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The Role of Transient Outward Current in Regulating Cardiomyocytes Electrical and Mechanical Functions

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The Role of Transient Outward Current in Regulating Cardiomyocytes

Electrical and Mechanical Functions

A dissertation submitted to the

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By

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Abstract

The transient outward current ($I_{to}$) is a major repolarizing current in the heart and is heterogeneously expressed across the ventricular wall. Marked reduction of $I_{to}$ density is consistently observed in human heart failure (HF) and animal HF models. It was proposed that this $I_{to}$ reduction contributes to a significant action potential duration (APD) prolongation and to the impaired contractility in failing heart. In addition, a high density of $I_{to}$ in the right ventricular (RV) epicardial myocytes has been suggested to play a critical role in the arrhythmogenesis for the Brugada syndrome, an arrhythmia that is responsible for up to 12% of sudden cardiac deaths. Due to the lack of a specific $I_{to}$ blocker, however, whether above suggestions are true is still under question. This dissertation, use the dynamic clamp to specifically simulate $I_{to}$ in ventricular myocytes, delineates the role of $I_{to}$ in regulating cardiomyocytes electrical and mechanical functions.

Firstly, to understand the role of $I_{to}$ in regulating the (AP) morphology and duration, we introduced simulated $I_{to}$ conductance in guinea pig and canine endocardial ventricular myocytes using the dynamic clamp technique. The effects of simulated $I_{to}$ in both types of cells were complex and bi-phasic, separated by a clear density threshold of about 40 pA/pF. Below this threshold, simulated $I_{to}$ resulted in a distinct phase 1 notch, and had little effect on or moderately prolonged the APD. $I_{to}$ above the threshold resulted in all-or-none repolarization and precipitously reduced the APD. We conclude that, in animals such as dogs and guinea pigs that have a broad cardiac AP, $I_{to}$ does not play a major role in setting the APD.
We next examined the influence of $I_{to}$ on the mechanical properties of canine ventricular myocytes. In endocardial myocytes, where the native $I_{to}$ is small, simulation of an epicardial-level $I_{to}$ by the dynamic clamp significantly suppressed cell shortening by 19%. The peak amplitude of Ca$^{2+}$ transient was also reduced in the presence of simulated $I_{to}$. Conversely, subtraction, or “blockade” of the native $I_{to}$ enhanced contractility in epicardial cells. These results agree with the inverse correlation between $I_{to}$ levels and myocyte contractility and Ca$^{2+}$ transient amplitude in epicardial and endocardial myocytes. AP clamp studies showed that phase-1 repolarization vs. peak $I_{Ca-L}$ relationship had an inverted-J shape; moderate-to-strong phase-1 repolarization decreased peak $I_{Ca-L}$ and markedly reduced early Ca$^{2+}$ influx. Our results show that $I_{to}$ acts as a negative, rather than positive regulator of myocyte mechanical properties in large animals.

Lastly, the cellular mechanism of the electrical abnormality in Brugada syndrome and the potential basis of the RV contractile abnormality observed in the syndrome were addressed. Tetrodotoxin was used to reduce cardiac Na$^+$ current ($I_{Na}$) to mimic a Brugada syndrome-like setting in canine ventricular myocytes. $I_{Na}$ reduction resulted in prolongation of APD or all-or-none repolarization in RV epicardial myocytes, and marked attenuation of myocyte contraction and Ca$^{2+}$ transient. Dynamic clamp and computational modeling were used to examine the interplay between $I_{Na}$ and the $I_{to}$ and its influence on AP morphology. Both reduction of $I_{Na}$ and increase of $I_{to}$ have similar biphasic effects on APD, and reduction of $I_{Na}$ shifts the APD-$I_{to}$ density curve to the left. As a result, in the presence of a large $I_{to}$, $I_{Na}$ reduction either prolongs or collapses the AP, depending on the exact density of $I_{to}$. Computational modeling showed that these
repolarization changes alter myocyte Ca\(^{2+}\) dynamics by reducing Ca\(^{2+}\) influx and sarcoplasmic reticulum Ca\(^{2+}\) load. As such, the contractile abnormality of the RV wall in Brugada syndrome may be secondary to the electrical abnormalities.

Together, this dissertation demonstrated the dynamic clamp as a powerful tool for studying the cardiac electrophysiology. More importantly, it quantitatively addressed the role of I\(_{to}\) in terms of its physiological functions and its potential contributions to arrhythmic diseases.
Acknowledgements

Looking back on the past 6 years of my Ph.D. training, I see myself having grown and developed, both as a person and as a scientist. It is impossible, however, that I would have made this progress without help from numerous people. I would like to take this opportunity to express my deepest appreciation to them.

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<tbody>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>AP(s)</td>
<td>Action potential(s)</td>
</tr>
<tr>
<td>APD</td>
<td>Action potential duration</td>
</tr>
<tr>
<td>APD&lt;sub&gt;90&lt;/sub&gt;</td>
<td>APD at 90% repolarization</td>
</tr>
<tr>
<td>AV node</td>
<td>Atrioventricular node</td>
</tr>
<tr>
<td>BayK</td>
<td>Bay-K8644</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>Cl&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Chloride</td>
</tr>
<tr>
<td>CICR</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-induced Ca&lt;sup&gt;2+&lt;/sup&gt; release</td>
</tr>
<tr>
<td>CPVT</td>
<td>Catecholaminergic polymorphic ventricular tachycardia</td>
</tr>
<tr>
<td>DAD</td>
<td>Delay after-depolarization</td>
</tr>
<tr>
<td>EAD</td>
<td>Early after-depolarization</td>
</tr>
<tr>
<td>EC coupling</td>
<td>Excitation-contraction coupling</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ENDO</td>
<td>Endocardial myocytes</td>
</tr>
<tr>
<td>EPI</td>
<td>Epicardial myocytes</td>
</tr>
<tr>
<td>ERP</td>
<td>Effective refractory period</td>
</tr>
<tr>
<td>FS</td>
<td>Fraction shortening</td>
</tr>
<tr>
<td>G$_{Na}$</td>
<td>Na$^+$ conductance</td>
</tr>
<tr>
<td>G$_{to}$</td>
<td>I$_{to}$ conductance</td>
</tr>
<tr>
<td>HF</td>
<td>Heart failure</td>
</tr>
<tr>
<td>I$_{CaL}$</td>
<td>L-type Ca$^{2+}$ current</td>
</tr>
<tr>
<td>I$_{Na}$</td>
<td>Cardiac Na$^+$ current</td>
</tr>
<tr>
<td>I$_{NaL}$</td>
<td>Cardiac late sodium current</td>
</tr>
<tr>
<td>I$_{K1}$</td>
<td>Inward rectifier K$^+$ current</td>
</tr>
<tr>
<td>I$_{Kr}$</td>
<td>Rapid delayed rectifier K$^+$ current</td>
</tr>
<tr>
<td>I$_{Ks}$</td>
<td>Slow delayed rectifier K$^+$ current</td>
</tr>
<tr>
<td>I$_{s}$</td>
<td>Sustained component of I$_{to}$</td>
</tr>
<tr>
<td>I$_{ti}$</td>
<td>Transient inward current</td>
</tr>
<tr>
<td>I$<em>{to}$ or I$</em>{to1}$</td>
<td>Transient outward current</td>
</tr>
<tr>
<td>I$_{to2}$</td>
<td>Ca$^{2+}$ dependent Cl$^-$ current</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ICD</td>
<td>Implantable cardioverter defibrillator</td>
</tr>
<tr>
<td>JSR</td>
<td>Junctional sarcoplasmic reticulum</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium</td>
</tr>
<tr>
<td>LAD</td>
<td>Left anterior descending coronary artery</td>
</tr>
<tr>
<td>LTCC</td>
<td>Cardiac L-type Ca²⁺ channels</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle or Left ventricular</td>
</tr>
<tr>
<td>LVED</td>
<td>Left ventricular end diastolic</td>
</tr>
<tr>
<td>LVES</td>
<td>Left ventricular end systolic</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NCX</td>
<td>N⁺-Ca²⁺ exchanger</td>
</tr>
<tr>
<td>PLN</td>
<td>Phospholamban</td>
</tr>
<tr>
<td>RRP</td>
<td>Relative refractory period</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle or Right ventricular</td>
</tr>
<tr>
<td>RVOT</td>
<td>Right ventricular outflow tract</td>
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<tr>
<td>RyR(s)</td>
<td>Ryanodine receptor(s)</td>
</tr>
<tr>
<td>SA node</td>
<td>Sinoatrial node</td>
</tr>
<tr>
<td>SERCA</td>
<td>Cardiac sarcoplasmic reticulum Ca²⁺ ATPase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VF</td>
<td>Ventricular fibrillation</td>
</tr>
<tr>
<td>Vm</td>
<td>Membrane potential</td>
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<tr>
<td>VT</td>
<td>Ventricular tachycardia</td>
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Chapter I: Introduction

1. Cardiac ventricular action potential and underlying ionic currents

1.1. Cardiac conductive system and ventricular action potential (AP)

The heart is a muscular organ which functions as a pump to deliver blood throughout the body. Triggered by electrical impulses, the heart contracts repeatedly and rhythmically to maintain a continuous supply of oxygen and nutrition to organs, including the heart itself. These electrical impulses, originated from the sinoatrial (SA) node, first spread through the atrial to the atrioventricular (AV) node, inducing contraction of the atrium. From there, the signals travel through the specialized fiber of the cardiac conductive system, the Bundle of His, which splits into two branches, the left and right bundle branch, in the interventricular septum. These bundle branches activate the left and right ventricles, respectively, and both branches taper out to produce numerous Purkinje fibers, which stimulate individual groups of myocardial cells to contract (Figure 1A).

From cell to cell, the electrical impulses are conducted through intercellular gap junctions. Once a cardiomyocyte is electrically stimulated, it begins a sequence of
actions involving the influx and efflux of multiple cations and anions that together establish the action potential (AP) of the cell. During this process, the ionic currents produced through sarcolemmal voltage- and ligand- dependent channels, as well as electrogenic ion transporters, determine the cardiac AP waveform, which differs significantly among different mammalian species and among different regions of the heart. In small animals, such as mouse and rat, the ventricular AP is characterized by a triangular waveform (Figure 1C, left), and is significantly shorter than those of large mammals, such as canine and human. In ventricular cardiomyocytes from large mammals, a spike-and-dome waveform is observed. For these spike-and-dome APs, 5 phases (phase 0-4) can be identified (Figure 1B). Phase 0 is the fast depolarizing phase, and is mediated by the cardiac Na\(^+\) channels. As an electrical impulse reaches a cardiomyocyte, Na\(^+\) channels are first activated, causing a rapid increase in the membrane conductance to Na\(^+\). The resultant inward Na\(^+\) current (I\(_{Na}\)) quickly depolarizes the membrane potential, producing the phase 0 upstroke. The Na\(^+\) channel then inactivates and the transient outward potassium (I\(_{to}\)) activates. I\(_{to}\) plays a major role in the early phase of repolarization, giving rise to a notable phase 1 notch. During phase 2, the inward L-type Ca\(^{2+}\) current (I\(_{Ca-L}\)) slightly depolarizes the cell, and then the rapid (I\(_{Kr}\)) and slow (I\(_{Ks}\)) delayed rectifier K\(^+\) currents activate. The depolarizing I\(_{Ca-L}\) balances with these two repolarizing K\(^+\) currents, holding the membrane potential momentarily stable, forming the phase 2 plateau. The I\(_{Ca-L}\) then inactivates and is overshadowed by the I\(_{Kr}\) and I\(_{Ks}\). I\(_{Kr}\) and I\(_{Ks}\) are joined by the inward rectifier potassium current (I\(_{K1}\)) during phase 3 and finally repolarize the cell back to the resting potential. When the membrane potential is restored to about -80 to -85 mV, the delayed rectifier K\(^+\) channels close, while I\(_{K1}\) remains open throughout phase 4, contributing to setting
Figure 1

A

B

C

Mouse

Guinea-Pig
Figure 1. The heart’s electrical conductive system and the action potential in human ventricular myocytes.

A, An electrical stimulus is generated by the sinus node (SA node) and then travels down through the atria to the atrioventricular (AV) node. The electrical impulse then continues down the conduction pathways via the Bundle of His into the ventricles. The Bundle of His divides into right and left pathways to provide electrical stimulation to both ventricles and causes them to contract. B, A cardiac ventricular AP consists of 5 phases. When an electrical impulse reaches a ventricular myocyte, it is activated by the phase 0 inward current carried by Na⁺. The transient outward current ($I_{to}$) then repolarizes the cells and is responsible for early (phase 1) repolarization, forming the prominent phase 1 notch. It is followed by a plateau at phase 2, during which inward L-type Ca²⁺ current ($I_{Ca-L}$) and delayed-rectifier K⁺ outward currents ($I_K$) are relatively balanced. The two components of $I_K$, rapid ($I_{Kr}$) and slow ($I_{Ks}$) delayed-rectifier K⁺ currents, further repolarize the cell during phase 3. The inward-rectifier K⁺ current ($I_{K1}$) then contributes to terminal repolarization and sets the resting membrane potential (~ -80 mV) during phase 4. C, AP profile of ventricular myocytes from mouse (left) and guinea pig (right).

(Fig 1A is adapted from St. John’s Mercy Health Care website http://www.stjohnsmercy.org/services/heartcenter/proced/saekg/default.asp; Fig 1B is modified from Pourrier et al, 2003; Fig 1C is from our own recordings; Rosati et al. 2008)
the resting membrane potential until the next electrical stimulus. Compared to the spike-and-dome AP waveform in canine and human, Guinea pig ventricular myocytes exhibit an AP without the phase 1 notch (Figure 1C, right; Niwa & Nerbonne, 2009). Because of the absence of an I_{to} current in these species, their AP is characterized by a limited phase 1 repolarization and the absence of a notch, followed by a prominent plateau (phase 2) (Linz & Meyer, 2000).

1.2. Effective refractory period (ERP) and relative refractory period (RRP)

During phases 0, 1, 2, and part of phase 3, inactivation of the Na^{+} channels results in a period during which the cell is refractory to initiation of new APs. This period is termed the effective refractory period (ERP). During the ERP, stimulation of the cell does not produce new APs. The ERP acts as a protective mechanism in the heart by preventing multiple, premature APs from occurring. In addition, the length of the refractory period limits the frequency of APs (and therefore contractions) that can be generated. Following the ERP is the relative refractory period (RRP), during which the membrane potential is close to full repolarization. The membrane potential in RRP is negative enough to allow the activation of Na^{+} channels, but only by a stimulus much stronger than usual. The subsequent premature excitation can be conducted to other parts of the myocardium, potentially developing into arrhythmias.

1.3. The cardiac Na^{+} current (I_{Na})

Being responsible for the AP upstroke during phase 0, I_{Na} is activated when transmembrane potential depolarizes into the range in which channel openings can occur (~ -60 mV). The opening allows an inward flow of Na^{+}, causing additional
depolarization. As aforementioned, $I_{Na}$ is the main depolarizing current during a cardiac AP. The activation time constant of $I_{Na}$ is very rapid (0.1-0.2 msec; Lee et al. 1979). By the time Na$^+$ channels are activated, the inactivation has also started to proceed. The quick inactivation limits the amplitude of $I_{Na}$ and contributes to the extreme brevity of $I_{Na}$ (Bers, 2001). Recovery of $I_{Na}$ from inactivation requires the membrane be repolarized to near the resting level for a period of time (~100 ms at -72 mV). Thus, Na$^+$ channels do not recover very rapidly until repolarization is nearly complete. This is a main cause of the electrical refractory period ERP (Bers, 2001).

The cardiac Na$^+$ channel consists of a main α subunit Na(v)1.5 (SCN5A) and auxiliary β subunits β1-β4 (Abriel & Kass, 2005). The α subunit is the principle component forming the channel pore and is essential for the gating property of the channel. Encoded by the SCN5A gene, the Na(v)1.5 protein has 2016 amino acids and has a molecular mass of ~240 kDa. The Nav1.5 protein has four homologous domains (DI–DIV), with each domain containing six transmembrane segments (S1-S6). Isoforms other than Na(v)1.5, including Na(v)1.1, Na(v)1.2, Na(v)1.3, Na(v)1.4, and Na(v)1.6, are found in the heart and may contribute up to 10% of $I_{Na}$ in canine ventricle, while the Nav(1.5) channel generates the rest 90% of $I_{Na}$ (Maier, 2002; Haufe et al. 2005).

