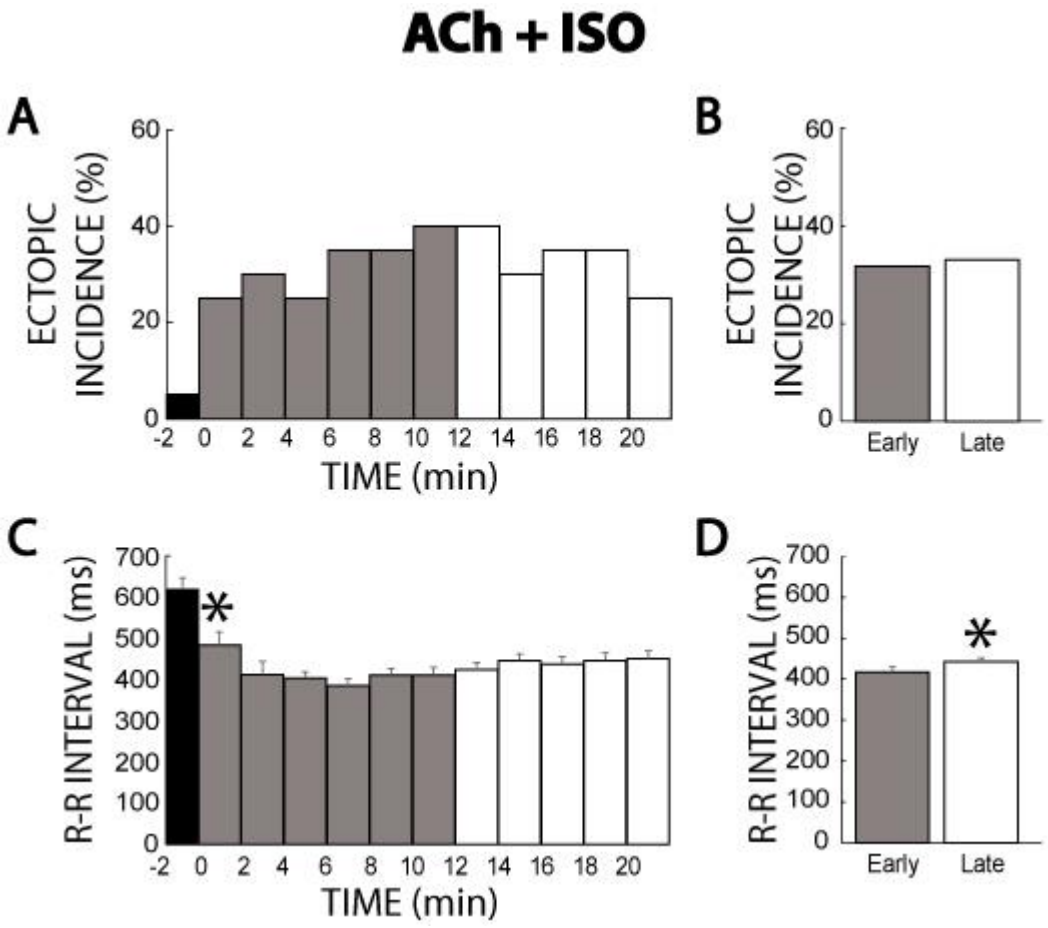

Figure 3.6: ACh + ISO ectopic incidence and R-R interval time course. A) ACh produced two ectopic beats in one heart (time -2). The addition of ISO elicited more ectopic beats which persisted for the entire perfusion period. **B)** No significant differences in ectopic incidence were found between the early and late stages (32% vs 33%, n=10). **C)** ISO shortened the R-R interval within 2 minutes (*). **D)** R-R interval gradually prolonged as evidenced by a significantly shorter R-R interval during the early relative to the late stage (417±10 vs 442±8 ms, *).

