REGULATION OF CARDIAC VOLTAGE GATED POTASSIUM CURRENTS IN HEALTH AND DISEASE

DISSERTATION

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By

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ABSTRACT

Cardiovascular disease (CVD) is a major cause of mortality and morbidity worldwide. CVD accounts for more deaths than all forms of cancer in the United States. Hypertension, Heart Failure and Atrial Fibrillation are the most common diagnosis, hospitalization cause and the sustained cardiac arrhythmia respectively in the US. Sudden cardiac death is the one of the most common causes of cardiovascular mortality after myocardial infarction, and a common cause of death in heart failure patients. This has been attributed to the development of ventricular tachyarrhythmias. In addition, most forms of acquired CVD have been shown to produce electrophysiological changes due to very close interactions between structure, signaling pathways and ion channels. Due to the increased public heath burden caused by CVD, a high impetus has been placed on identifying novel therapeutic targets via translational research. Identification of novel therapeutic targets to treat heart failure and sudden death is underway and is still in a very nascent stage. In addition, ion channel blockers, more specifically “atrial-specific” ion channel blockers have proposed to be a major therapeutic target to treat atrial fibrillation without the risk of ventricular pro-arrhythmia. This dissertation addresses these important therapeutic issues from the standpoint of cellular electrophysiology. All experiments were amphotericin-B
perforated whole cell patch-clamp experiments performed on isolated cardiac myocytes at 36 ± 0.5°C.

Chapter 2 addresses a very important issue of identification of a purportedly “atrial-specific” ion current in the canine ventricle. The findings suggest that the current is not atrial-specific and has properties similar to the atrial ultra-rapid delayed rectifier current (I_{kur}). This might have important implications for the use of I_{kur} blockers for the treatment of atrial fibrillation.

Hypertension leads to ventricular hypertrophy, and ionic and structural remodeling. Chronic hypertension leads to reduced ventricular compliance and if untreated can precipitate heart failure. Chapter 3 focuses on alterations in diastolic currents (I_{k1} and I_f) and their contribution to altered cardiac excitability in hypertensive heart failure.

Chapter 4 is the first study to document ionic remodeling in a well characterized canine model of sudden cardiac death. Our findings suggest that K^+ current remodeling (predominantly a complete absence of I_{Kr}) causes prolongation and increased variability of the action potential duration and early after-depolarizations. This study provides a basis for examining the potential benefits of I_{Kr} activators as a therapeutic target to prevent arrhythmias and sudden death.

Chapter 5 and Chapter 6 assess ventricular and atrial ionic remodeling in chronic heart failure. Chapter 5 presents some provocative preliminary data on
the electrophysiologic reverse remodeling after cardiac resynchronization therapy. Chapter 6 focuses on atrial ionic remodeling in chronic heart failure. The results suggest that duration of heart failure is a very important predictor of persistence of atrial fibrillation in heart failure. In addition, preliminary data suggesting specific oxidative processes that regulate atrial K⁺ currents are presented. Some of these effects are reversible, while others are irreversible with acute myocyte anti-oxidant (glutathione) replenishment. These studies provide a foundation for examining a future research direction where the use of specific anti-oxidant interventions could be tested to assess prevention of atrial remodeling and therefore atrial fibrillation.

We propose that these studies aid in understanding of important processes of K⁺ current remodeling in CVD. Understanding these mechanisms is important to devise new therapeutic targets for prevention/treatment of arrhythmias in CVD.
Dedicated to Family,
Friends and Gurus
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As I start this section of my dissertation, I can only look back at gratitude at each and every person who has contributed to my education and well-being. A lot of wishes, blessings and good-will has gone into me becoming a Doctor of Philosophy.

“For things to go the way we want it in life, there is only one primary requirement. Blessed to be at the right place, at the right time, to form the right association”. I have been blessed that way in a lot of ways during this lifetime. Call it luck, blessing or hardwork. I believe that its all due to one altruistic superpower called God (which I believe has no form, no religion but one that guides all that we do in a lifetime) for being extremely kind to me.

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(The term borrowed from my father’s dictionary of life). With their blessings and support, I hope to continue the good work in the future to benefit therapeutics and healthcare suffering from disease.
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PREFACE

The human heart is the very first organ to develop at the embryological stage (at 23 days of conception). The cardiovascular system is the first organ system to become fully functional in utero, at approximately 8 weeks after conception. The heart is traditionally described as the “seat of love and emotion” and serves as the organ that pumps blood throughout the body. The latter clause is true in any vertebrate animal known today (fish, frog, man, horse and a whale).

The adult human heart pumps ~80 mL (3 oz) of blood per beat. At rest, the heart pumps approximately 5 L of blood per minute. In a 70-year lifetime, the heart will pump about 200 million L (more than 1 million barrels) of blood, good enough to fill three super tankers. In 1 day, the heart exerts enough power to lift a 1-ton weight vertically to 12.5 m (41 ft). The changing realization from the heart as a mechanical pump to an electro-mechanical pump, took centuries to evolve. The heart beats rhythmically due to a specialized tissue called the pacemaker (SA Node) and this impulse travels down specialized conduction fibers to the whole heart; causing cardiac contraction to expel the blood from the heart. Today, with advent of many sleek tools and animal models that scientists use, we have an ever expanding, deeper, knowledge about how the electrical and the mechanical function of the heart is intricately linked in health and disease. More importantly, the search for a good animal model to study human electrical phenomena has led to the use of canines as a surrogate. Canines and human share a remarkable similarity in the ion channels that contribute to the generation of the heartbeat. This dissertation will
focus exclusively on studying how the cardiac electrical impulse (action potential or AP) is regulated in health and disease. More importantly, it deals with specific pathways called ion channels (K⁺ channels will be exclusively discussed) in the cell membrane.

There is a wide spectrum of 6-8 different K⁺ channels in the mammalian heart depending on the species under study. These K⁺ channels are absolutely critical to bring the electrical state of the heart back to the resting state, a process termed repolarization. The duration of repolarization determines the strength of contraction and alterations in one or more of the K⁺ channels can dramatically alter contraction and/or might produce AP prolongation with or without arrhythmia. The repolarization patterns differ between different chambers of the heart as well (with atrial repolarization (therefore atrial APD) being a lot shorter than the ventricular repolarization). In other words, the repolarization gradient is much higher due to higher densities of K⁺ currents in the atrium. One example of this K⁺ current is the ultra-rapid delayed rectifier K⁺ current (I_{Kur}) which has been shown by previous studies to be abundant in the canine and human atria. This channel was thought to be almost exclusive to the atria and has been proposed to a good candidate for treating a fast arrhythmia of the atrium called atrial fibrillation. The idea behind this proposed therapeutic option is that since many ion channels are similar between atria and ventricles, blockade of an exclusively “atrial-selective” channel might provide therapy with mild or no ventricular arrhythmias. This idea of “atrial-selectivity” of I_{Kur} blockade in large mammals is refuted in chapter 2, where the author found evidence of an “I_{Kur}-like” current in the canine left ventricle.
An estimated 79.5 million Americans suffer from one or another form of cardiovascular disease (excluding congenital cardiac defects), and this figure reaches epidemic proportions worldwide. Of all the causes for cardiovascular diseases, hypertension, myocardial infarction and heart failure accounts for 72, 7, and 6 million patients, respectively, in the US. All or some of the above mentioned conditions can co-exist. A lot of impetus has been laid on prevention with healthy diet, exercise and lifestyle modifications. However, the number of patients who have and will have cardiovascular disease will exponentially rise over the next 50 years. Therefore, understanding mechanisms that promote one risk factor like hypertension and/or myocardial infarction leading to heart failure or sudden death is of paramount importance. For example, a balloon (completely filled with air/water) possesses a high wall stress that will hinder further filling. A chronically hypertensive heart behaves in a similar manner. Hypertension leads to adaptive changes in the heart that promote hypertrophy. Hypertrophy, in turn leads to higher wall stress and promotes impaired filling and relaxation that eventually are precursors to heart failure if left untreated. Chapter 3 will address this issue where effects of untreated hypertension on electrical properties (with focus on K⁺ currents) that promote altered excitability is studied and data is presented. The resultant electrical remodeling produce a substrate for arrhythmias is studied as at different time points during the progression of hypertensive heart failure.

“Sudden Cardiac Death (SCD)” is defined as sudden unexpected mortality resulting due to an underlying cardiac cause. Almost 80% of the patients who die due to SCD have an underlying structural cardiac
disease. Continuous electrical monitoring (Holter) of the heart reveals that ventricular arrhythmias are a leading cause of SCD. In patients with lethal ventricular arrhythmias, there is a 50% incidence of healed scar due to a previous myocardial infarction. Chapter 4 focuses on understanding the mechanisms that predispose to the development of a ventricular arrhythmia (termed “ventricular fibrillation”) and eventually sudden death after myocardial infarction.

Heart failure (HF) affects 5 million Americans and has an incidence of 1 in 10 in people over 65 years of age. Heart failure has been the number one U.S. hospital discharge diagnosis for patients over the age of 65 for the past 12 years. It is a complex syndrome that takes months to years to develop and lack of chronic disease models has posed a major problem to understanding the disease pathogenesis and treatment. Cardiac re-synchronization therapy (CRT) is an emerging treatment modality for heart failure but the underlying molecular mechanisms are unknown at the present time. Chapter 4 discusses preliminary data from a chronic canine tachypacing HF model. Electrical reverse remodeling data after CRT is presented.

HF produces a significant risk for the development of atrial fibrillation (AF). AF increases mortality in HF by 4.5-5.9 fold. HF produces impaired relaxation and contractile function which is reflected in the left atrium as a higher left atrial pressure. This produces left atrial stretch and fibrosis that present a structural substrate for re-entry and AF. Ionic remodeling in human AF is another factor that helps in the substrate formation for AF development and perpetuation. Another interesting caveat is that in humans AF during HF is often persistent and permanent. The present animal models that study AF in HF result
in AF lasting for ~600 seconds.\textsuperscript{9} This can be attributed to shorter, more severe (completely non-clinical) and reversible HF. Chapter 5 discusses ionic remodeling that results from \textit{chronic minimally reversible} HF. AF in this model is persistent and ionic mechanisms are studied in the left atrial appendage myocytes. A very interesting and emerging area of research has emerged from this research, which is to understand oxidative modifications of ion channel proteins in normal and diseased myocytes. The possibility of modifying cellular redox status (and electrophysiology) by improving the anti-oxidant defense is proposed.

The over-all theme of this dissertation is to understand how myocardial K\textsuperscript{+} channels are altered in health and disease. Understanding how these channels are altered, offers very exciting insights into potential manipulations of these K\textsuperscript{+} channels to offer therapeutic benefit in patients with cardiac arrhythmias.
CHAPTER 1

INTRODUCTION
1.1 HISTORY OF THE STUDY OF THE HEART

As we know today, the heart is the electromechanical, muscular pump that pumps oxygenated blood throughout the body via the aorta. The realization that heart was indeed the organ in the mammalian body that pumped the blood took centuries to evolve. The very first origin of knowledge of heart (probably the circulatory system) according to many textbooks was from the observation, study and recording of pulse in ancient China (6th century BC). Aristotle (a noted Greek philosopher) in the fourth century BC identified the heart as the first important organ formed during development, based on his observations of chick embryos. Aristotle proposed the heart to be the seat of intelligence, motion, and sensation – “a hot, dry organ” and described the heart as a three-chambered organ that was the center of vitality in the body. Other organs surrounding it (e.g. brain and lungs) simply existed to cool the heart. Around the same time, Herophilus – a Greek physician and anatomist reported pulse in living prisoners and relied on experimental dissection of cadavers from prisons to show that heart was a muscular organ. The next individual whose immense contribution to field of anatomy of the heart cannot be forgotten is Galen. In the second century AD, Galen believed and taught his students that there were two distinct types of blood. ‘Nutritive blood’ was made by the liver and carried through veins to the organs, where it was finally consumed. ‘Vital blood’ was thought to be made by the heart and pumped through arteries to carry the "vital spirits." Galen believed that the heart acted not to pump blood, but to suck it in from the veins. Galen also believed that blood flowed through the septum of the heart from one ventricle to the other through a system of
tiny pores. He did not know that the blood left each ventricle through arteries.

1.1.1 CONTRIBUTION OF ANCIENT INDIA TO UNDERSTANDING THE STRUCTURE OF THE HEART

While Greek civilization was invading the rest of the world around 1400-1100 BC leading to propagation of their knowledge, knowledge about the heart (and the circulatory system) existed even before Galen and Herophilus and the Chinese physicians. In the Indus Valley basin (located presently in Northeastern Pakistan and Northern India - described as the oldest cradle of civilization), evidence of comparative anatomy studies have been found from excavations of archeological sites. After the European tribes called Aryans, invaded ancient India, the intermingling of these two civilizations lead to the formation of the oldest textbooks in the world called the "Vedas" around 1400 B.C. Vedas were a collection of four ancient scriptures (Rig, Yajur, Atharva and Sama) rendered by the ancient Indian saint, Veda Vyasa and preserved even to this day. He outlined in the Vedas the norms of living, science, astronomy and rituals; a lot of those facts remain true even today and these rituals are followed by the people of the Hindu religion. The Rig Veda mentions the heart, lungs, stomach and kidneys. The Atharva Veda lists various medicinal herbs, plants and also mentions "the wonderful structure of man". The Atharva Veda refers to the heart as "lotus with nine gates", an amazingly accurate description of the heart as we know it today. The heart as we know from anatomy could be compared to a lotus bud if held with its apex upwards. There are nine openings in all: 3 in the right atrium, 4 in the left atrium and one each in the right and left ventricles. The Atharva Veda refers to
dhamanis - which are ducts with thick walls equivalent to arteries; siras - which are ducts with thin walls equivalent to veins, and still finer ducts are referred to as snavas similar to capillaries. However some misconceptions existed and the Vedic scholars considered nerves also as hollow tubes or ducts, a concept which is quite far from reality. However, these amazingly correct hypothetical propositions were made well before the Greeks and the Chinese around 1500 BC, in the absence of cadaver dissection while treating patients in ancient India. These findings were later confirmed by Susruta (widely regarded by many as the father of applied anatomy and surgery) in his textbook Susruta Samhita.11

1.1.2 MODERN PHYSIOLOGY AND THE HUMBLE BEGINNINGS OF ELECTROCARDIOLOGY

Following the contributions of ancient Indian, Greek and Chinese civilizations, knowledge about the anatomy and function of the heart did not progress further until William Harvey (in 1615) proposed the mechanics of circulation.10 He used experimental dissection in animals to provide direct evidence that the circulatory system of the human body was composed of the muscular pump (the heart) feeding oxygenated blood into bigger vessels (arteries), and proposed in the absence of microscope that arteries branched into arterioles and capillaries. The de-oxygenated blood from the tissues was re-fed back to the heart via venules, feeding to bigger and thin walled (compared to the arteries) vessels called veins, which emptied into two big vessels (the vena cavae) into the right side of the heart. Thus, the concept of a four-chambered heart was born. All the observations of capillaries and venules were made in the absence of microscopy that is widely available to researchers today. He also
proposed that flow of blood in the body was in a closed circuit and calculated by emptying the human heart that the organ could hold approximately two ounces of blood. The direct evidence for the presence of arterioles, capillaries and venules came from Marcello Malpighi documented in his treatise named “De Polypo Cordis” in 1666, a few years after Harvey’s death (in 1657). Though Harvey’s observations were made in 1615, he waited until 1628 to validate his observations via correct experimental methods in his lectures titled “Exercitatio anatomica de motu cordis et sanguinis in animalibus”. However it must be remembered that, though Harvey’s work was the work that was not lost, there was a notable Spanish physician Michael Serveteus who discovered circulation a quarter century before Harvey. However he is not credited in modern day textbooks as his three manuscripts titled “Christianismi Restitutio” were lost in a fire.

Based on the above mentioned works, the idea of heart as a circulatory organ was widely proposed and accepted. However it was still unclear as to how the heart, considered as a muscular pump was able to pump blood regularly at a constant rate. This lead many physicians before 1883 to propose the idea of innervation (probably by the vagus identified by Eduard and Ernst Weber in 1845) of the heart providing the stimulus for the heart to contract and relax. This idea was refuted via the pioneering work of Gaskell (in 1883) proposing the myogenic origin of automaticity in the heart. Before Gaskell’s proposal, cardiac rhythms were analyzed and “refractoriness” was identified by Fontana (in 1700’s) and later by Schiff (1850) and Marey (1876). The Japanese physician, Tawara made seminal observations of the electrical conduction system of the heart via his observation of the Purkinje fiber network and the AV node in 1906.
Around the same time, other electrical phenomena called “paraarrhythmia” were proposed by Wenckebach in 1903 as the expression of activity of two independent centers in the heart. This was preceded by the description of the Wenckebach phenomenon in 1898. Abnormal electrical activity of the ventricle (ventricular fibrillation by Hoffa and Ludwig in 1850), atria (atrial fibrillation by Lewis, Rothberg and Winterberg in 1909 and atrial flutter by Jolly and Ritchie in 1911) was documented. All the knowledge of heart being an organ capable of generating its own electricity and propagation independent of nerves, culminated in the invention and demonstration via recordings using a string galvanometer by a Dutch physiologist Wilhelm Einthoven as early as 1895. He termed his recordings of the electrical activity of the heart the “electrocardiogram (ECG)” and thus modern day electrophysiology was born. He published a series of papers in 1903 with normal human subjects and in 1906 demonstrating the electrical activity of atrial and ventricular fibrillation. He proposed the equilateral triangle hypothesis and made his work public in the British journal Lancet in 1912 via a paper titled “The different forms of human electrocardiogram and their significance”. Einthoven’s ECG was composed of only three limb leads and this was expanded to 12 leads by the addition of six chest (pre-cordial) leads by Wilson in 1939 and the three unipolar leads introduced by Goldberger in 1942. Since the advent of modern electrophysiology, numerous physicians and physiologists like George Mines, Lewis, Norman Wilson and several others studied and contributed immensely to the early understanding of the abnormal rhythms of the heart termed “arrhythmias”. The next big step in understanding the conduction system was made by “Pick and Langendorff” who in their own words were seeking solutions to cardiac rhythm problems by “analysis and
They identified the differential diagnosis of supraventricular arrhythmias, ventricular tachycardia and developed an orderly classification of atrio-ventricular (A-V) dissociation.

The next major advancement in the understanding of cardiac electrophysiology occurred with the advent of microelectrodes to study the cellular basis of electrical potentials that occurs on the body surface as ECG. Though numerous advances in physiology, cardiology and cardiac surgery have occurred since Einthoven’s first recordings, the remainder of this section will focus on the electrical basis of impulse generation and propagation in the heart with due acknowledgement that advances in knowledge of vascular physiology, signaling systems, etc. has lead to a boom in understanding not only in cardiac physiology in the mechanical sense of the word, but also in the electrical nature of the heart and also the interplay of mechano-electric feedback in the heart.

### 1.1.3 ADVENT OF CELLULAR ELECTROPHYSIOLOGY

This ability to directly measure cellular electrophysiology has provided physiologists with a valuable and a versatile tool to study the mechanistic basis of electrical impulse generation and propagation using a reductionist approach. Seminal works lead to the understanding that electrical activity originated in the single cells (myocytes) of the heart which then propagated from one cell to another, and that the summation of these cellular potentials propagates from one region in the heart to a different region giving rise to the ECG on the body surface. Ling and Gerard (in 1948) at University of Chicago developed a method for pulling
glass microelectrodes to tip diameters of less than \( \leq 0.5 \mu m \) which would penetrate the cell membrane without actually injuring the cell.\(^{13}\) Future Nobel Prize winner, Alan L Hodgkin from Cambridge visited Ling and Gerard to learn the technique of recording cellular potentials using the pulled glass microelectrodes with isotonic KCl solution and replacing the cathode with a low voltage electrometer developed by Ling and Gerard. Hodgkin and his co-worker Andrew Huxley in a series of seminal papers (1949-1953) proposed the mechanisms of nerve impulse propagation. The voltage generated by the giant squid axon (nerve “action potential”) was measured, and by altering the experimental solution concentrations (the extracellular solution for baseline recordings being “sea water”) and by application of mathematical calculations demonstrated the presence of sodium and potassium as the primary ions necessary for nerve impulse. They also proposed long before ion channels were known that pathways for transport of ions existed in the cell membranes, and the movement of ions across the membranes resulted in the generation of voltage (as they had recorded). They even went one step further to propose, based on their mathematical deductions, that pathways that conduct sodium has open (“m” gate) and closed (“h” gate) states and thus the concept of “activation” and “inactivation” of ion channel permeation pathways was born.\(^{14}\) Around the same time, Silvio Weidmann along with Edward Corabeouf recorded the first action potentials from calf Purkinje fibers in 1949.\(^{15}\) Weidmann, considered by many as the “father of cardiac cellular electrophysiology”, published a series of elegant papers in the next six years outlining the application of ionic theory to cardiac muscle and differentiated cardiac action potential from a nerve action potential. While Weidmann carried out important studies in Purkinje fibers, Hoffman and
Suckling (1952) performed studies to record action potentials from atrial and ventricular muscle. Action Potentials (APs) were recorded from the sinoatrial (SA) node by West (1955), from His bundle by Alanis (1958), from transitional atrial fibers by Paes de Carvalho (1959) and from the AV node by Hoffman in 1959.

The years 1946-1959 lead to the realization of ion permeation pathways in cardiac muscle and provided a foundation for the nomenclature of “ionic currents”. This proposition was made long before ion channels were discovered. Calcium was identified to be important for cardiac contraction by Ringer in 1882. But the requirement of calcium for contraction, via an increase in inward calcium conductance, as the basis of the plateau phase of the cardiac AP came from the work of Harald Reuter in 1966. In addition to this observation in ventricular muscle, evidence for the AV node requiring calcium current for depolarization came from Zipes and Mendez (1973). Noma and Irisawa (1976) proposed that the same mechanism was in operation within SA node as well. The next big step in understanding ion movement across cell membranes came from work of Gadsby and Cranefield who demonstrated the presence of “ouabain -sensitive” ion exchanger in Purkinje fibers (ouabain is a plant-derived digitalis glycoside, which was used extensively for treatment of heart failure patients via increased cardiac contraction).

The next big wave after the microelectrode studies came with the development of high-fidelity patch clamp recordings from single ion channels located on cell membrane patches by Neher and Sakmann at the Max Planck Institute in 1976. They used high resistance seals (in the order of 10 -100 gigaohms) obtained by tighter membrane-pipette seals with smaller tip microelectrodes. With one microelectrode attached to the
muscle fiber for stimulation, and another microelectrode to record from the patch (portion of a membrane sucked and sealed onto the pipette), current was recorded from cultured muscle cells. Later on in 1980, current from a single voltage-gated sodium channel was measured and the propositions of Hodgkin and Huxley were verified and it was confirmed that the sodium channel indeed had 3 activation states (mentioned as gates by Hodgkin and Huxley) and 2 inactivation states. This technique reduced the background noise, enabling scientists to record conductance of unitary ion channels, to develop models of opening and closing (termed “gating) and to identify how various ion channels are modulated by ligands, membrane voltage and time dependence of gating. This lead to a boom in the understanding of ion channels mediating action potentials in various organs leading to discovery of many ion channels types with channel conductance varying between a few picoSiemens to more than 1 nanoSiemen. The following section will contain information relating the ion channels and action potentials in the heart and how differential ion channel expression is related to both impulse generation in various parts of the heart and propagation from one region to the other, ultimately generating a heartbeat.

1.1.4 ANATOMY OF THE SPECIALIZED CONDUCTION SYSTEM OF THE HEART: A HISTORICAL OVERVIEW

“Life must be lived forward, but understood backward” - Kirkegaard

The general belief was that nerve innervating the heart was delivering impulses to the cardiac muscle which made them to initiate contraction and this propagated via specialized conduction pathways. The heart generates electrical voltage which is myogenic in origin (as
described by Gaskell in 1883). The anatomy of the conduction system of the heart was initially understood from the lower conduction fibers in the hierarchy. Purkinje Fibers were the first fibers of the conduction system to be described by Johannes Purkinje in 1845. In microscopic sections of the heart, Purkinje observed a fiber formation (grey, gelatinous threads running between serous membranes of the heart) and described these fibers as composed of nucleated granules. However, at that point in time, the physiological significance of these fibers was unclear.

The next major discovery in understanding the anatomy of the conduction system was by Wilhelm His, Jr. in 1893 who described the Bundle of His. In his experiments with warm blooded animals, His found that separating the atria from the ventricles along the septum (in the line of AV groove) caused dissociation of cardiac rhythm. This specialized muscular bundle described by His as fasciculus atrioventricularis was later found to be rich in sarcoplasm and glycogen. Around the same time, Kent and Paladino independently described another conduction pathway which were anatomically different, and argued that in addition to the myogenic path that was described by His, another pathway existed. Years later, it was the Bundle of Kent-Paladino which was found to be an abnormal accessory pathway present in some people producing premature excitation of the ventricles.

Ludwig Aschoff and Sunao Tawara made a significant impact on the understanding of how the impulse once generated in the atria could traverse down to the ventricles. Aschoff and Tawara in 1906 described the AV node as the thick module of specific glycogen rich conductive myogenic tissue. They proposed that AV node was the only electrical connection that exists between the atria and the ventricles in a normal
heart. Therefore, at this time, studies described different types of conduction fibers with no consideration of how the electrical impulse spreads in the heart. Tawara and Ashcoff put together a comprehensive integration of earlier findings of His, Purkinje and their own studies to propose that the impulse travels down the AV node to the His Bundle then dividing into left and right bundles ending in the Purkinje Fibers. Tawara meticulously described these findings through systematic examination of a number of mammals and human hearts in Aschoff’s book titled – “The Conduction System of the Mammalian Heart. An Anatomic-Histologic study of the Atrioventricular Node and the Purkinje Fibers”.12

Arthur Keith and Martin Flack in 1907 described the pacemaker of the heart as we know it today. While the myogenic origin of automaticity has been known since 1883, a specific tissue structure that exhibited this property was not described. Keith and Flack were originally working on the bundles that Tawara had described a few years earlier in vertebrate hearts in which they consistently observed the rudiments of a primitive, annular, sinoatrial muscle which they named as the sino-auricular node.12 Walter Koch later named this structure the Sinus Node and clarified that this structure was 2-3 cm long and is located at a point where the superior vena cava opens into the right atrium.

Jean Bachmann from France described the presence of a specialized interatrial fiber which served as the electrical link between the right and the left atria in 1916.12 He was able to anatomic ally locate the position of these specialized bundles. Later on, it was found that two other specialized interatrial fibers were present. They are the anterior internodal tract of Bachmann, middle internodal tract of Wenckebach, and posterior internodal tract of Thorel. Some consider the Bachman bundle to be a
separate conduction pathway differing from the anterior internodal tract (figure 1.1), but evidence supporting this point is lacking.

The nodal tissues are complex structures and studies have described the slow and the fast pathways of the AV node\textsuperscript{17, 18} while even today the structure of the SA node is under investigation.\textsuperscript{19} Based on these studies we now know the hierarchy of pacemakers in the heart (Figure 1.1) with the SA node serving as the primary pacemaker of the heart (with higher automaticity). The impulse generated from the SA node travels through the specialized interatrial pathways and resulting in transmission of the impulse from the right to the left atria. From the base of the right atria, the impulse travels down the AV node to the specialized Bundle of His which splits into left and right bundles, which conduct the impulse to the respective ventricles. Since the mass of the electrical sink (in this case, the ventricles) is very much higher than the source (left and right bundle conduction fibers), nature has developed specialized conduction fibers on the endocardial surface, called Purkinje Fibers which are capable of ultrafast conduction, thereby ensuring efficient conduction to the endocardium. From the endocardium, the impulse conducts transmurally from endocardium to the epicardium.

The spread of the electrical impulse causes electrical stimulation of the ventricles and the resulting contraction which serves to empty the blood into the aorta. Since the heart is contracted due to depolarization from the electrical impulse, the heart relaxes by a process called repolarization which proceeds in the reverse direction from the epicardium to the endocardium. Of note, the apical epicardium repolarizes first, followed by the basal epicardium and finally the septum repolarizes.\textsuperscript{20} The
atria which was in systole (due to activation before the ventricular activation) relaxes during ventricular activation.

1.1.5 ION CHANNEL ACTIVITY CONTRIBUTES TO THE GENERATION OF IONIC CURRENTS AND THE ACTION POTENTIAL

Ion channels provide special membrane pathways that enable the movement of ions from the extracellular to the intracellular side and vice versa. Ion channels are membrane proteins which span the entire cell membrane thereby offering an electrophilic environment in the otherwise electrophobic environment of the cell membrane (composed of lipids). In very basic terms, ion channels serve as tightly packed hallways for ions to walk through the cell membrane with gates on either side (intracellular and extracellular) of the ion channel protein (protein conformation or specific sidechains of the proteins). Before moving onto ion channel biophysics that will enable the understanding of the ion channel physiology to start with, and later the cardiac ion channel biophysics, a few terms need explanation and clarification.

Most cardiac ion channels are voltage gated, while some electrogenic exchangers also play an important role as well. For the purposes of a review relevant to the content of this work, this discussion will be restricted to the voltage gated potassium channels alone. Voltage gated K\(^+\) channels exist as tetramers in the cell membrane as shown in the figure 1.2 (panel B). Each monomer of the channel possesses six membrane spanning \(\alpha\)-helices which are interconnected to each other. The segments of the \(\alpha\)-helices are numbered from one to six, and therefore referred to as \(S_1\), \(S_2\) and so on to \(S_6\). Most voltage gated K\(^+\) channels have a prominent amino (N-) and carboxy (C-) terminus. Both
play specific roles in the opening and closing mechanisms of the channel. One of the most widely studied voltage gated K⁺ channels is a member of the Shaker family isolated from the shaker gene locus of drosophila. This channel is homologous to many mammalian and eukaryotic K⁺ channels. Voltage gated K⁺ channels are tetramers formed from monomers. The topology of a monomer is shown in figure 1.2 (panel A). The most distinct and preserved part of the voltage gated K⁺ channel are the residues located in the pore loop between helices S5 and S6 (figure 1.2, panel C). These residues (GYG – shaded in grey in the figure) serve to determine the selectivity of the K⁺ channel and are conserved among both prokaryotic and eukaryotic K⁺ channels.