Mutations in the SCN5A gene are associated with several arrhythmic diseases, including long QT syndrome type 3 (LQT3), Brugada syndrome, and idiopathic ventricular fibrillation (Chen et al. 1998; Bezzina et al, 1999).

Many Na$^+$ channel blockers are used in clinical or experimental treatment for cardiac arrhythmias. They can be classified based upon their acting mechanisms. Alkaloid based toxins, such as tetrodotoxin (TTX), block the pore opening of the channel. A study has shown that in human atrial cardiomyocytes, TTX blocked the $I_{Na}$ with a Kd
of 1 µM (Sakakibara, 1992). Other drugs, including the "Class I" antiarrhythmic agents, such as quinidine and lidocaine, block Na⁺ channels by blocking from the intracellular side of the channel.

1.4. Late sodium current (I_{NaL})

Recently, a sustained component of the classical cardiac Na⁺ current, the late Na⁺ current (I_{NaL}), was described in canine as well as in human ventricular cardiomyocytes (Maltsev et al. 1998). It was found that as the majority of Na⁺ channels are quickly inactivated, I_{NaL} channels slowly inactivate and keep opening throughout the AP plateau (Undrovinas et al., 2002; Carmeliet, 2006). One study has shown that I_{NaL} has a 600-ms, voltage-independent inactivation time constant (Maltsev et al. 2001). Compared to the rapid I_{Na} current, which is responsible for the AP upstroke, I_{NaL} has a different physiological importance, mainly playing a role in the repolarization process. More evidence has shown that the I_{NaL} density is significantly up-regulated and the kinetics of inactivation are slowed in failing cardiomyocytes (Maltsev et al. 2007; Undrovinas et al. 1999). This increased I_{NaL} may both prolong APD and elevate [Na⁺]i, which in turn increase Ca²⁺ entry via Na⁺-Ca²⁺ exchanger (NCX) (Maltsev & Undrovinas, 2008).

1.5. The transient outward potassium current (I_{to})

The earliest phase of AP repolarization (phase 1) is the result of activation of the transient outward K⁺ current --- I_{to}, which gives rise to the prominent phase 1 notch in large animals. Also a voltage-dependent current, I_{to} is activated in response to depolarization positive to -20 mV from a holding potential of -60 mV in ventricular myocytes. It reaches its peak amplitude in approximately 10 msec and usually has a
rapid decay (time constants between 10-40 msec, depending upon the voltage applied) in canine ventricles (Dangman & Miura, 1991). $I_{to}$ is sensitive to 4-aminopyridine (4-AP) with a Kd of 0.2 mM (Castle & Slawsky, 1993). Recovery from inactivation of $I_{to}$ channels is slow. As a result, the current flow is suppressed with fast pacing, resulting in a shallower notch (Giles & Imaizumi, 1988). As a key repolarizing current, $I_{to}$ plays an important role in modulating morphology and duration of AP, and is also involved in the regulation of intracellular Ca$^{2+}$ handling and mechanical functions in cardiomyocytes. $I_{to}$ is one of the main focuses of my thesis research. A more detailed introduction of $I_{to}$ current and $I_{to}$ channel is in Introduction section 3 (page 28).

In some publications, the transient outward current was expressed as $I_{to1}$ in order to distinguish it from a Ca$^{2+}$ dependent Cl$^{-}$ current ($I_{Cl(Ca)}$), which is designated as $I_{to2}$. $I_{to2}$ is broadly found in cardiomyocytes as well as in neurons (Sorota, 1999). It is also activated and inactivated fast, though slower than that of $I_{to}$. This current is smaller than $I_{to}$ (Wang et al. 1997), and is larger in atria than in ventricles (Zygumunt & Gibbons, 1991).

**1.6. Cardiac L-type Ca$^{2+}$ current ($I_{Ca-L}$)**

So far, at least two classes of voltage-dependent Ca$^{2+}$ channels (L- and T-type) have been identified in cardiac myocytes (Wang & Cohen, 2003). T-type $I_{Ca}$ is negligible in most ventricular myocytes, whereas cardiac L-type ("L"ong lasting) Ca$^{2+}$ current ($I_{Ca-L}$) is the main depolarizing current during AP phase 2 (plateau) in ventricular cardiomyocytes. $I_{Ca-L}$ plays a crucial role in triggering cardiac contraction and serves as the initiator of cardiac excitation-contraction (E-C) coupling. The channels that conduct $I_{Ca-L}$, also called dihydropyridine receptors (DHPR), are voltage-dependent channels. They are sensitive to various 1,4-dihydropyridines or their derivatives, some of them
blocking (nifedipine, nicardipine) and some of them increasing (Bay K 8644) the $I_{\text{Ca-L}}$ (Kanmura & Kuriyama, 1984; Treinys & Jurevicius, 2008). $I_{\text{Ca-L}}$ is blocked by Cd$^{2+}$ and several other divalent alkaline ions. $I_{\text{Ca-L}}$ is normally activated by rapid depolarizations into the voltage range positive to -40 mV. Compared to $I_{\text{Na}}$, $I_{\text{Ca-L}}$ activates and inactivates more slowly. The activation and inactivation time constants of $I_{\text{Ca-L}}$ are 5-20 msec and 30-300 msec (depending upon the voltage applied), respectively (Jalife et al. 1999), whereas the activation time constant of $I_{\text{Na}}$ is only about 0.1 - 0.2 msec. In addition to the voltage-dependent inactivation, one feature of $I_{\text{Ca-L}}$ inactivation is that it is inactivated not only by voltage but also by Ca$^{2+}$, the channel's own charge ions (Eckert & Chad, 1984; Kass & Sanguinetti, 1984). This phenomenon is usually referred to as Ca$^{2+}$-dependent inactivation.

Cardiac L-type Ca$^{2+}$ channels (LTCC) are composed of four subunits ($\alpha_1$, $\alpha_2$, $\beta$ and $\delta$). The $\alpha_1$ subunit of 190 to 250 kDa is the largest subunit, and it incorporates the conduction pore, the voltage sensor, and the gating apparatus. Same with Na$^+$ channels, the $\alpha_1$ subunit consists of four repeated domains, with each domain containing six transmembrane segments (S1-S6). To date, at least 10 different $\alpha_1$ subunit isoforms have been identified, but only the Ca(v)$\alpha_1.2$ (encoded by CACNA1C) isoform is expressed at high levels in cardiac muscle in adults (Bodi et al. 2005). LTCCs is regulated by $\beta$-adrenergic stimulation. Catecholamine released from sympathetic nerve systems increases the current amplitude through cAMP-dependent phosphorylation, which results in an increase of channel open probability (Naguro et al. 2001).

1.7. Na$^+$-Ca$^{2+}$ exchanger (NCX)
Sodium-Calcium exchanger (NCX) is an antiporter membrane protein which allows Na\(^+\) and Ca\(^{2+}\) to cross the sarcolemma in opposite directions. It has been identified that three Na\(^+\) ions exchange for one Ca\(^{2+}\) ion with each transportation cycle, therefore each cycle produces one net ionic charge flux. The direction and amount of ion fluxes through NCX depend on the concentration of [Na\(^+\)] and [Ca\(^{2+}\)] on both sides of the membrane, and depend on the membrane potential (Vm). If there is more energy (both chemical and electrical) in the inward Na\(^+\) gradient (for three Na\(^+\) ions) than in the inward Ca\(^{2+}\) gradient (for one Ca\(^{2+}\) ion), forward mode of NCX will be favored. In this case, Ca\(^{2+}\) is transported out of the cell and Na\(^+\) is moved in, producing one net charge movement and an inward current (Sher et al. 2008). Conversely, in the reverse mode, three Na\(^+\) ions move out and one Ca\(^{2+}\) ion moves in, generating an outward current. During myocyte relaxation, four pathways mediate Ca\(^{2+}\) removal from the cytosol: 1) the SR Ca\(^{2+}\)-ATPase (SERCA), (b) NCX, (c) mitochondrial Ca\(^{2+}\) uniporter, and (d) sarcolemmal Ca\(^{2+}\)-ATPase (Bassani et al. 1994). The extrusion of Ca\(^{2+}\) by NCX in its forward mode is considered to be one of the most important cellular mechanisms for removing intracellular Ca\(^{2+}\) during diastole. The percentage of total Ca\(^{2+}\) decline through NCX Ca\(^{2+}\) extrusion differs in different species. NCX extrudes ~7% of the Ca\(^{2+}\) during relaxation in ventricular myocytes in rodents, while it is responsible for approximately 25% - 30% of Ca\(^{2+}\) decline in ventricular myocytes from larger mammals, including human, rabbit, cat, ferret, and dog (Bers et al. 1996; Shigekawa & Iwamoto, 2001). NCX function is complex because intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and membrane potential change dramatically during an AP. At an early phase of the AP (phase 0 & 1), membrane potential becomes positive and [Ca\(^{2+}\)]\(_i\) remains low, both of which favor the reverse mode. Consequently, a brief outward NCX current (Ca\(^{2+}\) influx) is generated.
during this short period. However, the reverse mode of NCX only exists for a very short period of the cardiac cycle and contributes very little to Ca\(^{2+}\) influx (Weber et al. 2002). Upon I\(_{\text{Ca,L}}\) activation and SR Ca\(^{2+}\) release, the intracellular Ca\(^{2+}\) concentration dramatically increases. The high [Ca\(^{2+}\)\(_i\)] switches the NCX to its forward mode (Ca\(^{2+}\) efflux) and an inward current is generated. NCX remains in this mode through the rest of AP and works as one of the main mechanisms of Ca\(^{2+}\) extrusion.

1.8. Other major cardiac K\(^{+}\) currents

Many K\(^{+}\) currents have been identified in cardiac cells (Snyders, 1999). In addition to I\(_{\text{to}}\), the following K\(^{+}\) currents play major roles in regulating cell electrical properties in ventricular cardiomyocytes.

1.8.1. Delayed rectifier potassium current I\(_{\text{Kr}}\) and I\(_{\text{Ks}}\)

The delayed rectifier K\(^{+}\) current (I\(_K\)) is a voltage- and time-dependent K\(^{+}\) current, comprised of at least two distinct currents, the rapidly activating current I\(_{\text{Kr}}\) and the slowly activating component I\(_{\text{Ks}}\) (Archer & Rusch, 2001). In addition to their gating kinetics, these two currents can also be separated pharmacologically, as I\(_{\text{Kr}}\) is blocked by class III antiarrhythmic drugs such as E-4031 and dofetilide (Carmeliet, 1992) and I\(_{\text{Ks}}\) is not. The I\(_{\text{Kr}}\) channel consists of α subunit HERG (KCNH2) and β subunit MiRP1 (KCNE2) proteins, while the I\(_{\text{Ks}}\) channel is formed by α subunit KvLQT1 (KCNQ1) and β subunit minK (KCNE1). Each α subunit is comprised of six membrane-spanning sequences (S1-S6). Four identical α subunits form the trans-membrane K\(^{+}\) pore. I\(_{\text{Ks}}\) does not show appreciable inactivation during the time course of AP. It keeps increasing throughout the plateau phase and decreases only during phase 3 due to a decrease in electrochemical driving force for K\(^{+}\) (Tristani-Firouzi et al. 2001).
1.8.2. Inward rectifier potassium current $I_{K1}$

$I_{K1}$ is believed to play a prominent role in setting the resting membrane potential and contributing to phase 3 repolarization (Barry & Nerbonne, 1996). At membrane potentials below the $E_K$, $I_{K1}$ is inward and above the $E_K$, $I_{K1}$ is outward (Dangman & Miura, 1991). When the membrane potential is above $E_K$, it generates an outward repolarizing current which facilitates the phase 3 fast repolarization. The pore-forming $\alpha$ subunit of $I_{K1}$ channels belongs to the Kir2 family (Zunkler, 2006; Dhamoon & Jalife, 2005). It only contains two transmembrane domains, M1& M2, which are highly homologous to the S5 and S6 of the delayed rectifier K$^+$ channels (Snyders, 1999; Dhamoon & Jalife, 2005).

1.9. Action potential duration (APD)

Action potential duration (APD) is the length of the AP. It is usually measured between the onset of the AP upstroke and the point at which the cell repolarizes to a certain fraction of the maximum repolarization, e.g., $\text{APD}_{50}$ refers to the APD measured at 50% repolarization and $\text{APD}_{90}$ to the APD at 90% repolarization. APD is related to the length of the interval between successive cardiac impulses. When heart rate increases (the interval between successive impulses decreases), repolarization is faster. Also, the AP is shorter in duration. As heart rates slow down, the APD lengthens (Woods, 2005). This rate-dependent behavior of AP in cardiac cell is called “AP adaptation” (Decker et al. 2009).

There is a significant species- and region- dependent heterogeneity of APD. In rodent animals, such as mouse and rat, APD is normally less than 100 ms due to the significant early repolarization. In large mammals, the endocardial cells (the inner layer
of the ventricular wall) have a longer APD than epicardial cells (the outer layer of the ventricular wall); while midmyocardial cells (M cells; cells between epicardium and endocardium) have the longest APD due to the small $I_{K_s}$ (Liu & Antzelevitch, 1995). It was shown that the transmural difference of APD is gradual except between the epicardial and subepicardial layer where the change is sharp (Antzelevitch & Fish, 2001).

It is important to maintain APD within physiological range to maintain normal electrical function of cardiomyocytes. The shorter APD of epicardium affords the normal sequence of repolarization from epicardium to endocardium, a direction that is opposite to the excitation sequence from endocardium to epicardium. It is believed that such a sequence prevents back-propagation of excitation from epicardium to endocardium, and thus protects against reentrant arrhythmias (reentrant arrhythmias are described on page 18). In addition, if the APD is too short, the heart is susceptible to tachycardia or fibrillation, while if it is too long, it frequently results in early afterdepolarizations (EADs) and Torsade de pointe (TdP) type of arrhythmias (Carmeliet, 2006). The genesis of EADs is largely believed to be caused by reactivation of $I_{Ca,L}$. Long APDs allow the recovery of $I_{Ca,L}$ from inactivation and hence increase the likelihood of EADs (Bers, 2006). The genesis of TdP was related to the EAD-induced triggered activity (Zipes, 2009).

APD is determined by the interplay between depolarizing and repolarizing currents that form the AP. As the depolarizing currents overpower the repolarizing currents, APD will be prolonged; as repolarizing currents overpower depolarizing currents, APD will be shortened. In many cardiac diseases, APD undergoes significant changes due to ionic remodelings. The contribution of $I_{to}$ on APD will be discussed in Introduction section 3.6. (page 32). In addition to the $I_{to}$ current, other currents also regulate the length of AP.
In ventricular M cells, APD is longer than that in epicardial cells. Liu & Antzelevitch found that the long APD in M cells is mainly due to a smaller \(I_{Ks}\) (1995). Also, a larger late Na\(^+\) current (Zygmunt et al. 2001) and a larger NCX current (Zygmunt et al. 2000) were found in M cells, suggesting that these currents may also be contributing factors to APD. In a modeling study, Winslow et al. identified that enhanced L-type Ca\(^{2+}\) current in HF contributes to the prolongation of APD (1999). However, whether \(I_{Ca,L}\) has any changes in HF settings is still controversial (Kaab et al. 1996; Li et al. 2004; Mewes & Ravens, 1994).

**1.10. Electrical heterogeneity in the heart**

Significant transmural electrical heterogeneity has been described in ventricles. One of the most well-known is the \(I_{to}\) gradient across the ventricular wall, with a prominent \(I_{to}\) density in epicardial myocytes but a small \(I_{to}\) density in endocardial myocytes (Litovsky & Antzelevitch, 1988; Liu et al. 1993). As the main topic of my thesis, more description of \(I_{to}\) heterogeneity is in Introduction section 3.2. (page 28). Due to the significant difference of \(I_{to}\), the AP morphology is also distinct in epicardium and endocardium. The epicardial AP is characterized by a prominent phase 1 notch, while the notch is shallow or almost absent in endocardial cells (Fig.17 in Results, page 99). In addition, the heterogeneity of APD between endocardial myocytes and epicardial myocytes, as aforementioned (page 12), has been broadly documented. A gradient of \(I_{Ca,L}\) density was described across canine ventricular wall by our laboratory (Wang & Cohen, 2003), although it was not universally observed (Volk et al. 1999; Cordeiro et al. 2004). In Wang & Cohen’s study, they found that the average peak current density at +10 mV in endocardial myocytes was 45% higher than that in epicardium. They did not find
significant difference in kinetic properties of the L-type current in epi- and endocardial cells. Xiong et al. described a transmural gradient in NCX current from epi- to endocardial in normal canine hearts, as NCX current density was significantly greater in epi- than midmyocardial and endocardial cells (2005). Moreover, the $I_{K1}$ was prominent in endo- but not in epicardial cells, whereas $I_K$ was more prominent in epi- than in endocardial myocytes due to a higher density of channels as well as a higher open probability of the channel (Furukawa et al. 1992).