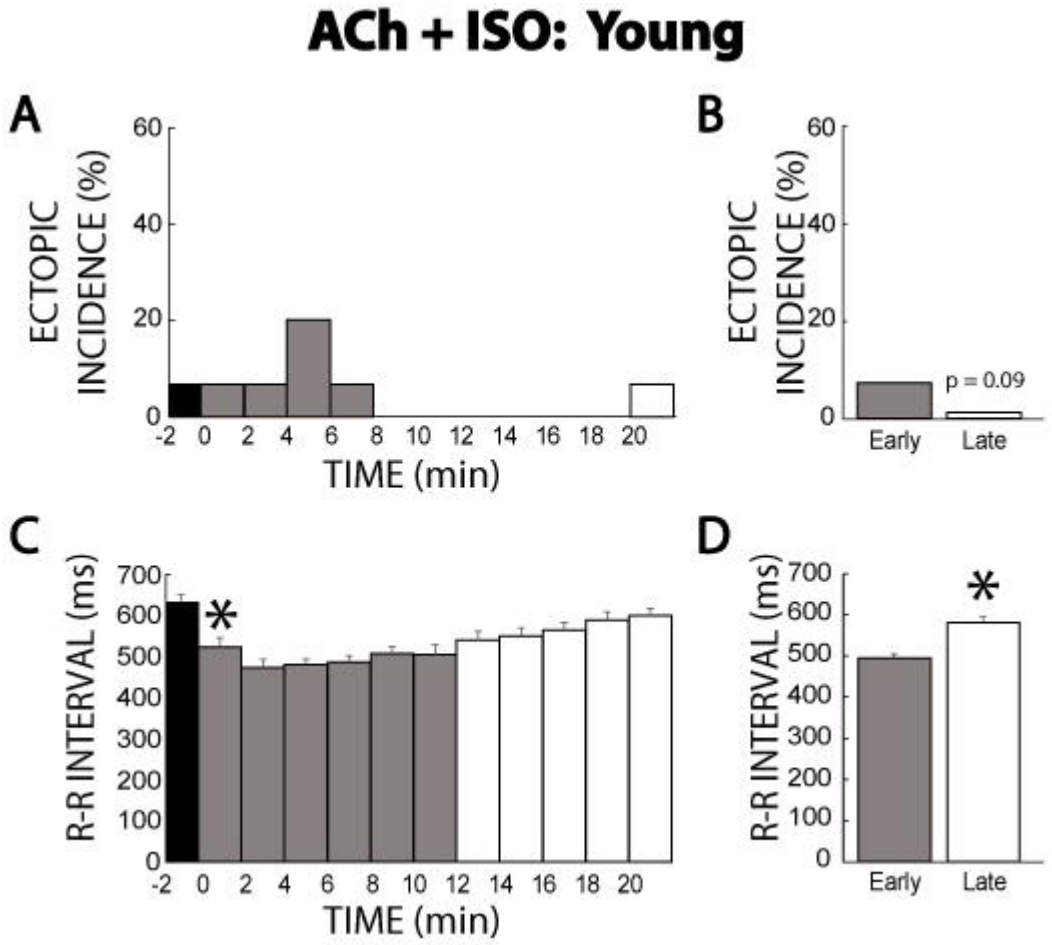

Figure 3.7: ACh + ISO ectopic incidence and R-R interval time course in young animals. **A)** One ectopic beat was produced with ACh (time -2). With ISO the ectopic beats were exhibited until 8 minutes, and only one ectopic beat was produced thereafter. **B)** The early stage had a 7% ectopic beat incidence, while the late stage had 1% (n=7, p=0.09). **C)** The R-R interval was shortened by ISO and then gradually prolonged over time. **D)** The early stage exhibited a shorter R-R interval relative to the late stage (496±8 vs 568±9 ms, *).