The first 20 amino acids of the N-terminus of the ion channel fold up to form a ball shaped structure while the remaining amino acids of the N-terminus form the chain shaped structure. This forms the "ball and chain" of the N-terminus which can act to block the channel pore on the cytoplasmic (intracellular) side and stop the ion current flow. This mechanism is referred to as N-type inactivation, so named due to the effect of the N-terminus of each monomer on inactivation. Also, another type of channel inactivation mechanisms occurs called C-type inactivation.¹⁴ This involves the combined movement of S₅ and S₆ helices in concert with the C-terminus to mechanically push into the lumen of the channel inwards thereby producing an electrostatic hindrance to the ion current flow through the channel opening. Since the voltage gated K⁺ channels can be opened by changes in membrane voltage, a specific process called activation occurs to open the ion channel pore to allow conduction. This was outlined by work of Hodgkin and Huxley and the exact mechanisms have been described in work form the laboratories of
various eminent scientists including Clay Armstrong, Bertil Hille and Francisco Bezanilla among others. In summary, a special voltage sensor in the ion channel pore has been proposed. This voltage sensor has a high number of positively charged amino acids, and the location has been confirmed in multiple studies to be the S4 transmembrane helix of the channel. Since a tetramer forms the channel, four such voltage sensors act in synergy to open the channel pore. This is brought about by the twisting and upward and outward motion of the S4 channel helix (figure 1.2) and this opens the channel by the mechanical pull of the S4 on its other helical counterparts. This tug induced by the S4 helices opens the channel and offers the appropriate activation properties to the channel with the degree of the movement controlled by the degree of change in the membrane voltage registered by S4. So, the higher the voltage perceived by the S4 voltage, the greater the movement, degree of activation and channel open times. This relationship is true of most voltage gated K\(^+\) channels which open more at positive potentials.

The great variety in these processes among various K\(^+\) channels as we know today, makes the voltage gated K\(^+\) channel family the most interesting ion channel family in the heart. In a very simplistic view, voltage gated ion channels exhibit three processes (activation, inactivation and deactivation) that are widely discussed in literature. A brief outline/description of these processes is described below.

**Activation:** Activation refers to the process of opening the ion channel as discussed above. It involves the process of channel opening in response to membrane voltage changes, and the kinetics of the whole process can be measured by fitting the activation current profile to a time constant.
This time constant describes how fast or slow the ion channel opens. As stated above, this process is voltage dependent. In some K⁺ channels (e.g. acetylcholine gated K⁺ channel), activation might be ligand- and voltage-gated).

**Inactivation:** This refers to the process by which an open channel undergoes molecular re-arrangement of its amino acid residues to limit the ion channel flow. This process is different from the closure of the ion channels pore as observed by crystallographic and other biophysical studies. All voltage gated ion channels cannot be open forever, and undergo a process called time dependent inactivation. This mechanism seems to be the nature’s way of adapting to limit the ion flux across the channel so as to limit changes in the intracellular milieu that might be detrimental to life.

**Deactivation:** This process is the transition from the open state of the ion channel to the closed state observed biophysically due to withdrawal of the stimulus to the channel. This process is again voltage dependent but is characteristically seen in ion channels whose current profile takes longer to activate compared to the test pulse used. This process of deactivation upon sudden withdrawal of the membrane voltage (assessed in patch clamp experiments) gives rise to the tail currents, and reflects two important factors. One is the amount of current available just prior to the withdrawal of the stimulus. The higher the current (ion flux) during the stimulus that is sustained during the test pulse, the greater the observed tail current. Second, the tail current gives a good indication of voltage dependence of the ion current being measured, which gives a measure of
the driving force present during a particular voltage stimulus. In most voltage gated K⁺ channels, higher channel open times result in increased ion current flux causing higher measured current amplitudes, therefore when stepped down to a lower (negative) potentials, a driving force still exists giving rise to the deactivation profile.

These three processes give an ion channel a specific signature and formed the basis for classifying voltage gated K⁺ channels in the heart. A typical voltage gated K⁺ channel in the heart allows ions to pass through in one direction (typically from in to out). Many different types of voltage gated ion channels similar to the channel structure described above exist in the heart. In addition, there are some channels which do not show the typical six membrane spanning domains in the membrane. Instead they might have only two membrane spanning domains, an example are the various voltage gated K⁺ channels in the heart called the inward rectifiers. The rectifying channels are called so, due to their properties which are similar to rectifiers in an electrical circuit, which allows current to preferentially pass in one direction (extracellular to intracellular). On biophysical examination, these channels also allow ion channel conductance to happen in the other direction as well (intracellular to extracellular), but this occurs in smaller magnitude compared to the preferential direction of the ion current flux. The contribution of these different ion channels to the generation of the cardiac action potential will be discussed in the next section.

1.1.6 ION CHANNEL NOMENCLATURE FOR K⁺ CHANNELS

Most ion channels can be broadly classified into two categories: voltage-gated and ligand-gated. The IUPHAR has developed a
nomenclature for ion channels, and understanding this nomenclature is critical to understanding the ion channel function as the names themselves contain valuable information about the ion channel.

Voltage-gated ion channel proteins are referred to as “I\textsubscript{vxx.x}” format. Here, “I” refers to the particular ion that will be conducted via the ion channel under reference. Sodium, calcium and potassium are referred as Na, Ca and K) without their ionic valences.

“v” refers to the channel being voltage-gated.

“x.x” refers to the particular channel subfamily. For example, K\textsubscript{v}4.3 will be a voltage-gated K\textsuperscript{+} channel, which carries K\textsuperscript{+} current belonging to the K\textsubscript{v}4 subfamily of which it is the third member. The current carried by these channels are denoted based on their biophysical activation kinetics. For example, I\textsubscript{to} means transient outward current, while I\textsubscript{Kr} and I\textsubscript{Ks} refer to the rapid and slowly activating delayed rectifier current.

The genes of the potassium channels are referred as “KCNxn” where the “x” can be any letter based on gene family and n is the specific number of that gene family in the order of discovery. For example, KCNA refers to the K\textsubscript{v}1 family, KCNB refers to K\textsubscript{v}2 family, and KCNA5 refers to fifth member of the K\textsubscript{v}1 family (K\textsubscript{v}1.5).

Ligand-gated ion currents are referred to as “I\textsubscript{xligand}” where ‘x’ stands for the particular ion that is carried by the ion channel. The ligand that serves to open the channel will be added after the ion. For example, I\textsubscript{KACH} refers to a channel carrying potassium current which opens in response to a ligand (in this case – “acetylcholine”). Likewise, I\textsubscript{KATP} refers to a K\textsuperscript{+} current, where the channel opens on decreased binding of ATP on its cytoplasmic surface.
In addition to pure voltage gated ion channels, rectifiers also form a class of voltage gated K$^+$ channels. The rectifier channels are referred as “Kir.x.x”. Where “ir” refers to inward rectifier and x.x refers to the subfamily they belong to in the order of discovery of the genes. For example, the cardiac inward rectifier channels belong to the subfamily Kir2.1. In some cases, overlap between subfamilies can occur and in those cases, the primary modality of channel opening will be referred to first. For example, $I_{K\text{ACH}}$ is a current carried by ligand gated K$^+$ channel that belongs to the inward rectifier subfamily. But since acetylcholine activated signaling pathway opens the channel, it is also referred to as a GIRK (G-protein coupled receptor). Some degree of overlap is still present in naming some K$^+$ channels and will be discussed in later sections.

1.1.7 HOW DOES THE ACTION POTENTIAL ARISE IN A WORKING VENTRICULAR MYOCYTE?

For sake of simplicity and due to the experiments (in this dissertation) on dog cardiac myocytes, this section will focus on the origin of the action potential in the dog ventricle which is very similar to the human ventricle. However, it must be noted that the action potential varies widely from one mammalian species to another due to differences in heart rate (a mouse resting heart rate is ~600/min while a man has a resting heart rate of 70/min). Species differences in action potentials will be pointed out in later sections.

The cardiac ventricular action potential (AP) originates with the spread of electrical impulse from adjacent cells spreading from the specialized conduction system to the ventricular muscle. The resting membrane of a ventricular cell (Phase 4; so called due to the resting state
after a previous AP) is permeable to K$^+$ ions primarily due to the current flow through the inwardly rectifying K$^+$ channels (I_{K1}). This ensures that the intracellular side of the membrane is rich in K$^+$ ions (usually around 140 mM) while the extracellular side is rich in Na$^+$, Ca$^{2+}$ etc. The impulse conducted from the adjacent cell travels to the cell of interest via specialized channels called gap junctional channels. This causes the voltage gated Na$^+$ channels to open and allows a rapid increase in membrane sodium conductance causing the cell to rapidly depolarize (move toward a more positive membrane voltage). This phase is denoted as Phase 0. Following the rapid activation of Na$^+$ channels causing rapid depolarization of membrane voltage, there is a rapid inactivation of Na$^+$ channels at positive voltages thereby reducing membrane sodium conductance. At this point in time, which occurs in less than 5-10 ms after channel activation, the membrane conductance through specialized voltage gated K$^+$ channels (producing a transient outward potassium current, I_{to}) increase, producing a rapid, early repolarization of the action potential.

Towards the end of Phase 1, the intracellular sodium concentration is very high, sodium calcium exchanger (NCX) participates in concert with Na$^+$$-$$K^+$ ATPase pump to pump the excess sodium out of the cell. This causes the return of the membrane voltage to approximately the ideal activation voltage for voltage gated Ca$^{2+}$ channels, producing activation of voltage gated Ca$^{2+}$ channels, i.e., the L-type calcium channels (I_{Ca-L}). This transient increase in I_{Ca-L} causes a calcium induced calcium release (CICR) from the sarcoplasmic reticulum via the ryanodine receptor; during CICR the free intracellular calcium concentration can rise to 1 µM (at peak systole) from 100 nM during the resting state (diastole). The influx of
Calcium through the sarcolemma produces the characteristic plateau phase of the AP (Phase 2) seen in dog and human ventricular myocytes. During the plateau, a very high membrane resistance is present and the plateau is maintained by a very delicate balance of inward and outward moving ions. During the plateau phase, around the same time as the calcium current activation, an ultra-rapidly activating \( K^+ \) current is activated (See chapter 3) and serves to counteract the increased positivity in the cell membrane. The likely atrial counterpart of this current is the ultra-rapid delayed rectifier \( K^+ \) current \((I_{Kur})\), due to its ultra-fast opening times with very slow inactivation times. Towards the end of the plateau, due to the increased intracellular Ca concentration, NCX operates in reverse mode, bringing in 3 sodium ions for each calcium ion extruded from the cell. Also, towards the end of the plateau, two very distinct voltage gated \( K^+ \) channels open and cause repolarization of the action potential (Phase 3). These \( K^+ \) currents are called rapidly activated \((I_{Kr})\) and slowly activated \((I_{Ks})\) delayed rectifier currents, so named due to their rapid (note: it is still rapid but slower than the ultra-rapid \( K^+ \) current, \( I_{Kur} \)) or slow activation kinetics. Towards the end of phase 3, the \( I_{Kr} \) and \( I_{Ks} \) currents start to inactivate and the terminal repolarization is carried by outward component of the current carried by the inwardly rectifying \( K^+ \) channel \((I_{K1})\). This returns the membrane potential back to resting membrane potential. At this stage, the current through the inwardly rectifying channel changes direction from being an outward current to an inward current (Phase 4), contributing to final repolarization.
1.1.8 CONTRIBUTION OF DIFFERENT K⁺ CHANNELS TO CARDIAC REPOLARIZATION: INSIGHTS FROM MICE AND RAT STUDIES

The dog and rabbit are widely considered to be good representative models of human cardiac disease due to a closer basal heart rate relationship to humans (compared to rats or mice). However, studies to understand the precise molecular mechanism by which different K⁺ channels contribute to AP repolarization came from very elegant studies from mouse and rat due to the relative ease of manipulation in smaller mammals. These manipulations are extremely limited in larger mammals and fraught with limitations. This section will deal with the evolution of our understanding of cardiac repolarization from the early days of cellular electrophysiology.

The first study to deal with cardiac K⁺ channels was to show the effect of α₁-adrenergic agonists on outward K⁺ currents. Application of α₁-agonists like phenylephrine, methoxamine or PKC suppressed the peak outward K⁺ currents. Depolarization of rat ventricular myocytes from -30mV to +50mV in 10 mV increments from a holding potential of -90mV elicited a distinct family of currents. This family of curves showed a voltage dependent outward K⁺ current that peaked within the first few milliseconds of depolarization. This peak outward current decayed to a steady state (the authors called this “the plateau” current). The latter current was voltage dependent but had smaller amplitude than the peak outward current. Apkon and Nerbonne demonstrated in a study of rat ventricular myocytes that two distinct components of K⁺ currents were present in rat ventricular myocytes. By voltage clamping rat ventricular myocytes from various holding potentials of -90, -70 or -50 mV, the authors identified variations in the amplitude of the elicited outward K⁺ currents. The highest
amplitude was observed at -90mV with a relatively smaller amplitude seen at -50mV. This study gave the first indication that voltage gated K⁺ channel activation depended on the resting membrane potential (in this case, holding potential) and therefore the idea of voltage dependence of activation of cardiac outward K⁺ channels was demonstrated. In addition, the authors demonstrated that depolarization-activated outward K⁺ currents do not close abruptly on stepping the test potentials back to holding potentials but in fact, closed with a characteristic pattern called deactivation, observed as tail currents. The tail currents were again a function of voltage. For example, stepping down from +20mV to a hyperpolarizing voltage steps of 10mV increments from -30mV to -140mV, greater tail current density could be found at more negative potentials (-70mV) than at positive potentials (-30mV), as the driving force for the movement was greater at negative potentials due to the higher voltage gradient (as computed form the Nernst equation). The pharmacological sensitivity of the peak outward current at the beginning of the test potential was tested and this peak component was highly sensitive to block by 3mM 4-AP, while the plateau current was not. The plateau current was however sensitive to 50mM tetra-ethyl ammonium (TEA), while the peak current was not affected. More detailed experimentation proved increased reduction of both peak and outward currents (due to inactivation) when the duration of the test pulse was increased from 100ms to 500ms to a 1 second pulse. Although the majority of peak outward current inactivated within 100ms of the test pulse, the plateau current was more sensitive to the duration of the test pulse. The authors also examined the effect of the preceding (conditioning) voltage to subsequent current to assess the voltage dependence of steady state inactivation. The protocol used was
depolarization steps in 10mV increments for a fixed time (10 seconds), followed by a fixed depolarization step to +30mV. Additional data also proved that two K⁺ current components could be separated by their recovery from inactivation. The peak outward K⁺ current from the rat ventricle was able to recover faster than the plateau current. Further proof to the voltage dependence of activation came from action potentials in control rat ventricular myocytes where eliciting action potentials from -88mV to -68mV had differing effects of action potential duration (APD). When the cell was depolarized, less outward K⁺ currents were available and therefore a longer APD was observed. The latter three experiments demonstrated that outward K⁺ currents (especially peak outward K⁺ current) was voltage and time dependent for both its activation and inactivation.

Boyle and Nerbonne²³ demonstrated similar findings in rat atrial myocytes. This study however provided detailed insights into the kinetic details of the two distinct depolarization activated K⁺ currents. It was found in atrial myocytes that the peak outward K⁺ current was kinetically separable into two components based on fitting the decay of the peak outward current (Figure 1.6). They named these two components I_Kf and I_Ks referring to the fast and slow components, with the fast component having an inactivation time constant around 180 ms while the slow component inactivated with a time constant of 3000 ms. Care must be taken not to confuse I_Ks with slow delayed rectifier current which are two different currents carried by two different ion channels. This confusion was later resolved by naming I_Kf and I_Ks components of peak outward current as I_{to,f} and I_{to,s}. 
The contribution of $I_{Kf}$, $I_{Ks}$ and the steady state ($I_{ss}$) was assessed and identified to be 32, 47 and 21 % respectively of the total outward current recorded. The recovery from inactivation of outward K$^+$ currents was assessed by applying test potentials at interpulse intervals of 3, 7, 20 or 52 seconds. $I_{Kf}$ component recovered faster with 3 second interpulse interval, while $I_{Ks}$ recovered slowly only at 52 second interpulse interval. This has two implications: At a steady-state heart rate in a rat, $I_{Kf}$ was the major component available for repolarization with a very minor contribution from $I_{Ks}$. Recovery from inactivation patterns for $I_{Kf}$ and $I_{Ks}$ were very similar to rat ventricular myocytes.\textsuperscript{22} Pharmacological testing with 1 and 5mM 4-AP and 50mM TEA showed that $I_{Kf}$ was half-blocked with 1mM 4-AP and more blockade occurred with 5mM 4-AP (~80%). The $I_{Ks}$ component was blocked 61% with 1mM 4-AP while 5mM 4-AP blocked ~97%. 50mM TEA did not significantly affect $I_{Kf}$ or $I_{Ks}$ in rat atrial myocytes. However, the authors concluded that the steady state current ($I_{ss}$) component was a non-inactivating component of $I_{Kf}$ and $I_{Ks}$ as the degree of blockade of $I_{ss}$ by 4-AP or TEA was similar to the effects seen on $I_{Kf}$ and $I_{Ks}$.

1.1.8.1 SUMMARY OF SIMILARITIES AND DIFFERENCES BETWEEN RAT ATRIA AND VENTRICLE

Kinetic analysis of activation and inactivation patterns revealed similarities between outward currents of rat atria and ventricle. They are the following:

1. A rapidly activating and inactivating component ($I_{K,fast}$ or $I_{Kf}$)
2. A rapidly activating and slowly inactivating component ($I_{K,slow}$ or $I_{Ks}$)
3. A rapidly activating, non-inactivating steady state current ($I_{ss}$)

Some interesting differences exist in the pharmacological sensitivities between rat atria and ventricle. In contrast to rat ventricle, a TEA sensitive component was smaller in the rat atria. While rat ventricular outward $K^+$ currents are sensitive to 4-AP (peak outward) and TEA (steady-state), the rat atrial peak outward currents were mainly susceptible to 4-AP alone, while only very minor effects were found with 50mM TEA making the authors suggest that rat atrial outward $K^+$ currents share different pharmacological sensitivities to 4-AP and TEA. However, it must be stated that the peak and steady state components were seen in both rat atrial and ventricular myocytes.

1.1.8.2 MOLECULAR CORRELATES OF RAT VENTRICULAR $K^+$ CURRENTS

Barry and Nerbonne$^{24}$ identified different $K^+$ channel proteins in adult rat ventricle. In immunohistochemical labeling studies of rat ventricular myocytes, the authors found significant labeling with anti-$K_{v}4.2$ and anti-$K_{v}1.2$. In addition, slightly more variable but consistent presence of $K_{v}1.5$ and $K_{v}2.1$ were found. No expression of $K_{v}1.4$ was found. The degree of protein expression (of $K_{v}4.2$, $K_{v}1.5$, $K_{v}2.1$) assessed by Western blots was identical in both rat atrial and ventricular myocytes. The rat atrial myocytes seemed to have greater $K_{v}1.2$ levels compared to rat ventricle.

However, the protein expression during development and in adulthood is not entirely consistent with the current densities observed in rat ventricle. Another elegant study by Xu et al$^{25}$, where currents, mRNA and protein levels were measured showed that discordance in these parameters does exist in nature. The authors examined post-natal rat
ventricular myocytes at (0, 5, 10, 15, 20, 25, 30 days post birth and adult rat ventricular myocytes). The peak outward K⁺ current (from here on, will be referred to as Ito) increases from day 0 to day 30 and the highest density was found at 30 day and adult ventricle. Cell size increased proportionally with age post-birth, consistent with the fact that with increasing cell size during development, more channels are being recruited to the membrane. The plateau (steady-state) however increased from post-natal day 0 to day 15 and then decreases until the adult ventricular myocyte plateau steady state current density was not different from day 5 density. When the mRNA levels were assessed from day 0 to day 30/adulthood, Kv4.2, Kv1.2, Kv1.5, Kv2.1 all increased during development. Kv1.2, Kv1.5 and Kv2.1 showed peak levels between day 5 and day 15 post birth. Kv4.2 increased from Day 0 to day 15 but the increase was much more modest compared to Kv1.2, Kv1.5 and Kv2.1. Between day 20 and adult stage, there was dramatic increase in Kv4.2 mRNA levels. Consistent with a previous study by Barry et al²⁴, Kv1.4 levels were highest between day 5-day10 post birth and declined gradually during development. The highest increase was found for Kv1.2 mRNA levels. Western blots analysis revealed slightly different results compared to the mRNA levels. The protein levels reached a steady state around day 15 – 20 post birth, consistent with the mRNA levels. A curious result was the discordance in the mRNA and protein levels of Kv2.1 levels. Kv2.1 protein decreased gradually from Day 5 to adulthood. This gives rise to the idea that mRNA expression might not always mirror the exact current densities; one hypothesis for this discrepancy is that some post-translational mechanisms might operate to regulate the protein levels.
Though the previous study by Xu et al.\textsuperscript{26} raised an interesting possibility that many proteins constitute the outward K$^+$ currents, the exact contributions of each protein to outward K$^+$ currents was not assessed. This formed the basis of another well-controlled study by Bou-Aboud et al.\textsuperscript{27} where antisense oligo deoxy-nucleotides (AsODN) against Kv4.2, Kv1.2, Kv1.5 and Kv2.1 were assessed in rat atrial myocytes. Since rat atria and ventricle share close similarity in the relative protein levels the results can be extrapolated to rat ventricle as well. Of all the antisense nucleotides, functional currents were blocked only when AsODNs were used against Kv4.2, Kv1.5 and Kv1.2. Kv2.1 AsODNs did not seem to alter any functional current in rat atrial myocytes. AsODNs against Kv4.2, Kv1.2 (homologous to Kv1.4) and Kv1.5 altered the density of currents measured and was proposed to encode $I_{\text{Kf}}$, $I_{\text{Ks}}$ and $I_{\text{ss}}$ respectively. The absence of effect of Kv2.1 AsODNs corroborate with the previous study where no TEA sensitive component was found.\textsuperscript{23}

Murine ventricular voltage gated K$^+$ channels are remarkably similar to rat ventricle.\textsuperscript{25} Murine ventricular $I_{\text{to}}$ is composed of two current components like the rat ($I_{\text{to,f}}$ and $I_{\text{to,s}}$). In addition to Kv4.2 encoded $I_{\text{to,f}}$ (found in all cells from left ventricle) and Kv1.4 encoded $I_{\text{to,s}}$ (found in septal cells), two additional current components can be identified. Both $I_{\text{to,f}}$ and $I_{\text{to,s}}$ are sensitive to millimolar 4-AP, but only $I_{\text{to,f}}$ was sensitive to heteropodatoxin 2 (HpTx2). One of them is $I_{\text{K,slow1}}$ or $I_{\text{ss}}$, due to its slow time constant of inactivation. $I_{\text{K,slow1}}$ was found to be encoded by the Kv1 family by two studies (one using a dominant negative construct and another replacing Kv1.5 with a 4-AP insensitive channel). This $I_{\text{K,slow1}}$ current component was susceptible to micromolar concentrations of 4-AP. In addition, a fourth current component was found which was sensitive to
≥ 25 mM TEA and this is considered to encode $I_{K_{\text{slow2}}}$. These current components were also found to be present in murine atrial myocytes.

1.1.8.3 KNOCKDOWN OF OUTWARD $K^+$ CURRENTS PRODUCE ARRHYTHMOGENIC PHENOTYPE

As studies mentioned above were delineating the various protein and its current correlates in ventricles in mice, the clear importance of different voltage gated $K^+$ channels to repolarization; and the role of these channels in preventing arrhythmogenesis due to reduced repolarization strengthened the view that these channels do play a major role in the heart. While these conclusions were derived exclusively from mice, it gave a direction for understanding the potential for arrhythmogenesis in disease(s) where one or more of these $K^+$ currents might be altered.

One of the earliest of these studies came from mice expressing a dominant negative (DN) $K_v2$ subunit. It must be remembered that $K_v2$ encodes the $I_{K_{\text{slow2}}}$ current. However, it must also be noted that in normal mouse ventricle, $K_v2$ encoded current forms only a small amplitude component of the total outward current. To test the hypothesis that if a small component of steady state $K^+$ current was altered it produced a different phenotype, transgenic mice overexpressing $K_v2$ DN were created. This produced only a selective attenuation of $K_v2$ encoded $I_{K_{\text{slow2}}}$ current, and demonstrated that $K_v2$ encoded current was indeed the TEA sensitive current (as demonstrated by the lack of effect of TEA on $K_v2$ DN transgenic mice). Abolishment of $I_{K_{\text{slow2}}}$ in $K_v2$ DN mice caused longer action potential duration (APD) and corrected QT interval (QTc) on the surface ECG, with occasional occurrence of afterdepolarizations. In addition, similar findings of prolonged APD and QTc were found in a
different mice overexpressing $\text{K}_v4.2$ DN (where $I_{\text{to},f}$) and $\text{K}_v1.1$ DN (where $I_{\text{K,slow}1}$) was attenuated.

The effects of altering multiple voltage gated $K^+$ channels were studied by Barry et al.\textsuperscript{28} and London et al.\textsuperscript{29} in mice overexpressing $\text{K}_v4.2/\text{K}_v1.1$ DN transgene. These mice had abolishment of $I_{\text{to},f}$ (that contributes to phase 1 repolarization of murine ventricular AP) and $I_{\text{K,slow}1}$ (that is sensitive to micromolar 4-AP). Mice overexpressing either one of the transgenes ($\text{K}_v4.2$ DN or $\text{K}_v1.1$DN) showed longer APD and QT\textsubscript{c} but this effect was more pronounced in mice overexpressing both $\text{K}_v4.2$DN/$\text{K}_v1.1$DN. The double DN transgenic mice had an increased susceptibility to the development of polymorphic ventricular arrhythmias. Interestingly it must be noted that instead of $\text{K}_v1.1$ DN, if $\text{K}_v1.5$ DN pore mutant was expressed, the mice did not have electrical or structural abnormalities. Therefore, it can be reasonably concluded that $\text{K}_v1.5$ attenuation does not produce an abnormal phenotype while a sub-family specific $\text{K}_v1.1$ DN which blocks all $\text{K}_v1$ sub-family channels ($\text{K}_v1.4$ and $\text{K}_v1.5$) produces abnormal electrophysiology suggesting a possibility that there are some channel subunits whose identities are still unknown.

Around the same time, Wickenden et al.\textsuperscript{30} reported that $\text{K}_v4.2$ DN expression leads to the development of dilated cardiomyopathy. This prompted Guo et al\textsuperscript{30} to study studied transgenic mice resulting from crossing $\text{K}_v4.2$ DN and $\text{K}_v1.4$ knockout where both $I_{\text{to},f}$ and $I_{\text{to},s}$ might be attenuated. Interestingly, the transgenic mice which showed complete lack of both $I_{\text{to},f}$ and $I_{\text{to},s}$ had no contractile or structural abnormality. In contrast to the studies of Wickenden et al, $\text{K}_v4.2$ DN mice of Guo et al\textsuperscript{30} did not show dilated cardiomyopathy suggesting that the $I_{\text{to},f}$ reduction as seen in many disease states might not be a causal disease mechanism.
However, in Kv4.2DN/Kv1.4<sup>−/−</sup> mice electrophysiological abnormalities (longer APD and early afterdepolarizations) were seen. In addition, these double transgenic mice revealed Mobitz type I AV block in 80% of the mice tested.

Similarly, attenuation of both Kv1 and Kv2 family of currents using a dominant negative overexpression approach produces longer APDs, QT<sub>c</sub> prolongation and ventricular arrhythmias. These studies lead to one consistent conclusion. Attenuation of one K<sup>+</sup> current has very modest effects on electrical phenotype, while reductions in multiple K<sup>+</sup> currents produce a clear repolarization abnormality. The most important implications comes from Kv4.2 DN mice where selective elimination of Kv4.2 does not produce electrical abnormalities, which excludes a potential role for Kv4.2 as a cause of electrical abnormalities in cardiac diseases like heart failure or myocardial infarction. Although block or elimination of Kv4.2 encoded I<sub>to,f</sub> prolongs APD, it does not necessarily precipitate arrhythmias in mice, suggesting I<sub>to,f</sub> reduction is more of a consequence than a causal mechanism as proposed by Wickenden <i>et al.</i>

### 1.1.9 ROLE OF HETEROMULTIMERS IN GENERATION OF CARDIAC VOLTAGE GATED K<sup>+</sup> CURRENTS

All the K<sup>+</sup> channels described in the sections above carry one important common feature. These K<sup>+</sup> channels as we know today are multimers of many different subunits. Some of these subunits are identified, while some are still elusive. The search for these heteromultimers accelerated as the currents from over-expression systems did not fully replicate the native currents recorded from the brain or the heart, raising important questions about the molecular constituents.
that contribute to native $K^+$ currents. The sequence of studies/events that led to our understanding of this concept will be discussed in this section.

1.1.10 INSIGHTS FROM RAT BRAIN AND CLONED NEURONAL CHANNELS

The first voltage gated $K^+$ channel was cloned by Lily Jan’s lab in 1987 from the shaker locus in drosophila. There has been an explosion in understanding the molecular correlates underlying the voltage gated $K^+$ channel since 1987. A significant step in understanding the stoichiometric relationships came from studies which indicated that RCK (rat brain $K^+$ channel forming) proteins RCK1 (Kv1 family as we know it today) and RCK4 (Kv4 family) can co-assemble in rat brain and result in currents which are similar to native $K^+$ currents recorded from rat brain. These studies confirmed the Kv4 and Kv1 families as primary mediators of voltage gated $K^+$ channels in the brain. In 1991, McKinnon proposed the idea of voltage gated $K^+$ channel as tetramers by studying the stoichiometric binding of scorpion toxin (charybdotoxin) to wild-type shaker channels.

Studies by Apkon and Nerbonne in rat ventricular myocytes and later in rat atrial myocytes identified similar characteristic of cardiac voltage gated $K^+$ channels which are similar to the currents recorded from the Kv1 and Kv4 family. The best understanding of heteromultimers is obtained by following the sequence of papers that examining the delayed rectifier current, which like Kv1 and Kv2 family (as explained above) form a major repolarizing current in bigger mammals like guinea-pigs, rabbits, dogs and humans.
1.1.10.1 THE STORY ABOUT HETEROMULTIMERS CHANNELS IN THE FORMATION OF DELAYED RECTIFIER CURRENT

In a very elegant study, Sanguinetti and Jurkiewicz demonstrated distinct voltage gated K⁺ currents in guinea-pig ventricular myocytes. Using specific channel blockers to inhibit ether-a-go-go gene (erg) encoded current (5 µM E-4031 and 100µM d-sotalol) identified two kinds of voltage gated K⁺ currents, E-4031 and d-sotalol sensitive and insensitive components within the composite delayed rectifier current (previously described as Iₖ). The drug sensitive current component of Iₖ had slightly faster activation kinetics with slightly slower inactivation kinetics and had a U-shaped current-voltage relationship with peak current recorded around 0 mV. In contrast, the d-sotalol and E-4031 insensitive component of Iₖ had slower activation kinetics. In a later study, Spector et al. reported that erg-encoded current (called I_{Kr}) had fast inactivation kinetics.