1.11. APD prolongation in HF and disease-induced electrical remodeling

HF is a condition in which with the heart can no longer supply sufficient blood flow to meet the body's needs. HF is the end phase of many cardiovascular diseases, and is one of the leading causes of death (Piacentino et al. 2003). More than 5 million patients are living with this illness in the US (Lloyd-Jones et al., 2009; Heart Disease and Stroke Statistics 2010 Update). Patient prognosis remains poor, with 20% mortality rate within one year of initial diagnosis, and more than a 45% five-year mortality rate (Lloyd-Jones et al., 2009; Heart Disease and Stroke Statistics 2010 Update). The pathophysiology of HF is complex, but is usually with three common features, including prolongation of the APD (Tomaselli et al. 1994), progressive reduction of basal cardiac contractility (Mason et al. 1970), and loss of inotropic reserve (Bristow et al. 1982).

One of the most consistent and significant electrical changes in failing hearts is the prolongation of APD, as it was found in HF patients and multiple animal HF models. For example, Beuckelmann and colleagues reported that, in end-stage HF patients ($n = 16$), APD in myocytes was significantly prolonged by 60% (APD$_{90}$, $1,038 \pm 223$ msec vs. $649 \pm 101$ msec) (Beuckelmann et al. 1993). In a fast pacing heart failure canine model,
$\text{APD}_{90}$ ranges from $842 \pm 56 \text{ ms}$ to $1097 \pm 73 \text{ ms}$, which is remarkably longer than a normal $\text{APD}_{90}$ (usually $< 400 \text{ ms}$; Kaab et al. 1996). $\text{APD}$ prolongation predisposes the heart to arrhythmias and sudden cardiac death (Carmeliet, 2006). Therefore, understanding the underlying mechanism of $\text{APD}$ prolongation in diseased cardiomyocytes is critical for developing future treatment or prevention of arrhythmic events. Extensive efforts have been made to study, both experimentally and theoretically, the mechanism of $\text{APD}$ prolongation. Generally speaking, it is caused by increases in the inward depolarizing current, and/or decreases in the outward repolarizing currents. However, the specific contribution of individual current to the $\text{APD}$ prolongation is not fully understood, and was one of the focuses of my thesis research.

HF is associated with many ventricular ionic changes that may predispose to arrhythmias and lead to $\text{APD}$ prolongation (for review, see Nass et al., 2008). In addition to the $I_{to}$ downregulation that is discussed in Section 3.5. (page 32), remodelings in other main ionic currents have also been found in the failing heart. $I_{K1}$ is reduced by approximately 40% in ventricular myocytes isolated from heart with end-stage HF, without obvious change in kinetics (Beuckelmann et al. 1993). As discussed above (page 11), $I_{K1}$ sets the resting membrane potential and contributes to the final phase of repolarization. Reduction of $I_{K1}$ may contribute to the prolongation of the final phase of repolarization in the AP and may favor membrane depolarization. Both the fast and slow component of delayed rectifier $K^+$ current ($I_{Ks}$ and $I_{Kr}$) were also found to be down-regulated in failing hearts (Tsuji et al. 2000; Li et al. 2004), which may contribute to the prolongation of $\text{APD}$. NCX function and expression level were found to be enhanced in a rabbit HF model, with NCX mRNA, protein, NCX current, and rate of $[\text{Ca}]_i$ decline during caffeine-induced sarcoplasmic reticulum (SR) $\text{Ca}^{2+}$ transient all
increased by about ~2-fold (Pogwizd et al. 1999). Recently, studies also show that chronic HF increases $I_{NaL}$ density and slows inactivation kinetics of $I_{NaL}$. As $I_{NaL}$ is a depolarizing current and a major contributor to the AP plateau (Maltsev et al., 1998), increases of $I_{NaL}$ will inhibit the cell repolarization and prolong the AP. However, the two main inward currents, L-type Ca$^{2+}$ current and Na$^+$ current, did not seem to undergo significant change in cells from failing heart (Kaab et al. 1996).

1.12. Ventricular arrhythmias and its molecular mechanism

The cellular mechanism of arrhythmogenesis in ventricles has been the subject of many studies. The following mechanisms have been established as possible causes of ventricular initiated arrhythmias:

1.12.1. Early after-depolarization (EAD) and delayed after-depolarization (DAD) triggered activity

In a rabbit HF model, Pogwizd showed that spontaneous occurrences of ventricular tachycardias (VTs) are initiated and maintained by triggered activity (Pogwizd, 1995). Triggered activity, by definition, occurs following a previous AP and is “triggered” by the previous impulse. Based on the relative timing of the triggered activity, it is classified into two types: early after-depolarization (EAD) and delayed after-depolarization (DAD). EADs interfere with or delay repolarization during phase 2 and/or phase 3 of AP, whereas DADs occur after full repolarization. These triggered APs lead to extrasystoles and may induce tachyarrhythmias.

The ionic mechanism of EADs involves the imbalance between depolarizing currents and repolarizing currents, normally an increase of $I_{Ca,L}$ and/or NCX, or a decrease of outward currents $I_{Kr}$ and $I_{Ks}$, or the combination of these two, resulting in
the prolonged repolarization phase (Podrid & Kowey, 2001). *The resulted long repolarization in phase 2 and phase 3 allow reactivation of I_{Ca,L} to depolarize the myocyte, leading to a second upstroke* (Zipes, 2009). The mechanism of EADs, as well as DADs, also involve the elevated [Ca]_{i} and spontaneous SR Ca^{2+} release which drive the NCX to its forward mode (Ca^{2+} efflux) and produce a transient inward current (I_{ti}). This inward current then depolarizes the cell and induces EADs or DADs. It was also identified that downregulation of I_{K1} seen in the failing heart is important in lowering the threshold for an I_{ti}-triggered DAD (Pogwizd, 2001). While NCX is almost entirely responsible for I_{ti}, it is believed that the reduced I_{K1} in HF allows the I_{NCX} to produce greater depolarization.

### 1.12.2. Reentry arrhythmias

Reentry arrhythmias represent one of the most common arrhythmogenesis mechanisms and are responsible for many arrhythmic diseases, including the Brugada syndrome and multiple types of VT/VF (Antzelevitch & Yan, 2000; Ebinger et al., 2005). Normal myocardium is protected from rapid rhythms by a long refractory period and high conduction velocity. For reentry to occur, there must be a unidirectional block of AP propagation and an area with abnormally slow conduction (Podrid & Kowey, 2001). Additionally, the ERP of the reentered region must be shorter than the propagation time around the loop. APs then can continue their propagation in a “circuit” movement. Once a reentry circuit is formed, it can continue on its path repetitively and induce arrhythmias (Figure 2).

It was proposed that the loss of spike-and-dome AP waveform and the existence of an all-or-none repolarization AP act as a mechanism of phase 2 reentry (Antzelevitch, 2001). It is believed that when I_{to} overwhelms the inward currents (I_{Ca}, I_{Na}), the AP
plateau (dome) is abolished at some epicardial sites but not other locations (such as endocardial cells), causing a marked dispersion of repolarization. Propagation of the AP dome from sites where it is maintained to sites where it is abolished can cause local reexcitation of the tissue. This mechanism, termed phase 2 reentry, produces extrasystolic beats which can then initiate cycles of circus movement reentry, in which a cardiac impulse travels around a circuit leading to reexcitation of the heart.
Figure 2. A model of reentry. Left panel: under normal condition, the electrical impulses transport by the cardiac conductive system to branch 1 & 2. The impulses then cancel each other out at site 3. In right panel, if branch 2 has a unidirectional block, then the AP traveling down branch 1, and branch 3, and then travel in retrograde fashion through the unidirectional block in branch 2 (dashed line). AP will then continue to travel to branch 1 again to form the reentry circus (Modified from http://cvphysiology.com/Arrhythmias/A008c.htm).
2. Cardiac excitation-contraction (EC) coupling and intracellular Ca\textsuperscript{2+} modulation

2.1. Excitation-contraction (EC) coupling and intracellular Ca\textsuperscript{2+} cycling

When the cardiomyocyte is electrically stimulated, the excitation is transferred to mechanical activities, mainly by the modulation of intracellular Ca\textsuperscript{2+} kinetics. When an electrical impulse reaches excitable cardiomyocytes, LTCCs in T-tubules are activated, resulting in a relatively small amount of Ca\textsuperscript{2+} entry. T-tubules are deep invaginations of the sarcolemma and are adjacent to the sarcoplasmic reticulum (SR), a special compartment for Ca\textsuperscript{2+} storage. The increased [Ca\textsubscript{i}] then ignites a larger amount of Ca\textsuperscript{2+} release (Ca\textsuperscript{2+} transient) from the SR Ca\textsuperscript{2+} channel, ryanodine receptor (RyR). This process is termed as “Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR)”. The large amount of Ca\textsuperscript{2+} then binds to troponin C, causing a conformational change in the troponin-tropomyosin complex. This movement exposes the site on actin where the myosin head binds. Slideing motion is then generated by myosin heads that project from the sides of the myosin filament and interact with adjacent actin filaments. This series of processes that recruit multiple Ca\textsuperscript{2+} handling proteins and ion channels through which the electrical impulses are able to be transferred to the mechanical movement is called “excitation-contraction (EC) coupling” (Fig. 3). EC coupling is critical to maintain the normal function of cardiomyocytes. During diastole, Ca\textsuperscript{2+} is reuptaken into the SR through Ca\textsuperscript{2+} ATPase (SERCA\textsubscript{2a}), which is regulated by phospholamban (PLN), or Ca\textsuperscript{2+} is pumped out of the sarcolemma through NCX. Through these processes, intracellular Ca\textsuperscript{2+}
Figure 3. Excitation–contraction (EC) coupling in the cardiomyocytes.

After the cell membrane is depolarized, voltage-gated L-type Ca\(^{2+}\) channels (LTCC) are activated. The small amount of Ca\(^{2+}\) influx through LTCC triggers a large-scale Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyR). The increase in cytoplasmic Ca\(^{2+}\) concentration will induce muscle contraction. To enable relaxation, intracellular Ca\(^{2+}\) is pumped back into the SR via SR Ca-ATPase (ATP), which is regulated by phospholamban (PLN), or extruded from the cell via the Na\(^+\)/Ca\(^{2+}\)-exchanger (NCX). Inset shows the time course of an AP (black line), Ca\(^{2+}\) transient (blue line) and contraction (red dash line) measured in a rabbit ventricular myocyte at 37°C. (Modified from Bers DM, 2002).
concentration returns to the level before the last excitation, and Ca\(^{2+}\) homeostasis is able to be maintained (Bers, 2002).

As aforementioned, the RyR is activated by sarcolemmal Ca\(^{2+}\) entry during EC coupling, resulting in the synchronized opening of the RyR channel and a large amount of Ca\(^{2+}\) release from SR. The [Ca\(^{2+}\)]\(_i\) is instantly and dramatically increased, usually from a diastolic level of 150 nM to over 1 \(\mu\)M at systole. This robust leap of [Ca\(^{2+}\)]\(_i\) is referred to as Ca\(^{2+}\) transient (Sandow A, 1952; Guatimosim et al., 2002). When the RyR channel is in a resting state during cell relaxation, an efflux of Ca\(^{2+}\) from the SR through RyRs has also been documented (Cheng et al. 1993). Using confocal fluorescent line scanning, this local and transient intracellular Ca\(^{2+}\) release can be captured as a spark. By studying the RyRs in planar lipid bilayers, Kettlun et al. showed that the Ca\(^{2+}\) sparks are generated by opening of multiple instead of single RyR channels (Kettlun et al. 2003). It is now believed that Ca\(^{2+}\) sparks reflect the synchronous activation of a cluster of 6 to 20 RyRs (Guo et al. 2006). Therefore, a Ca\(^{2+}\) spark is considered the smallest unit of Ca\(^{2+}\) release, while a Ca\(^{2+}\) transient can be understood as a coordinate formation of a large number of sparks.

While cardiac I\(_{CaL}\) is inactivated by its own charge ions (see Section 1.6), both the sarcolemmal Ca\(^{2+}\) entry through I\(_{CaL}\) and the Ca\(^{2+}\) released from the SR contribute significantly to I\(_{CaL}\) inactivation (Bers & Perez-Reyes, 1999). Quantitative analysis indicates that the rate of inactivation of I\(_{CaL}\) was linearly related to SR Ca\(^{2+}\) release and reduced when SR Ca\(^{2+}\) release was absent (Adachi-Akahane et al., 1996). As such, a stronger Ca\(^{2+}\) re-uptake would decrease I\(_{CaL}\) inactivation. Meanwhile, a diminished SR Ca\(^{2+}\) release or a stronger Ca\(^{2+}\) re-uptake reduce Ca\(^{2+}\) extrusion via NCX and this shortens APD and reduces I\(_{CaL}\) (Bers & Perez-Reyes, 1999).
2.2. β-adrenergic modulation of Ca$^{2+}$ handling in cardiomyocytes

Cellular Ca$^{2+}$ handling is highly regulated by the sympathetic nervous system. In response to neurohormonal stimulation, a catecholamine (such as norepinephrine) binds and activates β-adrenergic receptors, which are G protein-coupled receptors. The membrane-bound G protein consists of the Gα and the tightly associated Gβγ subunits. Four main families exist for Gα subunits: Gαs, Gαi, Gαq/11, and Gα12/13. Once the β-adrenergic receptor is activated, it functions as a guanine nucleotide exchange factor (GEF) that exchanges GTP in place of GDP on the Gαs. Gαs is then released to the cytosol and it then activates adenyl cyclase (AC), which enhance the catalysis of ATP to 3', 5'-cyclic AMP (cAMP). cAMP acts as a second messenger that interacts with and activates protein kinase A (PKA). PKA then phosphorylates Serine or Threonine residues on its downstream proteins, including the LTCC, the RyR, troponin I (TnI), phospholamban (PLN), protein phosphatase inhibitor-1 (I-1), myosin-binding protein C (MyBP-C), to modulate cardiac contractile function.

2.3. Transmural heterogeneity of mechanical function and Ca$^{2+}$ activity in canine ventricle

In addition to the electrical heterogeneity that has been discussed above (Introduction 1.10., page 14), transmural differences in mechanical and Ca$^{2+}$ activity have also been observed across the ventricular wall. Cordeiro et al. reported ~1.6-fold higher myocyte contractility in canine LV endo- than in epicardial cells (Cordeiro et al. 2004). Contrarily, the Ca$^{2+}$ transient was smaller in endo- than in epicardial cells in their study. At whole heart level, when paced at 5 Hz, diastolic and systolic intracellular Ca$^{2+}$ as well as the Ca$^{2+}$ transient were higher in endocardial than in epicardial myocytes.
in rat (Figueroedo et al. 1993). In a canine wedge preparation, the decay of Ca\(^{2+}\) transient was slower in the endocardial surface compared with the epicardial surface (Laurita et al. 2003), implying that epicardium has a greater Ca\(^{2+}\) reuptake than endocardium. Consistently, SERCA2a expression was higher in the subepicardial layers compared with the subendocardial layer in this study. Prestle et al. also observed that the expression level of SERCA2a is slightly higher in human epicardial cells. However, they found that the inhibitory regulator of SERCA2a, phospholamban (PLN) protein, was also slightly higher in human epi- than in endocardial cells (Prestle et al. 1999). Therefore, the function of SR reuptake in epi- and endocardial is complex. Hittinger et al. reported that the cardiac ryanodine receptor (RyR2) protein expression was nearly 2-fold higher in endo- than in epicardial myocytes in canine (1999), which may correlate to a larger SR release in endo- than epicardial cells. Collectively, current evidence demonstrated strongly that there is a spatial-dependent mechanical difference in the ventricular wall.