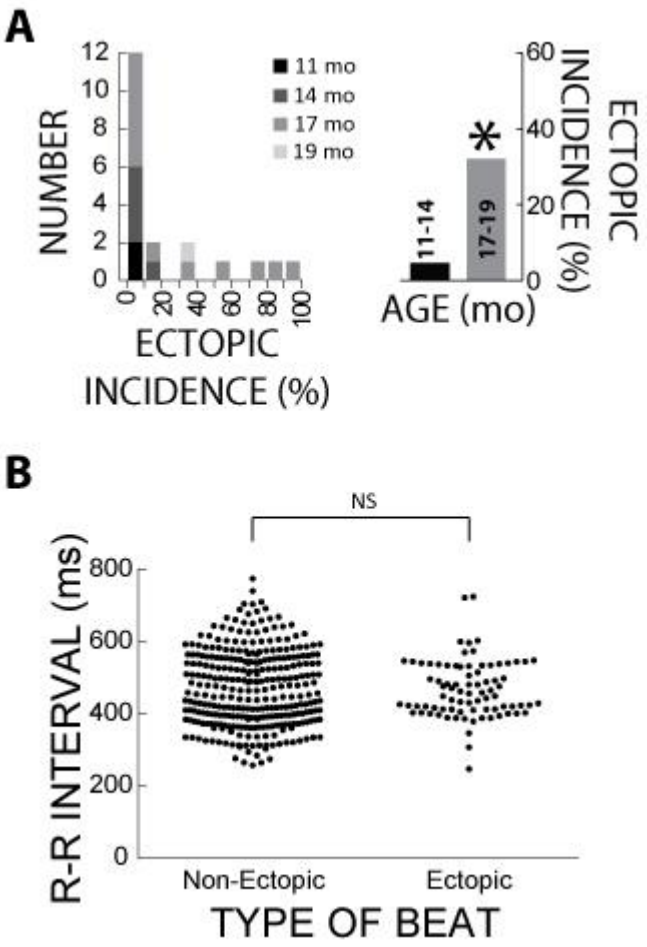

Figure 3.8: Age and R-R interval effects on ectopic behavior in ACh + ISO. A) Left Panel: Histogram of hearts that manifested differential ectopic beat incidences for the ACh + ISO experiments. Age is represented by the shade of grey. Right panel: Mature guinea pigs (17 – 19 months) manifested larger ectopic activity incidence relative to young guinea pigs (11 – 14 months, 31% vs 5%, n=13 vs n=7, *). **B)** The R-R interval following ISO was between ectopic and non-ectopic beats. There was no significant difference in R-R interval.