Around this time, cDNA clones for many K⁺ channels were identified. These channel proteins were cloned and identified to be large proteins of ~70 kDa and with six membrane spanning domains. Using heterologous expression systems, a gene putatively encoding for a voltage gated K⁺ channel was identified in heart, kidney and uterus which contained a single membrane spanning domain (~15kDa), in contrast to the well known six membrane spanning K⁺ channel. This protein was referred to as the minimal K⁺ channel subunit (minK). At this point in time, it was not entirely clear if minK formed a separate channel on its own, or had a regulatory role in conjunction with some specific voltage gated K⁺ channel. However, Hausdorff et al. successfully demonstrated that minK overpression in Xenopus oocytes produced a current with very
slow activation kinetics. Freeman et al. showed the first correlation of this channel to a cardiac voltage gated K⁺ current, Iₖₛ (which was earlier described as the E-4031 and d-sotalol insensitive component). Freeman et al., over-expressed minK in HEK203 cells and showed that the current characteristics were similar, but not identical, to Iₖₛ observed in guinea pig ventricular myocytes. This caused a significant gap in understanding of the delayed rectifier K⁺ current composition, as mink-encoded current was not entirely identical to cardiac slow delayed rectifier current (Iₖₛ).

Sanguinetti et al. cloned a different gene which was causing long QT syndrome, and it was clearly demonstrated that neither KvLQT1 nor minK gene encoded current separately could mirror cardiac Iₖₛ. In two separate studies by two different groups, it was shown that when KvLQT1 and minK (the current was referred to as Iₖₛ in 1990s) were co-expressed in CHO cells, it caused almost a 6-fold increase in current and therefore was identified to be the molecular correlate of slow delayed rectifier current (Iₖₛ).

1.1.10.2 HETEROMULTIMERS IN FORMING CARDIAC TRANSIENT OUTWARD (Iₜₒ) AND SUSTAINED (Iₖₛₜₜ) AND DELAYED K⁺ CURRENTS

Kv4 family members (section 1.1.8.2) have been identified as the molecular correlates of the fast activating and inactivating currents in mammalian brain and heart. But a major breakthrough that pioneered a whole new area of research came from the Antzelevitch laboratory whose studies brought new insights into understanding repolarization in the mammalian heart. Dr. Antzelevitch’s studies originated in a series of seminal experiments, where reflection as a mechanism for re-entrant
arrhythmias was being studied. While performing these studies, his laboratory observed supernormal conduction in ventricular epicardium but not in ventricular endocardium. This prompted a look into the action potential characteristics into the regions of the left ventricle and it was identified that action potentials were different in epicardium than in endocardium while the mid-myocardial region of the left ventricle had completely different action potential morphology and duration. The action potentials from the three regions differed in the duration and the shape of early repolarization during Phase 1 of the action potential. These findings were extremely critical because ion channel biophysicists before this period (early 1990s) were unable to reconcile as to why native \( I_{\text{to}} \) was so different in density, kinetics compared to overexpression systems. The findings of Antzelevitch’s studies opened the floodgates to new more exciting understanding of accessory subunits that modulate \( I_{\text{to}} \). This will be discussed in detail in the following section where electrical heterogeneity in the ventricle will be considered.

Two studies showed that \( I_{\text{to}} \) (\( I_{\text{to,f}} \) in this case) in rat heart could be attenuated by using antisense oligo-deoxy nucleotides against \( K_{v}4.2 \) or \( K_{v}4.3 \) or by using a \( K_{v}4.2 \) truncated subunit. Since evidence pointed to the role of \( K_{v}4.2 \) and \( K_{v}4.3 \) in formation of \( I_{\text{to}} \) in rat heart, it was unclear due to lack of direct evidence as to whether \( K_{v}4.2 \) or \( K_{v}4.3 \) alone or heteromultimer complexes (of \( K_{v}4.2 \) and \( K_{v}4.3 \)) encoded \( I_{\text{to}} \) in the heart. Guo et al. demonstrated using patch studies and immunoprecipitation that both \( K_{v}4.2 \) and \( K_{v}4.3 \) formed heteromultimeric complexes which encoded rat ventricular \( I_{\text{to}} \). However, it must be pointed out that in \( K_{v}4.2 \) dominant negative overexpression mice, there was no increase in \( K_{v}4.3 \) protein levels suggesting that though \( K_{v}4.3 \) associates with \( K_{v}4.2 \), \( K_{v}4.3 \) by
itself does not encode $I_{to}$ in rat ventricle. It also suggests that $K_v4.2$ is the predominant protein underlying rat ventricular $I_{to,f}$.

An et al\textsuperscript{44} demonstrated using a two hybrid assay that another protein subunit associated with $K_v4$ subunits in rat brain. They named this subunit KChIP ($K^+$ channel interacting protein). They identified four different isoforms of KChIP (isoforms 1-4). KChIPs are very similar to the neuronal calcium sensor family of proteins in possessing exclusive E-F hand domains for binding calcium.\textsuperscript{44} Co-expression of KChIP1-4 with Kv4 $\alpha$- subunits resulted in increased density of transient outward $K^+$ currents, slower inactivation and faster recovery from inactivation. In addition, it was shown that KChIP co-expression with $K_v4 \alpha$- subunits, increased membrane localization of $K_v4$ subunits, suggesting a regulatory trafficking of $K_v4$ channels by KChIP.\textsuperscript{44}

In addition to KChIP2 regulation of cardiac transient outward $K^+$ current density and channel expression, additional accessory subunits also interact with $K_v4\alpha$ subunits to make functional channels. One of the major classes of accessory subunits that interact with $K_v4 \alpha$ subunits are $K_v\beta$ subunits (named $K_v\beta$ 1-3).\textsuperscript{45, 46} They have identified to perform a variety of roles like: increasing the inactivation of $K_v$ channels, chaperoning function by promoting/stabilizing cell surface expression, and acting as redox sensors (due to their close similarity to oxidoreductase enzymes and binding to NADPH).\textsuperscript{47} $K_v4.2$ interacts with $K_v\beta1.2$, where it confers sensitivity to redox state of the cell. $K_v4.3$ in turn has been shown to interact with all $K_v\beta$ isoforms. Though studies in cultured systems indicate interaction of $K_v\beta$ subunits and Kv4 subunits, only one study has documented the direct role of $K_v\beta$ in mouse ventricle as a modulator of cardiac voltage gated $K^+$ currents. $K_v\beta^{/-}$ mice had reduced $I_{to,f}$ density with
increased \( I_{K,\text{slow2}} \) (TEA-sensitive component). The precise role of \( K_v \beta \) function in cardiac physiological and pathophysiological states warrants further investigation.

Recently, another accessory subunit has been shown to interact with cardiac Kv4 channels. This accessory subunit called DPPX (due to its similarity to dipeptidyl aminopeptidase CD-26 like protein, which plays a role in cell adhesion) increases cell surface expression and, accelerates speed of recovery from inactivation and causes a shift in voltage dependence of inactivation. Of all DPPX subunits, DPPX-6 is proposed to interact with \( K_v4.3 \) in the human heart.

**1.1.11 ELECTRICAL HETEROGENEITY IN THE HEART**

As outlined in section 1.1.4 of this chapter, cardiac function depends on proper timing of electrical impulses that are generated in the pacemaker tissues of the SA node. This impulse travels down specialized conduction pathways to atria and to the ventricles, in the process introducing a time delay of activation between the various regions of the heart, thereby allowing synchronous contraction and relaxation to aid the organ to pump blood through the aorta. An understanding of regional specialization of cardiac function came from studies of Stannius, who demonstrated that ligatures in the superior venacaval sinus region caused cardiac asystole in the frog. This happened even when the sinus node continued to beat. Since this discovery, various anatomists as described in section 1.1.4 have given us the current understanding of the cardiac conduction system.
1.1.11.1 SINOATRIAL (SA) NODE

The SA node is a tear-drop shaped cluster of tissue located at the roof of the right atrium at the junction of superior vena cava, inferior vena cava and crista terminalis; the SA node controls the rate of pacemaking in the heart via autonomic regulation. In man, SA nodal size varies from 7-20mm in diameter; while rabbit SA node is 2-4 mm in diameter and 7-20 mm in length. The SA node is extremely heterogeneous and made of specialized nodal cells, atrial myocytes and a large amount of connective tissue (varies between 50-90% depending on species and age). SA nodal APs have a diastolic potential of around -50 to -55 mV, a small phase 0 upstroke velocity ($V_{\text{max}} < 2$ V/s) and a prominent phase-4 depolarization. The tissue architecture of the SA node is very complicated by both radial (at the periphery) and longitudinal (towards the center) arrangement of fibers. Three cell types have been identified in the SA node. The central nodal cells are empty membranous bags also referred to as the P cells. From the center of the node, gradual transition occurs with cells becoming larger possessing larger mitochondria and clear striations. The third type of nodal cells is called spider cells. The role of spider cells is uncertain at the present time, but the most consistent hypothesis is that the extensive “dendritic” structure and large surface–volume ratio may help in propagation of the electrical wave within microdomains of the SA node and facilitate electrical coupling between various cells in the node. The characteristic electrophysiologic feature of the SA node is Phase 4 depolarization which has been identified as resulting from the presence of funny (pacemaker) current ($I_f$). $I_f$ is encoded by hyperpolarization-activated nucleotide gated cation channels (HCN), of which four isoforms have been identified (HCN1-4). HCN mRNA is 25 times more abundant in the SA
node than in the Purkinje cells and 140 times more abundant than in the ventricular myocytes.\textsuperscript{53} $I_f$ is called the funny current due to its non-selective conductance to sodium and potassium ions. All the channels discovered previously, had unique conductance to a particular ion ($K^+$, $Ca^{2+}$ or $Na^+$). HCN channels showed permeability to both $Na^+$ and $K^+$ at the ratio of 0.6-0.8.\textsuperscript{54} The channel C-terminus has a cyclic nucleotide binding domain (CBD); cAMP binding to this domain is thought to be the molecular basis for autonomic regulation of pacemaker function. When the beta-adrenergic system is activated, this increases intracellular cAMP which binds to the CBD domain of HCN channels, producing higher open probabilities and longer open times. In contrast, activation of muscarinic receptors can induce the opposite (less binding of cAMP); to cause a reduction in open probability and shorter open times to reduce $I_f$, causing reduced automaticity (Figure 7).

In addition, it must be remembered that HCN channel transcripts are present in SA node, Purkinje fibers and in atrial and ventricular muscle; the degree of protein abundance and isoform expression varies in different regions. HCN1 and HCN4 seem to be the most abundant isoform in rabbit SA node, while HCN2 is expressed in low amounts and HCN3 is absent. HCN2 and HCN4 constitute the ventricular isoforms. These isoform differences between different regions of the heart regulate the activation and deactivation properties of the current.\textsuperscript{55} The HCN isoforms are identical in their transmembrane protein sequence but differ in the N and C-terminus. Switching the C-terminus in artificial systems have shown that a given channel isoform's properties can be changed from one phenotype to another.\textsuperscript{56} A recent study documented the similarity of HCN1 current to that of native $I_f$ and therefore substantiates the role of HCN1 as
the primary current in rabbit SA node although the SA nodal current closely approximates HCN1/HCN4 chimeric channels. Additional evidence suggest that minK related protein-1 (miRP-1) can associate with HCN channels, but co-expression studies failed to identify changes in activation and deactivation kinetics with the addition of miRP-1. Based on these studies a fractional steady state activation of $I_f$ can be arrived at in different cell types. This is shown in figure 7 where data from a number of studies in SA node, ventricular myocytes and Purkinje myocytes are summarized. The graph demonstrates a greater fractional availability of $I_f$ in SA nodal cells at membrane potentials close to their resting membrane potentials. This strengthens the claim that $I_f$ is indeed the current that contributes to pacemaker current. A more recent study has outlined the stimulatory contribution of the NO-cGMP (Nitric oxide-cyclic guanylate cyclase) pathway during adrenergic stimulation. In the presence of adrenergic stimulation, NO donors, e.g., SNAP (S-nitroso pencillamine) significantly augmented $I_f$ current density. This effect was abolished by using a guanylate cyclase inhibitor, ODQ, and maintained with phosphodiesterase 2 inhibition. A direct augmentation of $I_f$ by beta-adrenergic stimulation is linked to beta-2 adrenergic receptor stimulation which are linked to caveolae, similar to the localization of beta-2 receptors in caveolae in ventricle.

In addition, to the pacemaker current ($I_f$) which causes phase 4 depolarization in the rabbit SA node, T-type calcium current has also been proposed to contribute to phase 4 depolarization. T-type calcium channels activate at more negative voltages (around -60 mV) and are found in rabbit, mice, guinea pig and pig sinoatrial cells. The upstroke of the SA nodal action potential is carried by L-type calcium current and the current
characteristics of the current are consistent with the abundant Ca\textsubscript{v}1.2 subunit. Although Ca\textsubscript{v}3.1 subunit mRNA has been identified in SA nodal cells, functional current from the associated gene product is yet to be demonstrated\textsuperscript{60, 61}.

The repolarization of the SA nodal action potential is carried by the delayed rectifier current I\textsubscript{K}. Though the precise molecular determinant(s) of I\textsubscript{K} in SA node is/are not clear, it can be hypothesized that this might be slow delayed rectifier K\textsuperscript{+} current (I\textsubscript{Ks}). This hypothesis is based on the observation of Brown and Noble\textsuperscript{62} where, in addition to demonstrating an increase in I\textsubscript{f}, they also showed an increase in I\textsubscript{K} current with adrenaline (consistent with known properties of I\textsubscript{Ks}) suggesting the a potential role of potassium current in the faster repolarization of the SA nodal action potential during adrenergic stimulation. Background sodium current and sodium calcium exchanger are also shown to be present in the SA nodal myocytes. Recent evidence has suggested that rapidly activating delayed rectifier current (I\textsubscript{Kr}) has been implicated in mouse SA node\textsuperscript{63} while guinea pig and rabbit SA node has been proposed to contain both I\textsubscript{Kr} and I\textsubscript{Ks}. Transient outward K\textsuperscript{+} current (I\textsubscript{to}), has also been shown to be present in rabbit SA nodal cells\textsuperscript{64, 65}. In summary, though the repolarizing currents in the SA nodal cells seem very similar to ventricular repolarizing currents (outlined in section 1.1.8), the SA nodal action potential morphology differs from atria or ventricle due to the lack of I\textsubscript{Na}, the presence of I\textsubscript{f} and I\textsubscript{Ca-L}, I\textsubscript{Ca-T} during the depolarization phase of the action potential.

1.1.11.2 ATRIAL ACTION POTENTIAL

From the SA node, the impulse travels to the atrial muscle and spreads from the right atria to the left atria via three atrial internodal tracts.
outlined in section 1.1.4. This section will focus on the cellular atrial electrophysiology with predominant focus on dog and human atrial myocytes. Appropriate references will be made to important differences between species.

Atrial myocytes possess a resting membrane potential (RMP) around -60 mV to -70 mV. This is in contrast to the ventricular myocytes which have RMP around -80mV to -90 mV. This is due to the lower $I_{K1}$ current density which allows increased resting membrane $K^+$ permeability thereby maintaining RMP at more hyperpolarized potentials in the ventricle than in the atria. $I_{K1}$ contributes to phase 3 repolarization in the ventricle. Therefore, a smaller $I_{K1}$ density in the atria implies less contribution from $I_{K1}$ to atrial phase-3 repolarization, therefore atrial action potentials show slower phase-3 repolarization. The molecular correlates of functional $I_{K1}$ in dog atrium are not entirely clear. Previous studies have pointed out that $K_{ir}2.1$ is less abundant in atria than in ventricle (~78%), while $K_{ir}2.3$ is of high abundance in the dog atrium compared to the ventricle (~228%). While there is evidence that different $K_{ir}$ isoforms can co-assemble to form heteromultimeric channels, direct evidence of this in native cells is not currently available.

After phase 4, when an appropriate timed stimulus arrives via gap junctional channels, sodium current, carried by Na$_v$1.5 carries the inward current required for phase 0 depolarization. This is followed by two very prominent currents of repolarization. First among these currents are the transient outward $K^+$ current ($I_{to}$) similar to the current found in ventricular myocytes. The second current, that contributes to early repolarization of the dog and human atrial myocytes is the ultra-rapid rectifier current ($I_{kur}$). In addition, to setting up faster repolarization in the atria, $I_{kur}$ seems
to be responsible for regulating action potential duration and setting the plateau potential. The molecular basis for atrial $I_{to}$ is Kv4.3 while atrial $I_{Kur}$ is encoded by Kv1.5.\textsuperscript{68, 71} It must be remembered that these $\alpha$-subunits of dog and human atrial $I_{to}$ are similar to those in rat atria. $I_{Kur}$ has been found exclusively in the atrial myocytes alone and therefore the current is considered to be “atrial-specific”.\textsuperscript{68, 69, 72} This finding will be refuted in Chapter 2 of this dissertation, as evidence supporting the presence of a canine ventricular $I_{Kur}$-like current with current characteristics similar to canine atrial $I_{Kur}$ will be presented (For details, see Chapter 2).

The plateau phase of the atrial action potential is less pronounced than in the ventricle. It can be hypothesized that this difference is due to faster repolarization resulting from higher $I_{Kur}$ in dog atria which gives less time for the L-type calcium current to open during an atrial AP. However, this has not been substantiated to date. The calcium current in both human and canine atria are carried by Ca\textsubscript{v}1.2 channels.\textsuperscript{60}

Following the plateau phase, two repolarizing currents can be readily detected in canine atrial myocytes. They are the rapidly activating ($I_{Kr}$) and slowly activating ($I_{Ks}$) delayed rectifier current. These currents have similar activation voltages to their ventricular counterparts and are believed to be encoded by the same channel subunits.\textsuperscript{73}

1.1.11.3 ACETYLCHOLINE GATED K$^+$ CURRENT

The canine and human atria are very distinct due to the variable and discontinuous degree of vagal innervation. Due to these differences in innervations, high degree of parasympathetic innervation in the atria has been thought to increase susceptibility to atrial fibrillation due to the resulting differential regional refractoriness.\textsuperscript{74-77} The distinctive feature of
atrial myocytes is the specific modulation of the action potential duration by cyclic adenine nucleotides. One of the early studies by Ragazzi et al \(^7^8\) identified specific activation of an atrial potassium current by acetylcholine and adenosine. The current was activated by adenosine monophosphate, while ATP was less potent in increasing the K\(^+\) current and producing atrial action potential shortening. Another study by Krapivinsky et al, \(^7^9\) showed that I\(_{\text{KAC}}\) was produced by a member of the G-protein gated inward rectifier K\(^+\) channel (GIRK) family and it is produced by heteromultimer of two inward rectifier channel subunits. One of the monomers was from GIRK-1 protein and the other was cardiac inward rectifier (CIR). This channel was found to be activated directly by external application of synthetic G\(_{\beta}\) subunits in excised patch membranes from guinea pig atrial myocytes. In contrast, G\(_{\alpha}\) did not activate the channel, providing the first evidence that breakdown of heteromeric G-proteins in response to muscarinic receptor stimulation causes breakdown of G-proteins to form G\(_{\alpha}\) and G\(_{\beta\gamma}\); the binding of G\(_{\beta\gamma}\) to the GIRK channel increases channel opening to increase the current and therefore produce faster atrial repolarization. \(^8^0\) I\(_{\text{KAC}}\) has been received attention recently due to its relative atrial specificity. I\(_{\text{KAC}}\) has been shown to be 6 times higher in the atria than in the ventricle. \(^8^1\) This can be easily demonstrated by superfusing cells with carbachol (an acetylcholine mimetic) (Figure 8) This has prompted the development of atrial specific ion channel blockers for treatment of atrial fibrillation. However, despite early studies documenting the presence of the I\(_{\text{KAC}}\) in guinea pig atria, GIRK4 mRNA and I\(_{\text{KAC}}\) are reduced in patients with atrial fibrillation (AF) suggesting that atrial fibrillation produces a chronic change in the atria by reducing GIRK4 mRNA levels to compensate for high activity and shorter action potential
duration of the fibrillating atria. More recently, it has been documented that $I_{K_{ACH}}$ is constitutively active in myocytes from patients with atrial fibrillation. This study also documented that while basal $I_{K_{ACH}}$ (caused by stimulation with carbachol) was lower in AF than in sinus rhythm, the channels were less sensitive to carbachol (suggesting constitutive activity) and possessed increased open probabilities. Newer studies have focused on $I_{K_{ACH}}$ as a potential therapeutic target for atrial fibrillation.

Canine atrial $I_{K_{ACH}}$ has been documented in myocytes from left atrium and pulmonary veins. The authors propose that $I_{KH}$ (Hyperpolarization activated $K^+$ Current) was identical to $I_{K_{ACH}}$ due to the sensitivity to tertiapin Q (a selective $I_{K_{ACH}}$ blocker). The same current has also been shown to be increased in canine atrial tachypacing model. However, it is worthwhile to point out some differences in the currents observed in the studies in human and canine atria. The latter study used a 800 ms depolarizing ramp protocol from a holding potential of -80mV to activate $I_{K_{ACH}}$. This is very similar to the current profile of $I_{K_{ACH}}$ studied in adult mouse atria. However, Ehrlich et al use longer test pulse durations to document a similar carbachol activated tertiapin sensitive current, which resembles $I_f$ more than $I_{K_{ACH}}$. Therefore, the studies so far documenting $I_{KH}$ in canine atria should be interpreted with a note of caution.

1.1.1.4 ELECTRICAL HETEROGENEITY IN THE VENTRICLE

One of the earliest documentations of heterogeneity in the heart arose from studies in dog papillary muscle. Variable action potential durations were observed in microelectrode recordings of papillary muscles at various depths from endocardium to subendocardium to deep-
myocardium, with the longest APDs observed in the deep-myocardium of the papillary muscle. Litovsky and Antzelevitch showed APD and AP morphology varied between canine left ventricular epicardium and endocardium in a perfused wedge preparation. The left ventricular epicardium had a prominent spike and dome morphology while the endocardium did not. The spike and dome morphology was attributed to a more accentuated phase 1 repolarization of the action potential in the epicardium than in the endocardium. In addition, the authors tested the restitution of the two regions. The restitution was faster in the endocardium than the epicardium, where the epicardial restitution was slower with a prominent spike and dome morphology re-appearing only with increasing diastolic intervals. The time for epicardial restitution was similar to that of the endocardium after the addition of 5mM 4-Aminopyridine, a potent blocker of I_{to}. Therefore the authors attributed the restitution pattern seen in the epicardium to the prominent phase 1, potentially arising from the higher density of I_{to}.

In addition, another line of observation pointed to the AP heterogeneity in the left ventricle. Before 1989, there was intense debate about the presence of supernormal conduction which was well known in the Purkinje fiber. But the understanding of this phenomenon in the left ventricle was controversial. Litovsky and Antzelevitch proved that supernormal conduction also occurred in the left ventricular epicardium. Using epicardial strips mounted on a sucrose gap, two impaled electrodes proximal and distal to the sucrose gap were applied to the epicardial strips. Then the basic stimuli were applied to the proximal segment and the resistivity of the sucrose gap was increased until conduction was blocked to the distal end. Following this conduction block, premature
stimuli applied to the epicardium at progressively increasing diastolic intervals seemed to conduct distal to the sucrose gap. This was attributed to the re-activation of $I_{io}$ (a current known to recover slowly). Only at longer diastolic intervals, spike and dome morphology reappeared, and this ensured supernormal conduction occurred. This finding propelled intense research into electrical heterogeneity in the left ventricle and identification of the gradient of $I_{io}$ in the left ventricle as discussed in section below on page 58.

Sicouri and Antzelevitch probed the action potential morphology in a wedge preparation of left and right ventricle. They found a distinct region in the deep myocardium which showed accentuation of the rate dependence of the action potential. The authors called this region the “M Cell” region. The M cell region showed longer action potentials at slower rates compared to epi- or endocardium. In addition, some major differences in AP morphology are worth pointing out. The M cell region had a more negative resting membrane potential (-90mV) than the epi- (-86 mV) or the endocardium (-87mV). Consequently, the velocity of propagation ($V_{max}$) was also higher in the M region than the other two regions. The M cell region phase 1 morphology and amplitude was similar, but still smaller than the epicardium, but greater than in the endocardium.

Liu et al reported the ionic basis for the action potential heterogeneity in the canine left ventricle. The major finding was the presence of higher $I_{io}$ in epicardium, followed by the midmyocardium and the endocardium. This was verified to true in rabbit, rat, and mice and human. The other ionic differences in the M cells compared to the epi- and endocardial cells include a smaller $I_{k_s}$ density and higher late sodium current ($I_{Na}$) (arising due to delayed re-activation of the sodium
current that was not partially inactivated). Interestingly, in spite of the absence of $I_{to}$, M cells were also identified in the guinea pig ventricle. This transmural dispersion in APD correlated well with the ion channel subunit expression in canine and human epi- and mid-myocardium. $K_{v}4.3$, $K_{v}1.4$ (both encoding $I_{to}$), $KChIP2$ (Accessory subunit for $K_{v}4.3$), and $K_{v}LQT1$ were higher in epicardium than the endocardium, while $Na_{v}1.5$ and $minK$ was lower in epicardium than the mid-myocardium. Other ion channels such as $K_{ir}2.1$, hERG and $Ca_{v}1.2$, and $MiRP-1$ were not different between the two regions.

The first study to evaluate the molecular mechanisms of transmural heterogeneity of $I_{to}$ was performed by Dixon and McKinnon. They identified a gradient of $K_{v}4.2$ mRNA level in the left ventricle from rat, with the highest $K_{v}4.2$ mRNA levels found in epicardium and the lowest in endocardium, mirroring the observed transmural differences in $I_{to}$ densities.

Rosati et al showed that in canine and human ventricle a gradient of $KChIP2$ (the cardiac isoform) underlies $I_{to}$ gradients in canine and human ventricle. This was in contrast to rat ventricle where a $K_{v}4.2$ gradient underlies $I_{to}$ differences between epicardium and endocardium. $KChIP2$ mRNA and protein in canine and human ventricle were highest in the epicardium, lowest in the endocardium and intermediate in the mid-myocardial region. In addition, further study of canine heart indicated that $KChIP2$ underlies $I_{to}$ density in the canine ventricle as shown in table 1.

In addition to the transmural heterogeneity in the left and right ventricle, regional differences in APD and repolarization exist in the ventricles. These differences include left and right ventricular differences as well apico-basal differences in the ventricle. Di Diego et al
showed that APD is shorter in myocytes from the RV epicardium compared to the left ventricular epicardium. Volders et al. showed that right ventricular midmyocardial cells have greater $I_{to}$ and $I_{ks}$ than left ventricular midmyocardial myocytes. The apico-basal differences in APD stems from elegant studies in mouse and human ventricle. Human left ventricular myocytes from the basal region have a longer APD than the apical cells. Consistent with observations in transmural regions of LV by Antzelevitch et al., the longer APD in basal LV myocytes was attributed to a smaller phase-1 amplitude in basal LV myocytes. Only two ion currents were found to be different between the apical and basal cells. $I_{to}$ and $I_{ks}$ were significantly higher in apical myocytes, thereby producing a shorter APD.

Similar to the $K^+$ current and APD differences in left ventricle, there is considerable evidence that differences other than $K^+$ currents exist in epi- and endocardium. Using square pulse voltage clamp pulses, Wang and Cohen found a greater density of peak L-type calcium current ($I_{Ca-L}$) in endocardial myocytes than the epicardial myocytes (smaller by ~45%). In addition, the authors also found a small expression of T-type calcium current in endocardial myocytes. In contrast, another study evaluating transmural $I_{Ca-L}$ density found no differences were found between canine epi-, M- and endo-cardial myocytes. It must be taken into account that square pulses give a good indication of kinetics and relative quantification between different cell types with similar properties (e.g: APD and AP morphology). However, since activation of $I_{Ca-L}$ depends on the voltage change created by phase-1 repolarization, changes in AP morphology especially in terms of Phase-1 amplitude could alter the activation properties of $I_{Ca-L}$. This was elegantly demonstrated to be true in canine
and human ventricular epicardial and endocardial myocytes by Banyasz et al,\textsuperscript{105} who showed that action potentials obtained from epicardium and endocardium when re-applied as AP clamp stimulation to the respective cells produced a distinct current voltage relationship. The endocardial cells produced only one prominent peak current (~ -4.8\,\text{pA/pF}) while the epicardial cells had two prominent peak (one immediately after the Phase 1 (~2.8\,\text{pA/pF}, and one during the plateau (~2.5 \,\text{pA/pF}), suggesting potential reactivation of $I_{\text{Ca-L}}$). The authors also proved that AP spike and dome morphology is coupled to the profile of $I_{\text{Ca-L}}$. This was achieved by applying an epicardial AP to an endocardial from which the original single peak of $I_{\text{Ca-L}}$ was observed. Switching from an endocardial AP to an epicardial AP produced a characteristic double peaked $I_{\text{Ca-L}}$, and the converse was also true. These findings were also verified in human ventricular myocytes.\textsuperscript{106} The contribution of these changes in $I_{\text{Ca-L}}$ to intracellular calcium handling was assessed by two studies in canine ventricle. Cordeiro et al,\textsuperscript{104} used fluorescent dye loaded myocytes isolated from epi-, M- and endo-cardial layers and showed that the time to peak intracellular calcium concentration (peak of the calcium transients) was slower in enodcardial cells compared to epicardial cells, and the sarcoplasmic reticulum Ca\textsuperscript{2+} load was higher in epicardial cells than in the endocardium. These findings were corroborated in another study using optical imaging (in canine perfused wedge preparation) by Laurita et al.\textsuperscript{107} The lower Ca\textsuperscript{2+} load in the endocardium was attributed to the smaller sarcoendoplasmic Ca\textsuperscript{2+} ATPase2a (SERCA2a) expression. In addition, more alternans were observed in subendocardium than the sub-epicardial region. No transmural differences in NCX expression were found.
1.1.12 THE CONCEPT OF REPOLARIZATION RESERVE

From sections described above, it's clear that cardiac ventricular action potential repolarization is under the influence of multiple repolarizing currents. Since the 1990s, a lot of time and effort have been invested in understanding the arrhythmogenic mechanisms that predispose a heart to arrhythmia when one of the major ion currents ($I_{Kr}$) is reduced due to pharmacologic blockade. Inhibition of $I_{Kr}$, which is encoded by hERG in the human myocardium, has been a notorious cause of acquired (drug-induced) ventricular arrhythmia. Many commonly used drugs (like erythromycin, quinine (to treat malaria)) have been shown to block $I_{Kr}$ and could precipitate a potentially lethal ventricular arrhythmia called Torsades de Pointes (TdP).\textsuperscript{108} Torsades de Pointes is a specific form of polymorphic ventricular tachycardia which occurs in the setting of a prolonged QT interval. However, not all drugs that block $I_{Kr}$ or the hERG-encoded current precipitate TdP.\textsuperscript{108} Arrhythmogenesis was proposed to occur only when $I_{Kr}$/hERG blockade was superimposed on the presence of other pathologies (like heart failure, hypertrophy) or subclinical ion channel or other mutations. This protection from arrhythmogeneis that was observed even during $I_{Kr}$ block was called “repolarization reserve”.\textsuperscript{109} Since the identification of two delayed rectifier currents in guinea pig ventricular myocytes (described above in section 1.1.10.1),\textsuperscript{39} intense research has gone into identifying the mechanisms by which drug interaction(s) with normal and/or mutated ion channels could precipitate arrhythmia in the whole heart. More importantly, it must be kept in mind that delayed rectifier currents ($I_{Kr}$ and $I_{Ks}$) are not high amplitude currents. The relative amplitudes of $I_{Kr}$ and $I_{Ks}$ in normal ventricular myocytes range from 20-50 pA.\textsuperscript{110} In spite of this relatively small current
amplitude, the importance and the effect of $I_{Kr}$ and $I_{Ks}$ blockade has attracted significant debate and discussion. $I_{Kr}$ blockade is universally accepted to prolong APD; an effect that is easily demonstrated at slower heart rates (~2000ms or slower basal cycle length). This effect has been hypothesized (and shown in some experimental studies) to precipitate APD prolongation, QT interval prolongation and even arrhythmias. The cellular mechanism that predisposes to TdP has been identified as early afterdepolarizations caused by reduced repolarization during the terminal phases of the plateau. Some forms of long QT syndromes (specific congenital forms of reduced repolarization) were identified as genetic mutations in $I_{Kr}$ and $I_{Ks}$. These mutations precipitated arrhythmic episodes in patients due to mutations in hERG or in $K_vLQT1$ genes. This was puzzling since specific blockade of $I_{Kr}$ prolongs APD while $I_{Ks}$ does not. In fact, in-vivo data from LQT1 patients (who possess $K_vLQT1$ mutations) indicates that arrhythmic episodes are precipitated only during excitable periods (like exercise, loud noise etc) which increases catecholamine levels. Increased sympathetic stimulation has been shown to increase $I_{Ks}$, so in LQT1 patients (mutations in $K_vLQT1$), higher catecholamine levels does not produce the normally expected increase in $I_{Ks}$, thereby precipitating arrhythmias. LQT2 patients (hERG mutations) have arrhythmic episodes when plasma $K^+$ levels fell below a specific threshold value. But despite these effects, not every single period of stress (exercise in LQT1) or low serum $K^+$ (in LQT2) produced arrhythmia. This raised a very important question, as to what protects the compromised heart from arrhythmias in some situations, while in some cases, arrhythmias can be easily precipitated. The concept of repolarization reserve arose to offer an explanation to this issue. Since canines and human are close in their ion
channel expression and effects of ion channel blockade, data supporting repolarization reserve in these two species will be presented in this section.