2.4. Altered Ca\(^{2+}\) handling in HF

Ventricular cardiomyocyte mechanical properties are significantly depressed in HF, with depressed Ca\(^{2+}\) transients and reduced contractions (Pogwizd & Bers, 2004). There are three factors that may contribute to the depressed mechanics: 1) reduced sarcolemmal Ca\(^{2+}\) entry; 2) reduced EC coupling gain (efficiency of Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR)); 3) reduced SR Ca\(^{2+}\) content. Numerous studies have focused on alterations of L-type Ca\(^{2+}\) current, the main sarcolemmal Ca\(^{2+}\) entry source, in HF patient and animal models (Kaab et al. 1996; Mewes & Ravens, 1994; Li et al. 2004; Ouadid et al. 1995; Tsuji et al. 2000). \(I_{Ca,L}\) was found to be unchanged in a study using a pacing-induced canine HF model (Kaab et al. 1996) and in HF human patients (Li et
al. 2004; Mewes & Ravens, 1994), but was found to be reduced in other studies, including in a pacing-induced rabbit HF model (Tsuji et al. 2000) and in HF patients (Ouadid et al. 1995). Therefore, whether Ca\textsuperscript{2+} entry through I_{Ca,L} is changed in HF is still controversial. Another theory of I_{Ca,L} alteration under the disease condition is that the density of I_{Ca,L} is determined by the stage of HF (Jin et al. 2008, review). Based on experimental data (Richard et al., 1998; Hill, 2003), Jin et al. concluded that I_{Ca,L} is increased in mild-to-modest hypertrophy, and is decreased in the end stage of hypertrophy and HF. Another source of sarcolemmal Ca\textsuperscript{2+} entry is NCX current in its reverse mode. It was found that the functional expression of NCX is increased in the HF model (Pogwizd et al., 2001), which may lead to an increase of Ca\textsuperscript{2+} entry in the early phase of AP. This may also lead to an increased NCX current (forward mode) to pump out the Ca\textsuperscript{2+} from the cell and induce a transient inward current (I_{ti}). The I_{ti} is one of the main ionic mechanisms for DAD development (Pogwizd & Bers, 2004). The upregulated NCX current in HF, along with downregulation of SERCA function (Pogwizd et al., 2001), can contribute to a reduction of SR Ca\textsuperscript{2+} content, which has been consistently found in human HF as well as in animal HF models (Hobai & O'Rourke, 2001; Pogwizd et al., 2001; Piacentino et al., 2003). An unchanged EC coupling gain, i.e. the efficiency of CICR, has been reported in HF in several studies (Pogwizd & Bers, 2004, review). All of the above data suggest that the contractile dysfunction in various forms of HF is due mainly to reduced SR Ca\textsuperscript{2+} load.

Increased diastolic spontaneous Ca\textsuperscript{2+} release (Ca\textsuperscript{2+} leak) has been reported in the ventricular myocytes from multiple HF animal models (Shannon et al., 2003; Marx et al., 2000). The consequence of this change is the abnormally high [Ca\textsuperscript{2+}]i during diastole, which may, in addition to the enhanced NCX in HF, induce the transient
inward current ($I_{hi}$), and trigger the arrhythmic activity. The linkage between the $\text{Ca}^{2+}$ leak and triggered arrhythmias was supported by the fact that inherited RyR2 mutations cause sudden cardiac deaths. Lehnart and colleagues showed that a missense RyR2 mutation found in the patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) results in exercise-induced arrhythmias and sudden cardiac death in a mouse model (Lehnart et al., 2008). With higher RyR channel open probability, an increase of the amplitude of the $\text{Ca}^{2+}$ transient is expected in HF condition. However, a study showed that this effect disappears within a few beats because the increased sarcolemmal efflux of $\text{Ca}^{2+}$ decreases SR $\text{Ca}^{2+}$ content (Eisner et al., 2009). It was believed, therefore, that the leaky RyR does not play a dominant role in modulating the mechanical force of cardiomyocytes. Conversely, a study showed that in cardiomyocytes with pharmacologically-induced SR $\text{Ca}^{2+}$ leak, the amplitude of cell shortening and of $\text{Ca}^{2+}$ transients was decreased (Mackiewicz & Lewartowski, 2008).
3. The transient outward potassium current (I\(_{to}\)) and its effects on cardiac electrical and mechanical properties

3.1. Cardiac ventricular I\(_{to}\) phenotypes

The early phase of AP repolarization (phase 1) in cardiac ventricles is the result of activation of the transient outward current --- I\(_{to}\), which gives rise to the prominent phase 1 notch in large animals. There are some common characteristics of this current in cardiac ventricles. First, I\(_{to}\) currents display high K\(^+\) selectivity (P\(_{Na}\)/P\(_{K}\) < 0.1), and hence are repolarizing currents. Second, ventricular I\(_{to}\) currents activate and inactivate rapidly in a voltage-dependent manner, with time constants of the order of milliseconds and tens to hundreds of milliseconds, respectively (Zipes, 2009). While this current is 4-aminopyridine (4-AP) sensitive, 4-AP is non-specific in blocking I\(_{to}\) (Patel & Campbell, 2005).

3.2. Species- and location- dependent heterogeneity of I\(_{to}\)

I\(_{to}\) has been identified in human, dog, rabbit, rat, and mouse ventricles, but not in guinea pig (Findlay, 2003; Missan et al., 2004). Although two papers reported an I\(_{to}\)-like current in isolated myocytes of the guinea pig ventricle, a recent study excluded the possibility that guinea pig has a voltage-dependent, transient outward K\(^+\) current (Findlay, 2003). I\(_{to}\) density is different in different species. In ventricular myocytes in small animals, I\(_{to}\) density is about 60pA/pF at +40mV, where in large animals, this value is only about 20pA/pF in epicardial myocytes (Sun & Wang, 2005). This robust outward I\(_{to}\) density in rodents is believed to over-power the depolarizing current in the end of phase 1 and abolish the “dome,” resulting in the triangular shape of AP waveform.
(Greenstein et al., 2000). In large animals, I_{to} is responsible for the phase 1 notch and causes the “spike-and-dome” waveform. It was found that the higher the I_{to} density, the deeper the notch (Sun & Wang, 2005; Greenstein et al., 2000).

I_{to} density also varies in different regions of the ventricular wall in some species, such as canine (Litovsky & Antzelevitch, 1988; Liu et al., 1993), cat (Furukawa et al., 1992), ferret (Brahmajothi, 1999), and human (Wettwer et al, 1994; Nabauer et al, 1996). In canine left ventricles, for example, I_{to} density is 5- to 6-fold higher in epicardial and midmyocardial cells than in endocardial cells (Liu et al., 1993; Litovsky & Antzelevitch, 1988). This heterogeneity is proposed to be the reason for the shorter APD and early repolarization in epicardial than in endocardium (Litovsky & Antzelevitch, 1998). In addition, the right ventricular epicardial cells seem to have a more pronounced I_{to} than the left ventricular cells (Di Diego et al., 1996). It was found that at +20mV, the average peak density of I_{to} is significantly higher in canine RV epicardial cells than in endocardial cells (17.1 ±3.5 and 13.7±5.6 for RV and LV epicardial cells, respectively).

3.3. Molecular correlates of I_{to} in cardiomyocytes

The I_{to} channel, same as the cardiac Na\(^+\) channel, consists of pore-forming (α) and accessory (β) subunits. The α subunits belongs to the shal-type Kv4.x family, including Kv4.2, and /or Kv4.3 proteins (Nerbonne, 2000). Each subunit has six transmembrane domains (S1-S6), including one domain (S4) that senses transmembrane voltage and a K\(^+\) selective pore (Figure 4A; Tristani-Firouzi et al. 2001). Both the N and C terminal of the protein are located in the intracellular side of the membrane.

Four Kv α subunits form a tetramer I_{to} channel (Nerbonne, 2000; Figure 4B). The
Figure 4. Structure of voltage gated Kv4.x channel.

A, Each Kv4.x α subunit protein has six membrane-spanning regions, a K⁺-selective pore and a highly charged S4 domain. B, Four Kv α subunits form a tetramer Iₒ channel.
composition differs in different species. It is now clear that $I_{to}$ consists of Kv4.2 and Kv4.3 in rodent, while only Kv4.3 subunit (encoded by KCND3 gene) is responsible for $I_{to}$ in the canine and human heart (Birnbaum et al., 2004; review). A study showed that coexpression of Kv4.2 and Kv4.3 (ratio 1:1) produces an $I_{to}$ that is similar to the native $I_{to}$ in mouse ventricles (Guo et al., 2002), implying that the native tetrameric $I_{to}$ channel in mouse is more likely composed of two Kv4.3 subunits and two Kv4.2 subunits.

### 3.4. Interacting subunit of Kv4.2 & Kv4.3

Several interacting subunits of Kv4 channels have been identified and have been shown to modulate the properties and/or the expression of the Kv α-subunit (Birnbaum et al., 2004; review). For example, when coexpressed in HEK-293 cells, Kvβ1.2 confers O$_2$ sensitivity to Kv4.2-induced K$^+$ currents (Perez-Garcia et al., 1999).

Among these subunit proteins, KChIP2 (encoded by KCNIP2) has been identified as an important ancillary subunit for Kv4.2 and Kv4.3 in the heart. It was first cloned by An et al. in 2000 and was shown to specifically bind with the Kv channel at its N-terminus (An et al., 2000). Coexpression of KChIP2 with Kv4.3 increases $I_{to}$ amplitude (Pourrier et al., 2003; review). In addition, KChIP2 is also shown to modulate the trafficking of the channels from the ER to the cell membrane (Kuo et al., 2001). Patel et al. also reported that KChIP2 alters the gating properties of Kv4 channels: slowing of inactivation and facilitating recovery from inactivation (2002).

A study showed that the KChIP2 mRNA and protein level but not those of Kv4.3 parallels the heterogeneous $I_{to}$ density in the ventricle (Rosati et al., 2003), suggesting that KChIP2 is responsible for the $I_{to}$ density gradient across the canine and human...
ventricular wall. However, another study showed that both Kv4.3 and KChIP2 mRNA as well as protein levels correlate to the I_{to} functional expression (Zicha et al., 2004).

3.5. Downregulation of I_{to} density in failing ventricular cardiomyocytes

I_{to} density has been consistently found to be downregulated in failing cardiomyocytes, with a more obvious downregulation in the epicardial than in the endocardial cells (Kaab et al., 1996; Tsuji et al., 2000; Beuckelmann et al., 1993; Li et al., 2002). Downregulation of I_{to} and APD prolongation are considered as the two electrical hallmark changes in the failing cardiomyocytes. Some studies showed that the I_{to} changes are without significant kinetic or steady-state gating changes (Xiao et al., 2008), while others showed slowed I_{to} recovery from inactivation, positively shifted inactivation voltage dependence, and reduced conductance in a fast-pacing canine HF model (Yu et al., 1999). Studies have shown that the molecular identity of I_{to} downregulation in HF is mostly due to the diminished Kv4.3 but not KChIP2 expression, both at gene and protein levels (Rose et al., 2005). Increasing evidence has also suggested that intracellular [Ca^{2+}]_{i} plays a critical role in downregulation of I_{to}. It was found that increased Ca^{2+}/calmodulin binding triggers calcineurin, a Ca^{2+}-dependent phosphatase, to dephosphorylate the transcription factor NFATc3 regulatory domain. I_{to} downregulation is caused by this increased calcineurin/NFATc3 signaling in a variety of physiological contexts, including tachycardia, increased extracellular Ca^{2+} (Perrier et al., 2004), endocardial tissues (Rossow et al., 2006), and myocardial infarction (Rossow, 2004).

3.6. Role of I_{to} in modulating AP morphology and duration
$I_{to}$ is a key repolarizing current. The influence of $I_{to}$ on APD, though clearly elucidated in small animals, is still unclear in large animals. In small animals such as mice and rats, the density of $I_{to}$ is around 60pF/pA, which is about 4-5 fold of that in large animals. This remarkable $I_{to}$ has been unanimously believed to be responsible for the short APD and the triangular AP shape in rodents (Brunner et al., 2001; Qin et al., 1996; Sun and Wang, 2005). However, the influence of $I_{to}$ on APD in large animals is more controversial. It was proposed that $I_{to}$ downregulation contributes to the prolongation of APD in a failing heart (Beuckelmann et al., 1993), and that $I_{to}$ is a potential therapeutic target in preventing and treating HF-related arrhythmias (Kaab et al., 1996). However, as extensive efforts focused on this question using various methods, different results were produced. The main methods and the major findings of these works are:

1) Pharmacological studies, mostly using $I_{to}$ blocker 4-AP, showed inconsistent results. High concentration of 4-AP (3-5 mmol/L) prolongs the APD in Ca$^{2+}$-buffered human (Beuckelmann et al., 1993) and canine (Kaab et al., 1996) ventricular midmyocytes. However, low concentration of 4-AP (1 mmol/L), in the absence of Ca$^{2+}$ buffers, shortens APD in canine ventricular mid- (Zygmun et al., 1997) and epicardial cells (Litovsky & Antzelevitch, 1988). In addition to this disparity, any conclusions from the pharmacological studies are compromised by the lack of specificity of $I_{to}$ blockers. The most commonly used blocker, 4-AP, also affects other currents. It has been shown that 4-AP blocks another repolarizing current --- the delayed rectifier K$^+$ current ($I_k$) (Ridley et al., 2003) and increases the $I_{Ca,L}$ current (Wang et al., 2006). Along with these findings, we can predict that 4-AP can prolong the duration of AP
and enhance sarcolemmal Ca$^{2+}$ entry through its effects on other currents but not necessary through $I_{to}$.

2) Studies using computational simulation also produced conflicting results (Winslow et al., 1999; Greenstein et al., 2000; Priebe & Beuckelmann, 1998; Hund & Rudy, 2004). For example, Greenstein et al. showed a threshold dependent relation between $I_{to}$ and APD: below a certain $I_{to}$ threshold, increasing $I_{to}$ density modestly prolonged APD, whereas APD was significantly decreased when $I_{to}$ density was larger than the threshold (Greenstein et al., 2000). On the other hand, Priebe & Beuckelmann showed that decreasing $I_{to}$ density progressively increased APD. Although a powerful tool, computer simulation studies are limited by their abilities of thoroughly mimicking the complicated cellular background.

3) Guinea pigs lack a native $I_{to}$ current, resulting in the absence of the phase 1 notch (Sanguinetti & Jurkiewicz, 1990, 1991). By expression of the $I_{to}$ channel encoding Kv4.3 gene, the effect of an $I_{to}$-like current on AP was tested in guinea pig ventricular cardiomyocytes (Hoppe et al., 2000). Introducing this $I_{to}$-like current did not generate a prominent phase 1 notch; instead, it progressively suppressed the AP plateau and shortened the APD over the physiological range of $I_{to}$. However, whether this Kv4.3-overexpression produced a current that represents the native $I_{to}$ current is not clear. The $I_{to}$ channel subunit KChIP2 protein has been shown to change the $I_{to}$ amplitude (Pourrier et al., 2003; review) and alter the kinetics of $I_{to}$ currents (Patel et al., 2002). Expression of Kv4.3 protein alone failed to fully reconstitute the gating kinetics of the native cardiac $I_{to}$ current (Patel et al., 2004). Notably, the phenotype of the $I_{to}$ current in this guinea pig study has a sustained inward current component which is not normally present in native cells and may have influence on APD.
4) Our laboratory used the dynamic clamp technique to study the role of $I_{to}$ in regulating the AP morphology and duration in the canine left ventricle (Sun & Wang, 2005). We showed that $I_{to}$, while being a key regulator of phase 1 repolarization, is not a major regulator of the APD over a wide current density range. Only when $I_{to}$ densities are above a threshold (a value that is higher than the physiological $I_{to}$ levels in canine LV epicardial cells), $I_{to}$ significantly shortens the APD and results in all-or-none repolarization. A notable limitation of this study is the use of the whole-cell patch clamp and intracellular Ca$^{2+}$ buffer, which can alter Ca$^{2+}$ intracellular handling and affect the AP properties (Dong et al., 2006).