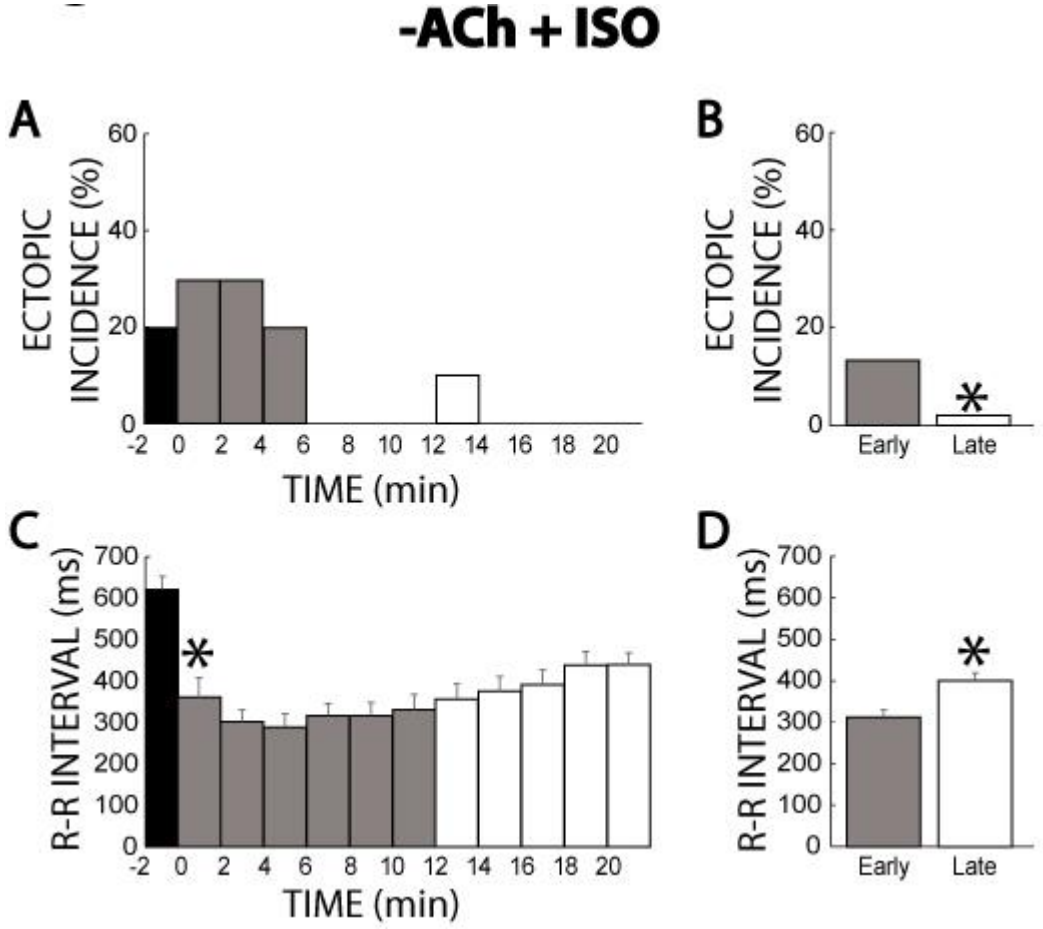

Figure 3.9: -ACh + ISO ectopic incidence and R-R interval time course. A) Without ISO, ACh produced few ectopic beats. Adding ISO produced ectopic beats for 6 minutes, but then ectopic activity effectively ceased **B)** Ectopic beat incidence was significantly larger for early (grey bar, 13%, n=5) relative to the late stage (*, white bar, 2%). **C)** ISO rapidly shortened the R-R interval within 2 minutes (*). Over time, the R-R interval gradually prolonged. **D)** R-R interval was significantly shorter for the early stage relative to the late stage (313±13 vs 400±15 ms, *).

Chapter 4: Conclusion

The role of ectopic activity in sudden cardiac death remains a work in progress. Some studies have linked ectopic beat burden with sudden cardiac death,[1] while others have shown that even with decreased ectopic burden sudden cardiac death remains prevalent.[2,3] Furthermore, in scientific laboratories it remains challenging to study ectopic activity as their incidence and timing remains unpredictable. The temporal nature of these events reveals important insights into the underlying mechanisms of arrhythmogenesis, and manipulation of the timing of these events can reveal the mechanisms that can increase arrhythmogenesis.

Summary

The first goal was to create a methodology that would distinguish ectopic beats from non-ectopic beats in guinea pig ventricular myocardium, as discussed in Chapter 2. Rapid pacing produces two types of recovery beats following rapid pacing: a period of quiescence longer than the native rhythm (overdrive suppression, OS) or a shortened interval (overdrive excited, OE). These are associated with normal automaticity and arrhythmogenic triggered activity, respectively. Previous studies in whole-heart preparations have used the lack of atrial depolarization[4] to distinguish ectopic activity from normal rhythm in ECG recordings. However, in our preparations we removed the atria in order to decrease the rate of spontaneous rhythm, allowing more time for ectopic beats to occur as the native intrinsic rhythm slowed. Therefore, P waves, or atrial depolarization, were absent for all beat types in our preparations, and presence or absence could not be used to differentiate between beats. Shortened cycle lengths have also been used to distinguish between types.[5] However, this remains challenging as, at slower pacing rates, it is possible that ectopic and non-ectopic beat cycle lengths may overlap, thereby leading to confusion. In our methodology we proposed using both the cycle length and QRS duration of the recovery beat.

A preliminary analysis using a k-means 2-cluster algorithm was used to stratify the data into two clusters using the absolute values of cycle length (RC) and QRS duration of the first

recovery beat following rapid pacing, but the data was preferentially clustered based on RCL values. Additionally, pharmacologic intervention and inter-animal variability could affect both the cycle length and QRS duration, leading to confusion when differentiating ectopic beats. Normalizing the recovery beat's RCL and QRS duration to the pre-paced spontaneous rhythm cycle length (BCL) and QRS duration, respectively, helped account for that variability. The k-means 2-cluster analysis was again performed and the data stratified into two populations. The Cluster 1 group exhibited RCL ratio values of ≥1 and QRS ratios of ~1, similar to overdrive suppressed behavior and a characteristic of normal automaticity.[6-8] The Cluster 2 group produced RCL ratio values of <1, exhibiting overdrive excitation, or arrhythmogenic behavior.[9,10] QRS ratios of Cluster 2 were >1, suggesting that overdrive excited beats occurred lower in the ventricular conduction system than the normal rhythm, and consistent with previous studies.[4,9,11] Moreover, across the pharmacologic interventions the Cluster 2 group exhibited smaller RCL and wider QRS ratios relative to Cluster 1, again suggestive of ectopic behavior. Both normothermic and hypothermic control conditions produced only Cluster 1 beats, or beats that manifested automaticity, which is to be expected.[4] Additionally, the RCL ratio of the Cluster 2 group was inversely proportional to pacing rate, a mechanistic identifier of triggered activity.[4,5,12] Also, the maximum coefficient of a cross-correlation analysis of QRS complexes has previously been used to stratify ectopic beats from non-ectopic beats.[13] The Cluster 2 group had a smaller maximum correlation coefficient relative to the Cluster 1 group, or in other words the Cluster 2 QRS morphologies were less similar to native rhythm than Cluster. This is expected of arrhythmogenic beats. Finally, when validated against the cross-correlation analysis the sensitivity and specificity of our methodology was very high. Taken together, this data confirms that our methodology can distinguish between ectopic and non-ectopic beats.