Early studies on $I_{Kr}$ and $I_{Ks}$ in human left ventricular myocytes suggested that $I_{Kr}$ was the only consistent current that could be observed while $I_{Ks}$ was not found.$^{113, 114}$ This was directly in contrast to studies by Li and Nattel, who observed $I_{Kr}$ and $I_{Ks}$ in right ventricular myocytes from explanted failing hearts. Similar results were obtained by Gintant in his studies on canine left ventricular myocytes.$^{73}$ This was resolved in two studies by Varro et al, who used undiseased human left ventricular myocytes to demonstrate $I_{Kr}$ and $I_{Ks}$. These two studies also pointed to the significant shortcomings of these studies by Li and Nattel,$^{72, 115, 116}$ in that the kinetics (of deactivation) can be significantly altered by using divalent cations (to block $I_{Ca-L}$ which was done in their studies). Varro’s findings using nisoldipine, rather than a divalent cation to block $I_{Ca-L}$, corroborated those of Gintant’s study on canine $I_{Kr}$ and $I_{Ks}$ respectively; thus both $I_{Kr}$ and $I_{Ks}$ are contributors to human ventricular repolarization.

The effect of $I_{Ks}$ blockade on canine ventricular repolarization was questioned by studies in action potentials in rabbit, guinea pig, dog and humans.$^{101, 110, 117}$ These studies showed in isolated myocytes and tissue microelectrode recordings that APD prolongation occurred only with $I_{Kr}$ blockade. $I_{Ks}$ blockade with relatively specific blockers, did not seem to alter the APD. This was in contrast to the effect seen in in-vivo experiments, where injection of an $I_{Ks}$ blocker (chromanol 293B) produced QT prolongation. This was verified to be due to low basal levels of circulating catecholamines in-vivo which might increase both $I_{Ca-L}$ and $I_{Ks}$. When microelectrode AP recordings were performed in the presence of
100 nM isoproterenol and $I_{Ks}$ blockade, it produced significant APD prolongation. Thus, the effects of $I_{Ks}$ blockade on the APD are seen only during sympathetic stimulation which was attributed to reduced $I_{Ks}$ available during the augmented plateau (arising from increased $I_{Ca-L}$). The effect of sympathetic stimulation on $I_{Ca-L}$ enhances the calcium release from the intracellular Ca$^{2+}$ stores and shifts the plateau to higher voltages, thereby producing a higher voltage for activation of $I_{Ks}$ which can then activate faster to repolarize the AP. But during $I_{Ks}$ blockade or reduced $I_{Ks}$ sympathetic stimulation could result in the AP having a longer time at a higher plateau voltage which might paradoxically precipitate a longer APD.

These findings on adrenergic modulation of the APD provided new insights into the prevailing views on the contribution of $I_{Ks}$ to APD at that time. Studies in guinea pig had shown that deactivation of $I_{Ks}$ was slow and this therefore left greater $I_{Ks}$ current density during the subsequent action potential and therefore this in effect, increased the net $I_{Ks}$ current and therefore could produce rate dependent APD shortening. Studies by Gintant and Varro in human and canine ventricle proved that unlike guinea-pig ventricular $I_{Ks}$, canine and human ventricular $I_{Ks}$ had faster deactivation kinetics. Later studies by Volders and Varro’s group identified a relatively minor role of $I_{Ks}$ in determining normal APD. This was nicely corroborated in another study by Stengl et al, who demonstrated that under baseline conditions (in the absence of sympathetic stimulation), the beat to beat accumulation of $I_{Ks}$ in canines was absent unlike the guinea-pig. In the presence of 100 nM isoproterenol, significant accumulation of $I_{Ks}$ was observed suggesting the potential for $I_{Ks}$ blockade that could be present during sympathetic stimulation. Jost et al made a very critical observation, while measuring
the action potential duration in the presence of $I_{Kr}$ and/or $I_{Ks}$ blockade. $I_{Kr}$ blockade with E-4031 (1 µM) or d-sotalol (30 µM) produced AP prolongation, but similar to the findings of Volders et al, the $I_{Ks}$ blocker (chromanol 293B) failed to prolong APD. However in human ventricle, when $I_{Ks}$ blocker was added on top of $I_{Kr}$ blockade and in the presence of sympathetic stimulation, significant APD prolongation occurred. This effect was found to be true when an $I_{Ks}$ blocker was applied on top of $I_{Kr}$ blockade even in the absence of isoproterenol suggesting that $I_{Ks}$ forms a large part of the “repolarization reserve” (figure 10). In addition, the same study also pointed out that blocking the outward component of $I_{K1}$ (with 10 µM BaCl$_2$) lengthened the APD but when this is coupled with $I_{Kr}$ blockade, it produced a greater prolongation in APD (figure 11). Therefore these studies suggest that $I_{Kr}$, $I_{Ks}$ and outward $I_{K1}$ play a major role in preventing excessive AP prolongation when one repolarizing current is reduced. When multiple repolarizing currents are reduced, repolarization becomes impaired which has potential implications for arrhythmogenesis. This concept is absolutely critical to the understanding of arrhythmias in pathological conditions, where one or more of these currents might be reduced.

Future chapters (4, 5, and 6) will examine the role of repolarization abnormalities during animal models of human heart disease.
Figure 1.1: Pictorial representation of the anatomy of the cardiac conduction system\textsuperscript{121}
**Figure 1.2:** Panel A shows the surface membrane topology of a single monomer of a voltage gated $K^+$ channel. The six membrane helices are marked S1 to S6 and shown as cylinders. The inter-helical loops connecting each helix are also shown. Note the longer chain length of the pore loop between S5 and S6. Panel B shows the top-view of channel (each monomer containing six helices (S1 to S6) is shown in different colors for clarity. The black ball represents a $K^+$ ion trapped in the pore of the ion channel formed by the tetramers.\(^4^7\)(From Long SB, Campbell EB, MacKinnon R. Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. *Science* 2005 August 5;309(5736):897-903. Reprinted with permission from AAAS). Panel C shows the conserved amino acid sequence (in grey) in the pore loop of different voltage gated $K^+$ channels which have a eukaryotic homologue in the heart, vasculature and the brain\(^1^2^2\)(Reprinted by permission from Macmillan Publishers Ltd: *EMBO J* 2003 Aug 15;22(16):4049-58 copyright 2003).
Figure 1.3: Panel A: Crystal structure of voltage gated K+ channel (Kv1.2) with its accessory subunit (β2). The β subunit as it will be discussed in later sections has a NADH binding domain shown in black. Panel B shows the interactions (viewed sideways) between the cytosolic interface of transmembrane (TM) helices and the tetramerization (T1) domain which links the TM helices and the β subunit. Panel C shows the top down view of the K+ channel structure. Note each channel subunit (monomer) is colored differently for clarity.47 (From Long SB, Campbell EB, MacKinnon R. Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. Science 2005 August 5;309(5736):897-903. Reprinted with permission from AAAS).
Figure 1.4: Ion channel processes commonly observed and measured using patch clamp techniques. Note that both the inactivation and deactivation processes can be fitted with a time constant to gain insight into the speed of the processes.
**Figure 1.5**: Representative action potential drawing from a canine ventricular myocyte is shown with different colors representing different phases of the action potential with the corresponding ion currents shown in the same color for clarity. The protein encoding each ion current is shown on the left and right respectively.
Figure 1.6: Representation of fitted time constants to the decay of the outward $K^+$ current, which aids in determining the fast ($I_{Kf}$) and slow ($I_{Ks}$) components of $I_{to}$ and $I_{ss}$. 
Figure 1.7: Panel A shows the SA nodal action potential at baseline (solid lines) and after norepinephrine (NE) stimulation (dotted lines). Panel B shows the structural correlates of the C-terminal domain which binds to cAMP.⁵⁴ (Adapted by permission from Elsevier Publishing group: Trends. Cardiovasc. Med Cardiac HCN channels: structure, function, and modulation copyright 2002) Panel C shows the activation curves of $I_f$ in different tissues (ventricle, Purkinje and SA node). In addition, note the shift in the activation voltage with sympathetic and muscarinic stimulation (Redrawn from Excitation-Contraction Coupling and Cardiac Contractile Force, Eds. D M Bers, Copyright 2001).³
**Figure 1.8:** Evidence for the presence of $I_{KACH}$ in the atrium, but not in the ventricle. Data obtained from canine atrial and ventricular myocytes. Superfusion with a muscarinic agonist (Carbochol 10 µM) produces dramatic shortening of action potential which has been proposed to play a role in development of atrial fibrillation. Panel B shows no effect on a canine left ventricular myocyte. Similar data was obtained from 10 atrial myocytes and 5 ventricular myocytes. X-Y Scale bars are the same for both panels.
**Figure 1.9:** Electrical heterogeneity in the ventricular myocardium. The figure shows action potentials on the left recorded from epicardial (Epi), mid-myocardial (Mid) and endocardial myocytes (Endo) myocytes from a canine ventricle. The dotted lines are drawn to show the differences in the action potential repolarization between the different regions. To the right are the corresponding transient outward $K^+$ current ($I_{to}$) recordings from the myocytes in the corresponding region. Note the change in the notch morphology and the corresponding $I_{to}$ amplitude.
**Figure 1.10**: Evidence for the presence of repolarization reserve in human ventricle. Panel A shows the action potential tracings at baseline (control) and after $I_{Ks}$ blockade (10 µM chromanol 293B and 100 nM HMR-1556) and $I_{Kr}$ blockade (1 µM E-4031). Panel B shows the ability of $I_{Ks}$ to prevent AP lengthening after $I_{Kr}$ block with dofetilide. In presence of adrenergic stimulation, $I_{Ks}$ blockade produces higher prolongation of the APD which might be pro-arrhythmic. The results are summarized as bar graphs to the right.\textsuperscript{110} (Reprinted by permission from Macmillan Publishers Ltd: Br J Pharmacol 2002 Oct;137(3):361 copyright 2002)
Figure 1.11: Evidence of repolarization reserve in the canine ventricle. Panel A shows the summary data and the raw traces corresponding to $I_{Kr}$ blockade alone or a combined $I_{Kr}/I_{Ks}$ block. Panel B shows the evidence for a combined reduction in outward $I_{K1}$ (with Barium) and $I_{Kr}$ confirming that reduction in multiple repolarizing currents challenges the cardiac ventricle to completely repolarization, thereby producing excessive action potential prolongation which might be potentially pro-arrhythmic\textsuperscript{120}.

**Table 1.1:** Regional differences in Kv4 and KChIP2 subunit distribution in the canine ventricular myocardium.

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CHAPTER 2

4-AMINOPYRIDINE-SENSITIVE PLATEAU OUTWARD CURRENT IN CANINE VENTRICLE: A CONSTITUTIVE CONTRIBUTOR TO VENTRICULAR REPOLARIZATION

This is a modified version of the final manuscript accepted in British Journal of Pharmacology, 2007
2.1 INTRODUCTION

Cardiac action potential (AP) repolarization occurs through a multitude of voltage-gated K\(^+\) channels with differing activation and inactivation patterns \(^{123}\). The transient outward potassium current (I\(_{to}\)) activates and inactivates rapidly; thereby contributing to early phase 1 repolarization \(^{124}\). The rapid (I\(_{Kr}\)) and slow (I\(_{Ks}\)) delayed rectifier K\(^+\) currents activate towards the end of the action potential plateau (phase 2) and contribute significantly to phase 3 repolarization. In rat, canine and human atria, and in rodent and guinea pig ventricle, an ultra rapid delayed rectifier current (I\(_{Kur}\)) has been described \(^{125-128}\). I\(_{Kur}\) is a determinant of the normal canine atrial action potential duration (APD). I\(_{Kur}\), like I\(_{to}\) activates very rapidly, but in contrast to I\(_{to}\) inactivates very slowly; I\(_{Kur}\) along with I\(_{to}\) contributes to phase 1 repolarization in the atria \(^{129}\).

Despite the abundant K\(_v\)1.5 protein expression in canine and human ventricle, previous studies have not demonstrated functional ventricular I\(_{Kur}\) in canine or human ventricular myocytes \(^{130,131}\). More recently, I\(_{Kur}\) has received increased attention due to the “atrial specific” expression of the current (in canines and humans), providing a novel therapeutic target for the treatment of atrial arrhythmias without the risk of ventricular proarrhythmia \(^{132}\).
I_{Kur} is sensitive to micromolar concentrations of 4-aminopyridine (4-AP), while I_{lo} is inhibited only at millimolar concentrations\textsuperscript{133,134}. This difference in sensitivity to 4-AP has been used to define I_{Kur} in canine and human atria, and in rodent ventricle. We elicited a current that is sensitive to micromolar concentrations of 4-AP which is activated during the plateau voltages of the action potential in canine left ventricular midmyocardium. This outward current has a constitutive role in ventricular repolarization.

2.2 MATERIALS AND METHODS

2.2.1 ANIMAL PROCEDURES AND MYOCYTE ISOLATION

Twenty-three adult hound type dogs (age 9 months - 5 years) weighing between 8 -20 kilograms were used for the experiments. All animal procedures were approved by the Institutional Lab Animal Use and Care committee of The Ohio State University.

Dogs were verified to have normal cardiac function by routine electrocardiograms and echocardiographic examinations during butorphanol tartarate (0.5 mg kg\textsuperscript{-1} intramuscularly) sedation. On the day of the experiments, dogs were euthanized by intravenous injection of pentobarbital sodium (Dosage: 120 mg kg\textsuperscript{-1} for the first 4.5 kilograms and 60 mg kg\textsuperscript{-1} for every 4.5 kilograms thereafter) via cephalic vein. Following this, the hearts were rapidly excised via thoracotomy and perfused with
cold cardioplegic solution (containing 5% Glucose, 0.1% Mannitol, 22.4 mM NaHCO₃, 30 mM KCl) injected into the coronary ostia. The left circumflex artery was cannulated for myocyte isolation as previously described. Following the washout of blood from the heart, collagenase (Worthington type 2, 0.65 mg ml⁻¹) and protease-free bovine serum albumin (0.65 mg ml⁻¹) were added to the perfusate (100 ml). After 30-45 minutes of collagenase perfusion, the digested mid-myocardial section of the lateral wall of the left ventricle was separated from the epicardial and endocardial sections; digested tissue was shaken in a water bath at 37°C for an additional 5-10 minutes. This typically yielded 70-90% rod shaped myocytes with staircase ends and sharp margins. The myocytes were stored at room temperature in a standard incubation buffer solution containing (in mM) NaCl 118, KCl 4.8, MgCl₂ 1.2, KH₂PO₄ 1.2, glutamine 0.68, glucose 10, pyruvate 5, CaCl₂ 1, along with 1 µmol l⁻¹ insulin, and 1% BSA until use.

2.2.2 SOLUTIONS AND CHEMICALS
All chemicals for buffer and stock solution preparation were purchased from Fisher Scientific (USA), Sigma Aldrich (St. Louis, MO, USA) and Invitrogen Inc. (Carlsbad, CA, USA). Stock solutions of nifedipine, amphotericin B and 4-aminopyridine were prepared fresh on
the day of each experiment. DPO-1 (2-isopropyl-5-methylcyclohexyl
diphenylphosphine oxide, Tocris), a relatively new, selective $I_{kur}$ blocker\textsuperscript{136} was used for a separate set of experiments, and was prepared from a stock solution (10mM) in DMSO prepared on the day of each experiment. Isoproterenol solutions were prepared daily from commercially available injectable solutions (0.2mg ml$^{-1}$), which were stored at 4°C until use. All nifedipine, isoproterenol, DPO-1 and amphotericin B solutions were protected from exposure to light.

2.2.3 ELECTROPHYSIOLOGICAL PROTOCOLS

Myocytes were placed in a laminin coated cell chamber (Cell Microcontrols, Norfolk, VA) and superfused with bath solution containing (in mM): 135 NaCl, 5 MgCl$_2$, 5 KCl, 10 Glucose, 1 CaCl$_2$, 5 HEPES, pH 7.40 with NaOH, at a temperature of 36 $\pm$ 0.5°C. For action potential (AP) recordings, the same bath solution was used with CaCl$_2$ increased to 1.8 mM. During voltage clamp experiments to measure potassium currents, L-type calcium current was blocked by 2 µM nifedipine. Solutions were changed with a six-port gravity flow system (~1 ml min$^{-1}$). Borosilicate glass micropipettes (tip resistance between 1.5 to 3 MΩ) were filled with pipette solution containing (in mM): 100 K$^+$-aspartate, 40 KCl, 5 MgCl$_2$, 5 EGTA, 5 HEPES, pH adjusted to 7.2 with KOH. Perforated whole cell...
patch clamp (using amphotericin B) was used to minimize alterations in intracellular milieu. For voltage clamp experiments, only recordings with an access resistance < 20 MΩ were included in the analyses. For determination of drug-sensitive currents, only cells with less than a 20% change in access resistance were included in the analyses. Series resistance compensation (~70%) was used for current recordings. All 4-AP sensitive currents were recorded after 3-5 minutes of 4-AP superfusion.

APs were recorded with perforated whole cell patch techniques, as described above. APs were measured as the average of the last 10 (steady-state) APs, recorded during a train of twenty five APs at each stimulation rate. To analyze beat-to beat variability in the AP recordings, standard deviation and coefficient of variation (CV) of the AP duration at 90% repolarization was calculated. The amplitude of phase 2 was measured as the maximum potential following phase 1 of the action potential.

Transient outward potassium current (I_{to}) and the rapid component of the delayed rectifier current (I_{Kr}) were elicited using voltage protocols as shown in the insets of Figure 2. Currents were recorded both in the presence and absence of 100 µM 4-AP to examine potential inhibition of I_{to} and I_{Kr} by 4-AP.
Sustained outward potassium current was elicited from a holding potential of -40 mV with a 80 ms prepulse to +30 mV to inhibit I_{to}, followed by 300 ms voltage steps from -20 mV to +50 mV. The interval between each voltage step was three seconds. 4-AP sensitive plateau outward current was measured as the steady-state difference current after a minimum of four minutes of superfusion with 4-AP. The activation time constant of the 4-AP sensitive current was determined by fitting the activation to a monoexponential function in Clampfit (v 8.0, Axon Instruments, Union City, CA, USA).

We tested two concentrations of 4-AP: 50 µM and 100 µM, based on previously published observations 137, 138. An envelope of tails test was adapted from a previously published method used to evaluate I_{Kur} in canine atria 139. The envelope of tails protocol started from a holding potential of -40 mV with a prepulse to +30 mV, followed 30 ms later by a variable-duration test pulse (60 to 240 ms) to +20 mV, followed by a step to -30 mV to elicit the tail current. The interval between test pulses was three seconds. The constancy of the ratio of the step current to tail current was evaluated as discussed below.

Data acquisition was performed with Clampex 8.0 software (Axon Instruments, Union City, CA, USA) and an Axopatch 200A patch clamp amplifier (Axon Instruments Inc, CA, USA).
2.2.4 STATISTICAL ANALYSIS

Acquired data was analyzed using Clampfit 8.0 (Axon Instruments) and Origin 6.1 (OriginLab, Northampton, MA USA). Currents were normalized to cell capacitance in picofarads (pF) and are expressed as pA pF\(^{-1}\). All data are presented as mean ± SE.

Action potential durations obtained at baseline and during drug exposure were analyzed by one way ANOVA (SAS for Windows v9.1, Cary, NC, USA). 4-AP sensitive, isoproterenol-modulated and baseline 4-AP sensitive current densities at each test voltage were statistically compared using Student’s t-tests. Dose response curves for 4-AP were constructed using the Hill equation in Origin 6.1 (OriginLab Corp., Northampton, MA, USA). To test the envelope of tails data, a linear regression was performed (SAS for Windows, v.9.1, SAS Inc., Cary NC, USA) to calculate the slope and to test whether the slope was statistically different from zero. A p-value of less than 0.05 was the criterion for statistical significance for all tests.
2.2.5 COMPUTATIONAL METHODS

Because the patch clamp data suggested little inactivation of the current, we used a three-state Markov model:

\[
\begin{array}{c}
C_2 \xleftarrow{\alpha_2} \xrightarrow{\beta_2} C_1 \xleftarrow{\alpha_1} \xrightarrow{\beta_1} O
\end{array}
\]

with two closed states (C_1, and C_2), and one open state (O) to fit the data. All state transition rates (\(\alpha_1, \alpha_2, \beta_1, \beta_2\)) are of the form \(P_x \exp(P_y \cdot V)\), where \(P_x\) and \(P_y\) are parameters and \(V\) is the membrane potential in mV.

From the recorded difference current, a training data set for the model fitting was constructed. First, data during the pre-pulses and data after the 300 ms pulses were discarded. Second, data demonstrating a capacitance transient were excluded to remove any artifactual changes in the current.

Optimization of the model parameters began with a set of randomly selected parameters. The same voltage clamp protocol that was used in the experiment was applied to the model. The resulting simulated current was compared to the training data. A cost function, \(CF(M)\), was defined where \(M\) is the vector of the parameters in the model that were to be optimized. This cost function is defined by

\[
CF(M) = \frac{\sum_i (Train_{\text{data}}, - Sim_{\text{data}},(M))^2}{N}
\]
Here $\text{Train\_data}_i$ is the training data at the $i$th time point, $\text{Sim\_data}_i(M)$ is the simulation value at the $i$th time point using the parameters given by $M$. The sum is taken over $i = 1$ to $N$, the total number of time points in the recording. The goal of the optimization process was to find the parameter values that minimize the cost function $CF(M)$ by iteratively changing the parameters, simulating the model, and evaluating the cost function until the simulation nearly matched the data.

The optimization routine used a combination of global and local methods. The global method, Differential Evolution $^{140}$, used the following strategy: A population of parameter sets was generated randomly, and the cost of each parameter set was evaluated. New parameter sets were generated by first adding a weighted difference between two randomly selected parameter sets to a third random parameter set, and then by exchanging a fraction of the resulting parameter set with a member of the population according to a cross-over probability. The cost of the new parameter set was then evaluated and compared to the cost of a randomly chosen set in the population. The lower cost set was kept in the next generation of the population. The optimization strategy consisted of using Differential Evolution for a total of four thousand generations. After each thousand generations, the Levenberg-Marquardt $^{141,142}$ local method was
applied to each parameter set in the population to quickly take each parameter vector to a local minimum.

All simulations and optimizations were run on a Dell Inspiron 9100 computer and a 16-node Linux cluster of Intel Xeon dual processors using custom written C++ computer code. Each model is represented by a set of differential equations of the form \( \frac{dx}{dt} = f(x,t,p) \), where \( x \) is a vector describing the current state of the system, \( t \) is time, and \( p \) is a vector of parameters. The corresponding differential equations are usually quite stiff in the sense that they have widely separated time scales: some variables change rapidly under small perturbations while others change slowly. To improve the accuracy of our simulations, we used the CVODES package from Lawrence Livermore National Laboratories with the backwards differentiation formula, designed for stiff systems. We also used automatic differentiation to calculate the Jacobian derivative of the function \( f \) for use with the dense Newton based solver that is included as part of CVODES.

2.3 RESULTS

Myocyte capacitance was 163.8 ± 7.7 pF (n=56). Figures 2.1A and 2.1B show representative action potentials recorded at 0.5 and 1 Hz at baseline, in the presence of 100 µM 4-AP and during washout.
Superfusion with 100µM 4-AP decreases the net outward current evident during Phase 1. Consistent with 100 µM 4-AP block of an outward current (activated at plateau potentials), phase 2 amplitude was increased from baseline values (Table 2.1). Figure 1C summarizes the action potential duration data obtained at baseline and after 4-AP superfusion at all tested stimulation frequencies. There was a statistically significant prolongation of action potential duration at 50% (APD_{50}) and 90% (APD_{90}) repolarization seen at 0.5 Hz and 1 Hz (Figure 2.1C and 2.1D). No significant change in the APD was seen at a stimulation rate of 2 Hz during 4-AP superfusion. This effect was reversible after prolonged washout of seven to ten minutes (Table 2.1). The resting membrane potential was not affected by 50 µM or 100 µM 4-AP (-76.5 ± 0.5 mV at baseline, -75.7 ± 0.8 mV and -75.2 ± 0.4 mV, with 50 and 100 µM 4-AP, respectively, p = NS). The baseline CVs of the APD_{90} values were 4.2, 5.9, and 5.8% at 0.5, 1 and 2 Hz respectively. Superfusion with 100 µM 4-AP did not change the CVs of the APD_{90} values (3.4, 5.8, and 6.7% at 0.5, 1 and 2 Hz respectively).

K^+ current-dependent AP prolongation in the canine ventricle has been attributed to two currents: I_{to}, where blockade with 2mM 4-AP prolongs both APD_{50} and APD_{90} or I_{Kr}, where blockade selectively prolongs APD_{90}. Therefore, we sought to exclude these possibilities...
and determine whether the 4-AP concentrations used in our experiments affected either $I_{to}$ or $I_{Kr}$.

Figures 2.2A and 2.2B show $I_{to}$ and $I_{Kr}$ recorded at baseline and during superfusion with 100 µM 4-AP, respectively. There was no significant inhibition of either $I_{to}$ or $I_{Kr}$ amplitude by this concentration of 4-AP, confirming that the observed AP prolongation did not result from blockade of either $I_{to}$ or $I_{Kr}$.

The 100 µM 4-AP sensitive difference current, which was obtained by digital subtraction, shows rapid current activation following membrane depolarization (Fig 2.3A). The 4-AP sensitive current does not display significant inactivation during the 300 ms test pulse. The current density-voltage relationship of the 100 µM 4-AP sensitive current is shown in figure 2.3B. The outward current begins to activate at -10mV and increases with increasingly positive test potentials. The threshold for activation (figure 2.3B) is consistent with the voltage range occurring during phase 1 of the action potential. The activation time constant was 16.7 ± 11ms at -10mV, 4.7 ± 0.81 ms at +10mV, and 3.96 ± 1.44 ms at +50mV.

Notably, we were unable to measure a 4-AP (100 µM) sensitive current in 12 of the 41 myocytes tested with this protocol. This finding was evaluated further in secondary experiments. Action potentials were
recorded first, followed by voltage clamp experiments (with nifedipine exposure of 3-4 minutes) in the same cells, to record baseline currents (n=4). Then, 4-AP superfusion (perfusate calcium at 1.8 mM) was performed to washout nifedipine and record action potentials during 4-AP exposure in the same myocytes. Following the second AP recordings, voltage clamp experiments were repeated to determine the presence of any 4-AP sensitive current. No action potential prolongation during 4-AP treatment was seen in myocytes lacking a 4-AP sensitive current. The converse was also true; in myocytes (n=3) exhibiting 4-AP-dependent action potential prolongation, we were able to consistently elicit a 4-AP sensitive sustained outward current. These results argue against either a non-specific effect of 100 µM 4-AP on action potential duration or current rundown during the duration of our experiments.

We fit the concentration-response data to evaluate the inhibition of the sustained potassium current by 4-AP (figure 2.3C). This analysis revealed an IC$_{50}$ value of 24.2 µM. At 50 µM and 100 µM 4-AP, this fitted curve predicts 78.7% and 96.4% inhibition, respectively. The tested concentrations were therefore close to the maximal blocking concentration, which explains the lack of a significant difference when comparing results from the two concentrations (figure 2.1C). We tested only up to a concentration of 500 µM, as a recent publication 147 has
shown inhibition of $I_{K_r}$ with millimolar concentrations of 4-AP, and $I_{to}$ is known to be blocked at 1-1.5 mM $^{148}$.

Figure 2.3D shows a representative envelope of tails test using the protocol shown. The ratio of the peak tail current to the average steady-state step current as a function of the step duration is shown in figure 3E. Visual examination of the data revealed a constant ratio as a function of time. This was confirmed statistically by linear regression analysis, which revealed a slope of $0.021 \pm 0.015$, which did not differ significantly from a slope of zero ($p = 0.17$); this is consistent with a single current component in the 100 µM 4-AP-sensitive current.

In canine atria, $I_{Kur}$ is augmented by β-adrenergic stimulation $^{149}$. To test the β-adrenergic modulation of the 4-AP-sensitive plateau outward current in the ventricle, we used 100 nM and 1 µM isoproterenol (a nonspecific $\beta_1$ and $\beta_2$ adrenergic receptor agonist), followed by isoproterenol and 4-AP to obtain the 100 µM 4-AP sensitive current. Data were obtained after 6-8 minutes of exposure, which we found in preliminary experiments to result in steady-state activation of the sustained outward $K^+$ current.