*Understanding the role of $I_{to}$ in regulating APD is of importance since it will improve our knowledge on the arrhythmogenesis in the failing heart, and will provide insights for developing future treatments for HF. Overall, the above studies represent a significant controversy and limitation of available techniques and previous studies in addressing this issue. In my thesis study, the role of $I_{to}$ in regulating APD is addressed using an improved dynamic clamp method.*

### 3.7. Role of $I_{to}$ in modulating intracellular Ca$^{2+}$ handling and mechanical function

$I_{to}$ is the major player in early phase repolarization and a key determinant of the AP morphology. It overlaps with the $I_{Ca,L}$ during AP time-course and may modulate the magnitude of $I_{Ca,L}$ and EC coupling (Zygmunt et al., 1997). Recently, evidence has suggested a tight relation between $I_{to}$ and intracellular Ca$^{2+}$ dynamics. Greenstein et al. showed that increasing $I_{to}$ density leads to an increase both in the open probability of L type Ca$^{2+}$ channel and L type Ca$^{2+}$ current ($I_{Ca,L}$) amplitude in a modeled canine
ventricular cell (Greenstein et al., 2000). Experimentally, implementation of an epicardial AP waveform in a canine cardiomyocyte was associated with a higher cell fractional shortening (FS) than when the cell was imposed to an endocardial AP waveform (Cordeiro et al., 2004). A study further suggested that Ito has a positive inotropic effect by showing that fast repolarization enhanced Ca$^{2+}$ influx and SR Ca$^{2+}$ release in rat myocytes (Sah et al., 2002), predicting that Ito downregulation may contribute to the impaired intracellular Ca$^{2+}$ handling and contractility in a failing heart. However, other studies have yielded contrary results. Bouchard et al. reported that Ca$^{2+}$ entry along with Ca$^{2+}$ transient and myocyte contractility were enhanced after pharmacological Ito blockade in rat ventricular myocytes (Bouchard et al., 1995). Supporting this, increased I_{Ca,L} was observed after pharmacological blockade of Ito (Wang et al., 2006) or under AP clamp with Ito blocker-induced AP morphology (Zygmunt et al., 1997). Collectively, the above studies demonstrate an important but unsettled controversial role for Ito in regulating intracellular Ca$^{2+}$ handling.
4. The Brugada syndrome and its underlying ionic mechanism

4.1. General characterization and epidemiology of Brugada syndrome

The Brugada syndrome was first described by the Brugada brothers in 1992 (Brugada & Brugada, 1992). They reported that 8 patients, 6 males and 2 females, had recurrent episodes of ventricular tachycardia (VT) or ventricular fibrillation (VF) and that the arrhythmias could not be explained by any known diseases. These patients had two consistent ECG characteristics: 1) right bundle branch blocks which indicate a defect in the right ventricular electrical conduction system; 2) persistent ST-segment elevation in the right precordial leads V1-V3. No histological abnormalities were found in these patients.

After the first description and recognition, the number of reported cases has been increasing in recent years. The disease is now believed to be responsible for up to 20% of sudden death in subjects without accompanied cardiomyopathy (Benito et al., 2008). The syndrome usually presents during adulthood, with a mean age of sudden death of 41 ± 15 years (Antzelevitch et al., 2005). The prevalence of Brugada syndrome is estimated at 1-5 per 10,000 people worldwide, with the highest frequency in Southeast Asia (≥ 5 per 10,000), such as Thailand and the Philippines, where the syndrome is one of the main causes of sudden cardiac death in young male adults (Antzelevitch, 2006). Males represent the majority of the patients and they display a higher risk profile and worse prognosis than women (Benito et al., 2008).

Three types of ST segment elevation in the right precordial ECG leads have been described in Brugada syndrome patients: coved type (type 1), saddleback with ≥ 1mm elevation (type 2), and saddleback with < 1mm elevation (type 3). A type 1 ST segment
elevation larger than 2 mm in more than 1 right precordial lead (V1-V3) is proposed as definitively diagnosed criteria in the Brugada Syndrome Consensus Conference (Antzelevitch, 2006). The ST segment is a short section of ECG baseline that begins at the end of the QRS complex and ends at the beginning of the T wave. It represents the early phase of repolarization of the ventricle (Antzelevitch et al., 1999). The ECG abnormalities are predominantly identified in the right precordial ECG leads, indicating that Brugada syndrome is a right ventricular arrhythmic disease.

4.2. Genetic factors underlying Brugada syndrome

Brugada syndrome is an inherited channelopathy transmitted in an autosomal dominant mode. The first and a well-known gene linked to this disease is SCN5A, the gene that encodes the α subunit of cardiac Na⁺ channels. As mentioned above (page 5-6), cardiac Na⁺ channels mediate depolarizing Iₙa and are TTX sensitive. Since the first SCN5A gene mutation was found (Chen et al., 1998), more than one hundred SCN5A mutation variants have been identified up to the present (Antzelevitch, 2006; Remme et al., 2008). Some of these mutations have been studied in expression systems and shown to result in the attenuation of Iₙa by changing Na⁺ current kinetics or trafficking of the Na⁺ channel proteins to the membrane (Cordeiro et al., 2006). Studies have shown that the homozygous mutants usually resulted in complete loss of function. In heterozygous mutants, however, the percentage of Iₙa amplitude reduction is different based upon different mutations. For example, Smits et al. showed about 60% reduction in peak current with mutant channels compared to the control channels (Smits et al., 2005). Another study found that heterozygous expression of SCN5A mutants resulted in 74% ± 6% less peak macroscopic Iₙa, when compared with wild-type channels (Pfahnl et al.,
Two other studies showed that coexpression of mutants with wild type channels resulted in a nearly 50% reduction of Na\(^{+}\) currents’ amplitude with no significant alterations of biophysical properties (Baroudi et al., 2004; Keller et al., 2005). Overall, these studies indicated a 50 - 75% reduction in I\(_{\text{Na}}\) of SCN5A heterozygous mutants.

Although Brugada syndrome has been linked to SCN5A, SCN5A gene mutations are found in only 18 - 30% of patients (Antzelevitch et al., 2005). Promoter gene or alternative splicing mutations have also been demonstrated to contribute to the disease. Hong et al. (2005) provided the first report of a dysfunctional Na\(^{+}\) channel created by an intronic mutation giving rise to cryptic splice site activation in SCN5A in a family with Brugada syndrome. Bezzina and colleagues recently provided evidence, which supports that SCN5A promoter polymorphisms may contribute to the differing prevalences of Brugada syndrome in Asian, Caucasian or Africa patients (Yang et al., 2008). In addition to the mutations found in the SCN5A gene, a second mutation in the glycerol-3-phosphate dehydrogenase 1-like gene (GPD1-L), which disrupted trafficking of SCN5A, was found in a family with Brugada syndrome (Van Norstrand et al., 2007). Recently, the third and fourth genes associated with Brugada syndrome were reported and shown to encode the \(\alpha1\) (CACNA1C) and \(\beta\) (CACNB2b) subunits of the L-type Ca\(^{2+}\) channel. These mutations in the \(\alpha\) and \(\beta\) subunits of the Ca\(^{2+}\) channel can also lead to a shorter than normal QT interval, creating a new clinical entity consisting of a combined Brugada/short-QT syndrome (Antzelevitch, 2007). A recent study also identified a mutation in KCNE3 (MiRP2 protein) in a patient with Brugada syndrome. This mutation was suggested to result in increased I\(_{\text{to}}\) densities (Delpon et al., 2008).

4.3. Proposed electrophysiological mechanism for Brugada syndrome

39
Brugada syndrome is primarily considered an electrical disease. Under this concept, two ionic mechanisms underlying this disease have been proposed: the repolarization disorder model and the depolarization disorder model. Both of them provide explanations for the characteristic ST segment elevation and high propensity for VT/VF.

### 4.3.1. The repolarization disorder model

The repolarization disorder model has received the widest support, both from experimental and clinical studies. It was first demonstrated by Yan and Antzelevitch in a canine right ventricular wedge preparation (Yan & Antzelevitch, 1999). They simultaneously recorded the APs in 2 epicardial sites and 1 endocardial site, together with transmural ECG recordings. As shown in Figure 5A, in control RV tissues, APD is shorter in epi- than in endo- and M cells, corresponding to a positive T wave in ECG. The ST segment remains isoelectric because of the absence of transmural voltage gradients at the level of the AP plateau. As discussed above (page 28-29), the AP notch is more profound in RV epicardium with a prominent Ito in this region, forming the spike-and-dome waveform. When the tissue was perfused with Na+ channel blocker to mimic the INa reduction or with K+ channel opener to increase the K+ current, the phase 1 notch was accentuated in epicardium, but not in endocardium, which resulted in a marked transmural voltage gradient and reversed the APDs in epi- vs. endocardium, and lead to the development of a coved-type ST segment elevation and a negative T wave (Fig. 5B).

When the prominent Ito overpowered the reduced INa, loss of the AP dome was produced at some epicardial sites, but not in others. This gave rise to a marked dispersion of repolarization within the epicardium and transmurally, between epicardium and endocardium (Fig. 5C). The heterogeneous loss of the dome provided a window for re-excitation via a phase 2 reentry mechanism (for details, see Introduction 1.12.2., page 18).
and underlaid the VT/VF in Brugada syndrome (Fig. 5D). Using optical imaging, a study further supported this model. Kimura et al. found that epicardium developed the most significant dispersion in repolarization after giving $I_{Na}$ blocker or $K_{ATP}$ opener, and the extrosystole originated from a site with a short recovery time (Kimura et al., 2004). A later clinical study recorded a spike-and-dome AP with a deep notch in all 3 Brugada syndrome patients but not in any control patients (Kurita et al., 2002). The deeply notched AP would be expected to induce the loss of dome and transmural dispersion.

4.3.2. The depolarization disorder model

The depolarization disorder model proposed that the APs from right ventricular outflow tract (RVOT) is delayed with respect to the RV AP, and that the ST elevation is not due to the different AP shapes, but due to the conduction delay in RVOT. This model is mostly supported by clinical studies (Tukkie et al., 2004; Coronel et al., 2005; Postema et al., 2008; Lambiase et al., 2009; Nishii et al., 2010). For instance, using mapping measurements, conduction delay but not transmural repolarization differences was found in an explanted heart from a Brugada syndrome patient (Coronel et al., 2005). This conduction delay originated at the RVOT endocardium caused the ECG signs and was the origin of ventricular fibrillation.

4.4. Wall motion abnormalities in Brugada syndrome and possible underlying mechanism

After the first identification of Brugada syndrome in 1992, many controversies have surrounded this disease. One of them is whether this disease represents a new clinical entity or it is just a subtype of the arrhythmogenic right ventricular cardiomyopathy/
Figure 5

A Normal

Transmembrane Action Potentials

ECG (V2)

200 msec

B Brugada Syndrome (coved)

Transmural voltage gradient

Endo Epi

C Brugada Syndrome (Heterogeneous loss of AP dome)

Transmural dispersion of repolarization

Endo Epi

D Brugada Syndrome (Phase 2 Reentry)

Epi (Phase 2 Reentry)
Figure 5. Schematic representation of RV epicardial action potential changes thought to underlie the ECG manifestation and arrhythmogenesis of Brugada syndrome.

A, In control tissues, ST segment remains isoelectric and the APD is shorter in epi- than in endo- and M cells, which creates a positive T wave; B, Accentuation of the phase 1 notch in epicardium, but not in endocardium, results in a marked transmural voltage gradient and reverses the APDs in epi- vs. endocardium, leading to the development of a coved-type ST segment elevation and a negative T wave; C, Loss of the AP dome at some epicardial sites, but not in others, produces dispersion of repolarization within the epicardium and transmurally; D, The significantly shortened APs in some epicardial cells provide a window for re-excitation via a phase 2 reentry mechanism (Adapted from Antzelevitch et al., 2003.)
dysplasia (ARVC/D) (Martini et al., 1993; Naccarella, 1993). Structural abnormalities, a characterization of ARVC/D, were also reported in patients with Brugada syndrome (Takagi et al., 2001; Papavassiliu, 2004; Frustaci et al., 2005). ST segment elevation and polymorphic VT which characterize Brugada syndrome have also been found in ARVC/D (Corrado et al., 2001). ARVC/D is a type of nonischemic cardiomyopathy that involves primarily the right ventricle. It is characterized by hypokinetic areas with fibrofatty replacement and associated arrhythmias originating in the right ventricle. The Brugada syndrome, however, was described as a primary electrical disease in the Brugada syndrome Consensus Conference (Antzelevitch et al., 2005). ECG, angiography, MRI, and biopsy usually show no overt structural heart disease in Brugada syndrome patients. The conference, though, did not exclude the possibility that mild fibrosis may happen in Brugada syndrome patients and precipitate the arrhythmic events.

In addition to the structural abnormality, right ventricular motion abnormalities were found in Brugada syndrome patients. Using the electron beam CT, Takagi et al. demonstrated morphological abnormalities of the right ventricle in 21 (81%) of 26 Brugada syndrome patients, but in only two (9%) of 23 controls (2001). The same research group also showed that after giving disopyramide, a Na+ channel blocker, wall motion abnormalities appeared in the right ventricular outflow tract region in eight (62%) of the 13 patients, suggesting that Na+ channel dysfunction may induce the contractile changes (Takagi, 2003). Using magnetic resonance imaging (MRI), Papavassiliu et al. found decreased RV ejection fraction (EF) compared with controls, even though the difference did not reach significance (Papavassiliu et al., 2004). Recently, a Chinese group reported wall motion abnormalities at the interventricular septum detected by two-dimensional echocardiography (Huang et al., 2007).
These contractile abnormalities are commonly considered as an indication of structural changes, and it was proposed that such localized morphological changes may contribute to the arrhythmogenic substrate in Brugada syndrome (Razmi, 2004; Meregalli et al., 2005). For this reason, the mechanism of the contractile abnormality in Brugada syndrome is of significance for it is relevant to the arrhythmogenesis of the syndrome. On the other hand, Dr. Antzelevitch speculated that contractile abnormalities can be explained by the primary electrical dysfunction responsible for Brugada syndrome (Antzelevtich, 2002). He suggested that the all-or-none repolarization believed to happen in Brugada syndrome patients causes the L-type Ca^{2+} channel to inactivate very soon after it activates. Consequently, Ca^{2+} entry is drastically reduced, and the cell becomes depleted of Ca^{2+} within seconds and all contractile function ceases. This hypothesis, however, is not supported by any experimental evidence, which represents an important gap in our current understanding of this clinical entity.

4.5. Right ventricular outflow tract (RVOT)

The RVOT is the portion of the right ventricle that leads up to the pulmonary valve and pulmonary artery. When the right ventricle contracts, blood is forced along this RVOT region to the pulmonary artery. There is growing clinical evidence that the RVOT is the origin of arrhythmias in patients with Brugada syndrome (Shimada, 1996; Ogawa, 2001; Takagi et al., 2001; Papavassiliu et al., 2004; Coronel, 2005; Yokokawa, 2006). One example is from an explanted heart from a Brugada patient who underwent cardiac transplantation. Activation mapping showed that the induced VF was originated in the RVOT (Coronel, 2005).
What causes the RVOT to be a hotspot of arrhythmias in the patients with Brugada syndrome? Using an optical mapping method, Morita et al., has shown that the RVOT epicardial cells have a deeper baseline phase 1 notch than other areas do, which causes them to have a higher tendency for all-or-none repolarization (Morita et al., 2007). The abnormal AP profile then produces great electrical heterogeneity, mainly the repolarization heterogeneity, either within the RVOT epicardium or between epi- and endocardium, and induces the phase 2 reentry arrhythmias. Using the contact electrode method, Kurita et al. recorded a spike-and-dome configuration of AP from RVOT epicardium in all 3 Brugada patients but not in any control patients (2002). Morita et al. further showed that RVOT has a deeper phase 1 notch at baseline than the RV does in isolated canine heart preparations (2007). In their later study, they found that inducing Brugada syndrome by $I_{Na}$ blocker produced two types of APs at the same time within the RVOT epicardium: prolonged AP without all-or-none repolarization and an abbreviated one with all-or-none repolarization (Morita et al., 2008). Consistently, they found that phase 2 reentry originated from the epicardium in 88% of RVOT preparations, while none originated in RVOT endocardium and RV.