We also investigated the characteristics of the ectopic beats. Both normothermic digoxin and normothermic hypercalcemia produced ectopic beats, as would be expected.[4,14] However,

neither generated enough ectopic beats to allow for statistical comparison or discussion. Interestingly, the RCL variance did not significantly change with pacing rate for digoxin-induced ectopic beats. This in contrast with a previous study which demonstrated that spontaneous calcium release timing variability decreased with rapid pacing.[15] The discrepancy between the data sets could be due to the differences in the mechanisms that result in spontaneous calcium releases and fully propagated triggered activity. Further investigation is needed to elucidate these mechanisms, however for the first time these results demonstrate that ectopic beat variability is not dependent on pacing rate.

Hypothermic conditions were also observed to determine their effect on ectopic activity. Hypothermia has previously been linked with augmented calcium handling,[16] and thereby may create conditions for arrhythmogenesis. However, hypothermia did not significantly change the ectopic incidence for either hypercalcemia or digoxin. Interestingly, normothermic and hypothermic digoxin did produce significantly more ectopic beats normothermic and hypothermic hypercalcemia, suggesting that digoxin is a better intervention for creating ectopic beats.

We also sought to determine the effect of tetrodotoxin-sensitive sodium channel inhibition, on ectopic beat timing. Previous studies have demonstrated that sodium channel inhibitors can delay the timing of spontaneous calcium releases in isolated cardiomyocytes,[17] and act as antiarrhythmic agents by decreasing delayed afterdepolarization amplitude.[18] Importantly, our data demonstrated that ectopic beat RCL ratio was prolonged with TTX, without altering the native QRS duration and QRS ratio of ectopic beats. This would suggest that the delay in onset of ectopic beats was not due to altered membrane excitability. Another possible mechanism is that TTX prevents cytosolic calcium accumulation.[19] While further investigation is needed to understand the mechanisms by which TTX can affect ectopic beat timing, these exciting results do demonstrate that sodium channel inhibition can delay the timing of ectopic beats.

Additional discoveries of OS beat behavior were discussed in Chapter 2. As expected, normothermic control conditions only produced beats classified as overdrive suppressed (RCL>1), a characteristic of normal automaticity. Furthermore, the RCL ratio of these beat types proportionally increased with pacing rate, consistent with other studies.[4,20] However, OS beats became insensitive to pacing rates under hypercalcemia. Nevertheless, this is consistent with expectations, as purkinje fibers have previously been shown to recover more quickly from pacing-induced hyperpolarization during hypercalcemia.[6] One possible explanation for this finding is that hypercalcemia may increase intracellular calcium during rapid pacing,[21] leading to further calcium extrusion via the sodium calcium exchanger and increasing the resting membrane potential.[22] By making the tissue hyperexcitable, spontaneous rhythm may have occurred relatively early following rapid pacing. Finally, OS beats produced with digoxin were also insensitive to pacing rates. This is to be expected, however, as digoxin inhibits the sodium-potassium ATPase, effectively preventing hyperpolarization.[23]

As with normothermic control, OS beat RCL ratio increased with pacing rate during hypothermic control. Surprisingly, RCL ratio decreased with pacing rate during hypothermic hypercalcemia. As this has previously been used as a mechanistic identifier of triggered activity,[4,12] we again compared our methodology to the QRS complex cross-correlation analysis and found that our sensitivity and specificity were high for hypothermic hypercalcemia. This would suggest that our methodology was correctly stratifying between ectopic and non-ectopic beats. Furthermore, our methodology and the cross-correlation analysis *both* suggested that RCL ratio for OS beats decrease with rapid pacing. As discussed above, hypercalcemia may increase intracellular calcium, thereby increasing sodium calcium exchanger activity and depolarize the membrane. Hypothermia has also been linked with increased sodium calcium exchanger activity,[16] and may further depolarize the membrane during rapid pacing, resulting in spontaneous beats that occur earlier. However, this hypothesis has not been validated and requires further

investigation. In contrast, hypothermic digoxin remained insensitive to pacing rates. This may be due to digoxin increasing intracellular sodium, and therefore may prevent the sodium-calcium exchanger from significantly depolarizing the membrane as intracellular sodium and calcium rise concurrently.