The baseline 100 µM 4-AP-sensitive current recorded in the absence of isoproterenol (figure 2.4A) and isoproterenol stimulated, 4-AP sensitive current (Figure 2.4B) is shown. Isoproterenol (1 µM) significantly ($p<0.05$)
increased the measured current amplitude at +50 mV from 0.51 ± 0.10 pA pF⁻¹ to 1.53 ± 0.37 pA pF⁻¹ (Figure 2.4C). Isoproterenol did not significantly alter the activation time constant of the current measured at +50mV, which was 3.96 ± 1.4 ms at baseline vs. 1.3 ± 0.9 ms with 1 µM isoproterenol (p=0.09).

In separate experiments, action potentials were recorded in the presence and absence of DPO-1 (0.3 and 1 µM). We observed significant prolongation of APD₅₀ with DPO-1 (p<0.05) at 1 Hz and 2 Hz (Figure 2.5B), while significant prolongation of APD₉₀ was evident at all three stimulation frequencies (Figure 2.5C).

2.3.1 RESULTS FROM COMPUTER SIMULATIONS

A Markov model structure was used to develop a computer simulation of the 4-AP-sensitive “Iₖur-like” current. Parameters in the model were estimated by using the nonlinear optimization routine described above to fit the model output to experimental data from patch clamp recordings. Multiple parameter vectors with low cost were obtained after the optimization. The lowest cost parameter vector (Table 2.2) was used to generate the results below. No significant differences were observed for the other low cost parameter vectors (data not shown). Figure 2.6A
compares the resulting ion current model and the experimental patch clamp data. The lowest cost ion current model was then incorporated into a canine AP model by replacing the $I_{K_p}$ current originally in that model. APs were generated from the model at the same cycle lengths as in the experiment (basic cycle lengths of 2000, 1000 and 500 ms). To study the effect of the “$I_{Kur}$-like” current on APD, control AP simulations were compared with simulations of “$I_{Kur}$-like” current block (Figures 6B, 6C, 6D), and to simulations of $I_{to}$ block (Table 2.3). Table 2.3 summarizes the effects of $I_{to}$ blockade versus “$I_{Kur}$-like” current blockade in two different canine AP models. Blockade of “$I_{Kur}$-like” current in the AP models results in significantly more APD prolongation than that of $I_{to}$ blockade.

2.4 DISCUSSION

We found that a 4-AP-sensitive plateau outward current occurs in the majority of left ventricular mid-myocardial canine myocytes. Based on pharmacologic response and activation and inactivation properties, we suggest that the observed 4-AP-sensitive current has canine “$I_{Kur}$-like” properties. Furthermore, this current plays a functional role in AP repolarization in normal canine LV mid-myocardial myocytes, as assessed both in-vitro and in-silico.
Ultra-rapid delayed rectifier current has been found in rodent atria and ventricle, and canine and human atria\textsuperscript{155, 156}. This current has been shown to initially activate in phase 1 of atrial action potential and blockade of this current results in prolongation of APD\textsubscript{50} and APD\textsubscript{90} in canine atria\textsuperscript{157}. In addition, guinea-pig ventricular myocytes exhibit a similar outward K\textsuperscript{+} current which activates at plateau voltages\textsuperscript{158}. This current was named I\textsubscript{Kp} due to its unique open probabilities at plateau potentials, with no detectable inactivation. Similarities between I\textsubscript{Kp} and I\textsubscript{Kur} in canine and human atria have been noted\textsuperscript{159}.

Low concentrations of 4-AP (50-100 µM) have been previously used to block I\textsubscript{Kur} in canine and human atria, and in rodent ventricle\textsuperscript{160}, and the similar sensitivities to micromolar 4-AP concentrations suggests blockade of the same channel subunit\textsuperscript{161}. The 4-AP IC\textsubscript{50} for plateau outward “I\textsubscript{Kur-like}” current we measured from canine left ventricle was 24.2 µM, which is similar to that previously reported for I\textsubscript{Kur} in canine (5.31±0.74 µM) and human (49 µM) atria\textsuperscript{162, 163} and in Kv1.5 expression systems (50 µM)\textsuperscript{164}. As reported for canine atrial I\textsubscript{Kur}\textsuperscript{165}, we observed that the plateau outward current is rapidly activating, and did not show significant inactivation during the 300 ms test pulse. While previous reports\textsuperscript{166, 167} on I\textsubscript{Kur} demonstrate that the current inactivating more rapidly at physiologic temperatures (compared to room temperature), there is still sufficient I\textsubscript{Kur}
at physiologically relevant temperatures to contribute to action potential repolarization.

Kv1.5, the protein encoding I_{Kur}, is present in canine atria and ventricle \(^{168, 169}\). While Kv1.5 encoded current has not been thought to be normally expressed in the ventricles of large mammals, we note one report of a C9356-sensitive current (a Kv1.5 blocker) in ventricular myocytes isolated from a post-infarction canine model \(^{170}\). In this canine post-infarction model, Kv1.5 has been shown to lateralize from the intercalated disks in the peri-infarct region \(^{171}\). Due to the “I_{Kur}-like” nature of the 4-AP sensitive plateau outward current observed, we suggest that the 4-AP sensitive current may be carried by Kv1.5 channels. Further studies are required to address this issue.

2.4.1 A ROLE FOR I_{Kur} IN CANINE AND HUMAN VENTRICLE?

We tested two different concentrations of 4-AP on APD at 0.5, 1 and 2 Hz. Both 50 and 100 µM 4-AP produced a reverse use dependent prolongation of APD\(_{50}\) and APD\(_{90}\) consistent with a previous study \(^{172}\), where block of Kv1.5 by 4-AP was relieved at faster stimulation rates, which was attributed to rate-dependent relief of closed channel block. In addition, we evaluated the effects of DPO-1, reportedly a use-dependent, selective I_{Kur} blocker, \(^{173, 174}\) to provide additional confirmatory evidence, of
the existence of an outward plateau (“I_{Kur}-like”) current in canine midmyocardial left ventricular myocytes. We observed similar effects on the APD with both DPO-1 and 4-AP, and these were quantitatively similar at a stimulation frequency of 1 Hz. In contrast to a previous report, we did not observe use-dependent effects of DPO-1 on APD^{175}. The reported use-dependent properties of DPO-1 are in contrast to the known reverse use-dependent effects of 4-AP. Our simulations revealed a similar degree of frequency-independent prolongation of APD_{90} during I_{Kur}-like current block (Table 2.3). This suggests that it is the block of the outward plateau current by 4-AP which is reverse-use dependent, as has been reported for I_{Kur}^{176}, rather than the current itself being fundamentally reverse use-dependent.

Our data are in contrast to data obtained in an in vivo study on the effect of I_{Kur} blockade on canine ventricular repolarization, where dogs were anesthetized with pentobarbital^{177}. Pentobarbital is a known blocker of repolarizing currents, and also prolongs canine ventricular APD_{50} and APD_{90} at concentrations required for general anesthesia^{178}. Thus, any potential effect of I_{Kur} blockade in this study of in vivo canine ventricular repolarization may have been obscured by anesthesia; and the in vivo effects of I_{Kur} blockers on in vivo canine ventricular repolarization therefore remain poorly defined.
A study in myocytes from failing explanted human hearts used 50 µM 4-AP to detect $I_{Kur}$, which could be detected in atrial, but not in right ventricular myocytes. The authors suggested that the absence of $I_{Kur}$ in ventricular myocytes may have resulted from limited sampling (n=5 myocytes) or possible differences in regional distribution. A recent report with a mixed $I_{Kur}$ and $I_{to}$ blocker did not find significant effects on the canine right ventricular APD; the difference between this report and our observations could potentially result from differences in the selectivity of the drugs, or differences between right and left ventricular electrophysiology.

DPO-1, is reported to cause rate dependent blockade of $I_{Kur}$ and AP prolongation in human atrial myocytes, with no AP prolongation noted in a human ventricular myocyte, obtained from an end-stage heart at the time of transplantation. However, the source of the ventricular myocyte (right vs. left ventricle) was not stated, and the results are again limited by a small sampling of myocytes. In summary, the contribution of $I_{Kur}$ to normal human left ventricular repolarization is undefined at the present time. Notably, recent reports confirm the expression of $Kv1.5$ mRNA in the ventricle of normal, undiseased, human hearts, suggesting a potential role for $Kv1.5$ encoded currents in the human ventricle. In both human atria and ventricle, immunohistochemical studies reveal high expression of
Kv1.5 which is localized to the intercalated disks, similar to the distribution in canine atrial myocytes\textsuperscript{184, 185}.

2.4.2 4-AMINOPYRIDINE AND BLOCKADE OF OTHER K\textsuperscript{+} CURRENTS

hERG current and canine ventricular I\textsubscript{to} are blocked by 4-AP, with an IC\textsubscript{50} of 4.4 mM and 1.15 mM respectively\textsuperscript{186, 187}. We found no inhibition of either I\textsubscript{to} or I\textsubscript{Kr} at the 4-AP concentrations used in our experiments. Our I\textsubscript{to} measurements are consistent with a previous report\textsuperscript{188}, where a concentration of 50 \textmu{}M 4-AP, only blocked \textasciitilde{}1\% of ventricular I\textsubscript{to}\textsuperscript{189}. The following lines of evidence: 1) the previously reported lack of significant inhibition of ventricular I\textsubscript{to} with concentrations up to 200 \textmu{}M 4-AP\textsuperscript{190}; 2) the characteristics of the 4-AP sensitive current we recorded; 3) the use of I\textsubscript{to} inactivating prepulses, and 4) the absence of I\textsubscript{to} inhibition with 100 \textmu{}M 4-AP in our myocytes, all suggest that the 4-AP sensitive current which we report was not contaminated by I\textsubscript{to}. Similarly, the results of the envelope of tails test and the absence of 4-AP block of I\textsubscript{Kr} both suggest that the 4-AP sensitive plateau current was not contaminated by I\textsubscript{Kr}. 

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2.4.3 BETA-ADRENERGIC MODULATION OF “IKur-LIKE” CURRENT

Beta-adrenergic modulation of $I_{\text{Kur}}$ has been previously reported in human atrial myocytes. $Kv1.5$ has been shown to interact with the accessory channel subunits, $Kv\beta1.2$ and $Kv\beta1.3$, which alter the adrenergic responsiveness of the channel. Using the $\beta$-adrenergic agonist (isoproterenol) we found that the ventricular 4-AP sensitive plateau current was also modulated by $\beta$-adrenergic stimulation. These results suggest a possible role for the current in modulating ventricular repolarization during sympathetic stimulation.

2.5 LIMITATIONS

We did not examine the presence of this current in either right ventricular or atrial myocytes, and our measurements were confined to one region of the LV myocardium. The purpose of the current study was to establish the presence and functional role of this “$I_{\text{Kur}}$-like” current in the canine left ventricle, and thus, we did not examine myocytes from other regions in these experiments.

The model used for the in silico experiments is based on experimental data derived from canine right and left endocardial tissues, and therefore the model does not emulate the action potentials from the left ventricular lateral midmyocardium. The modeling results are
therefore more qualitative than quantitative, and serve to confirm that
block of a small amplitude plateau current increases AP plateau voltage
and prolongs the APD.

The length of our recorded action potentials is longer than that
previously reported in canine ventricular muscle \(^{194}\). One potential
explanation for this difference is the use of isolated myocytes, rather than
multi-cellular preparations, since the APD is modulated by intercellular
coupling\(^{195}\). Alternatively, the APD may vary with site of origin of the
myocytes\(^{196}\). We isolated myocytes from the left lateral midmyocardium of
the LV free wall, and previous reports have primarily focused on myocytes
of anterior LV origin.\(^{197, 198}\). Notably, while our control APD\(_{90}\) values are
longer than those in some previous reports, they are shorter than control
values previously published in isolated canine myocytes\(^{199}\). Lastly, there is
the potential for alterations in APD due to the myocyte isolation procedure,
which cannot be excluded in our experiments.

We did not examine the effects of 4-AP on \(I_{Ks}\). However, in control
myocytes (in the absence of \(\beta\)-adrenergic agonists), there is no
contribution of \(I_{Ks}\) to repolarization of canine or human ventricle\(^{200-203}\). Our
experimental protocols (250-300ms test pulses) were designed to
minimize the role of \(I_{Ks}\) during the current.\(^{204}\) Furthermore, we did not
observe any time-dependent increases in the amplitude of the rapidly
activating, 4-AP sensitive, current, as would be expected if $I_{Ks}$ were present.

2.6 CONCLUSIONS

Our study demonstrates the presence of a plateau outward current in canine ventricle which has several "$I_{kur}$-like" properties. This current activates rapidly at depolarized potentials, and block of the current causes prolongation of both $APD_{50}$ and $APD_{90}$. In addition, the β-adrenergic mediated increase in current density suggests a potential role in rate-adaptation of the action potential duration during periods of increased sympathetic stimulation. Our findings suggest that $I_{kur}$ blockers (e.g. DPO-1) may affect ventricular as well as atrial repolarization. This may have implications for the use of $I_{kur}$ blockers for the treatment of atrial tachyarrhythmias.
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<td></td>
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</tr>
<tr>
<td>APD$_{50}$(ms)</td>
<td>392.8±34.6</td>
<td>543.8±56.6*‡</td>
<td>367.8±90.5</td>
</tr>
<tr>
<td>APD$_{90}$(ms)</td>
<td>576.3±33.9</td>
<td>784.6±60.5*‡</td>
<td>518.9±106.2</td>
</tr>
<tr>
<td>Phase 2</td>
<td>27.5±2.6</td>
<td>36.3±3.0*‡</td>
<td>27.4±2.0</td>
</tr>
<tr>
<td>Amplitude(mV)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                |          |            |         |
| **1 Hz**       |          |            |         |
| APD$_{50}$(ms) | 328.9±26.6| 434.8±23.7*‡| 328.7±36.9|
| APD$_{90}$(ms) | 498.4±21.7| 633.3±34.6*‡| 483.4±51.9|
| Phase 2        | 26.4±2.7  | 33.1±3.1*  | 29.4±1.3 |
| Amplitude(mV)  |          |            |         |

|                |          |            |         |
| **2 Hz**       |          |            |         |
| APD$_{50}$(ms) | 243.7±9   | 253.1±26.8 | 242.5±16.4|
| APD$_{90}$(ms) | 373.8±9.5 | 412.1±14.2 | 386.6±22.2|
| Phase 2        | 21.6±4.4  | 31.4±5.2*  | 22.4±4.7 |
| Amplitude(mV)  |          |            |         |

*- p<0.05 vs. Baseline, ‡ - p<0.05 vs. Washout (n=11-13 cells in 0.5 and 1 Hz, n=4-9 cells in 2 Hz group)

**TABLE 2.1:** 100 µM 4-AP prolongs APD and increases Phase 2 amplitude at 0.5 and 1 Hz.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_K$</td>
<td>-94.88 mV</td>
</tr>
<tr>
<td>$G_{Kur}$</td>
<td>0.1453 mS/uF</td>
</tr>
<tr>
<td>$\alpha_{1x}$</td>
<td>0.04957 msec$^{-1}$</td>
</tr>
<tr>
<td>$\alpha_{1y}$</td>
<td>0.007138 mV$^{-1}$</td>
</tr>
<tr>
<td>$\alpha_{2x}$</td>
<td>0.002703 msec$^{-1}$</td>
</tr>
<tr>
<td>$\alpha_{2y}$</td>
<td>0.0001 mV$^{-1}$</td>
</tr>
<tr>
<td>$\beta_{1x}$</td>
<td>0.2348 msec$^{-1}$</td>
</tr>
<tr>
<td>$\beta_{1y}$</td>
<td>-1.708 mV$^{-1}$</td>
</tr>
<tr>
<td>$\beta_{2x}$</td>
<td>0.02055 msec$^{-1}$</td>
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**TABLE 2.2**: Ion current model parameter values from the lowest cost fit.
<table>
<thead>
<tr>
<th>Model</th>
<th>Fox-Gilmour</th>
<th>Hund-Rudy</th>
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<tbody>
<tr>
<td></td>
<td>2000ms</td>
<td>1000ms</td>
</tr>
<tr>
<td>Basic cycle length</td>
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<td></td>
</tr>
<tr>
<td>Control APD (ms)</td>
<td>166</td>
<td>163</td>
</tr>
<tr>
<td>Control plateau (mV)</td>
<td>-4.4</td>
<td>-5.3</td>
</tr>
<tr>
<td>I$_{to}$ blocked APD (ms)</td>
<td>161</td>
<td>163</td>
</tr>
<tr>
<td>I$_{to}$ blocked plateau (mV)</td>
<td>7.0</td>
<td>8.0</td>
</tr>
<tr>
<td>I$_{Kur}$-like current blocked APD (ms)</td>
<td>227</td>
<td>227</td>
</tr>
<tr>
<td>(%increase to control)</td>
<td>(36%)</td>
<td>(39%)</td>
</tr>
<tr>
<td>I$_{Kur}$-like current blocked plateau (mV)</td>
<td>5.4</td>
<td>5.8</td>
</tr>
</tbody>
</table>

**TABLE 2.3:** Effects of I$_{to}$ blockade versus “I$_{Kur}$-like” current blockade on APD$_{90}$ and plateau amplitude in *in-silico* models
FIGURE 2.1: Blockade of $I_{\text{Kur}}$ using 50 or 100 µM 4-AP causes reverse use-dependent action potential prolongation. Representative AP recordings are shown at 0.5 Hz (Panel A) and 1 Hz (Panel B). Panel C: Summary data for $\text{APD}_{50}$ plotted as a function of stimulation frequency. Panel D: Summary data for $\text{APD}_{90}$ plotted as a function of stimulation frequency. The numbers on each bar indicate the number of cells tested. (* p<0.05 compared to baseline recordings).
FIGURE 2.2: 100 µM 4-AP does not block $I_{to}$ or $I_{Kr}$. Panel A shows representative transient outward $K^+$ current ($I_{to}$) traces from a ventricular myocyte (cell capacitance – 154 pF) recorded at baseline (left), after superfusion with 100 µM 4-AP (middle, voltage protocol is inset) and the averaged $I_{to}$ I-V curves (right). Panel B shows the peak ramp $I_{Kr}$ from a ventricular myocyte (Cell capacitance – 148 pF) recorded at baseline and 100 µM 4-AP using the ramp protocol (inset). Current was measured as the peak outward current during the voltage ramp. For purposes of clarity, only the traces obtained at the last test pulse (plateau duration = 80 ms) are shown. The current densities plotted as function of the plateau duration are shown on the right. The dashed lines represent the zero current line. The interpulse interval for $I_{to}$ and $I_{Kr}$ was 2 and 7 seconds respectively.
FIGURE 2.2
FIGURE 2.3: 100 µM 4-AP inhibits canine ventricular sustained outward $K^+$ current. Panel A shows the baseline (top), 100 µM 4-AP (middle) and difference currents from a ventricular myocyte (Cell capacitance – 166 pF) using the voltage clamp protocol shown in the inset. The dashed lines represent the zero current line. Panel B shows the averaged I-V curve for the 4-AP sensitive sustained outward $K^+$ current (from myocytes exhibiting measurable 4-AP sensitive plateau outward current), with current measured as the average during the last 20 ms of the test pulse. Panel C shows the dose response relationship for 4-AP inhibition of sustained outward $K^+$ current, fit to the Hill equation ($r^2 = 0.99$, $n=5-8$ myocytes at each concentration). Panel D shows a representative 4-AP sensitive current trace from a ventricular myocyte using the voltage clamp protocol shown in the inset. Circles on the step current trace indicate the time at which the tail currents were elicited. Panel E shows the mean envelope of tails test data where the ratio of tail to step current amplitude is plotted as a function of step pulse duration.
FIGURE 2.3
FIGURE 2.4: Canine ventricular plateau outward current is augmented by isoproterenol (Iso). Representative 100 µM 4-AP sensitive current traces from myocytes recorded at baseline (Panel A, 166 pF) and after exposure to isoproterenol (Panel B, 154 pF). The dashed lines represent the zero current line. Summary I-V curves are shown in panel C. (* p < 0.05 vs. baseline control values).
FIGURE 2.5: DPO-1, an I_{Kur}-selective blocker, prolongs APD in canine left ventricular myocytes. Panel A: Representative action potential trace recorded at 1Hz before and after superfusion of 1µM DPO-1. APD_{50} and APD_{90} recorded at 0.5 Hz (Panel B), 1 Hz (Panel C) and 2 Hz (Panel D) are shown. Controls are shown in open bars, 0.3 µM DPO-1 in grey and 1 µM DPO-1 in black respectively. (* p < 0.05 vs. control).
FIGURE 2.6: “I_{Kur}-like” current blockade prolongs canine ventricular AP in-silico. Panel A:
Fitting of a computer model of the 4-AP sensitive (“I_{Kur}-like”) current to current data from patch clamp experiments. For purposes of clarity, current fitting to test steps to -10 mV, 0 mV, 20 mV and 40 mV are shown. Model parameters after fitting are in Table 2. The fitted model was incorporated into the Fox-Gilmour and Hund-Rudy canine AP models. Steady-state action potential tracings from the Fox-Gilmour model obtained at 2000 (Panel B), 1000 (Panel C) and 500 ms (Panel D) basal cycle lengths. Control AP traces are shown in black and APs in the presence of “I_{Kur}-like” current blockade are shown in grey.
CHAPTER 3

ABNORMAL DIASTOLIC CURRENTS IN VENTRICULAR MYOCYTES FROM SPONTANEOUS HYPERTENSIVE AND HEART FAILURE (SHHF) RATS

3.1 INTRODUCTION

Hypertension can lead to compensatory hypertrophy in cardiac muscle. Chronically, these compensatory mechanisms can become maladaptive and cardiomyopathy and heart failure (HF) can result. Experimental animal studies in hypertrophy and heart failure have shown abnormalities in ion channel function; altered expression and function of proteins involved in excitation-contraction coupling; and an increased propensity for cardiac arrhythmias. Clinically, ventricular arrhythmias occur in 80% of HF patients. However, the mechanisms of arrhythmogenic electrophysiologic remodeling during hypertension and hypertensive HF are not fully elucidated.

Spontaneous Hypertensive Heart Failure (SHHF) rats develop hypertension at an early age. In contrast to spontaneously hypertensive rats (SHRs), the SHHF rats consistently develop reproducible, hypertensive heart failure, in an age-dependent manner. Echocardiographic studies in the SHHFs demonstrate LV dysfunction (left ventricular ejection fraction <40%) at 17 -18 months of age. There is also evidence of progressive cardiac hypertrophy in the SHHFs, evidenced as an increase in heart weights.
Hypertension and heart failure can increase the pacemaker current ($I_f$) in the ventricular myocardium.\textsuperscript{213-216} This can pathologically alter the diastolic phase of the action potential and enhance abnormal automaticity. In addition, aging has been shown to increase $I_f$ density in both normal and hypertensive (SHR) rat ventricles.\textsuperscript{217}

Normally, the diastolic membrane potential is primarily regulated by the inward rectifier $K^+$ current ($I_{K1}$). $I_{K1}$ is reduced in heart failure and consequently may contribute to increased excitability of the ventricle.\textsuperscript{218} We hypothesized that chronic hypertension and the resultant heart failure would lead to altered diastolic membrane currents, providing a substrate for abnormal excitability and automaticity. We measured $I_{K1}$ and $I_f$ in SHHF rats during the development of hypertension and heart failure in comparison to age-matched controls. Our observations suggest that changes in $I_{K1}$ and $I_f$ provide a mechanism for the initiation of ventricular arrhythmias during hypertension, heart failure and aging.

3.2 METHODS
SHHF rats were obtained from the colony produced by Dr. Sylvia McCune at the Ohio State University.\textsuperscript{219} Only phenotypically lean SHHF rats of either sex were used for this study. SHHF rats of either sex were studied at two and eight months of age, and after the development of overt
heart failure (HF). As previously described, HF was identified between 16-22 months of age, and was defined as a left ventricular ejection fraction < 40% by echocardiography, with signs of labored breathing, cyanosis, subcutaneous edema, piloerection, cold extremities (tails), and orthopnea. Age-matched Wistar-Furth rats or Wistar rats (Harlan, Indianapolis, IN) of either sex were used as controls.

Animals were deeply anesthetized with sodium pentobarbital (IP) and the heart was rapidly removed. After flushing the heart, the heart was weighed and the heart mass / body mass ratio was calculated. All animal procedures were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

3.2.1 VOLTAGE-CLAMP RECORDINGS

Ventricular myocytes were isolated using a previously described method. Myocytes were stored at room temperature under an oxygen hood until studied. Only cells with clear striations and sharp margins were studied. All data were acquired within 8 hours of cell isolation to minimize potential time-dependent changes in the currents.

Cells were placed in a dish (Cell Microcontrols, Virginia Beach, VA) and superfused with test solutions; the bath temperature was maintained at 35°C with a temperature controller (Cell Microcontrols). Solutions were
changed with a six-port gravity flow system (~1 mL/min). Data acquisition was performed with pClamp software (V. 8, Axon Instruments, Union City, CA) and an Axopatch 200A patch-clamp amplifier (Axon Instruments).

The amphotericin B-perforated, whole-cell, patch-clamp technique was used for all recordings to minimize alterations in the intracellular milieu. Patch pipettes (2 – 5 MΩ) were used and after seal formation, changes in the capacitative response to a –10 mV step were used to monitor perforation of the patch by amphotericin B. Series resistance compensation was applied (40-60%) to minimize voltage errors. Only cells with low access resistance (< 20 MΩ) which were stable (< 20% change in series resistance) were included in the data analysis.

The bath solution contained (mM) NaCl 134; MgCl₂ 1; KCl 5; HEPES 5, CaCl₂ 1; D-glucose 5 mM; pH adjusted to 7.40 with NaOH. Nifedipine (2 μM) was added to the bath solution to block L-type calcium current. The pipette solution contained (mM) KCl 130; MgCl₂ 5; HEPES 5; EGTA 5; pH adjusted to 7.2 with KOH.

A holding potential of −40 mV was used for all voltage clamp experiments to inactivate sodium current. A series of 100 msec test potentials from −140 to + 40 mV, with a 20 mV increment, were used to
elicit $I_{K1}$. The current was measured at the end of each 100 msec test potential. $I_{K1}$ was defined as barium-sensitive current, and was measured as the difference current between that in control solution and in bath solution with 2 mM BaCl$_2$. $I_{f}$ was measured following the addition of BaCl$_2$ to the bath solution. $I_{f}$ was elicited using one second voltage steps from -130 to -50 mV applied at 5 second intervals. $I_{f}$ was measured as the difference between the instantaneous inward current and that at the end of each voltage step.

3.2.2 ACTION POTENTIALS

In separate experiments, we used current clamp recordings to evaluate resting potential and abnormal automaticity. For these recordings, the bath solution contained (mM) NaCl 134; MgCl$_2$ 1; KCl 5; HEPES 5, CaCl$_2$ 1.8; d-glucose 5 mM; pH adjusted to 7.40 with NaOH. Cells were stimulated at 0.2, 0.5, 1, and/or 2 Hz. The proportion of myocytes exhibiting abnormal automaticity (defined as phase 4 depolarization events) was calculated. Zatebradine (10 μM, ) was used as an $I_{f}$ blocker.\textsuperscript{224, 225}

3.2.3 REAGENTS
Zatebradine was supplied by Boehringer Ingleheim Pharma (Germany). All other reagents were supplied by Sigma Chemical (St. Louis, MO).

3.2.4 DATA ANALYSIS  The measured currents were expressed as current density (pA/pF) after normalization for cell capacitance. $I_{K1}$ inward conductance (mS/cm²) was determined by calculating the slope of the linear portion of the current density-voltage relationship from –140 mV to –100 mV. Peak outward $I_{K1}$ density was measured at -60 mV ($I_{-60}$). The $I_{K1}$ rectification ratio (RR) was calculated as $((I_{-100}) - (I_{-60}))/ (I_{-100})*100$.\cite{227, 228}

$I_I$ current density (in pA) was normalized to the cell capacitance (pF) and expressed as pA/pF. All data were included for the current-voltage and current density-voltage relationship analyses.

To assess the physiologic relevance of $I_I$, only cells classified as having significant $I_I$ were included in the analysis of current differences at -90 mV. The presence of significant $I_I$ was classified following histogram analysis of normalized $I_I$ at -120mV from all WF and SHHF myocytes studied.\cite{229} Based on this, a cut off value of 0.60 pA/pF at -120 mV was established to classify the presence or absence of significant $I_I$. 

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Initial statistical analysis revealed no sex-dependent statistical differences; male and female data were pooled for further analyses. Two \( \text{way ANOVA} \) with post-hoc Student Neumann-Keul’s test was used to test for differences between groups. One \( \text{way ANOVA} \) was used to test for age-dependent cardiac hypertrophy in the SHHFs. Differences in the proportion of cells demonstrating \( I_f \) (as defined above) were assessed using the Pearson Chi-square test. Data are presented as mean ± SE. Statistical significance was defined as \( p<0.05 \). Statistical analysis was performed using SAS for Windows (v.8.0.1 SAS Systems, Cary, NC).

3.3 RESULTS

SHHF heart size increased as a function of age with wet weights being 1231±55 mg at two months, 1575 ± 80.5 at eight months, and 2442 ± 220mg in heart failure (\( p<0.05 \)). Significant myocyte hypertrophy only occurred in the oldest group of controls (Figure 3.1). However, hypertrophy was evident in the SHHFs as early as eight months and did not increase further after the progression to heart failure.

3.3.1 INWARD RECTIFIER K⁺ CURRENT

Representative current tracings and mean data from SHHF and control myocytes are shown in Figure 3.2. In contrast to controls, in SHHF
myocytes there was a decrement in inward $I_{K1}$ conductance at eight months, which was decreased further after the onset of heart failure ($p<0.05$). Notably, even in control myocytes, there was a reduction in inward $I_{K1}$ conductance at an advanced age, however, inward $I_{K1}$ conductance in controls was still substantially larger compared to the age-matched SHHFs ($p<0.05$).

Analysis of peak outward $I_{K1}$ showed no age-dependent change in the controls. The SHHF rats showed a substantial reduction in peak outward $I_{K1}$, but only during heart failure. The rectification ratio (RR) was decreased in an age-dependent manner in controls, consistent with reduced rectification of the current as a function of “normal” aging. In the SHHFs, there was a significantly larger reduction in the rectification ratio at the oldest age compared to age-matched controls, consistent with a further decrement in current rectification with heart failure. In summary, normal aging, hypertension and hypertrophy, and heart failure all appeared to reduce the $I_{K1}$ rectification ratio.

3.3.2 PACEMAKER CURRENT ($I_f$)

Figure 3.3 depicts raw current recordings and summary data for ventricular $I_f$ in WFs and SHHFs. There was an age-dependent increase in $I_f$ in WFs. In SHHFs the $I_f$ was larger at all ages compared to two month
controls. There were no age- or heart failure-dependent increases in \( I_f \) density in the SHHF groups. When only cells expressing significant \( I_f \) were analyzed (Figures 3.3E and 3.3F), there were significantly more myocytes expressing \( I_f \) in the SHHF groups at both two and eight months of age. However, at \( \geq \) seventeen months of age, there was no difference between controls and SHHFs in the proportion of myocytes expressing \( I_f \). The amplitude of \( I_f \) increases as a function of myocyte size (Figure 3.3F, \( p<0.035 \)).