4.6. Treatment options for Brugada syndrome

Various devices and pharmacological therapies have been tested in patients with Brugada syndrome. Currently, implantable cardioverter defibrillator (ICD) is proven to be effective for the disease (Antzelevitch et al., 2005). In a multicenter study in which 258 patients received an ICD, the cumulative efficacy was 38% at 5 years of follow up. The pharmacological approach has been focused on reducing the phase 1 notch and restoring the AP dome in the right ventricular epicardial cells. Therefore, Quinidine, a
class IA antiarrhythmic and I_{io} blocker, has been proposed to be protective. Clinical
evidences supported this hypothesis, showing that quinidine normalized ST segment
elevation in Brugada syndrome patients (Alings et al., 2001) and reduced arrhythmias in
high-risk patients with Brugada syndrome (Belhassen et al., 2004). β-adrenergic agonist
isoproterenol and phosphodiesterase III inhibitor cilostazol have also been shown to be
effective, presumably because their ability to increase L-type Ca^{2+} current and decrease
the depth of phase 1 notch (Tanaka et al., 2001; Silva et al., 2004).

5. Mathematical modeling of cardiac myocyte and the dynamic clamp

5.1. Hodgkin–Huxley model of membrane current

The electrical behavior of ion channels can be described by mathematical equations,
a concept that was first introduced by the Nobel Laureates Hodgkin and Huxley in 1952
(Hodgkin and Huxley, 1952). They numerically defined a membrane current using
empirical kinetics data, and the mathematical equation they developed is able to
characterize specific channel states (e.g. open, closed, inactivated). They used a series of
hypothetical gates to describe the open- and close-state of the channel. The conductance
is a function of these gates and also a function of the maximum conductance of the
membrane for an ion type:

\[ I = g \cdot m \cdot h \cdot (V_m - E_m) \]  

(1)

\( g \) is the maximum conductance (mS/µF). \( V_m \) is the membrane potential. \( E_m \) is the
reversal potential of the ion current. \( V_m - E_m \) is the driving force for the particular ion.
The open probability of the activation and inactivation gate is usually assigned as \( m \) and \( h \), respectively. \( m \) and \( h \) range from 0 (all gates closed) to 1 (all gates open), and the time-dependent change in \( m \) or \( h \) is described by the first-order differential equation:

\[
m'/dt = \alpha_m \cdot (1-m) - \beta_m \cdot m
\]

\[
h'/dt = \alpha_h \cdot (1-h) - \beta_h \cdot h
\]

where \( m \) and \( (1-m) \) are the open and closed probabilities of the gate, \( t \) is time, and \( \alpha \) and \( \beta \) are opening and closing transition rate constants and are the functions of voltage but not time. If \( V \) is changed, \( \alpha_m, \beta_m, \alpha_h, \) and \( \beta_h \) instantly change values appropriate to the new voltage. Hodgkin and Huxley solved equations (2) and (3) as \( m=m_0 \) and \( h=h_0 \) at \( t=0 \):

\[
m=m_\infty-(m_\infty-m_0) \exp (-t/\tau_m)
\]

\[
h=h_\infty-(h_\infty-h_0) \exp (-t/\tau_h)
\]

where \( m_\infty= \alpha_m/(\alpha_m+\beta_m) \) and \( \tau_m=1/(\alpha_m+\beta_m) \)

\[
h_\infty= \alpha_h/(\alpha_h+\beta_h) \) and \( \tau_h=1/(\alpha_h+\beta_h) \)

By analyzing experimental voltage clamp recordings, they were able to get values of \( m_\infty, h_\infty, \tau_m, \tau_h, \alpha_m, \beta_m, \alpha_h, \beta_h \) at various voltages, and then fit the values of \( \alpha_m, \beta_m, \alpha_h, \beta_h \) to mathematical equations of the function of the voltage. For example, the \( \alpha_m \) of \( I_{Na} \) was expressed as:

\[
\alpha_m=0.1 \cdot (V+25)/\exp ((V+25)/10)-1
\]

They therefore are able to predict the current values at different voltage levels.

**5.2. Mathematical formulation for \( I_{to} \)**

The main focus of my thesis research is the role of \( I_{to} \) current in regulating cardiomyocyte electrical and mechanical properties. Quantitative study of \( I_{to} \) using
mathematical simulation is valuable, especially since specific $I_{to}$ blockers are unavailable.

Several mathematical formulations of $I_{to}$ have been developed (Gima & Rudy, 2002; Hund & Rudy, 2004; Dumaine et al., 1999; Priebe & Beuckelman, 1998; Courtemanche et al., 1998). All of those formulations are based on experimental recordings and follow Hodgkin and Huxley’s formalism (Hodgkin & Huxley, 1952). Based on these models, we developed our $I_{to}$ formulation and this formulation is described in the Methods Section (page 65).

5.3. Integrative models of ventricular cardiomyocyte

Based on Hodgkin and Huxley’s formalism, in the past decades, simulation studies have developed from modeling ionic current and simple APs into models of an entire cardiomyocyte (Luo & Rudy, 1994; Jafri et al., 1998). The first model for ventricular myocyte, usually called the Luo-Rudy phase-1 model, was developed by Luo and Rudy in 1991. Based on available experimental data from single-cell or single-channel preparations, they developed this model incorporating (a) the fast inward Na$^+$ current $I_{Na}$; (b) a slow inward current $I_{si}$, i.e. the L-type Ca$^{2+}$ current ($I_{Ca,L}$); (c) a time-dependent delayed rectifier K$^+$ current $I_K$; (d) a time independent potassium current $I_{K1}$; (e) a K$^+$ current activated at plateau potential $I_{Kp}$, later known as the ultra-rapid delayed-rectifier current ($I_{Kur}$) ; and (f) a background K$^+$ current. The simulated AP presented with some phenomenon consistent with experimental observations, such as effects of K$^+$ concentration on APD and the resting potential. The main limitation of this phase-1 model is that it did not include a sarcolemmal pump and exchanges that also contribute to changes in intracellular ionic concentrations. Also, intracellular Ca$^{2+}$ handling was missed in this phase-1 model.
Luo and Rudy soon introduced an improved model (Luo & Rudy, 1994), the Luo-Rudy Phase-2 model, which has become the most extensively used cardiac ventricular cell model. In this model, they incorporated intracellular Ca\(^{2+}\) cycling, sarcolemmal Na\(^+\)-Ca\(^{2+}\) exchange, Na\(^+\)-K\(^+\) pumping, and temporal variation of intracellular ion concentrations that were not described in the phase-1 model. Also, they separated the delayed rectifier current I\(_K\) into rapid- and slow-activating components I\(_{Kr}\) and I\(_{Ks}\). In order to simulate the dynamic process of Ca\(^{2+}\) release and reuptake by SR, they divided SR into two subcompartments, a network SR (NSR) and a junctional SR (JSR). Functionally, they designated that Ca\(^{2+}\) enters the NSR and then translocates to the JSR following a monoexponential function of Ca\(^{2+}\) concentration. SR Ca\(^{2+}\) release occurs at JSR and can be triggered by two different mechanisms, Ca\(^{2+}\)-induced Ca\(^{2+}\) release (I\(_{rel}\)) and spontaneous release (I\(_{leak}\)). In 2004, Hund and Rudy further improved the model by adding dynamic CaMKII activity and its regulation on intracellular Ca\(^{2+}\) handling (Hund & Rudy, 2004). Also, they incorporated 1) the late Na\(^+\) current, I\(_{NaL}\); 2) the Ca\(^{2+}\)-dependent transient outward current, I\(_{to2}\); 3) dynamic intracellular Cl\(^-\) concentration changes; and 4) a novel I\(_{rel}\) formulation. Figure 6 shows a generic dynamic model of the canine cardiac ventricular cell. The main components are 1) sarcolemmal ionic currents, pump, and transporters; 2) SR release and reuptake; 3) CaMKII modulation on Ca\(^{2+}\) dynamics; 4) Ca\(^{2+}\) buffers.

A concept of microdomains of Ca\(^{2+}\) inside the cell was proposed and modeled by several authors (Jafri et al., 1998; Winslow et al., 1999; Shannon et al., 2004). Jafri et al. simulated a restricted subspace where the RyRs and L-type Ca\(^{2+}\) channels interact (1998). They suggested that in this subspace, [Ca\(^{2+}\)] rises more rapidly and reaches a higher level (10-30 µM) than the bulk cytosolic Ca\(^{2+}\) (peak [Ca\(^{2+}\)]\(_i\) is about 1 µM).
Shannon et al. similarly suggested a subsarcolemmal space based on experimental evidence that the NCX, \( I_{Na} \), and \( Na^+/K^+ \)-ATPase sense local ionic concentrations different from the bulk cytosolic \([Ca^{2+}]_i\) (2004). They proposed that the ion flow between these compartments follows a simple gradient diffusion law. This subsarcolemmal model probably represents a better description of local \( Ca^{2+} \) handling, and has been shown to be suitable for studying EC coupling process (Shannon et al., 2003).

5.4. Applications of single myocyte modeling

Modeling studies can help us to solve questions that cannot be answered due to practical restraint of available technology or our current knowledge of the biological system, and have significantly advanced our understanding of cardiomyocytes electrical properties as well intracellular \(Ca^{2+}\) handling (Greenstein et al., 2000; Saucerman & Bers, 2008; Nakamura et al., 2007; Decker et al., 2009; for review see Winslow et al., 2000). Some examples of applying this method in ventricular myocytes studies includes:

1) the influence of ions or transporters on AP duration and shape (Greenstein et al., 2000);
2) consequences of ion-channel mutations or electrical remodeling at the cellular level (Clancy & Rudy, 1999; Seemann et al., 2007);
3) ionic mechanisms of delayed after-depolarizations (DADs) and early after-depolarizations (EADs) (Zeng & Rudy, 1995);
4) effects of cell signaling and regulatory pathways on the electrophysiological behavior of cardiomyocytes (Hund et al., 2008);
5) mechanism of cardiac action potential rate-adaptation (Decker et al., 2009). As an example, in a study entitled “Mechanisms of altered excitation-contraction coupling in canine tachycardia-
Figure 6
**Figure 6. Hund-Rudy dynamic model of the canine epicardial myocyte.**

SR, sarcoplasmic reticulum; CT$_{KCl}$, K$^+$-Cl$^-$ cotransporter; CT$_{NaCl}$, Na$^+$-Cl$^-$ cotransporter; $I_{NaL}$, slowly activating late Na$^+$ current; $I_{Na}$, Na$^+$ current; $I_{Nab}$, background Na$^+$ current; $I_{NaCa,i}$, Na$^+$/Ca$^{2+}$ exchanger (localized to plasma membrane); $I_{Cab}$, background Ca$^{2+}$ current; $I_{pca}$, sarcolemmal Ca$^{2+}$ pump; $I_{to1}$, 4-aminopyridine-sensitive transient outward current; $I_{Kr}$, fast component of delayed rectifier K$^+$ current; $I_{Ks}$, slow component of delayed rectifier K$^+$ current; $I_{Ki}$, time-dependent K$^+$ current; $I_{NaK}$, Na$^+$/K$^+$ pump current; $I_{leak}$, NSR leak current; $I_{Ir}$, Ca$^{2+}$ transfer from NSR to JSR; $I_{rel}$, JSR release current; $I_{Ca,L}$, L-type Ca$^{2+}$ current; $I_{to2}$, Ca$^{2+}$-dependent transient outward Cl$^-$ current; $I_{up}$, Ca$^{2+}$ uptake from myoplasm to NSR; PLB, phospholamban; SER, sarcoplasmic reticulum Ca$^{2+}$-ATPase; CaMKII, Ca$^{2+}$/calmodulin-dependent kinase; NSR, network SR; JSR, junctional SR. The shaded areas indicate the presence of Ca$^{2+}$ buffers (Modified from Hund & Rudy, 2004).
induced heart failure”, Winslow et al. (1999) established a model of the canine ventricular AP and Ca\(^{2+}\) transient. They incorporated Ca\(^{2+}\) handling protein expression changes that have been found in failing heart into the computational model one by one and used the model to predict the functional consequences of each alteration in this disease. This study showed that the altered expression of Ca\(^{2+}\) handling proteins plays a significant role in prolongation of APD in failing canine myocytes. They further examined the nature of the interplay between APD and Ca\(^{2+}\) handling in the model. The model indicated that reductions in SERCA expression and increased expression of the NCX in the failing heart both contribute to a reduction of JSR Ca\(^{2+}\) load. This reduction in JSR Ca\(^{2+}\) load consequently produces a smaller Ca\(^{2+}\) release from SR, reduced Ca\(^{2+}\) induced I\(_{Ca,L}\) inactivation, and therefore enhanced Ca\(^{2+}\) current, which then contributes to the prolongation of APD. The significance of this study lies in that it identified the critical role of HF-induced Ca\(^{2+}\) overload in contributing to the APD prolongation, and to the increased risk of EADs and arrhythmias. This study represents an example of the importance and power of the modeling method in studying the electrical as well as the EC coupling properties in normal and disease cells.

### 5.5. Basic concept of the dynamic clamp

Dynamic clamp is a technique that combines the modeling study with the classical patch clamp. In the traditional patch clamp, “current clamp” or “voltage clamp” protocols are usually used to assess the electrical properties of excitable cells. Under current clamp mode, a current stimulus, typically a brief rectangular pulse of current, is injected into a myocyte to induce an AP while the membrane potential is being recorded. In voltage clamp, the membrane potential is held at pre-determined levels, and the
transmembrane currents that reflect the voltage-dependent properties of the conductance are recorded. Both techniques are powerful and have contributed significantly to the understanding of the electrical behavior of different types of cardiomyocytes. The measurement of voltage-dependent properties of an ionic current has also facilitated the development of mathematical models that characterize individual ionic currents (Hodgkin & Huxley, 1952).

In addition to the traditional patch clamp techniques, a novel and powerful technique that is based on the patch clamp is the real-time dynamic clamp. This technique was first described in a peer reviewed publication in 1993 by Sharp et al. (Sharp et al., 1993). Since the first description, this method has been extensively and successively used in neuroscience and cardiac electrophysiology (Carter & Regehr, 2002; Prinz et al., 2004; Sun & Wang, 2005; Dong et al., 2006). It allows injecting voltage- and time-dependent currents into a cell, so as to simulate the presence of specific ionic conductance in the cell membrane.

Implementation of the dynamic clamp requires an analog-to-digital converter (ADC)/digital-to-analog converter (DAC) interface (so that the computer can acquire voltage signals and generate current commands), and software programs that calculate the current equations and operate the interface (Pinto et al., 2001). As shown in Figure 6, the dynamic clamp uses a standard patch clamp set-up with a computer interface to implement the dynamic clamp commands. The membrane potential Vm of a single myocyte is continuously sampled by the intracellular electrode and transmitted to the computer through an ADC. According to the Vm and the mathematical equation describing the conductance of interests, the current (I) that would be generated is calculated and is sent to the amplifier/DAC to inject this current into the myocyte. The
power of the dynamic clamp technique lies in its combination of computer simulation with experimental biology. Effectively, programmable artificial conductances can be introduced in living cells and the effects of this introduced conductance on the cells can be studied.

5.6. Applications of the dynamic clamp in cardiac electrophysiology

The dynamic clamp has been proven to be a powerful technique in cardiac electrophysiology research, demonstrated by our and others’ studies (Sun & Wang, 2005; Dong et al., 2006; Berecki et al., 2005, 2006; Wang, 2006; Wagner et al., 2004). In our studies, we simulated a specific membrane current ($I_{\text{to}}$) into ventricular cardiomyocytes and studied its effects on AP morphology and duration, as well as on myocyte mechanics and Ca$^{2+}$ kinetics. The dynamic clamp can also be used to investigate the intercellular electrical coupling of myocytes (Wilders, 2006). In two recent studies, Berecki et al. used the “dynamic action potential clamp” configuration (Berecki et al., 2005; Berecki et al., 2006). They recorded the free-run AP in an isolated myocyte (cell 1) but pharmacologically blocked the native conductance of interest. Simultaneously, a mutant or a wild type current recorded from the gene-manipulated cell (cell 2) was measured in voltage clamp mode and was introduced to cell 1. By doing so, the influence of this mutant current to AP was compared with wild type current.
Figure 7

Figure 7. Configuration of dynamic clamp.
The dynamic clamp uses a standard patch clamp set-up with a computer interface to measure membrane potentials and control the current injected. The membrane potential $V_m$ of a single myocyte is continuously sampled by the intracellular electrode and transmitted to the computer through an analog-to-digital (A/D) converter. According to the $V_m$ and the mathematical equation describing the conductance of interest, the current ($I$) that would be generated is calculated and is sent to the amplifier to inject this current into the myocyte. Effectively, programmable artificial conductances can be introduced into living cells and the effects of this introduced conductance on the cells can be studied.
6. Dissertation scope and objectives

Tremendous efforts have been made towards understanding the mechanisms that control cellular processes in the heart. However, many questions still remain unanswered. My dissertation, using the dynamic clamp technique as the central technique, attempts to elucidate the role of a main repolarizing current, the transient outward current ($I_{to}$), in regulating electrical and mechanical properties in heart physiology and pathophysiology. Within this category, two related studies have been pursued. The rationale for these studies and the specific objectives are:

1) Heart failure is characterized by both electrical and mechanical dysfunctions of the heart. A number of ion channels and Ca$^{2+}$ handling proteins undergo alterations to compensate for or contribute to the impaired heart function during the progression towards HF. Among them, one of the most notable electrical remodelings is the downregulation of the transient outward current ($I_{to}$). This remodeling is consistently observed in failing and diseased cardiomyocytes in human as well as animal models. Previous studies have suggested that this downregulation of $I_{to}$ plays an important role in modulating electrical and mechanical properties in ventricular cardiomyocytes. However, due to the limitations of available techniques, this role is rather controversial and unclear. Here, using the dynamic clamp technique, which is able to specifically and quantitatively simulate an artificial conductance in a real biological cell, I intend to elucidate the role of $I_{to}$ in these three perspectives:
a. The role of I$_{to}$ in regulating AP morphology and duration in normal cardiomyocytes.

b. The role of I$_{to}$ in regulating myocyte mechanical properties.

c. The role of I$_{to}$ in regulating AP morphology and duration in cardiomyocytes from a canine HF model.