In summary of Chapter 2, we were able to differentiate between ectopic and non-ectopic beats using a simple and robust methodology. The next goal was to create a consistent model for ectopic activity. Hypercalcemia, however, seldom produced ectopic beats. Furthermore, while hearts with digoxin did manifest many ectopic beats, the intervention was also susceptible to ventricular tachycardia or fibrillation. Consequently, we decided to use another model to create ectopic activity.

Sympathetic agonists are associated with augmented calcium handling and have previously been used to induce spontaneous calcium releases and triggered activity.[24,25] Therefore we perfused isoproterenol (ISO), a sympathetic agonist, to induce ectopic activity. Hearts were rapidly paced every minute following ISO perfusion to determine the temporal nature of the ectopic activity. Although ISO did initially produce ectopic activity, this was followed by a gradual decrease and then ectopic activity ceased. Furthermore, when heart rate was evaluated it was shown to produce a transient response as well. In contrast, control conditions without an intervention did not produce ectopic beats. Isoproterenol, therefore, created few ectopic beats.

Similarly, parasympathetic activity has also been associated with arrhythmogenesis.[26] We therefore perfused acetylcholine (ACh), a parasympathetic agonist, to determine its effect on arrhythmogenesis. Still, ACh produced little ectopic activity.

However, transition phases from parasympathetic to sympathetic and vice versa have previously been linked with increased sudden cardiac death and ectopic activity incidence.[27,28] Therefore, we sought to determine the role of simultaneous sympathetic and parasympathetic stimulation on the temporal nature of ectopic activity. ACh following ISO reintroduced ectopic

activity, but the relationship was still transient, while heart rate slowing was maintained. Importantly, ISO following ACh increased and *sustained* ectopic activity. Furthermore, ISO initially increased heart rate, but this was followed by a small but significant slowing in heart rate. Of note, this slowing in heart rate was not significantly different from that seen under control conditions. Therefore, ISO following ACh persistently increased ectopic activity and heart rate. The question then arises as to what mechanisms were responsible for the sustained response.

Rapid intrinsic heart rates may contribute to the formation of ectopic activity. To determine whether the native heart rate could affect ectopic beat emergence we compared the pre-paced heart rates of pacing protocols that did or did not produce ectopic beats for the intervention of ISO following ACh. In short, the native heart rate did not significantly change between ectopic and non-ectopic beats, suggesting that heart rate was not playing a significant role in triggered activity.

In addition, age has previously been shown to have an important role in the autonomic nervous system,[29] and so we sought to determine if the sustained effect shown during ISO following ACh would occur in younger animals. Animals that had been used thus far were aged 17 – 19 months. Younger animals (11 – 14 months) were used, and interestingly ISO following ACh no longer produced persistent arrhythmogenesis. Mature animals (17 – 19 months) manifested greater ectopic incidences relative to the young animals. Furthermore, heart rate once again manifested a transient response. These results would indicate that age is an important factor in autonomic-induced arrhythmias and the persistent response induced by ISO following ACh. However, further investigation is needed to determine the mechanisms that lead to these response differences.

Finally, we also sought to determine if concurrent parasympathetic stimulation was needed to elicit the sustained response with ISO following ACh in mature animals. When ACh was washed out, ISO produced ectopic activity initially, but then this disappeared. Furthermore, ISO initially increased heart rate but this gradually decreased as well. Therefore, withdrawal of

ACh with ISO perfusion produced a transient response. This would suggest that concurrent parasympathetic stimulation is needed to for sympathetic stimulation to create a persistently arrhythmogenic substrate.