### 3.3.3 PHYSIOLOGIC IMPACT OF ALTERED DIASTOLIC CURRENTS: ABNORMAL AUTOMATICITY

To evaluate the role of \( I_f \) at physiologic resting membrane potentials, we conducted an additional analysis at a test potential of -90 mV. There was a significant difference between groups at two months of age (-0.06 ± 0.04 vs. -0.27± 0.36 pA/pF in controls and SHHFs, respectively). At eight and \( \geq \) seventeen months of age there was no significant difference between groups in the \( I_f \) at -90 mV.

We recorded action potentials (APs) from two month (10 cells) and eight month (3 cells) SHHFs. APs were recorded in 11 myocytes from 2-6 month Wistar rats; no age-dependent differences in action potential characteristics were found in the control myocytes, and these data were
pooled. The resting membrane potential was reduced in the 8 month SHHF myocytes compared to controls (p<0.05). In addition, the resting membrane potential in the eight month SHHF myocytes was reduced compared to that in the two month SHHF myocytes (-72.2 ± 1.8 mV vs. -80.6 ± 1.2 mV respectively, p<0.05). There was no difference between 2 month SHHF myocytes and control myocytes in the resting membrane potential.

In control myocytes, we did not observe any evidence of abnormal automaticity (zero of eleven). In contrast, in the two month SHHF myocytes, we observed abnormal automaticity in three of five myocytes at 0.2 Hz. At a faster stimulation rate of 0.5 Hz, none of the myocytes (zero of eight) from the two month SHHF rats exhibited abnormal automaticity; in contrast, two out of three myocytes from the eight month SHHF rats exhibited abnormal automaticity. Abnormal automaticity was abolished by zatebradine (I_f channel blocker) and reappeared after washout of zatebradine (Figure 3.4). At stimulation rates of 1 and 2 Hz, no abnormal automaticity (0/13) was evident in the SHHF myocytes.

3.4 DISCUSSION

There is an increasing prevalence of ventricular arrhythmias (premature ventricular depolarizations and nonsustained VT) observed
The frequency and complexity of ventricular arrhythmias is increased in hypertensive patients, even in the absence of left ventricular hypertrophy.\textsuperscript{233} The presence of left ventricular hypertrophy in hypertensive patients results in an increased complexity and frequency of ventricular arrhythmias, including sudden cardiac death.\textsuperscript{234-236} Interpretation of studies evaluating arrhythmias in patients with hypertensive left ventricular hypertrophy is potentially confounded by the use of pharmacotherapy with the potential to cause electrolyte imbalances, and thus, result in increased arrhythmia risk. During heart failure, ventricular arrhythmias occur in $\sim$80\% of patients\textsuperscript{237} and many with heart failure die of sudden cardiac death. We used the SHHF rat model and age-matched controls to evaluate the ventricular diastolic membrane currents, $I_{K1}$ and $I_{f}$, and found differential regulation of these currents by hypertension, heart failure and aging.

SHHF rats are a reliable model for human hypertension and heart failure. Systolic blood pressures are consistently elevated in the SHHFs but are decreased after the progression to significant heart failure (which typically occurs in phenotypically lean SHHFs at 15-30 months of age).\textsuperscript{210,238} Myocyte size is increased as early as two months of age in SHHFs, and progressive myocyte and cardiac hypertrophy have been measured through 12 months of age in the SHHFs.\textsuperscript{239} During heart failure,
SHHFs have intracardiac thrombi, myocyte hypertrophy and increased cardiac interstitial fibrosis.\textsuperscript{240} Wistar and Wistar-Furth rats have been used as a suitable control for SHHFs.\textsuperscript{241} Wistar-Furths have normal blood pressures during early to middle adult ages,\textsuperscript{242} although recently the systolic blood pressure in aged WFs (21 months) was reported to be elevated, ~160 mm Hg.\textsuperscript{243} Thus, it is possible that hypertension may have contributed to the increased myocyte capacitance and increased $I_f$ in the aged controls.

We observed an age-dependent decrement in $I_{K1}$ in the control group. This is in contrast to previous reports where no change in $I_{K1}$ was reported when comparing ventricular myocytes from 3 and 9 month\textsuperscript{244} or young and senescent (24-25 months) Wistar rats.\textsuperscript{245} Although we did observe a reduction in the aged control rats, there was a significantly greater reduction in $I_{K1}$ with age and disease progression in SHHFs. When compared to SHHFs, the age matched WFs expressed a statistically decreased inward conductance only in the $\geq 17$ month age group. To our knowledge, this decrease in inward $I_{K1}$ during aging has not been previously reported and could contribute to a depolarized diastolic membrane potential.

The peak outward $I_{K1}$ was significantly altered only in end stage SHHFs, consistent with a heart failure-induced response. This reduction...
would be expected to result in a prolongation of terminal repolarization, and has been previously described in other heart failure models.\textsuperscript{246, 247} We observed a significant reduction in the $I_{K1}$ rectification ratio (RR) with age in both controls and SHHFs. The RR decrease was more pronounced in SHHFs than in WFs. Rectification of $I_{K1}$ is modulated by testosterone, intracellular magnesium, and intracellular polyamines;\textsuperscript{248-250} alterations in these regulators have been reported during aging, hypertension and heart failure,\textsuperscript{251-254} and may be the basis of this observation.

In normal human and rat hearts there is a low level of ventricular pacemaker current, and the proportion of cells with significant $I_f$ is less in controls, when compared to myocytes from failing hearts.\textsuperscript{255, 256} Ventricular $I_f$ is increased in both amplitude and prevalence with both normal aging and hypertension in rats.\textsuperscript{257, 258} The age-dependent increase we observed in the proportion of myocytes expressing $I_f$ in controls is similar to that reported in normal Wistar-Kyoto rats.\textsuperscript{259} Notably, the age-dependence of the proportion of myocytes we observed with $I_f$ in the SHHFs differs from that previously reported in spontaneous hypertensive rats (SHRs), where the occurrence of $I_f$ in young SHRs did not differ from that in young controls.\textsuperscript{260} The reason for this difference is unclear; it is interesting to note that while both SHRs and SHHFs are hypertensive at a young age, only SHHFs consistently progress to develop heart failure at an early age.
Similar to the observed increases in $I_r$ in both control and SHHF rats, ventricular $I_r$ is increased in human myocytes as a result of normal aging, hypertension and HF.\textsuperscript{261, 262} We observed a significant age-dependent increase in $I_r$ current and current density in the WFs which is consistent with that previously reported in aged Wistar-Kyoto rats.\textsuperscript{263} We found that the amplitude of $I_r$ was positively correlated with cell size. This relationship explains why we did not observe an increase in current density with progressive cardiac hypertrophy in the SHHFs.

The early occurrence of $I_r$ in the SHHFs is consistent with the known response to hypertension.\textsuperscript{264} Thus, our data suggest that hypertension can mediate increased $I_r$ even at an early age in the SHHF rat. Progression from hypertension to hypertensive heart failure increases $I_r$ amplitude, but not current density since amplitude is tightly correlated with myocyte size. This differs from a previous report by Cerbai and colleagues in spontaneous hypertensive rats (SHRs) where cell size did not correlate with $I_r$ amplitude, although they did observe a significant correlation in controls.\textsuperscript{265}

The physiologic relevance of the $I_r$ expression in the SHHFs can be evaluated by comparing the current at -90 mV (to approximate a normal resting potential). This reveals a significant increase in current amplitude
as a function of age in both the controls and SHHFs, although the current was always larger in the SHHFs.

$I_f$ is a time-dependent current, so we used variable stimulation rates to assess the physiologic impact of $I_f$. Our data indicate that increased $I_f$ alone is sufficient to result in abnormal automaticity, only at very low stimulation rates (0.2 Hz). Based on the data from both two and eight month SHHF myocytes, both an increase in $I_f$, and a reduction in inward $I_{K1}$, is necessary for abnormal automaticity to occur at a faster stimulation rate (0.5 Hz). Our data suggest that altered excitability through a combination of decreases in $I_{K1}$ and increases in $I_f$ could precipitate arrhythmias during aging, hypertension and heart failure.

3.5 LIMITATIONS

We did not directly measure blood pressure in our cohort of animals. However, the SHHFs have reproducible blood pressures at the time points used in this study, which we obtained from the literature. The SHHFs in this study were phenotypically lean, a mixture of animals heterozygous for or lacking the cp gene. This factor, and the use of both males and females, resulted in a more variable onset of heart failure than would be seen in genotypically lean rats of one sex. To control for this variable, we used echocardiography to define the presence of heart
failure, using previously established criteria.\textsuperscript{266, 267}

\subsection*{3.6 CONCLUSION}

By using young adult and aged SHHF\textsubscript{s} and control rats, we found that aging, hypertension, and heart failure have variable effects on diastolic membrane currents. Reductions in $I_{K1}$ and increases in $I_{f}$ are both arrhythmogenic, and the magnitude of these changes is affected by age, hypertension and heart failure. The finding of reduced $I_{K1}$ in aged controls is interesting, as there are increased ventricular arrhythmias during normal aging in humans.\textsuperscript{268, 269} Our results indicate that the underlying cause of arrhythmias may vary during the progression from hypertension to heart failure. $I_{K1}$ is functionally regulated by testosterone, intracellular magnesium, and intracellular polyamines, while $I_{f}$ is regulated by the autonomic nervous system. This suggests that therapeutic interventions targeting each of these ionic currents may be required to minimize ventricular arrhythmias during the progression of hypertensive heart failure.
Figure 3.1: Cell capacitance as a function of rat strain and age. There is an increase in myocyte size in the Wistar-Furth controls. Myocyte size is larger in the SHHFs than in the controls at 8 and ≥17 months. * p<0.05 compared to 2 month WF; † p<0.05 compared to 8 month WF, ‡ p<0.05 compared to 2 month SHHF; § p<0.05 compared to ≥17 month WF.
Figure 3.2: $I_{K1}$ is altered by age in controls and by the progression to heart failure in SHHF rats. Panel A: Raw current recordings from 2 month WF (left) and 17 month WF (right), cell capacitances – 109 and 114.5pF respectively. Panel B: Representative raw current traces from 2 month SHHF (left), cell capacitance and ≥17 months SHHF (right), cell capacitances – 116 and 118pF respectively. Panel C: I-V relationships normalized to cell capacitances of WF and SHHF shown. (□- 2 month WF, ○ - 8 month WF, △ - 17 month WF, ■ - 2 month SHHF, ● - 8 month SHHF, ▲ - > 17 months SHHF). Panel D: Normalized inward $I_{K1}$ conductance for WF (white) and SHHF (black) at different ages. Numbers on each bar indicates total cells in each group. Panel E: Peak outward current at -60mV as a function of strain at different ages. Panel F: Rectification ratio for WF and SHHF. (* - age dependent change from 2 month WF, + - different from 8 month WF, ‡ - age dependent change from 2 month SHHF, § - different from 8 month SHHF, # - different from 17 month WF at p<0.05).
FIGURE 3.2.
Figure 3.3: $I_f$ is altered by age in controls and by hypertension and heart failure in SHHF rats. Panel A: Current recordings from a 2 month WF myocyte (left) and a 17 month WF rat (right), cell capacitances – 109 and 141pF respectively. Panel B: Raw current traces from 2 month SHHF (left) and end stage SHHF (right), cell capacitance – 109pF and 186pF respectively. Panel C and D: I-V curves for WF and SHHF respectively at different ages. (□ - 2 month WF, ○ - 8 month WF, △ - ≥ 17 month WF, ■ - 2 month SHHF, ● - 8 month SHHF, ▲ - ≥ 17 month SHHF). * p<0.05 for current density-voltage relationship compared to that in 2 month WF controls. Panel E Proportion of myocytes expressing significant $I_f$ in each group. *p<0.05 compared to age-matched controls. Panel F: Relationship between cell size and $I_f$ amplitude in control (open symbols) and SHHF (closed symbol) myocytes. Current amplitude increases as cellular hypertrophy develops ($r^2 =0.74$ and p<0.035).
FIGURE 3.3
Figure 3.4. Abnormal automaticity in myocytes from SHHF rats isabolished by an If blocker (zatebradine). Representative action
potential recordings (25 traces) in a 2 month SHHF myocyte at 0.2 Hz
(panel A) after 3 minutes of superfusion with zatebradine (Panel B), and
after 5 minutes of washout of zatebradine (panel C).
CHAPTER 4

REPOLARIZATION ABNORMALITIES AND
AFTERDEPOLARIZATIONS IN A CANINE MODEL OF SUDDEN
CARDIAC DEATH.
4.1 INTRODUCTION

Sudden Cardiac Death (SCD) is a major cause of cardiovascular mortality in the United States, accounting for ~500,000 deaths annually. Holter analysis has established that the vast majority (>80%) of these deaths result from tachyarrhythmias that culminate in ventricular fibrillation (VF). Post-mortem examinations of SCD subjects indicates that scar tissue due to a previous myocardial infarction (MI) is present ~1/3 of the time. It has been estimated that up to 80% of SCD results from myocardial ischemia or its sequelae.

Numerous studies have identified electrophysiologic abnormalities in the hours to days following MI in canine models. At 5 and 14 days post-infarction, there is shortening of the action potential duration (APD) in the epicardial peri-infarct border zone. Specifically, post-MI decrements in $I_{to}$ occur at 5 and 14 days post infarction in the epicardial peri-infarct border zone (EBZ); however, at 2 months post-infarction, $I_{to}$ reportedly returns to control values. Jiang et al. reported a reduction in both $I_{Kr}$ and $I_{ks}$ in myocytes isolated from the EBZ of 5 day post-MI canine hearts. Notably in these previous studies, stratification for, and the occurrence of, lethal arrhythmias were not described, and the specific mechanisms
predisposing to post-MI SCD during recurrent ischemia remain poorly defined.

In this study, we utilized a post-MI canine model of sudden cardiac death\textsuperscript{282}, to examine the cellular electrophysiologic abnormalities which pre-dispose to the development of ventricular tachyarrhythmias and SCD. Our results suggest that downregulation of repolarizing K\textsuperscript+ currents and afterdepolarizations provide a substrate for SCD following myocardial infarction.

4.2 MATERIALS AND METHODS

All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. Sixteen mongrel dogs (M/F; 2-3 years of age) had a surgically induced myocardial infarction by occlusion of the left anterior descending coronary artery, as previously described\textsuperscript{282}. At the time of surgery, a pulsed Doppler flow transducer and vascular occluder were placed on the left circumflex artery and the chest was closed. After recovery, arrhythmia susceptibility was determined using a combined exercise with ischemia test, where animals exercised on a treadmill to a target heart rate (~210 beats/min or 70\% of the maximum heart rate). During the final minute of exercise, the circumflex was occluded via the implanted occluder; occlusion was maintained for one
additional minute after the cessation of exercise. This method induced VFI in approximately 60% of the animals, which were classified as “VF+”; the remainder of the animals were classified as “VF-”. Two-D B- and M-mode echocardiograms were performed in a subset of dogs (3 VF+ and 2 VF-) under butorphanol sedation (0.5 mg kg⁻¹, IM) at baseline and 8 weeks following surgery. Ten age-matched dogs served as controls. In the same subset, 24 hour ambulatory ECG was recorded by Holter monitor.

Myocytes were isolated 8-10 weeks post-infarction from the antero-lateral left ventricular mid-myocardial wall. A minimum 5 days elapsed between the final arrhythmia testing and myocyte isolation. For myocyte isolation, dogs were anesthetized by intravenous injection of pentobarbital sodium (Dosage: 120 mg kg⁻¹ for the first 4.5 kilograms and 60 mg kg⁻¹ for every 4.5 kilograms thereafter); after achieving anesthesia the hearts were rapidly excised and perfused with cold cardioplegic solution (containing 5% Glucose, 0.1% Mannitol, 22.4 mM NaHCO₃, 30 mM KCl) injected into the coronary ostia. The left main coronary artery was cannulated for myocyte isolation. Following the washout of blood from the heart, collagenase (Worthington type 2, 0.65 mg ml⁻¹) and protease-free bovine serum albumin (0.65mg ml⁻¹) were added to the perfusate (100 ml). In hearts from the both the VF groups, a clear margin of the infarct was visible as scar tissue. After 30-45 minutes of perfusion, the digested mid-
myocardial section of the left ventricle up to 3-6 centimeters from the infarct center (scar tissue) was separated from the epicardial and endocardial sections; digested tissue was shaken in a water bath at 37°C for an additional 5-10 minutes. This typically yielded 50-70% rod shaped myocytes with staircase ends and sharp margins. The myocytes were stored at room temperature in a standard incubation buffer solution containing (in mM) NaCl 118, KCl 4.8, MgCl₂ 1.2, KH₂PO₄ 1.2, glutamine 0.68, glucose 10, pyruvate 5, CaCl₂ 1, along with 1 µmol l⁻¹ insulin, and 1% BSA until use. All myocyte experiments were conducted within ten hours of isolation.

4.2.1 SOLUTIONS AND CHEMICALS

All chemicals for buffer and stock solution preparation were purchased from Fisher Scientific (USA), Sigma Aldrich (St.Louis, MO, USA) and Invitrogen Inc (Carlsbad, CA, USA). Stock solutions of nifedipine, amphotericin B and 4-aminopyridine were prepared fresh daily. Nifedipine (3.5mg) was dissolved in 1 ml of ethyl alcohol (10mM stock). This stock solution (200 µL) was added to 800 µL of bath solution to yield a 2 mM stock. The 2 mM stock solution was subsequently diluted (1:1000) in cell bath solution (listed below). Amphotericin B (6 mg) was dissolved in 100 µL of di-methyl sulfoxide (DMSO). Following this, 20 µL of the
amphotericin-B stock was added to 5 mL of pipette solution (see below). The pipette solution was vortexed, sonicated and re-vortexed prior to filling the glass micropipettes for patch clamp measurements. Isoproterenol solutions were prepared daily from commercially available injectable solutions (Sanofi Winthrop Pharmaceuticals, New York NY). D-sotalol was obtained from Merck Research Laboratories, West Point PA. All nifedipine, isoproterenol, and amphotericin B solutions were protected from exposure to light.

4.2.2 ELECTROPHYSIOLOGICAL PROTOCOLS

Myocytes were placed in a laminin coated cell chamber (Cell Microcontrols, Norfolk, VA) and superfused with bath solution containing (in mM): 135 NaCl, 5 MgCl₂, 5 KCl, 10 mM Glucose, 1 mM CaCl₂, 5 mM HEPES, pH adjusted to 7.40 with NaOH, at a temperature of 36 ± 0.5°C. For action potential (AP) recordings, the concentration of CaCl₂ in the bath solution was increased to 1.8 mM. During potassium current measurements, L-type calcium current was blocked by the addition of 2µM nifedipine to the superfusate. Solutions were changed with a six-port gravity flow system (~1 ml min⁻¹). Borosilicate glass micropipettes (tip resistance of 1.5 - 3 MΩ) were filled with pipette solution containing (in mM): 100 K⁺-aspartate, 40 KCl, 5 MgCl₂, 5 EGTA, 5 HEPES, pH adjusted
to 7.2 with KOH. Perforated whole cell patch clamp (using amphotericin B) was used to minimize alterations in intracellular milieu. For voltage clamp experiments, only recordings with an access resistance < 20 MΩ were included in the analyses. Series resistance compensation (~70%) was used for current recordings. For determination of drug-sensitive currents, only cells with less than a 20% change in access resistance were included in the analyses. All drug sensitive currents were recorded after 3-5 minutes of drug superfusion, which in our pilot experiments resulted in current blockade.

APs were recorded with perforated whole cell patch techniques, as described above. APs were measured as the average of the last 10 (steady-state) APs, obtained during a train of twenty five APs at each stimulation rate. To analyze beat-to beat variability in the AP recordings, the standard deviation of the AP duration at 90% repolarization in each myocyte was calculated for beats 15 to 25. The amplitude of phase 2 was measured as the maximal potential following phase 1 of the action potential. We observed arrhythmia in some cells. Myocytes exhibiting cellular arrhythmias were excluded from APD measurements. In a second series of current clamp experiments, cellular arrhythmias were quantified by recording action potentials in the presence and absence of isoproterenol.
Transient outward potassium current ($I_{to}$) was elicited by a series of 100 ms test potentials from –20 to +50 mV, from a holding potential of –60 mV. $I_{K1}$ was elicited by voltage steps from -140 to +40 mV from a holding potential of -40 mV. The current was measured at the end of each 100 msec test pulse. $I_{K1}$ inward conductance (mS/cm$^2$) was determined by calculating the slope of the linear portion of the current density-voltage relationship from –140 mV to –100 mV$^{284}$. Peak outward $I_{K1}$ density was measured as the current at -60 mV ($I_{-60}$).

Rapid ($I_{Kr}$) and slow ($I_{Ks}$) components of the delayed rectifier current were elicited using 10mV incremental voltage steps from -40mV to +50 mV from a holding potential of -50 mV. $I_{Kr}$ was measured as the d-sotalol-sensitive (100µM) current, while d-sotalol-insensitive tail currents were used for tail $I_{Ks}$ measurements$^{285,286}$. During $I_{Kr}$ and $I_{Ks}$ recordings the bath solution also contained 4-Aminopyridine (100 µM) to prevent any potential contamination with “$I_{Kur}$-like” plateau current.

Sustained outward potassium current was elicited from a holding potential of -40 mV using 10mV voltage steps from -20mV to +50 mV. A combination of -40 mV holding potential and 80 ms prepulse to +30mV was used to inactivate $I_{to}$. A sustained 4-AP-sensitive plateau current was measured as the steady-state difference current, recorded after a minimum of four minutes of superfusion with 4-AP.
D-sotalol was used at concentrations of 30 and 100 $\mu$M to inhibit $I_{Kr}$ during action potential recordings in VF-myocytes and control myocytes. These concentrations have been previously reported to selectively inhibit $I_{Kr}$ in canine ventricular myocytes\textsuperscript{287, 288}.

Data acquisition was performed with Clampex 8.0 software (Axon Instruments, Union City, CA, USA) and an Axopatch 200A patch clamp amplifier (Axon Instruments Inc, CA, USA).

### 4.2.3 STATISTICAL ANALYSIS

Acquired data were analyzed using Clampfit 8.0 (Axon Instruments) and Origin 6.1 (OriginLab, Northampton, MA USA). Currents were normalized to cell capacitance and are expressed as pA pF$^{-1}$. Action potential durations and current densities were analyzed by ANOVA with post hoc least significant difference testing as appropriate (SAS for Windows v9.1, Cary, NC, USA). All data are presented as mean $\pm$ SE.

### 4.3 RESULTS

There was no evidence of impaired global LV structure or function after infarction, as has been previously reported in this model\textsuperscript{282}. Echocardiograms showed that LV fractional shortening was unchanged (41$\pm$1.6% vs. 40.6$\pm$0.9%, baseline and 8 weeks post-MI, respectively,
p=NS); LV diameters at end systole (2.43±0.05cm vs. 2.40±0.03cm, baseline and 8 weeks post-MI, respectively, p=NS) and end diastole (4.14±0.14cm vs. 4.13±0.05cm, baseline and 8 weeks post-MI, respectively, p=NS) were unchanged. However, myocyte capacitance was significantly increased post-MI in both VF+ and VF- groups (p<0.05) compared to controls (Controls: 157±19; VF+: 202±14; VF-: 191±8), but did not differ as a function of susceptibility to VF.

In ambulatory ECGs, we observed frequent ventricular premature depolarizations and episodes of non-sustained ventricular tachycardia in the VF+ group (Figure 4.1). In experiments to quantify cellular arrhythmias, we observed early afterdepolarizations (EADs) in 8/12 VF+ myocytes (Figure 4.1). We observed no evidence of EADs in eleven control and eight VF- myocytes. When treated with isoproterenol, there was no significant change in the number of cells showing EADs (9/12 in presence of isoproterenol vs. 8/12 at baseline in the VF+ group). No delayed afterdepolarizations were observed in any experiments.

VF+ myocytes exhibited a significant increase in action potential durations at 50% (APD_{50}) and 90% (APD_{90}) repolarization at both 0.5 and 1 Hz (p<0.05), while the VF- myocytes did not differ from controls (Figure 4.2). There was a trend toward an increased plateau potential in the VF+
myocytes compared to both the control and the VF- group (27.3±0.8 mV, 35.6±2.8 mV, 31±1.6 mV at 0.5 Hz in control, VF+ and VF- groups, respectively, p=0.08). The resting membrane potential was not different between the three groups (-82±2.6 mV in control, -79.6±0.9 mV in VF+ and -79.8±0.4 mV in VF- group respectively).

There was increased beat-to beat variability in the action potential duration from the VF+ myocytes; variability was quantified by measuring the standard deviation (SD) of APD$_{90}$ (Figure 4.2). The VF+ myocytes exhibited a significantly increased SD of APD$_{90}$ at both 0.5 and 1 Hz compared to both control and VF- myocytes (p<0.05).

$I_{to}$ was reduced to a similar extent in both the VF+ and VF- groups (figure 4.3) compared to control values (p<0.05). No differences in the kinetics of inactivation or recovery from inactivation of $I_{to}$ were found between groups (data not shown). Inward $I_{k1}$ slope conductance was significantly reduced in the VF+ group compared to both the control and VF- groups (Figure 4.4). The peak outward component of $I_{k1}$ did not differ between the three groups.

$I_{kr}$ was significantly reduced in myocytes from the VF+ group to levels which were almost undetectable (p<0.05, Figure 4.5); $I_{kr}$ density was unchanged in VF- compared to controls. The slow component of the delayed rectifier current ($I_{ks}$) did not differ between the three groups.
We recently identified a 4-AP sensitive “\(I_{\text{Kur}}\)-like” plateau current in a majority (~70%) of left ventricular mid-myocardial myocytes\(^{290}\). The VF+ myocytes had a reduced density of “\(I_{\text{Kur}}\)-like” plateau current compared to controls and the VF- groups (\(p<0.05\), Figure 4.6). The VF-myocytes generally did not differ from control values in 4-AP-sensitive plateau current density.

To verify that loss of \(I_{\text{Kr}}\) is a critical contributor to AP variability and afterdepolarizations, a series of separate experiments were conducted in control and VF- myocytes. \(I_{\text{Kr}}\) was inhibited by superfusion of d-sotalol, a selective \(I_{\text{Kr}}\) blocker (Figure 4.7). In control myocytes, both concentrations of d-sotalol resulted in APD prolongation (data not shown). Superfusion with 30 \(\mu\)M sotalol did not induce EADs in control myocytes (\(n=6\)) at 0.5, 1 and 2 Hz, while 100 \(\mu\)M d-sotalol resulted in a single isolated EAD in one of five myocytes, stimulated at 0.5 Hz. No EADs were observed during treatment with 100 \(\mu\)M d-sotalol at either 1 or 2 Hz respectively. In the VF-myocytes, superfusion with 30 and 100 \(\mu\)M d-sotalol consistently resulted in early afterdepolarizations at all frequencies (4 of 4 myocytes at both concentrations tested, compared to 0 of 4 at baseline, Figure 4.7).
4.4 DISCUSSION

Sudden cardiac death is the leading cause of cardiovascular mortality, and pre-existing coronary artery disease is a major risk factor for SCD. In myocytes from a model of experimental MI with reproducible ischemia-induced sustained ventricular tachyarrhythmias\textsuperscript{282, 291}, we observed a distinct form of electrophysiologic remodeling. Our key findings are that post-infarction, myocytes from dogs exhibiting lethal arrhythmias demonstrate early afterdepolarizations, action potential prolongation and increased beat-to-beat variability in repolarization, which are attributable to specific $K^+$ current abnormalities.

Our data suggest that early afterdepolarizations may trigger reentrant ventricular tachyarrhythmias in this model of sudden death. Repolarization depends on the balance between multiple currents ($K^+$ currents, late $Na^+$ current, NCX and $I_{Ca}$); alterations in this balance can result in prolongation of the action potential. During action potential prolongation, early afterdepolarizations may occur during phase 2 or phase 3 of the ventricular action potential. Reductions in a single repolarizing $K^+$ current, $I_{Kr}$, may be sufficient to result in AP prolongation in the canine ventricle\textsuperscript{292}. Recently, it was shown that block of $I_{Kr}$ in isolation is insufficient to prolong the APD unless $I_{Kr}$ is also inhibited in normal
canine ventricular myocytes. This requirement for the block of multiple currents has been attributed to the presence of “repolarization reserve”, where the presence of multiple repolarizing K currents provides sufficient capacity for repolarization in the absence of a single repolarizing current. The early afterdepolarizations we observed on the background of reduced Ito, Ik, and a 4-AP sensitive “Ikur-like” current in the VF+ myocytes do not clearly indicate which defect or defects in repolarization are required for the initiation of afterdepolarizations in this model of SCD. However, block of Ik in the VF- myocytes suggests that either inhibition of Ik alone or combined reductions in Ito and Ik are sufficient to induce early afterdepolarizations in this post-infarction canine model. Since d-sotalol (30 μM) did not induce EADs in the control myocytes, it appears that APD prolongation due to Ik inhibition alone is not sufficient to induce EADs consistently. Thus, suggesting that the presence of repolarization reserve in normal canine ventricular myocytes prevents induction of EADs at physiologically relevant stimulation rates (0.5 and 1 Hz) following inhibition of Ik in isolation. Based on the induction of EADs in the VF- myocytes (where Ito is reduced, Figure 4.3), our data suggest that reductions in both Ito and Ik in combination are sufficient to induce EADs. This suggests that in the VF+ myocytes,
pathophysiologic inhibition of multiple repolarizing K⁺ currents results in APD prolongation, APD variability and induction of EADs, thus providing a substrate for the initiation of ventricular tachyarrhythmias in this model of SCD.

Our myocytes were all derived from the left ventricular anterior mid-myocardium, and I_{Kr} blockers have been shown to produce greater action potential prolongation in the mid-myocardium compared to the epicardium or the endocardium. While AP prolongation is more prominent at slower stimulation rates, an elegant study by Hua and Gilmour demonstrated the contributions of I_{Kr} to rate-dependent action potential dynamics in canine endocardial myocytes, with “baseline” I_{Kr} (current activated preceding the upstroke of the action potential during continuous AP clamp stimulation) increasing significantly at faster stimulation rates. Conversely, they observed that “peak” I_{Kr} was increased at slower stimulation rate. Furthermore, decreasing I_{Kr}, both in isolated myocytes and in-silico, increased the amplitude of APD alternans. In a separate study, these authors demonstrated the converse, that hERG overexpression in canine ventricular myocytes successfully abolished APD alternans. Additional studies have linked the loss of I_{Kr}, and APD alternans, to ventricular fibrillation. Although we did not observe APD alternans in the VF+ myocytes, we did observe a significant increase in the variability of APD_{90}. 
Notably, increased variability of repolarization has also been associated with the development of SCD in a different canine model of heart disease, one of chronic AV block\textsuperscript{302}. The action potential variability we observed, which is attributable at least in part, to the absence of $I_{Kr}$, manifested as EADs in 8/12 VF+ myocytes tested. Interestingly, the absence of $I_{Kr}$ in left ventricular midmyocardial myocytes corresponds with a known lack of efficacy of $I_{Kr}$ blockers in preventing VF in this model\textsuperscript{282, 303}.