We hypothesized that I$_{to}$ is not a significant modulator to the APD. Instead, it plays a critical role in regulating cardiac I$_{Ca-L}$ and EC coupling. Our earlier study have shown that I$_{to}$, though a dominant current in phase 1 repolarization, is not a significant determinant of APD in canine ventricular myocytes (Sun & Wang, 2005). In the current study, we improved our method to mimic a more physiological condition. In addition, as the major player of the early phase repolarization and a key determinant of the AP morphology, I$_{to}$ overlaps with the I$_{Ca-L}$ during AP time-course and may modulate the magnitude of I$_{Ca-L}$ and EC coupling.

2) Further, the role of I$_{to}$ in arrhythmogenesis in Brugada syndrome was illustrated in this dissertation. Brugada syndrome, a malignant RV arrhythmia, is precipitated by loss-of-function mutations in the cardiac Na$^+$ channel-encoding gene SCN5A, which results in reduction of cardiac I$_{Na}$. In addition to I$_{Na}$ reduction, a second and necessary contributing factor is believed to be the robust I$_{to}$ in the right ventricular epicardial myocytes. It was suggested that the prominent I$_{to}$ and the reduced I$_{Na}$ caused alteration of AP morphology, leading to a loss of the AP plateau. However, this proposed ionic basis of the disease is based mostly on tissue studies using non-specific Na$^+$ and I$_{to}$ channel blockers. In addition, structurally defective heart and motion abnormalities
have been observed in Brugada patients. The basis of these mechanical abnormalities and their implications for the arrhythmogenesis of the syndrome remain controversial. In this part of the study, we work on delineating the role of $I_{to}$ in the cellular electrical abnormalities as well as in the cellular contractile abnormality associated with the Brugada syndrome. The cellular mechanism of any impacts of $I_{to}$ on the development of these abnormalities was explored.

We hypothesized that $I_{to}$ plays an important role in arrhythmogenesis in Brugada syndrome and the contractile abnormality in Brugada syndrome can be accounted for by the cellular electrical alterations, and is not necessarily an indication of morphological abnormalities. We proposed that the reduced $I_{Na}$ contributes to all-or-none repolarization by leaving $I_{to}$ unopposed. In addition, the loss of the dome causes the $I_{CaL}$ dramatic reduction. The cell becomes depleted of Ca$^{2+}$ within seconds and all contractile function diminished. Hence, wall motion abnormalities are to be expected as a result of the primary electrical disease responsible for the Brugada syndrome. We also examined the molecular basis for a higher phase 1 notch in RVOT than in RV. Our hypothesis is that $I_{to}$ encoding gene and $I_{to}$ channel proteins are highly expression in the RVOT epicardium.
Chapter II: Material and Methods

1. Cell dissociation

1.1. Isolation of guinea pig cardiomyocytes

Handling and usage of animals were in accordance with protocols approved by the University of Cincinnati Institutional Animal Care and Use Committee. Adult guinea pigs weighing 200-250mg of either sex were anaesthetized by intraperitoneal injection of sodium pentobarbital (150mg/kg body weight). Hearts were then quickly excised and rinsed to remove blood. The heart was then mounted on a Langendorff perfusion apparatus, and perfused with oxygenated Ca$^{2+}$-free and then Ca$^{2+}$-containing (1.5 mM) solution for 5 minutes each. The perfusion solution contained (in mM): NaCl 112, KCl 5.4, NaH$_2$PO$_4$ 1.7, MgCl$_2$ 1.63, NaHCO$_3$ 4.2, HEPES 20, Glucose 5.4 and Taurine 10 (pH = 7.6). This was followed by perfusion with the same solution containing zero Ca$^{2+}$ and 85 unit/ml collagenase (type II, Worthington) at 37°C until the hearts became flaccid. The ventricles were removed and minced and pipette-triturated in oxygenated Kraft-Brühe (KB) solution containing (in mM): KCl 83, K$_2$HPO$_4$ 30, MgSO$_4$ 5, Na-pyruvate 5, β-OH butyric acid (sodium salt) 5, taurine 20, creatine 5, glucose 10, EGTA 0.5, HEPES 5, and Na$_2$ATP 5 (pH = 7.4). Cells were stored in the KB solution at room temperature and used on the day of isolation.
1.2. Isolation of canine left and right ventricular cardiomyocytes

All animal usage and handling followed protocols approved by the Institutional Animal Care and Use Committee of University of Cincinnati. Adult dogs, weighing 25-35 Kg, of either sex were used in the experiments. After the dog was euthanized by intravenous injection of sodium pentobarbital (80 mg/kg body weight, Vortech, Dearborn, MI), the heart was quickly removed from the chest and was rinsed by a pre-chilled high K⁺ Tyrode solution containing (in mM): NaCl 129, KCl 12, NaH₂PO₄ 0.9, NaHCO₃ 20, CaCl₂ 1.0, MgSO₄ 0.5 and glucose 5.5 (pH = 7.4). Wedge-shaped left and right ventricular free walls were dissected separately and prepared for cannulation. The left anterior descending coronary artery and the right coronary artery were chosen for cannulation in the left and right ventricles, respectively. The tissues were then perfused on a Langendorff system with an oxygenated solution containing (in mM): NaCl 135, KCl 5.4, MgCl₂ 1.0, NaH₂PO₄ 0.33, HEPES 10, and glucose 10 (pH = 7.4) at 37°C. After the dribbles became pink-less, 140 unit/ml collagenase (type II, Worthington, Lakewood, NJ), 25 μM leupeptin, and 0.32 unit/ml protease were added and the tissues continued to be perfused for 10-15 min. Small pieces of tissue (<2 mm in thickness) were then cut off from the epicardial and endocardial surface, minced and gently shaken in the presence of a lower concentration of collagenase (110 unit/ml). Isolated myocytes were harvested and stored in a standard Tyrode solution containing 0.1 mM Ca²⁺ at room temperature or 4°C, for recordings on the same day or the following day. All chemical and drugs used in this study were from Sigma (St Louis, MO) unless otherwise stated.

2. Electrophysiology recording
2.1. Action potential measurement

Isolated cells were perfused with Tyrode's solution containing (in mM): NaCl 140, KCl 5.4, MgCl₂ 1, CaCl₂ 1.8, HEPES 5, and glucose 10 (pH = 7.4). Perforated patch-clamp recordings were used for action potential recordings and the dynamic clamp studies in order to preserve the intracellular Ca²⁺ handling. Electrodes were pulled from borosilicate glass using conventional one-stage pullers (P-97 horizontal pipette puller, Sutter Instruments, USA). The glass pipettes were then back-filled with a pipette solution containing (in mM): K-aspartate 110, KCl 20, NaCl 8, HEPES 10, MgCl₂ 2.5, CaCl₂ 0.1, and 240 mg/ml amphotericin B (pH adjusted to 7.2 with KOH), and have a resistance of 1.5 – 2.0 MΩ. The liquid junction potential was adjusted to zero before a high-resistance seal between pipette and cell was formed. Cells were studied once stable series resistance <7 MΩ was achieved; cells with unstable or a higher series resistance were rejected. Series resistance was fully compensated under the current clamp. Action potentials were triggered with just-threshold 2-ms rectangular current steps at 1Hz or 3Hz and recorded at steady-state. All experiments were performed with an Axopatch-200B amplifier at 34°C. Data was collected using PCLAMP9 software through an Axon Digidata 1322A data acquisition system. APD was qualified as the duration at 90% repolarization (APD₉₀), and phase-1 repolarization was quantified as the notch voltage measured at the lowest point of the notch, and notch amplitude was measured as the voltage difference between the peak of the AP and the lowest point of the notch.

2.2. Transient outward current (Iₒ) and delayed rectifier K⁺ current (I_K) measurement
For $I_{\text{to}}$ and $I_{K}$ current recordings, whole-cell patch clamp recordings were used and 0.2 mM CdCl$_2$ was added to the AP external solution (listed above) to block the Ca$^{2+}$ current. Pipette solution for $I_{\text{to}}$ recording contained (in mM): K-aspartate 110, KCl 20, EGTA 10, HEPES 10, MgCl$_2$ 2.5, NaCl 4, CaCl$_2$ 1, Na$_2$-ATP 2, and Na-GTP 0.1 (pH adjusted to 7.2 with KOH). For $I_{\text{to}}$ recordings, which were carried out at 34°C, the cells were activated by depolarizing steps from a holding potential of –70 mV to voltages ranging from –30 to +50 mV, in 10 mV increments. $I_{K}$ current was recorded in response to depolarizing steps ranging from -30 to +50 mV in 10 mV increments from a holding potential of -40 mV. $I_{K}$ recordings were carried out at room temperature (24°C). Tail currents were recorded at -40 mV.

2.3. L-type Ca$^{2+}$ current recordings

For measurement of the L-type Ca$^{2+}$ current, myocytes were maintained at room temperature (24°C) and perfused with Tyrode's solution containing (in mM) NaCl 140, KCl 5.4, MgCl$_2$ 1, CaCl$_2$ 1.8, HEPES 5, and glucose 10 (pH = 7.4). To eliminate contamination from other ionic and exchange currents, the perfusion solution was switched to a Na$^+$- and K$^+$-free solution that contained (in mM) TEA-Cl 137, CsCl 5.4, CaCl$_2$ 2, MgCl$_2$ 1, HEPES 5, glucose 10, and 4-aminopyridine 3 (pH = 7.4) after the membrane was ruptured. Glass pipettes were filled with solution containing (in mM) Cs-aspartate 115, CsCl 20, EGTA 11, HEPES 10, MgCl$_2$ 2.5, Mg-ATP 2 and Na-GTP 0.1 (pH = 7.2), and had a resistance of 1.5 – 2.5 MΩ. Cells were clamped at –50mV for 5 minutes to allow dialysis of the intracellular solution and stabilization of the Ca$^{2+}$ currents before measurement began. The L-type Ca$^{2+}$ current was activated by depolarizing steps from a holding potential of –50mV to voltages ranging from –40 to +60mV, in 10mV.
increments. Data collection and analysis were performed using pCLAMP software (Axon Instruments, Foster City, CA).

2.4. Action potential voltage clamp measurement

AP waveforms at steady-state were recorded in current clamp using conventional whole-cell patch clamp technique as described above. Resting voltage values were subtracted from the trace to bring the resting membrane potential to zero and the files were saved as command protocol files. The protocol files were then applied to a whole-cell patch clamp recording as the command voltage in voltage clamp mode.

3. Implementation of the dynamic clamp

A modified version of the Windows-based DynClamp software was used in the dynamic clamp studies (Pinto et al., 2001). The program was installed on a Dell PC with a 1.6 GHz processor and 256 MB of memory. It was dedicated solely to the dynamic clamp simulation to ensure a high calculation speed and had an update rate of about 5 kHz. A second PC ran the PClAMP software and data sampling and acquisition. Membrane potential, Vm, was continually sampled and transferred to a personal computer. Based on Vm, a command signal was calculated using a model of I_{to} described below and output was sent to the amplifier to be converted to a transmembrane current signal. Voltage sampling of the dynamic clamp software and output of the current injection command were through an Axon Digidata 2100 board.

I_{to} was defined as a rapidly and fully inactivating outward current, and was formulated based on our previous canine epicardial I_{to} model (Sun & Wang, 2005).
Modifications were made to account for shifts of the voltage-dependence of \( I_{to} \) gating properties by external \( \text{Cd}^{2+} \) (Stengel et al., 1998; Wickenden et al., 1999). The \( I_{to} \) conductance was of the usual Hodgkin–Huxley form (Hodgkin & Huxley, 1952) and was derived from that of Dumaine et al.’s (1999):

\[
I_{to} = g_{to} m h R(V_m - E_K) \tag{1}
\]

\[
R = \exp \left( \frac{V_m}{300} \right) \tag{2}
\]

\[
\frac{dm}{dt} = \alpha_m (1-m) - \beta_m m \tag{3}
\]

\[
\frac{dh}{dt} = \alpha_h (1-m) - \beta_h h \tag{4}
\]

\[
\alpha_m = \frac{4}{1 + \exp \left( \frac{(V_m - 25)/20}{-20} \right)} \tag{5}
\]

\[
\beta_m = \frac{3.5}{1 + \exp \left( \frac{(V_m + 110)/29.5}{-29.5} \right)} \tag{6}
\]

\[
m_\infty = \frac{1}{1 + \exp \left( \frac{(V_m + 13.9)/-8}{-8} \right)} \tag{7}
\]

\[
\alpha_h = \frac{0.016}{1 + \exp \left( \frac{(V_m + 59)/5.5}{5.5} \right)} \tag{8}
\]

\[
\beta_h = \frac{0.11}{1 + \exp \left( \frac{(V_m + 27)/-6.5}{-6.5} \right)} \tag{9}
\]

\[
h_\infty = \frac{1}{1 + \exp \left( \frac{(V_m + 50.3)/3.9}{3.9} \right)} \tag{10}
\]

\[
\tau_m = (\alpha_m + \beta_m)^{-1} \tag{11}
\]

\[
\tau_h = (\alpha_h + \beta_h)^{-1} \tag{12}
\]

\( g_{to} \), maximum conductance of the channel. \( m \) and \( h \), activation gate and inactivation gate, respectively. \( R \), outward rectification factor for the channel. \( V_m \), membrane potential. \( E_K \), the reversal potential for \( \text{K}^+ \), was set to \(-85\) mV. \( \alpha_m \) and \( \beta_m \),
voltage-dependent opening and closing rate constants of activation gate (m·sec\(^{-1}\)). \(\alpha_h\) and \(\beta_h\), voltage-dependent opening and closing rate constants of inactivation gate (m·sec\(^{-1}\)). The electrode junction potential was 12 mV, and was corrected on-line in the computation. Action potentials were triggered with just-threshold 2 ms current steps and recorded at steady-state. APs were re-controlled after each simulation. Because \(g_{to}\) in equation (1) does not equal the true simulated \(I_{to}\) conductance, the true peak current density in response to a depolarizing step from –80 mV to +40 mV is given to indicate the amplitude of the simulated \(I_{to}\) unless otherwise stated.

4. Computational simulations

The computational model was constructed in MATLAB\textsuperscript{®} based on the Hund-Rudy dynamic (HRD) model (Hund & Rudy, 2004, see Introduction on page 49 & Fig. 6). Modifications were made to update the Na\(^+\)-Ca\(^{2+}\) exchanger (NCX) based on advances made by other studies (Greenstein et al., 2006; Sher et al., 2008). The modifications made were including the different types of NCXs which sense Ca\(^{2+}\) in three compartments: 1) between T-tubule and SR, 2) sub-membrane, and 3) global sarcoplasm or cytosol. The \(I_{to}\) current was modified to reflect the high density of \(I_{to}\) in RV epicardial myocytes, with a peak of approximately 30 pA/pF. All model runs were taken to steady state (50 APs).