There are several mechanisms by which parasympathetic stimulation followed by sympathetic stimulation can induce a persistent arrhythmogenic substrate and sustained heart rate elevation. Parasympathetic stimulation may induce internalization of β adrenergic receptors (β−AR), and thereby blunting the ISO-induced negative feedback mechanism. Our results would argue against this, however, because if this was the mechanism then ISO following ACh washout should have similar effects as ISO following ACh. A previous study has shown that the time course to 50% of β−AR recovery following internalization is about 20 minutes,[30] and therefore it should take a significant amount of time for the negative feedback to take place following ACh washout. Another possible mechanism is that parasympathetic stimulation may affect the desensitization of the β−ARs by inhibiting cAMP production, and phosphorylation of the β2 adrenergic receptor and the resultant desensitization. However, the presence of β2 receptors in guinea pig ventricular myocardium reveals controversial,[31,32] and thus further investigation is needed to determine the part of β2 phosphorylation in ISO following ACh sustained arrhythmogenesis. Finally, downstream factors may have a role in sustained arrhythmogenesis. cAMP is degraded by phosphodiesterases and this enzyme contributes to sympathetic agonist-induced β−AR desensitization, as it is activated by Protein Kinase A. Parasympathetic stimulation prevents Protein Kinase A activation, and therefore should also blunt phosphodiesterase activation and cAMP degradation. Thus, parasympathetic stimulation may help induce a sustained response from sympathetic stimulation by preventing full activation of phosphodiesterases. Interestingly, phosphodiesterase activity decreases with age in human cardiac tissue.[33] If the same relationship is found in guinea pig, this may help explain the age-

dependent responses to ISO following ACh. While these are intriguing hypotheses, these require further investigation.

Significance

This dissertation has demonstrated that a simple and robust methodology can be used to differentiate between beat types. Optical mapping and electrode array studies may consider using this method when ectopic beats do not initiate within the field of view, or when only ECG leads are available. In the clinical setting, ectopic beats are generally identified by physicians. However, our presented method may provide a quick indicator for differentiating between beat types in which a high volume of clinical ECG data has been collected. To do so, sinus rhythm would need to be identified to use for QRS and RCL normalization. As our data was obtained using guinea pig hearts and pacing-induced ectopy, simply using centroids of the clusters from our data and applying to these studies to distinguish ectopic beats from sinus rhythm would likely result in inaccurate classification. In short, future studies should consider applying a 2-cluster k-means analysis to their own data set to determine centroids. In summary, future clinical and basic research studies should consider using this methodology.

Interestingly, in Chapter 2 neuronal sodium channel blockade delayed the formation of ectopic beats without affecting QRS duration. Non-specific sodium channel blockers have proven to be pro-arrhythmic despite decreasing ectopic beat burden because they decreased membrane excitability.[34] These and other[17,35] data suggest that neuronal sodium channel blockade may be an effective anti-arrhythmic therapy that ameliorates ectopic propensity without affecting membrane excitability.

In Chapter 3 our data suggests that transition periods from parasympathetic to sympathetic stimulation and vice versa will be susceptible to arrhythmogenesis, and is supported by clinical studies which show that rest after exercise and waking after sleep are associated with augmented arrhythmogenesis and sudden cardiac death.[36-38] Importantly, preceding and

concurrent parasympathetic stimulation with sympathetic stimulation created persistent ectopic activity. This data suggests that preventing parasympathetic activity induced preservation of sympathetic sensitivity may be a new target in preventing sudden cardiac death. Furthermore, as it has remains a challenge to reproducibly create ectopic activity, future studies investigating the underlying mechanisms leading to ectopic activity should consider using this model. However, the mechanisms that by which muscarinic agonists can affect β–AR sensitivity have not been fully explored in this dissertation. As parasympathetic stimulation has been shown to have both pro- and anti- arrhythmic properties, future studies may consider carefully studying the time and dose dependent effects of muscarinic agonists on β–AR sensitivity.

Conclusions

In this dissertation we have taken the steps to evaluate the temporal nature of ectopic activity. First, we created a simple and robust methodology for distinguishing ectopic beats from non-ectopic beats. We also created a reproducible model for ectopic activity using concurrent sympathetic and parasympathetic stimulation. Finally, we also evaluated the temporal nature of ectopic activity by sodium channel inhibition and sympathetic and parasympathetic stimulation. This research may assist other scientists who want to evaluate the underlying mechanisms of ectopic activity as this model was reproducible and had a low incidence of VT/VF. Importantly, we showed that sympathetic following parasympathetic stimulation created a persistent model for arrhythmogenesis.

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