4.4.1 COMPARISONS TO OTHER EXPERIMENTAL MI STUDIES IN DOGS

Multiple studies have documented the cellular electrophysiological remodeling process occurring in the hours to days post MI in dogs. Most of the pioneering studies in this area have focused on the epicardial border zone and the Purkinje fibers as sites of re-entry initiation\textsuperscript{304, 305}. In contrast to these previous studies, we studied dogs with healed MIs and a known predisposition to ischemia-induced sudden death, and our action potential measurements were restricted to the mid-myocardial layer where we found increases in APD$_{50}$ and APD$_{90}$. It is possible that additional regional differences in repolarization within the LV, either transmural or regional, may contribute to arrhythmogenesis.
We found a decrement in $I_{to}$ density in both post-MI groups, which was independent of the arrhythmia phenotype. This is consistent with a previous report of reduced $I_{to}$ on day 5 post-infarction in the epicardial border zone (EBZ)$^{306}$. In a separate report from these investigators, $I_{to}$ was reduced at 14 days post-infarction in EBZ myocytes, but restored to control values two months post-infarction$^{307}$. In contrast to this report, we found a significant reduction in $I_{to}$ at 8-10 weeks post-MI in both the VF+ and VF- groups. However, our data suggests that $I_{to}$ plays only a very minor role in modulating APD, as has been previously suggested$^{308}$. This interpretation is supported by the observed reduction in $I_{to}$ density in the VF- myocytes, which exhibited normal APDs; and the comparable reductions in $I_{to}$ in both VF+ and VF- myocytes, which exhibit significant differences in APDs.

Delayed rectifier currents have been studied extensively$^{309,310}$ in canine post-infarction models. Jiang et al reported a reduction in both $I_{Kr}$ and $I_{Ks}$ in myocytes isolated from the EBZ of 5 day post-MI canine hearts$^{311}$. In the same model, Dun et al reported a more complex form of remodeling in myocytes from the infarcted EBZ$^{312}$. They reported an upregulation in the TEA-sensitive component of delayed rectifier current (possibly $K_{r,2.1}$). Notably, similar to our finding in the mid-myocardial region of a 4-AP-sensitive plateau current, Dun et al reported a C9356-
sensitive (C9356 is a selective blocker of Kv1.5 channels) current in myocytes from both the EBZ and normal zone of canine hearts. We recently reported the presence of a similar current which activates at plateau voltages and modulates canine midmyocardial APD\textsuperscript{290}. Due to its similarities with canine atrial \( I_{Kur} \) (both in properties and inhibition with micromolar 4-aminopyridine), we suggest that this is a 4-aminopyridine sensitive “\( I_{Kur} \)-like” plateau current. “\( I_{Kur} \)-like” current was selectively reduced in myocytes from VF+ group alone, which has the potential to alter the plateau potential as well as the APD.

4.5 LIMITATIONS

Our myocyte studies were limited to those isolated from the LV midmyocardium. Therefore, the contribution of electrophysiologic abnormalities in other ventricular regions to the arrhythmic phenotype remains undefined. Our studies were focused on repolarizing K\(^+\) currents as modulators of the action potential, additional studies will be required to determine the potential roles of Na\(^+\) current or abnormalities in Ca\(^{2+}\) cycling to arrhythmogenesis in this model.

The myocardium undergoes a process of dynamic remodeling following a myocardial infarction. We only studied animals that were 8-10 week post-infarction. This time point was selected as one where
ventricular remodeling is complete\textsuperscript{313-315}, and we did not examine any time-dependent pathologic remodeling of myocyte electrophysiology.

In humans, medications are typically given post-MI to minimize ventricular remodeling and sudden death. In our cohort of animals, no such medications were administered. Additional studies would be required to examine the effects of such medications (e.g. angiotensin converting enzyme inhibitors, beta-adrenergic antagonists) on arrhythmogenic electrophysiologic remodeling.

4.6 SUMMARY

Our study suggests that non-acute post-MI lethal arrhythmias arise from repolarization abnormalities which are manifested as prolonged action potentials, increased beat-to-beat variability in repolarization and cellular arrhythmias. The repolarization abnormalities which predispose to lethal arrhythmias are associated with a profound reduction in $I_{Kr}$. Further studies are warranted to examine other potential contributors to arrhythmogenesis (e.g. abnormalities in myocyte calcium handling or autonomic modulation of electrophysiology) in this model.

4.7 ACKNOWLEDGEMENTS

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**FIGURE 4.1: Evidence of arrhythmia in-vivo and in isolated myocytes.** Panel A shows a Holter recording from a VF+ animal with two normal sinus beats followed by focal initiation (premature beat, marked with downward arrow) of nonsustained ventricular tachycardia at rest. Panel B shows evidence of cellular arrhythmia from the same VF+ dog recorded at 1 Hz.
FIGURE 4.1
FIGURE 4.2: VF+ myocytes exhibit increased APD and variability in APD$_{50}$ and APD$_{90}$. Panel A and Panel B show representative action potential tracings from control (black), VF + (red) and VF- (Blue) recorded at 0.5 and 1 Hz respectively. The line indicates zero mV potential. Panel C depicts the summary APD$_{50}$ and APD$_{90}$ values at the two stimulation rates on the three groups. Panel D shows the averaged standard deviation in APD$_{90}$ measured from each myocyte from the three groups plotted as the function of stimulation frequencies (* p<0.05 vs. control, § p<0.05 vs VF-).
FIGURE 4.3: Transient outward K⁺ current (I_{to}) is reduced in both VF+ and VF- myocytes. Panel A depicts traces recorded from control, VF+ and VF- myocytes using the voltage protocol shown in the inset. Panel B depicts the average I-V relation for I_{to} in the three groups. The numbers within parentheses in panel B indicate the number of animals used to obtain the data, while n is the number of myocytes. Panel C shows the slope conductance of I_{to} measured from the linear part of the I_{to} I-V curve (panel B) from +10 mV to +50 mV (* p<0.05 vs. control).
FIGURE 4.4: Inward $I_{K1}$ is reduced in the VF+ myocytes and preserved in the VF- myocytes.

Panel A shows representative raw current tracings recorded in response to a hyperpolarizing voltage step to -140mV in the three groups. Panel B shows the summary I-V relation for the myocytes from the three groups. The numbers within parentheses indicate the number of animals used to obtain the data, while n indicates the number of myocytes. Panel C. $I_{K1}$ slope conductance is reduced in the VF+ group compared to control values. Panel D. Outward $I_{K1}$ is not different between the three groups. (* p<0.05 vs. control)
FIGURE 4.5: $I_{Kr}$ is significantly reduced in VF+ myocytes. Panel A shows representative current traces from the three groups. Panel B shows the summary I-V relationships from myocytes in the three groups. $I_{Kr}$ is substantially reduced in myocytes from VF+ animals, while VF- animals have comparable $I_{Kr}$ to controls (* p<0.05 vs. control, § p<0.05 vs. VF-).
FIGURE 4.6: $I_{\text{Ks}}$ is unchanged, with the 4-AP sensitive plateau current is reduced in the VF+ myocytes. Panel A depicts $I_{\text{Ks}}$ traces obtained from myocytes from the three groups with the summary current density-voltage relationships shown at right (p=NS). Panel B depicts 4-AP sensitive plateau current, which is reduced in VF+ myocytes; the summary current density-voltage relationships shown at right (* p<0.05 vs. control; † p<0.05 vs. VF-). Note that the percentage of cells expressing this current was the same in all groups.
FIGURE 4.7: Inhibition of \( I_{Kr} \) with d-sotalol (30 \( \mu \)M) in VF- myocytes results in the development of early afterdepolarizations at 0.5 and 1 Hz. Afterdepolarizations are marked with an *. This was a consistent finding and occurred in four of four VF- myocytes tested. In separate experiments in VF- myocytes, zero of eight myocytes displayed EADs at baseline, and zero of six control myocytes displayed EADs with 30 \( \mu \)M d-sotalol.
CHAPTER 5

IONIC REMODELING DURING CHRONIC HEART FAILURE IN CANINES: EFFECT OF CARDIAC RESYNCHRONIZATION THERAPY
5.1 INTRODUCTION

Heart failure (HF) continues to result in significant morbidity and mortality. There are an estimated 5 million Americans aged 20 years or older with HF.\textsuperscript{316} The incidence of HF approaches 1 per 100 after the age of 65 and accounts for approximately 57,000 deaths every year.\textsuperscript{316} In HF patients, sudden cardiac death (due to arrhythmic events) accounts for approximately 40-50\% of HF-related mortality.\textsuperscript{317} Cardiac resynchronization therapy (CRT) utilizes atrial-synchronized, bi-ventricular pacing to optimize cardiac synchrony and function. Recent studies have found that CRT is beneficial in patients with chronic HF.\textsuperscript{318} Specifically, CRT reduces LV size and hospitalizations, while improving symptoms, exercise capacity, systolic function, and survival.\textsuperscript{7, 318, 319} Although CRT exerts a beneficial effect in heart failure, the underlying mechanisms contributing to improved ventricular function remain poorly understood. In HF patients, prolongation of the QRS interval or signs of mechanical dyssynchrony by echocardiography are most frequently used as a criterion for CRT in clinical trials and symptomatic HF patients.

Myocardial remodeling during HF involves a number of changes that could lead to a progressive deterioration of contractile function,
including contractile protein isoform switching, desensitization of beta-adrenergic signaling, changes in extracellular matrix, alterations of electrophysiological properties, and abnormalities in intramyocyte calcium handling. In part, these are manifested as prolongation of repolarization and impaired excitation-contraction coupling, with a characteristic reduction in the amplitude of the myocyte calcium transient. This reduced calcium transient is believed to result from a decrease in intra-sarcoplasmic reticulum calcium stores, and may contribute to impaired myocyte contractility.

Clinical trials have shown that CRT results in improvements of left ventricular ejection fraction, functional status (six minute walk test) and quality of life (≥ 1 NYHA classification) and reduced ICD shock frequency in HF patients. Inspite of these clinical, precise cellular reverse remodeling that occurs during CRT is unknown at the present time. This might be important to understand how reduced incidence of arrhythmias occur after CRT. We used a chronic model of canine nonischemic cardiomyopathy (greater than one year) to study cellular myocyte remodeling after CRT. We previously reported that this chronic model of cardiomyopathy results in left ventricular dysynchrony, prolongation of in vivo repolarization, and impaired exercise capacity, measured by the six minute walk test (6MWT). In our in-vivo studies we found a reduction in
left ventricular mass and volumes and improved functional capacity (6MWT) after 6-9 months of CRT. This study was designed to study the changes in myocyte electrophysiological properties after CRT.

5.2 METHODS

All animal procedures were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. We used three groups of adult mongrel dogs (ages 1.5 – 3 years; weights 20-33 kg): normal controls (n=8), HF for 24 months (n=6) or HF (15 months) with subsequent CRT for 9 months (n=3).

5.2.1 ANIMAL MODEL

All control dogs were verified to have normal baseline cardiac function by examination by a veterinary cardiologist, followed by ECG (Biopac, MP100 software, Biopac Systems Inc, Santa Barbara, CA), 2-D and M-mode echocardiograms (GE Vivid 7) during butorphanol (0.5 mg kg\(^{-1}\) intramuscularly) sedation. These procedures were all conducted during normal sinus rhythm.

Serial six minute walk tests (6MWT) were done as previously described in a non-sedated state.\(^{328}\) These standardized exams were
conducted under controlled conditions (standard time of day, standard location, single observer, etc.)

After confirming normal cardiac function, as described above, male hound-type mongrel dogs (24.2-28.2 kg) were chronically instrumented with a Prevail 8086 pacemaker (Medtronic, Minneapolis, MN) with the pacing lead (Medtronic Model 4092) placed in the RV apex. RV apical pacing was done from a single site for up to 2 years. Pacing was performed at: 180 bpm for 2 weeks, 200 bpm for 6 weeks, 180 bpm for 2 months, 160 bpm for 6 months, then 120 bpm thereafter to achieve and maintain stable, clinically moderate LV dysfunction (i.e., minimal dyspnea, cardiomegaly, systolic murmurs of mitral regurgitation, LV FS <11.8%), as previously reported.323 Notably, 120 bpm is within the normal canine daily heart rate range.329 After 15 months of RV tachypacing, single-site RV pacing was continued in half the dogs as HF controls. The HF and CRT dogs were rate-matched at 120 bpm, while the additional control dogs were in normal sinus rhythm. Repeat examinations were obtained in all HF dogs (untreated and CRT) at six and nine months after randomization and included ECG, echocardiograms, and 6MWT.

5.2.2 CRT DEVICE IMPLANTATION
The day prior to CRT device implantation, a fentanyl transdermal patch (100 mcg) was placed (72 hours of treatment). Preoperatively, dogs received morphine sulfate (1-4 mg/kg, IV) and surgical anesthesia was induced and maintained with isoflurane (2-5%). Through a left lateral hemithoracotomy (the 5th intercostal space) and pericardiotomy, the heart was exposed and a left ventricular pacing lead (Medtronic Model 4968 or 5071) was sutured to the epicardium (posterior-lateral wall), and the chest incision was closed. A bipolar intravenous atrial pacing lead (Medtronic Model 4568) was inserted through the jugular vein into the right atrium for atrial endocardial pacing. The pacemaker for chronic RV pacing was removed and switched to a CRT pacing device (Insync III 8042 pacemaker, Medtronic Inc., Minneapolis, MN) using the same RV apical lead. CRT was started (by pacing the right atrium, right ventricular endocardium, and left ventricular epicardium. Atrioventricular (AV) delays were 110 ms, 90 ms and 100 ms, optimized by maximal aortic stroke volume (velocity-time integral). V-V delays between stimulation of the right ventricular endocardium and left ventricular epicardium were 12 ms, 4 ms and 4 ms, respectively, optimized by tissue velocity imaging to minimize mid-ventricular LV dyssynchrony.
5.2.3 MYOCYTE ISOLATION

After completing the in vivo phase of the study, ventricular myocytes were isolated from the midmyocardial layer of the LV mid-lateral free wall. After achieving adequate anesthesia, the hearts were rapidly excised via thoracotomy and perfused with cold cardioplegic solution (containing 5% Glucose, 0.1% Mannitol, 22.4 mM NaHCO₃, 30 mM KCl) injected into the coronary ostia. The left circumflex artery was cannulated for myocyte isolation as previously described. This typically yielded 50-70% rod shaped myocytes with staircase ends and sharp margins. The myocytes were incubated at room temperature in a standard incubation buffer solution containing (in mM) NaCl 118, KCl 4.8, MgCl₂ 1.2, KH₂PO₄ 1.2, glutamine 0.68, glucose 10, pyruvate 5, CaCl₂ 1, along with 1 µmol l⁻¹ insulin, and 1% BSA and until use. Only quiescent myocytes with sharp margins and clear striations were used for electrophysiolgic study.

5.2.4 ACTION POTENTIALS

Myocytes were placed in a laminin coated cell chamber (Cell Microcontrols, Norfolk, VA) and superfused with bath solution containing (in mM): 135 NaCl, 5 MgCl₂, 5 KCl, 10mM Glucose, 1.8 mM CaCl₂, 5mM HEPES, pH 7.40 with NaOH; at a temperature of 36 ± 0.5°C. Borosilicate glass micropipettes (tip resistance between 1.5 to 3 MΩ) were filled with
pipette solution containing (in mM): 100 K+-aspartate, 40 KCl, 5 MgCl$_2$, 5 EGTA, 5 HEPES, pH adjusted to 7.2 with KOH. Perforated whole cell patch clamp (using amphotericin B) was used to minimize alterations in intracellular milieu.

5.2.5 ELECTROPHYSIOLOGICAL PROTOCOLS

Myocytes were placed in a laminin coated cell chamber (Cell Microcontrols, Norfolk, VA) and superfused with bath solution containing (in mM): 135 NaCl, 5 MgCl$_2$, 5 KCl, 10mM Glucose, 1mM CaCl$_2$, 5mM HEPES, pH adjusted to 7.40 with NaOH, at a temperature of 36 ± 0.5ºC. For action potential (AP) recordings, the concentration of CaCl$_2$ in the bath solution was increased to 1.8 mM. During potassium current measurements, L-type calcium current was blocked by the addition of 2µM nifedipine to the superfusate. Solutions were changed with a six-port gravity flow system (~1 ml min$^{-1}$). Borosilicate glass micropipettes (tip resistance of 1.5 - 3 MΩ) were filled with pipette solution containing (in mM): 100 K+-aspartate, 40 KCl, 5 MgCl$_2$, 5 EGTA, 5 HEPES, pH adjusted to 7.2 with KOH. Perforated whole cell patch clamp (using amphotericin B) was used to minimize alterations in intracellular milieu. For voltage clamp experiments, only recordings with an access resistance < 20 MΩ were included in the analyses. Series resistance compensation (~70%) was
used for current recordings. For determination of drug-sensitive currents, only cells with less than a 20% change in access resistance were included in the analyses. All drug sensitive currents were recorded after 3-5 minutes of drug superfusion, which in our pilot experiments resulted in steady-state current blockade.

APs were recorded with perforated whole cell patch techniques, as described above. APs were measured as the average of the last 10 (steady-state) APs, obtained during a train of twenty five APs at each stimulation rate.

Transient outward potassium current (I_{to}) was elicited by a series of 100 ms test potentials from –20 to +50mV, from a holding potential of –60 mV. I_{K1} was elicited by voltage steps from -140 to +40mV from a holding potential of -40 mV. The current was measured at the end of each 100 msec test pulse. I_{K1} inward conductance (mS/cm²) was determined by calculating the slope of the linear portion of the current density-voltage relationship from –140 mV to –100 mV. Peak outward I_{K1} density was measured as the current at -60 mV (I_{-60}).

Rapid (I_{Kr}) and slow (I_{Ks}) components of the delayed rectifier current were elicited using 10mV incremental voltage steps from -40mV to +50 mV from a holding potential of -50 mV. I_{Kr} was measured as the d-sotalol-sensitive (100µM) step current, while d-sotalol-insensitive tail currents
were used for tail $I_{KS}$ measurement. During $I_{Kr}$ and $I_{KS}$ recordings the bath solution also contained 4-Aminopyridine (100 µM) to prevent any potential contamination with “$I_{Kur}$-like” plateau current.

Data acquisition was performed with Clampex 8.0 software (Axon Instruments, Union City, CA, USA) and an Axopatch 200A patch clamp amplifier (Axon Instruments Inc, CA, USA).

5.2.6 SOLUTIONS AND CHEMICALS

All chemicals for buffer and stock solution preparation were purchased from Fisher Scientific (USA), Sigma Aldrich (St.Louis, MO, USA) and Invitrogen Inc (Carlsbad, CA, USA). Stock solutions of nifedipine and amphotericin B were made fresh on the day of the experiments. Nifedipine (3.5mg) was dissolved in 1 ml of ethyl alchohol (10mM stock). This stock solution (200 µL) was added to 800 µL of bath solution to yield a 2 mM stock. The 2 mM stock solution was subsequently diluted (1:1000) in cell bath solution (listed below). Amphotericin B (6 mg) was dissolved in 100 µL of di-methyl sulfoxide (DMSO). Following this, 20 µL of the amphotericin-B stock was added to 5 mL of pipette solution (see above). The pipette solution was vortexed, sonicated and re-vortexed prior to filling the glass micropipettes for patch clamp measurements. Isoproterenol solutions were prepared daily from commercially available
injectable solutions (Sanofi Winthrop Pharmaceuticals, New York NY). D-

sotalol was obtained from Merck Research Laboratories, West Point PA. All nifedipine, isoproterenol, and amphotericin B solutions were protected from exposure to light.

5.2.7 STATISTICAL ANALYSIS

Acquired data were analyzed using Clampfit 8.0 (Axon Instruments) and Origin 6.1 (OriginLab, Northampton, MA USA). Currents were normalized to cell capacitance (measured as picofarads (pF)) and are expressed as pA pF⁻¹. Action potential durations and current densities were analyzed by ANOVA with post hoc least significant difference testing as appropriate (SAS for Windows v9.1, Cary, NC, USA). All data are presented as mean ± SE.

5.3 RESULTS

Compared to control myocytes (145 ± 6 pF), HF resulted in significant myocyte hypertrophy (211 ± 12 pF, p<0.05), indicated as an increase in membrane capacitance. There was not a significant reduction in membrane capacitance in the myocytes from the CRT dogs compared to the untreated HF dogs (211 ± 12 pF vs. 230 ± 12 pF, respectively, p=0.14).
We observed significant HF-induced prolongation of LV myocyte action potential durations (Figure 5.1). CRT reversed the HF-induced action potential prolongation, resulting in action potential durations which did not differ from normal control myocytes. These experiments also revealed a significant reduction in the resting membrane potential (RMP) in the myocytes from the HF dogs compared to controls (-75.8 ± 1.3 mV vs. -79.4 ± 0.9 mV, respectively; p=0.03). In contrast, the resting potential in the CRT myocytes (-76.9 ± 0.7 mV) did not differ from controls (p=NS). These results suggest that subsequent to CRT, there is a re-stabilization of the diastolic membrane potential, and a normalization of repolarization time in isolated myocytes from the LV midmyocardium.

A summary of the inward rectifier current (I_{K1}) data is shown in figure 5.2. Consistent with the depolarization of the RMP, a significant decrement in the inward I_{K1} slope conductance during HF was found (p<0.05 vs. control and CRT). CRT treatment restored this to values not different from control. Outward I_{K1} was not different between the three groups.

Transient outward K^+ current (I_{to}) data is shown in figure 5.3. I_{to} was reduced during heart failure. Interestingly, CRT treatment did not alter the I_{to} densities compared to HF. Time constants of inactivation was not different between the three groups (p = 0.092).
\( I_{Kr} \) was significantly reduced in HF compared to control (\( p<0.05 \) (Figure 5.4) while HF did not induce a change in \( I_{Ks} \) tail current density (Figure 5.5). There was no difference in the activation time constants for \( I_{Ks} \) (886±58ms vs. 875±92ms in control and HF respectively, \( p=0.23 \)). We were unable to assess differences in \( I_{Kr} \) and \( I_{Ks} \) densities after CRT due to limited sampling.

5.4 DISCUSSION

Chronic heart failure is associated with multiple manifestations; at the myocyte level it is associated with impaired ventricular repolarization and impaired excitation-contraction coupling.\(^{321, 322}\) Cardiac resynchronization therapy (CRT) has been shown to be beneficial for the treatment of symptomatic HF in patients with abnormal ventricular activation; specifically, those patients that have dyssynchronous activation of the left ventricle.\(^{7, 318}\) We used our previously described canine model of non-ischemic heart failure for this study. This model results in stable, non-reversible cardiomyopathy, and emulates the chronic non-ischemic human HF population, which is the most likely to respond favorably to CRT.\(^{323, 330}\) Similar to this human HF population, our cohort of dogs with chronic HF exhibit prolongation of the QRS and ventricular dyssynchrony. We have previously reported that in this chronic canine model of HF, there is
substantial reduction in intra-SR calcium stores which is attributable to an increase in diastolic calcium leak from the calcium release channels (ryanodine receptors).\textsuperscript{322}

In left ventricular midmyocardial myocytes, we observed the well-known HF-induced prolongation of repolarization of the action potential. CRT normalized HF-induced prolongation of myocyte repolarization. Our APD results correlated well with our \textit{in vivo} data, where a trend toward an attenuation in the QTc interval was seen (277 ± 7 ms vs. 267 ± 3 ms, for HF and CRT groups, respectively, $p = 0.08$)\textsuperscript{331}. This suggests that altering \textit{in vivo} activation of the ventricle can modulate myocyte electrophysiology. In a previous report, Spragg and colleagues studied the electrophysiologic consequences of ventricular dyssynchrony using a canine model of left bundle branch block. In their model, although LV systolic function was maintained, regional abnormalities in the APD occurred in the ventricle.\textsuperscript{332} Thus, altered mechanical synchrony of ventricular contraction appears to be sufficient to modulate myocyte electrophysiology, in particular repolarization. Further studies will be necessary to determine the contributions of improved ventricular synchrony vs. overall improvements in heart failure as the mediator of improved myocyte function during CRT.
5.4.1 REGULATION OF K⁺ CURRENTS BY CRT

Our studies revealed two very interesting findings. The first observation is the restoration of inward \( I_{K1} \) conductance with CRT treatment. The second observation is a similar degree of \( I_{to} \) reduction in HF and CRT groups. The reduction in \( I_{to} \) density persists even after normalization of APD in the CRT group. Our study presents the first evidence where improving synchrony with CRT in HF substantially improves RMP. \( I_{to} \) has been shown to play an important role in determining APD in mice.\(^{333-335}\) Previous reports in canine HF have suggested that reduced \( I_{to} \) is a contributor to HF-induced APD prolongation. Our study suggests that in canines, CRT-induced normalization of the APD occurs without improvements in \( I_{to} \) density. Evidence from rodent studies suggest that \( I_{to} \) has a major role in determining APD.\(^{333}\) In contrast, computational studies suggest that \( I_{to} \) has a minor role in determining human and canine APD.\(^{336, 337}\) We provide the first experimental proof in a disease model supporting the computational studies suggesting a minimal role for \( I_{to} \) in regulation of APD in canine ventricle.

Our studies show that \( I_{Kr} \) is downregulated, while \( I_{Ks} \) is unaltered in HF providing a causal mechanism for APD prolongation in our HF model.
Our existing evidence points to the restoration of inward \( I_{K1} \) alone. Based on our findings in our current study, we hypothesize that CRT might restore \( I_{Kr} \) and this warrants further experimentation.

5.5 CONCLUSIONS

CRT improves cellular electrophysiology. Reverse ionic remodeling occurs after CRT and is manifested as restoration of APD. This is accompanied by restoration of inward \( I_{K1} \) conductance, while the reductions in \( I_{to} \) still persist. These results suggest that \( I_{to} \) is not a critical mediator of APD. Additional studies will be needed to address if \( I_{Kr} \) or \( I_{Ks} \) are involved in mediating the improvements seen in APD after CRT.

5.6 LIMITATIONS

It is possible that the heart rate of 120 bpm may have attenuated the CRT-induced reverse remodeling in our dogs, compared to that occurring in humans with a normal heart rate. Since heart rate is a critical modulator of cardiomyopathy in this model, this required a matched heart rate for the two treatment groups. We have previously reported this heart rate is sufficient to maintain stable impairment of LV function after 12-15 months of RV tachypacing;\(^{330}\) and notably it is well within the daily range...
in normal dogs. Furthermore, the use of RV rather than right atrial
tachypacing means that dyssynchronous ventricular activation, rather than
solely heart rate, may be contributing to the cardiomyopathy. However,
due to the high vagal tone in resting dogs, right atrial pacing cannot
feasibly maintain a rapid ventricular rate, necessitating RV pacing.

Due to technical limitations with the placement of the LV pacing lead, we placed the lead on the posterior-lateral epicardium of the left ventricle. The majority of patients have the LV pacing lead placed via the coronary sinus, and our methodology in these experiments does not replicate the site used in these patients. While the epicardial lead placement does not replicate the site used in the majority of patients, we note that this approach is used in a subset of patients. Most importantly, either approach to lead placement results in similar electrical activation of the LV epicardium.

This heart failure model is a non-ischemic (minimally reversible) cardiomyopathy model, and it is unknown if our observations would extend to an ischemic cardiomyopathy population. Moreover, our treated animals were managed with CRT alone, and any potential interactions or synergies between pharmacologic HF therapies and CRT were not addressed.
Since only myocytes from the lateral LV midmyocardium were studied, it is unknown if similar changes occur in other layers or regions of the ventricles. Additional studies will be required to determine the generalizability of these results to other regions of the ventricles and to the treatment of other forms of cardiomyopathy.

One computational study proposed an accelerated time course for $I_{\text{Ks}}$ under conditions where $I_{\text{Kr}}$ could be downregulated.\textsuperscript{338} But our study indicates an unchanged $I_{\text{Ks}}$ activation time course. However, it must be pointed out that this might be due to limited sampling (n=6 myocytes) and further studies to obtain higher sample size is needed.

We did not assess $I_{\text{Kr}}$ or $I_{\text{Ks}}$ densities after CRT in this study. CRT mediated APD shortening in HF might be due to changes in $I_{\text{Kr}}$ or $I_{\text{Ks}}$ current densities and/or protein changes. This will be addressed in future studies.
FIGURE 5.1: Normalization of HF-induced prolongation of action potential duration with CRT. Panel A shows the representative raw action potential traces obtained from a myocyte from each group (Control in black, HF in blue, CRT in magenta). Panel B shows the average action potential durations at 50% (APD<sub>50</sub>) and 90% (APD<sub>90</sub>) in myocytes from the three groups (* p<0.05 vs. control, § p<0.05 vs CRT).
FIGURE 5.2: The inward component of inward rectifier current (I_k1) normalized following CRT treatment. Panel A shows representative normalized current current traces from myocytes isolated from the 3 groups. Panel B shows the summary I-V relationships for all myocytes tested. Panel C shows the inward conductance in the 3 groups (* p<0.05). Panel D shows the peak outward current measured at -60 mV (NS = not significant).
FIGURE 5.3: Transient outward $K^+$ current ($I_{to}$) is unaltered by CRT.
Panel A shows representative normalized current traces from the three groups in response to a depolarizing voltage step to +50 mV from a holding potential of -60 mV. Panel B shows the summary I-V relationships from the three groups (* p<0.05 vs. control).
FIGURE 5.4: Rapid component of the delayed rectifier current ($I_{Kr}$) is reduced in chronic HF. Panel A shows the d-sotalol sensitive raw current recordings of $I_{Kr}$ from a control (black) and chronic HF myocytes (blue) in response to a depolarizing voltage step to +40 mV from a holding potential of -50 mV. Panel B shows the average $I_{Kr}$ densities in both the groups (* $p<0.05$ vs. control).
FIGURE 5.5: Slow component of delayed rectifier current ($I_{Ks}$) is unchanged in chronic HF. Panel A shows normalized $I_{Ks}$ traces recorded in response to a voltage step to +50 mV from a holding potential of -50 mV. Panel B shows the I-V relationship for $I_{Ks}$ tail current density for the two groups.
CHAPTER 6

ATRIAL IONIC REMODELING IN CHRONIC HEART FAILURE: ROLE OF REDOX AND ANTIOXIDANT PROCESSES
6.1 INTRODUCTION

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and is the leading contributor to stroke in the United States.\textsuperscript{4} Heart failure (HF) is a major pandemic and accounts for approximately half a million new cases every year in the US.\textsuperscript{4} In HF patients, atrial fibrillation results in a 4.5-5.9 fold increase in mortality.\textsuperscript{4} Our laboratory and others have demonstrated that oxidative stress plays a significant role in the pathogenesis of heart failure and AF.\textsuperscript{339-341} The right ventricular (RV) tachypacing model of heart failure has been proposed to be a promising animal model for studying heart failure.\textsuperscript{342} Several experimental studies have used RV tachypacing at very high rates (220-250 beats per minute) to study heart failure.\textsuperscript{321, 343-349} However, once the RV pacing is stopped, the animals revert to the baseline hemodynamic values within 2 weeks.\textsuperscript{344} Though the fibrosis and conduction abnormalities induced by short term RV tachypacing in the atria and the ventricle remains after the cessation of tachypacing, the maximum duration of AF observed (unlike the clinical situation) was not sustained typically lasting only approximately 750 seconds.\textsuperscript{9, 343, 344} AF during HF is often persistent and HF is a clinical syndrome that takes months to years to develop. The lack of knowledge
about the substrate for persistent AF during chronic HF is an important gap in our understanding.

We hypothesized that unlike short term HF, atrial remodeling in HF is different in chronic HF. We have developed a model of chronic canine heart failure (16-18 weeks of RV tachypacing with a modified induction of heart failure). Our pilot experiments indicate that this model produces an excellent substrate for persistent AF. Our results differ from previous studies due to the chronicity of HF which induces substrate for persistent and permanent AF.

Our hypothesis was that oxidative stress plays a critical role in chronic atrial remodeling in HF and we show that certain aspects of HF induced atrial ionic remodeling are reversible, suggesting a potential role for redox processes in atrial remodeling in heart failure.

6.2 METHODS

All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. Sixteen hound type dogs (M/F; 2-3 years of age) had an RV pacemaker lead implanted in the RV apex. The sixteen dogs were divided into three groups of nine, three and four dogs respectively. The pacemaker was implanted in a subcutaneous pocket in the chest wall and leads were tunneled subcostally to the
pacemaker. The chest was closed and animals were allowed to recover.

Following surgical recovery, the RV was paced using the following protocol as showed below to induce heart failure. Electrocardiograms, 2D B- and M-mode echocardiograms were performed in all dogs during butorphanol sedation (0.5 mg kg⁻¹, IM) at baseline and during the pacing protocol, during sinus rhythm. Five age-matched dogs served as controls.

In the first group of dogs (n=9 dogs), reversibility of HF was assessed by monitoring echocardiograms after cessation of tachypacing. RV tachypacing was terminated in nine dogs after 16 weeks of tachypacing and serial echocardiograms were performed to assess the LV fractional shortening to assess the recovery from tachypacing. In the second group of dogs (n=3), at the end of the 16 week pacing protocol, AF inducibility was tested using a standard S₁-S₂ protocol in the right atrium. In the last group (n=4 dogs), myocytes were isolated from the left atrial appendage using collagenase perfusion via the left circumflex artery.

Briefly, dogs were anesthetized by intravenous injection of pentobarbital sedation (0.5 mg kg⁻¹, IM) at baseline and during the pacing protocol, during sinus rhythm. Five age-matched dogs served as controls.

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Briefly, dogs were anesthetized by intravenous injection of pentobarbital sedation (0.5 mg kg⁻¹, IM) at baseline and during the pacing protocol, during sinus rhythm. Five age-matched dogs served as controls.
sodium (Dosage: 120 mg kg\(^{-1}\) for the first 4.5 kilograms and 60 mg kg\(^{-1}\) for every 4.5 kilograms thereafter); after achieving anesthesia the hearts were rapidly excised and perfused with cold cardioplegic solution (containing 5% Glucose, 0.1% Mannitol, 22.4 mM NaHCO\(_3\), 30 mM KCl) injected into the coronary ostia. The left circumflex coronary artery was cannulated for myocyte isolation. Following the washout of blood from the heart, collagenase (Worthington type 2, 0.65 mg ml\(^{-1}\)) and protease-free bovine serum albumin (0.65mg ml\(^{-1}\)) were added to the perfusate (100 ml). After 30-45 minutes of perfusion, the left atrial appendage was excised and the digested tissue was shaken in a water bath at 37ºC for an additional 10-15 minutes. This typically yielded 40-60% rod shaped myocytes with staircase ends and sharp margins. The myocytes were stored at room temperature in a standard incubation buffer (IB) solution containing (in mM) NaCl 118, KCl 4.8, MgCl\(_2\) 1.2, KH\(_2\)PO\(_4\) 1.2, glutamine 0.68, glucose 10, pyruvate 5, CaCl\(_2\) 1, along with 1 µmol l\(^{-1}\) insulin, and 1% BSA until used. In some experiments, myocytes were stored in IB containing 10 mM N-acetyl cysteine (NAC), a membrane-permeant precursor of glutathione. All myocyte experiments were conducted within ten hours of isolation.
6.2.1 SOLUTIONS AND CHEMICALS

All chemicals for buffer and stock solution preparation were purchased from Fisher Scientific (USA), Sigma Aldrich (St.Louis, MO, USA) and Invitrogen Inc (Carlsbad, CA, USA). Stock solutions of nifedipine, amphotericin B and 4-aminopyridine were prepared fresh daily. Nifedipine (3.5 mg) was dissolved in 1 ml of ethyl alcohol (10 mM stock). This stock solution (200 µL) was added to 800 µL of bath solution to yield a 2 mM stock. The 2 mM stock solution was subsequently diluted (1:1000) in cell bath solution (listed below). Amphotericin B (6 mg) was dissolved in 100 µL of di-methyl sulfoxide (DMSO). Following this, 20 µL of the amphotericin-B stock was added to 5 mL of pipette solution (see below). The pipette solution was vortexed, sonicated and re-vortexed prior to filling the glass micropipettes for patch clamp measurements. Isoproterenol solutions were prepared daily from commercially available injectable solutions (Sanofi Winthrop Pharmaceuticals, New York NY). D-sotalol was obtained from Merck Research Laboratories, West Point PA. All nifedipine, isoproterenol, and amphotericin B solutions were protected from exposure to light.
6.2.2 ELECTROPHYSIOLOGICAL PROTOCOLS

Myocytes were placed in a laminin coated cell chamber (Cell Microcontrols, Norfolk, VA) and superfused with bath solution containing (in mM): 135 NaCl, 5 MgCl₂, 5 KCl, 10mM Glucose, 1mM CaCl₂, 5mM HEPES, pH adjusted to 7.40 with NaOH, at a temperature of 36 ± 0.5ºC. For action potential (AP) recordings, the concentration of CaCl₂ in the bath solution was increased to 1.8 mM. During potassium current measurements, L-type calcium current was blocked by the addition of 2µM nifedipine to the superfusate. Solutions were changed with a six-port gravity flow system (~1 ml min⁻¹). Borosilicate glass micropipettes (tip resistance of 1.5 - 3 MΩ) were filled with pipette solution containing (in mM): 100 K⁺-aspartate, 40 KCl, 5 MgCl₂, 5 EGTA, 5 HEPES, pH adjusted to 7.2 with KOH. Perforated whole cell patch clamp (using amphotericin B) was used to minimize alterations in intracellular milieu. For voltage clamp experiments, only recordings with an access resistance < 20 MΩ were included in the analyses. Series resistance compensation (~70%) was used for current recordings. For determination of drug-sensitive currents, only cells with less than a 20% change in access resistance were included in the analyses. All drug sensitive currents were recorded after 3-5
minutes of drug superfusion, which in our pilot experiments resulted in steady state current blockade.

Action Potentials (APs) were recorded with perforated whole cell patch techniques, as described above. AP durations were measured from the average of the last 10 (steady-state) APs, obtained during a train of twenty five APs at each stimulation rate.

Transient outward potassium current (Ito) was elicited by a series of 100 ms test potentials from –50 to +50 mV, from a holding potential of –60 mV (to minimize current inactivation). The peak current elicited early in 300 ms test pulse was subtracted from the steady state current (towards the end of test pulse) to measure Ito. The steady state current was used to define the sustained outward K+ current (IKsus). IK1 was elicited by a voltage step to – 80 mV from a holding potential of -60 mV. Inward IK1 was measured as the current at the end of each test pulse.

Rapid (IKr) and slow (IKs) components of the delayed rectifier current were elicited using 10mV incremental voltage steps from -40mV to +50 mV from a holding potential of -50 mV.350 IKr was measured as the d-sotalol-sensitive (100µM) current, while d-sotalol-insensitive tail currents were used for measure IKs (tail currents). During IKr and IKs recordings the bath solution also contained 4-Aminopyridine (50 µM) to prevent any potential contamination with IKur.
$I_{Kur}$, a 4-aminopyridine sensitive sustained outward potassium current, was elicited from a holding potential of -40 mV using 10mV voltage steps from -20mV to +50 mV. A combination of a -40 mV holding potential and an 80 ms prepulse to +30mV was used to inactivate $I_{to}$ and prevent other current contamination. The sustained 4-AP-sensitive $I_{Kur}$ was measured as the steady-state difference current, recorded after a minimum of four minutes of superfusion with 4-AP.

Data acquisition was performed with Clampex 8.0 software (Axon Instruments, Union City, CA, USA) and an Axopatch 200A patch clamp amplifier (Axon Instruments Inc, CA, USA).

6.2.3 STATISTICAL ANALYSIS

Acquired data were analyzed using Clampfit 8.0 (Axon Instruments) and Origin 6.1 (OriginLab, Northampton, MA USA). Currents were normalized to cell capacitance and are expressed as pA pF$^{-1}$. Action potential durations and current densities were analyzed by ANOVA with post hoc least significant difference testing as appropriate (SAS for Windows v9.1, Cary, NC, USA). All data are presented as mean ± SE.
6.3 RESULTS

Figure 6.1 (Panel A) shows the LV fractional shortening in a subset of dogs (n=9) which decreases gradually during RV tachypacing and at 16 weeks of RV tachypacing, stabilizes around 15%. Following cessation of RV tachypacing (16 weeks), the LV fractional shortening gradually recovers and stabilizes at 7 weeks of recovery, although the values at 7 weeks of recovery remain significantly lower than pre-pacing baseline fractional shortening (p<0.05). Panel B shows an ECG and atrial electrogram recorded after AF induction with a single premature stimulus (after 16 weeks of RV tachypacing) in the right atrium. AF was sustained for up to 3 months.

Myocytes from the HF left atrial appendage had higher capacitance compared to controls (HF: 146±11 pF vs. Control: 95±4 pF, p<0.05). Figure 6.2 shows the representative action potential traces from the three groups. Depolarization of the resting membrane potential was seen in the HF atria group compared to control (HF atria: -68±3 mV vs. Control: -74±2 mV, p <0.05). The action potential durations at 50% and 90% repolarization (APD$_{50}$ and APD$_{90}$ respectively) were significantly shorter in the HF atrial myocytes compared to controls. In the HF atrial myocytes treatment with NAC restored the APD$_{50}$ to values not different from
control. In contrast, NAC incubation did not affect \( APD_{90} \) values in the HF Atria + NAC group \( (p<0.05 \text{ vs. control}) \). After NAC incubation, the resting membrane potential and the cell capacitance was significantly different than the controls and did not differ from HF atria group \( \text{(RMP: -62±1.5 mV, Cell capacitance: 178±17 pF, } p<0.05 \text{ vs.control). Incubation of control atrial myocytes did not alter APD values (data not shown, } n=3 \text{ cells).} \)

Figure 6.3 shows the transient outward \( K^+ \) current \( (I_{to}) \) from the three groups. \( I_{to} \) was significantly augmented in atrial myocytes from the HF group at all voltages positive to -20mV. NAC incubation reduced the \( I_{to} \) density to values that did not differ from control. The sustained outward \( K^+ \) current \( (I_{K_{sus}}) \) was reduced in HF atria and HF atria + NAC compared to control \( (p<0.05) \). Incubation of control atrial myocytes with NAC failed to alter \( I_{to} \) density \( \text{(data not shown, } n=3 \text{ cells)} \)

Figure 6.4 shows the representative inward component of the inward rectifier current \( (I_{K1}) \) traces from the three groups \( \text{(Panel A)} \) and the summary data in Panel B. Inward \( I_{K1} \) was significantly reduced in the HF atria and the HF atria + NAC group \( (p<0.05 \text{ vs.control}). \)

The 4-AP \( \) (50µM) sensitive \( I_{Kur} \) current traces are shown in figure 6.5 \( \text{(panel A)} \). The average I-V curve demonstrates a significant down-regulation of \( I_{Kur} \) in HF atria compared to control \( (p<0.05). \) Incubation of myocytes with NAC did not affect \( I_{Kur} \) density.
Figure 6.6 shows the raw traces of the d-sotalol (100 µM) insensitive slow component of the delayed rectifier current (I_{ks}) (panel A). HF induces a reduction in I_{ks} tail current density compared to control (p<0.05). The rapid component of the delayed rectifier current was not different between control and heart failure atrial myocytes (Panel B).

Figure 6.7 shows the effect of peroxynitrite donor, SIN-1 on control atrial myocyte action potentials. Panel A shows the raw action potential traces at baseline and after superfusion with 200 µM SIN-1. Upon superfusion with SIN-1, the resting membrane potential of the atrial myocyte is reduced suggesting direct peroxynitrite effects on inward rectifier K⁺ current (Panel B). Action potential duration was significantly longer (p<0.05) after SIN-1 superfusion (Panel C). Incubation of control atrial myocytes with the peroxynitrite scavenger, urate (500 µM) alone or urate incubation followed by SIN-1 superfusion did not alter the atrial APD. This suggests acute peroxynitrite exposure to left atrial myocytes can prolong action potential duration and reduces the inward rectifier current; thereby causing depolarization of the resting membrane potential.

Figure 6.8 shows the effect of SIN-1 on control atrial transient (I_{to}) and sustained (I_{Ksus}) outward and K⁺ currents. SIN-1 superfusion did not alter the current densities measured.
6.4 DISCUSSION

HF results in a four to six fold increased risk for developing AF.\textsuperscript{4} In addition, studies indicate that AF is present in approximately 15-30% of HF patients.\textsuperscript{4} Using a chronic, minimally reversible model of canine tachypacing model of HF; we show that AF occurring in this HF model is sustained and permanent. Left atrial ionic remodeling as a consequence of chronic HF might offer a substrate, and in combination with structural changes, act to stabilize AF in chronic HF.

6.4.1 DIFFERENCES WITH PREVIOUS HF STUDIES

Experimental studies have used a canine short-term ventricular tachypacing for 2-4 weeks at very high rates (220-240 beats/min) to study the pathogenesis of HF and to understand the atrial ionic remodeling.\textsuperscript{9, 344, 347} HF is a chronic syndrome lasting months to years and is typically irreversible. Experimental studies using short-term tachypacing produces signs of HF (enlarged heart, pulmonary edema, ascites, etc.) but these clinical manifestations (e.g.; reduced fractional shortening) revert back to baseline values upon cessation of RV tachypacing.\textsuperscript{344} Our results indicate that RV tachypacing carried out over 4 months using our pacing protocol results in a steady decrement in LV fractional shortening and that even after cessation of RV tachypacing, LV fractional shortening is reduced.
significantly compared to baseline values (figure 6.1) and results in minimally reversible heart failure. Li et al showed that in short term HF, inducible AF lasted for 600-750 seconds. In our model, we show that AF was inducible and was persistent up to 3 months post induction. Our studies indicate that chronic HF results in a substrate for sustained AF.

We suggest that unlike short term HF, ionic remodeling during chronic HF results in a specific form of electrical remodeling characterized by shorter action potential durations (both APD$_{50}$ and APD$_{90}$) at all frequencies tested. This is in direct contrast to studies in short term HF where APD prolongation was seen in canines paced for 4 weeks at 240 beats per minute.

Our results indicate that reductions in APD$_{50}$ and APD$_{90}$ during heart failure can be partly reversed by incubating myocytes with the glutathione precursor, N-acetylcyesteine (NAC). Selective NAC reversal of HF-induced changes in APD$_{50}$ without altering APD$_{90}$ indicates that ion currents that regulate APD$_{50}$ ($I_{lo}$, $I_{Kur}$ and potentially $I_{Ca-L}$) might be redox-modulated.

6.4.2 REDOX MODULATION OF CARDIAC ION CURRENTS
Transient outward K⁺ current (I_{to}) was upregulated in chronic HF left atrial myocytes, and incubation of HF myocytes with NAC reversed higher I_{to} densities to values not significantly different from control. In contrast, the sustained outward K⁺ current (I_{KSUS}) was reduced in chronic HF and was not affected by NAC incubation. Redox modulation of I_{to} has been extensively studied in rat diabetic and myocardial infarction models. I_{to} has been shown to be reduced in rat diabetes and after myocardial infarction. Rozanski et al showed in a series of elegant studies that I_{to} density is metabolically regulated. Improving the cellular redox state by increasing reducing equivalents by incubating the diseased myocytes in insulin (to raise glucose uptake, therefore raising intracellular NADH levels), or in glutathione and/or its precursor (N-acetyllysisteine) produced a reversal of reduced I_{to} levels back to control values. These studies were the first to suggest that in diseased states where metabolic states are altered, I_{to} reductions are reversible. The authors suggested potential reversible cysteine oxidation of channel during conditions of oxidative stress that are readily reversed by reducing agents. Our studies provide similar results where I_{to} was increased in chronic HF, in contrast to reductions in I_{to} seen by Rozanski et al, but the changes were still modulated by myocyte redox balance. This suggests a very strong coupling between metabolic states of the cardiac myocyte and I_{to} density.
One potential mechanism is increased levels of superoxide in diseased heart as occurs in heart failure\textsuperscript{358-361} or diabetes\textsuperscript{351, 356} to produce direct cysteine oxidation on the Kv4 channel subunit. Another recent study documented that Kvβ accessory subunit binds to Kv4 \( \alpha \) subunit and modulated current density possibly by acting as a chaperone for membrane trafficking.\textsuperscript{45} Further studies have documented the Kvβ subunit has a NADH binding pocket and could act as the redox sensor for the Kv4 channels.\textsuperscript{47}

6.4.3 DELAYED RECTIFIER CURRENTS IN CHRONIC HF ATRIA

Ultra-rapid delayed rectifier current (\( I_{Kur} \)) constitutes a major component of \( I_{Ksus} \) in the canine atria. \( I_{Kur} \) assessed as 50 \( \mu \)M 4-AP sensitive current was reduced in HF and was not affected by NAC incubation. These results are similar to those obtained by Rozanski \textit{et al}, where \( I_{Ksus} \) was unchanged after incubation with reducing agents.\textsuperscript{351} The slow component of delayed rectifier current (\( I_{Ks} \)) was reduced in chronic HF atrial myocytes compared to control. Deciphering modulation of rapid component of the delayed rectifier current (\( I_{Kr} \)) in chronic HF atrial myocytes warrant further study.
These results indicate that certain aspects of atrial ionic remodeling are redox sensitive even in chronic HF. Studies focusing on redox regulation of cardiac ion channels have relied on indirect evidence (incubating myocytes with reducing equivalents\textsuperscript{351-357}) or used over-expression of ion channel subunits in cultured cells\textsuperscript{362, 363} to study the direct effects of specific free radical species like superoxide and nitric oxide on $K^+$ currents.

6.4.4 POTENTIAL COMPARTMENTALIZATION OF REDOX SPECIES TO ION CHANNELS

We performed pilot studies to understand why only $I_{to}$ was augmented by NAC incubation, while the other currents were not. Peroxynitrite (ONOO$^-$) is produced in heart failure from increased superoxide and nitric oxide levels. Recent studies have documented that nitric oxide synthase 2 (or inducible nitric oxide synthase) activity is increased in human and experimental HF\textsuperscript{364}. Under normal conditions and in the presence of co-factors like tetrahydrobiopterin (BH$_4$) and arginine, NOS2 makes NO in the process of converting arginine to citrulline\textsuperscript{365, 366}. Increased NOS2 levels in heart failure are thought to result in increased superoxide and NO production\textsuperscript{339, 364}. This results in production of peroxynitrite which produces irreversible nitration on tyrosine residues in
the cellular proteins. Our results indicate that increases in Kv4 encoded $I_{\text{to}}$ density can be modulated by increasing the intracellular glutathione. This argues against an irreversible protein nitration of Kv4 channels, i.e., potentially a reversible oxidative modulation. Other potential mechanisms might be a change in the subunit interaction of Kv$\beta$ subunits with Kv4 subunits is an intriguing possibility and merits further investigation.

We decided to study how different ion currents might be modulated by specific free radical species by testing if SIN-1 (an ONOO$^-$ donor) alters AP and $K^+$ currents in the canine atria. Our preliminary results indicate that SIN-1 causes a depolarization in the resting membrane potential of the atrial myocyte, and that ONOO$^-$ produces acute effects by potentially reducing $I_{K1}$ density (presumably through interactions with $K_{ir}$ channels). We failed to observe specific modulation of $I_{\text{to}}$ or $I_{K\text{sus}}$ by acute superfusion of 200 $\mu$M SIN-1. APD prolongation seen in our experiments might be explained by $I_{\text{Ca-L}}$ augmenting effects of SIN-1, as $K^+$ channels which mediate APD ($I_{\text{to}}$ and $I_{K\text{sus}}$) are not altered with SIN-1 superfusion. Potential explanations include cysteine oxidation of Kv4 channels, while $K_{ir}$ channels are nitrated with ONOO$^-$, thereby providing explanations for their upregulation and dowregulation (and reversibility or lack thereof) in
chronic HF. The mechanism by which \( I_{Kur} (Kv1.5) \) is modulated by other free radical species is unknown at the present time. One recent study points to nitric oxide mediated reduction in Kv1.5 channels where the channels can be S-nitrosylated due to direct binding of NO to cysteine residues.\(^{362}\) We have not evaluated that possibility in these studies.

### 6.5 CONCLUSIONS

Our results open up two potential areas of research that merit further investigation. First is the idea that certain aspects of ionic remodeling remain redox sensitive even in chronic HF. Second, the possibility of different ion channels being modulated by selective free radical species, potentially points to potential compartmentalization of free radicals to specific channels.

### 6.6 LIMITATIONS

Evaluation of NAC effects on the rapid and slow component of delayed rectifier currents in HF atria was limited due to availability of animals. The L-type calcium current and sodium calcium exchanger current which are shown to be reduced and increased respectively in short-term HF were not assessed in our studies due to limited availability of chronic HF animals. These issues need to be addressed in future studies. All our
experiments suggesting free radical species’ effects on cardiac ion current function are indirect. Direct studies to identify the residues altered by different species need to be identified and warrants further investigation.
FIGURE 6.1: Minimally reversible chronic HF model results in a substrate for inducible, persistent and permanent AF

Panel A shows the LV fractional shortening (%) from a subset of dogs assessed during the tachypacing protocol and after cessation of tachypacing (recovery). Panel B shows the surface ECG at the top and atrial electrogram recorded after a premature stimulus induced AF. Data was acquired at 3 months after AF was induced. See text for results and discussion.
FIGURE 6.2: Left atrial action potential shortening is seen in chronic HF. Panel A shows the representative action potential traces recorded from a control (black), HF atria (red) and HF atria incubated with NAC (blue). Action potential duration at 50% (APD_{50}) and 90% (APD_{90}) repolarization are shown in Panel B and Panel C respectively for the three groups. The numbers within parentheses indicate the numbers of animals used to obtain the data.
FIGURE 6.3: Transient outward K⁺ current (I_{to}) is reduced in HF and susceptible to redox regulation. Panel A shows the raw I_{to} traces recorded in response to a depolarizing voltage step to +50 mV from a holding potential of -60 mV. I_{to} density measured at different voltages from the three groups in panel B, while sustained outward K⁺ current is shown in panel C. The numbers within parentheses indicate the numbers of animals used to obtain the data.
FIGURE 6.4: Inward $I_{K1}$ is reduced in chronic HF and is insensitive to NAC incubation. Panel A shows the raw traces of inward $I_{K1}$ recorded in response to a hyperpolarizing voltage step to -80 mV from -60 mV. The average $I_{K1}$ densities from the three groups are shown in Panel B (n=7-8 cells/group, 4 animals).
FIGURE 6.5: 50 µM 4-aminopyridine sensitive $I_{\text{Kur}}$ is reduced in chronic HF and is insensitive to NAC incubation.

Panel A shows the 50 µM 4-AP sensitive $I_{\text{Kur}}$ traces from the three groups. Average I-V relations are depicted in panel B in the three groups.
FIGURE 6.6: Slow component of delayed rectifier current ($I_{ks}$) is reduced in chronic HF atria while rapid component ($I_{kr}$) is unaltered. Panel A shows the raw traces of D-sotalol (100 µM) insensitive $I_{ks}$ from control and HF atria respectively, with tail current densities shown in the inset for clarity. Tail $I_{ks}$ is reduced in chronic HF atria and is shown in Panel B. Panel C shows the D-sotalol sensitive $I_{kr}$ I-V relation in control and HF atria. The numbers within parentheses indicate the numbers of animals used to obtain the data.
FIGURE 6.7: The peroxynitrite donor (SIN-1) increases action potential duration and produces depolarization of the resting membrane potential (RMP) while scavenging peroxynitrite with urate prevents its effect on atrial AP. Panel A shows the raw traces at baseline (black) and after 200 µM SIN-1 superfusion (blue). The absolute changes in RMP are shown in panel B (use left axis) while % change in RMP is also depicted (use right axis) (n=3 myocytes). Panel C shows the % APD₅₀ and APD₉₀ prolongation after SIN-1 superfusion. Panel D shows effects of SIN-1 can be blocked by superfusion with the peroxynitrite scavenger urate in combination with SIN-1.
FIGURE 6.8: Transient (I_{to}) and sustained (I_{Ksus}) outward K^+ currents are not unaltered with SIN-1 superfusion. Panel A and B shows the family of raw current traces recorded before and after superfusion with SIN-1. The average I-V relation for I_{to} and I_{Ksus} is shown in Panel C and D.
CHAPTER 7

SUMMARY
The studies presented in this dissertation focus on cardiac repolarization with primary focus on voltage gated K$^+$ currents in the heart. Cardiac repolarization determines the duration of action potential. This in turn determines the effective refractory period and diastolic interval. Alterations in action potential repolarization reflect on the surface electrocardiogram. The studies presented in this dissertation address these action potential changes which have significance for the surface electrocardiogram during health and disease.

The second chapter of the dissertation focuses on the identification of “$I_{Kur}$-like” current in the canine ventricle. While previous studies in the literature have failed to elucidate this current due to inadequate sampling or due to myocytes obtained from end-stage failing hearts, this study focused on identification of “$I_{Kur}$-like” current in normal canine ventricle. The current has very similar properties to canine atrial $I_{Kur}$. Pharmacological blockade of this current has action potential prolonging effects but however, regional distribution in “$I_{Kur}$-like” current was not considered in this study. Further studies will be required to assess regional and inter-ventricular differences in this current. Inspite of this fact, it is evident from the data that “$I_{Kur}$-like” current blockade significantly
prolongs action potential duration and has a major role in ventricular repolarization.

Hypertension is the most common cardiovascular diagnosis in the US. If left untreated, hypertension can lead to reduced ventricular compliance, and heart failure might result. Chapter 3 deals with measurements of diastolic K+ currents (I_{K1} and I_f) that control excitability. Hypertension per se increases the expression of pacemaker current (I_f) in ventricular myocytes. It must be noted that pacemaker current is usually expressed in very low densities in normal ventricular myocytes. Chronic hypertension seems to drive the expression of I_f to high levels, and this in combination with a reduced inward rectifier K+ current (I_{K1} – the current that maintains resting membrane potential) could significantly alter myocytes excitability. Cellular arrhythmias is triggered in hypertensive myocytes starting at 2 months of age, but at 8 months (with long standing hypertension) a combination of higher I_f and reduced I_{K1} is enough to trigger delayed after depolarizations. Progression from hypertension to heart failure does not increase I_f further but produces a further downregulation of I_{K1}. This study is the first to document the changes in diastolic currents during progression from hypertension to heart failure.

Sudden cardiac death is most common mode of death in the first year after myocardial infarction. Holter monitoring reveals that ventricular
arrhythmias occur in 80% of sudden cardiac death patients. Our collaborator, Dr. George Billman’s laboratory has developed a canine sudden cardiac death model, where exercise induced ischemia superimposed on a healed myocardial infarction produces ventricular fibrillation (VF). This chapter is the first study to document the K+ current alterations that predispose to the susceptibility or resistance to VF. Our results indicate that the susceptible animals have undetectable rapid component of the delayed rectifier current (I_{Kr}). This in combination with reduced I_{to}, and I_{Kur}, reduces the repolarization reserve and produces cellular arrhythmias at physiological rates in vivo and in vitro. In addition, this study also provides indirect evidence to computational studies, which show that higher pacing rates require greater contribution from I_{Kr} in addition to higher catecholamine stimulated I_{Ks}. Since the susceptible myocytes have lower I_{Kr}, they tend to have higher incidence of arrhythmias during exercise during the lack of I_{Kr} augmentation during higher heart rates in susceptible animals. Our studies also provide a very interesting future therapeutic option of using I_{Kr} activators to increase I_{Kr} in susceptible animals to improve repolarization reserve and to reduce the incidence of VF.

Chapter 5 analyzes ionic mechanisms that occur during chronic heart failure (HF). One of the major pitfalls from HF models (in larger
animals which have heart rate closer to humans) is that HF is usually reversible, and not permanent. This is especially true of right ventricular tachypacing model to induce HF. K⁺ current data from a chronic irreversible tachypacing HF model (developed by Dr. Robert L. Hamlin’s laboratory) is presented. In addition, preliminary data supporting the efficacy of cardiac resynchronization therapy (CRT) is presented. Our study in CRT treated HF dogs present the first experimental evidence to computational studies that suggest that I_{1o} does not contribute to action potential repolarization.

Chapter 6 deals with HF induced changes in atrial ionic remodeling with special focus on redox regulation of cardiac atrial K⁺ currents. Atrial fibrillation is the most common sustained arrhythmia in the US. AF in HF increases mortality by 4.5-5.9 fold. HF is the most common hospital discharge diagnosis in the US in adults over the age of 65. Our laboratory has previously shown that oxidative stress plays a very important role in AF. Therefore this is a very important area of investigation and identification of new therapeutic strategies are warranted. Our study shows that even in chronic HF, certain aspects of cardiac ionic remodeling are redox-sensitive. This could potentially be exploited to test the efficacy of redox agents for the treatment of AF.
In summary, changes in cardiac repolarization could significantly alter the QT interval on the surface electrogram. However, exceptions to this rule do exist. In the cardiac ventricle, a great deal of apico-basal, transmural and interventricular heterogeneity is present. This makes it difficult to directly interpret patch clamp recordings from isolated myocytes to the whole heart due to the inherent regional repolarization heterogeneity in the cardiac ventricle. The use of isolated myocytes for patch clamp studies suffers from limited sampling from a single region of the heart. But inspite of these shortcomings, our patch clamping studies offers a very powerful and insightful view of K⁺ current abnormalities that occurs during disease remodeling which predispose to arrhythmic episodes.
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