5. Evaluation of single cell contractility and Ca\(^{2+}\) transient

5.1. Cell shorting measurement
Mechanical properties of the ventricular myocytes were assessed using a video-based edge-detection system (Crescent electronics, Sandy, UT) at a 60-Hz frame rate. Epi- and endocardial cells were placed in a chamber mounted on the stage of an inverted microscope and perfused with the standard Tyrode’s solution (listed above, 1.8 mM Ca\textsuperscript{2+}). A CCD camera was attached to the camera port of the inverted microscope, and the output was fed to a video-edge detector (Crescent electronics, Sandy, UT), which monitored changes in position of both the right and left cell edges. Cells were field stimulated by a Grass S48 stimulator (Grass instrument, Gincy, MA) at 0.5 or 0.2 Hz. Fractional shortening, maximal rates of contraction and relaxation were analyzed to evaluate the contractility and the kinetics of contraction using Clampfit 9 software.

5.2. Calcium transient measurement

Fluo-4 acetoxyethyl (AM) ester (Molecular Probes, Eugene, OR) was used for the measurement of the intracellular Ca\textsuperscript{2+} signal. A 1.0 mM stock solution was prepared by adding 45 μL of DMSO in a vial of 50 μg Fluo-4 AM (MW: 1096.95). The stock solution was then divided into 10-μl aliquots and wrapped with aluminum foil to prevent light exposure and stored at -20°C. When needed, the stock solution was diluted to 6 μM in a DMEM medium solution. Epi- and endocardial cells were loaded in the solution for up to 20 minutes at room temperature. After several washes with standard Tyrode’s solution to get rid of the extracellular fluo-4 AM, cells were then incubated for 10 minutes to allow de-esterification. Fluorescent signals were recorded by a ratiometric photometry system (Intracellular imaging Inc., Cincinnati, OH) at an excitation wavelength of 488 nm and a long pass 510 nm emission. The qualitative changes in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) were inferred from the fluorescence unit ΔF/F₀.
The time constant of decay of the Ca\textsuperscript{2+} signal was acquired by fitting the data to a single exponential decay function curve.

5.3. **Myocyte mechanics and Ca\textsuperscript{2+} kinetics simultaneously measured with action potentials**

Action potentials were induced by intracellular stimulations as described above. Cell shortening or Ca\textsuperscript{2+} transient was simultaneously recorded as described above. The electrode pipettes were clamped only in the central area of the cells to minimize the influence on cell contraction and to avoid the interference with edge tracking. Cell shortening and Ca\textsuperscript{2+} transient data were collected by pCLAMP9 and InCytPm1 software, respectively, and both data were analyzed by Clampfit 9.

6. **Real time PCR**

6.1. **Primer design**

After acquiring the gene sequence of interest from the website of the National Center for Biotechnology Information (NCBI), the exon/intron boundaries were searched at [http://genome.ucsc.edu/cgi-bin/hgBlat](http://genome.ucsc.edu/cgi-bin/hgBlat). The 2 exons that span the longest intron were picked and all primer pairs were designed to cross that intron. The primers were designed using Primer3 program at [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with a preferred Tm of 62°C.

6.2. **RNA isolation**
Snap-frozen hearts were pulverized, put in Trizol solution (~50 mg tissue/1 mL, Invitrogen, Carlsbad, CA), and immediately homogenized with a mini-tissue polytron homogenizer. Cardiac homogenate was transferred into a 1.5- microcentrifuge tube and centrifuged at 12,000X g for 10 minutes at 4 °C. Supernatant was incubated at room temperature for 5 minutes. RNA was extracted by phase separation with 200 µl chloroform per 1ml of the TRIzol Reagent, vortexed at the highest speed, and centrifuged at 12,000X g at 4°C for 15 minutes. The aqueous phase was transferred into a new tube and RNA was precipitated by adding one-half volume of isopropanol. The RNA was pelleted via centrifugation at 4°C for 10 minutes, and washed with 1 mL 75% ethanol to remove excess salt, and then centrifuged at 14, 000X g for one minute. The pellets were air dried for less than 1 minute and dissolved in 30 µL RNase/DNase free water. To assess the integrity of the RNA, an RNA sample, which contained 1 µL of RNA, 13 µL water and 6 µl 6X bromophenol blue loading dye mixture, was loaded onto a 2% agarose gel containing 0.01% ethidium bromide (Bio-Rad, Hercules, CA), run at 130 V for 40 mins and visualized for the appearance of two strong bands (28S and 18S) and a faint band (5S). The purity and concentration of RNA were determined by a spectrophotometer at an ultraviolet (UV) absorbance of 260 and 280 nm. Calculation of the RNA concentration was based on the absorbance at 260 nm, and RNA purity was determined from the 260 nm/280 nm ratio; a low ratio indicates contamination by protein. RNA samples were frozen and stored at -80°C until further use.
Table 1. Primers used in real-time PCR evaluation of gene expression level.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’→3’)</th>
<th>Product length</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN5A</td>
<td>sense</td>
<td>TTGTGGTTGTCATCCTCTCCA</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>GGACATCATGAGGGCAAAGAG</td>
<td></td>
</tr>
<tr>
<td>KCND3</td>
<td>sense</td>
<td>TCCCTGCCTCTTTTTGGTACA</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>TGCCTTTATCGGCTCTCTGAT</td>
<td></td>
</tr>
<tr>
<td>KCNIP2</td>
<td>sense</td>
<td>GTTTATCCGATTCCCGAGACC</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>AAGGCAGCAGCTTGAGGAAC</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>sense</td>
<td>ATTCTACCCACGGCAAAATCC</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td>TTCTCCATGGTGGTGAGACC</td>
<td></td>
</tr>
</tbody>
</table>
6.3. Reverse transcription

Reverse transcription was performed with the SuperScript™ Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Briefly, 1µg of total RNA was incubated at 65°C for 5 minutes with 250ng of random primers and 1 µL of 10 mM dNTP. RNA samples were quickly chilled on ice. To obtain a higher yield of full length cDNA, samples were incubated at room temperature for 2 minutes with 4 µL of 5X reverse transcription buffer (MgCl₂ included), 1 µL of 0.1 M DTT and 1 µL of RNaseOUT recombinant RNase Inhibitor. The first strand cDNA synthesis was performed by adding 1µL Superscript III reverse transcriptase (200 U/µl) in 25°C for 10 minutes and then in 50°C for 50 minutes. The reaction was terminated at 70°C for 15 minutes. After being chilled on ice, the cDNA products were diluted 1:10 or 1:20 and stored in -80°C for PCR reaction.

6.4. Real-time PCR

Direct detection of polymerase chain reaction (PCR) product was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green (Applied Biosystem, Foster City, CA) to double-stranded (ds) DNA. 9.2 µL of cDNA plus 10 µL SYBR mix, and 0.4 µL each of 5’ and 3’ primer (5 µM) were subjected to RT-PCR reaction. The following PCR protocol was used: initial denaturing step, cycle 1 (50°C for 2 minutes), cycle 2 (95°C for 10 minutes), followed by 40 cycles of denaturing (95°C for 15 seconds), annealing and extension (60°C for 1 minute). The amount of fluorescence that was incorporated into the PCR product was detected under the GeneAmp® 5700 Sequence Detection System. Relative quantity of a target gene was represented by fold
Figure 8

Figure 8. Agarose gel of real-time PCR products.

Representative electrophoresis in 2% agarose and staining with ethidium bromide demonstrated cDNA products for KCND3, KCNIP2, and SCN5A.
change against a housekeeping internal standard. Relative fold changes were calculated by the ∆∆CT (CT: cycle threshold) method. The products were run in an agarose gel to check the specificity and purity of the products. Figure 8 shows a representative agarose gel of the cDNA products. Clean bands appeared at the expected sizes.

7. **Quantitative immunoblotting**

7.1. **General protocol**

7.1.1. **Total and membrane protein extraction from heart tissue**

A thoracotomy was performed to harvest the heart after animals were euthanized. The heart was then quickly rinsed by ice cold PBS (for mouse and rat) or high K solution (for canine). Heart tissue was rubbed by Kimwipes to absorb liquid and flash-frozen in liquid nitrogen. A weight of about 200mg of frozen tissue was pulverized by mortar and pestle chilled in liquid nitrogen. Pulverized hearts were placed in a chilled glass-teflon homogenizer with ~1 mL homogenization buffer of the following composition (in mM): 250 sucrose, 1 PMSF, 1 EDTA, and 1 iodoacetamide. The tissue was then homogenized on ice by 10 strokes and centrifuged at 700 g for 10 min at 4°C. The supernatant was collected as the total protein. To get the membrane fraction, the supernatant was centrifuged at 100,000 g for 1 h at 4°C and the pellet was then resuspended in 200 µl buffer containing (in mM): 20 Tris, 1 EDTA, 1 PMSF, 1 iodoacetamide, and 1% SDS.

7.1.2. **Protein assay**

The protein assay protocol was based on the “microtiter plate protocols” from Bio-rad. Briefly, prepare the dye reagent by diluting 1 part Dye Reagent Concentrate with 4
parts distilled water. Filter through a Whatman #1 filter to remove particulates. Three to five dilutions of a protein standard were then prepared in the linear range of 0.05 mg/ml to approximately 0.5 mg/ml. Protein solutions were normally assayed in triplicate. 200 µl of diluted dye reagent was added into each microplate wells where 10 µl of standard or sample solution had been added. The sample and reagent were mixed thoroughly and incubated at room temperature for at least 5 minutes before the absorbance was measured at 595 nm by a Thermomax microplate reader (GMI, Inc., Ramsey, Minnesota,).

7.1.3. Running gel and membrane transfer

The desired amount of protein (see table 2) was mixed with sample buffer of the following composition: 0.125M Tris-HCl (pH 6.8), 20% glycerol (v/v), 4% SDS (w/v), 2% β-mercaptoethanol (v/v, add freshly), trace of Bromophenol Blue, in H₂O. The sample was boiled for 10 minutes at 97°C and was placed in ice to cool it down. The percentages of resolving gel for the specific proteins are listed in table 2. The gels were loaded with pre-stained protein marker and samples, and run at 120-150 mV for the length of time needed for the protein of interest. The gel was then rinsed and transferred to a 0.2µm nitrocellulose membrane (Bio-rad, Hercules, CA) using a transfer sandwich cassettes filled with 2 sponges, 2 pieces of filter paper, the membrane of desired size, and the gel. The transfer was carried out using the appropriate current and amount of time (see table 2).

7.1.4. Blocking, antibodies incubation, and washing
After transferring, membranes were blocked and incubated on a rocker for one hour in 5% milk (in PBS) to decrease non-specific binding of antibodies to the membrane. The membranes were then incubated with primary antibodies and secondary antibodies. After incubation with the antibody, the membranes were washed with PBS-Tween (0.1% Tween 20 in 1X PBS) to remove excess or not tightly bound antibody. Membranes were washed 3 times for 10 minutes each after incubation.

7.1.5. Detection

The membranes were developed by an enhanced chemiluminescence western blotting detection system (GE healthcare, Buckinghamshire, UK). Following the washing of the blots, the membranes were exposed to a luminol-based substrate (ECL) which reacts with horseradish peroxidase conjugated to secondary antibodies to create chemiluminescence. This chemiluminescence allowed for the visualization of the proteins of interest on the X-ray film. After the membranes were exposed to ECL, they were overlaid with film in a dark room. The amount of time the film was exposed to the membrane varied by antibody and was determined experimentally so film exposure was within the linear range of detection to ensure reliable quantification. All the protein levels were quantitated using an Alpha Innotech FlourChem8800 Imager and FlourChem 8900 software (Alpha Innotech, San Leandro, CA).

7.1.6. Stripping blot

After membrane detection was finished, those that needed to be reprobed with another antibody were stripped of the original antibody. The membranes were
Table 2. Conditions for western blotting

<table>
<thead>
<tr>
<th>Protein</th>
<th>Loading amount (μg)</th>
<th>Percentage of resolving gel</th>
<th>Transfer condition (current (mA) / time (hr))</th>
<th>Primary antibody (sources/amount)</th>
<th>ECL reagent (Product/source)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv4.3</td>
<td>80</td>
<td>8%</td>
<td>200/2</td>
<td>Neuromab (1μg/ml)</td>
<td>SuperSignal West Femto</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Maximum Sensitivity Substrate/Thermo Scientific</td>
</tr>
<tr>
<td>KChIP2</td>
<td>80</td>
<td>10%</td>
<td>200/2</td>
<td>Neuromab (1μg/ml)</td>
<td>SuperSignal West Femto</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Maximum Sensitivity Substrate/Thermo Scientific</td>
</tr>
<tr>
<td>Cav1.2</td>
<td>20</td>
<td>8%</td>
<td>250/4</td>
<td>Alomone (1:200)</td>
<td>Amersham ECL Western Blotting Analysis System/GE Healthcare</td>
</tr>
<tr>
<td>Calsequestrin</td>
<td>---</td>
<td>---</td>
<td></td>
<td>ABR (1:500)</td>
<td>Amersham ECL Western Blotting Analysis System/GE Healthcare</td>
</tr>
</tbody>
</table>
incubated in stripping buffer (62.5 mM Tris pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol) for 30 minute at 65°C. The membranes were then thoroughly washed in 0.1% PBS-Tween 3 times for 10 minutes each. Following the washes, the membranes were blocked with blotting solution again before incubation with the primary antibody of interest.

7.2. Specified protocols for different proteins

Specific antibody requires personalized western protocols. Table 2 lists the conditions for the different antibodies.

8. Canine heart failure model

All the procedures to establish the canine heart failure model were performed by our collaborator Dr. Lopshire at IUPUI. It is a combination of the microembolization-induced HF and a pacing-induced canine HF model. The experimental protocol consisted of two surgical procedures (Fig. 9).

8.1. Implantation of implantable cardioverter defibrillator (ICD) via right internal jugular vein

During the surgical procedures, the canine was be pre-anesthetized with sodium pentothal (25 mg/kg IV), and then intubated with a cuffed endotracheal tube, and ventilated with room air using a Narkomed 2 volume-cycled respirator. The canine was then anesthetized with 2.0-3.0% isoflurane. The ventral side of the neck was shaved and prepped with betadine, and a 2” cut down was performed in the right side of the neck, and blunt dissection was used to expose the right jugular vein, through which an active-fixation pacing and/or defibrilating lead was introduced and
advanced under fluoroscopic guidance into the right ventricular apex. The lead body was tunneled to the right side to a suitable position for device implantation. A subcutaneous pocket was created with blunt dissection into which the device was implanted. The pacemaker function was evaluated and the pocket was then closed by interrupted stitches. The pacemaker was set at a pacing rate of 40 bpm.

### 8.2. Baseline measurements

Heart rate, blood pressure, and EP measurements were obtained. A two-dimensional echocardiography was performed weekly to monitor changes in LV ejection fraction and to ensure the development of LV systolic dysfunction.

### 8.3. Creation of an anterior myocardial infarction

Once the canine was anesthetized, the right groin was shaved and prepped with betadine and the femoral artery was exposed by blunt dissection. An 8-Fr sheath was placed into the femoral artery. A fluid filled canula was connected to the sheath and to a transducer to monitor femoral arterial pressure. A 7-Fr Amplatz-2 angiography catheter (Boston Scientific SciMed, Inc., Maple Grove, MN) (or a similar catheter) was passed over a guide wire through the femoral artery sheath and through the aorta up to the aortic root under fluoroscopy guidance, and then the catheter was manipulated to engage the left main coronary artery. The tip of the guide catheter was then advanced into left anterior descending coronary artery (LAD). A small amount (0.2-0.3 cc) of polyvinyl alcohol foam remobilization particles (Cook Inc., Bloomington, IN) diluted in
Figure 9. Experimental protocol of the canine heart failure model.

LAD: left anterior descending coronary artery; LVEF: left ventricular ejection fraction; SCS: thoracic spinal cord stimulation; ICP: intracranial pressure; HR: heart rate; BP: blood pressure; Echo: echocardiography; CRP: C-reactive protein; BNP: Brain natriuretic peptide; NE: nonepinephrine; E: epinephrine; MI: myocardial infarction; LVEDP: left ventricular end diastolic pressure. All the procedures were performed by our collaborator Dr. Lopshire at IUPUI.
10 cc of mixture of contrast material and normal saline was then injected through the angiography catheter into the LAD. The angiography catheter was then completely withdrawn. The ECG and a transthoracic echo were then monitored for 1-2 hours and the animal was allowed to recover.

8.4. **Rapid ventricular high rate pacing to induce HF**

After a 2-week recovery period, rapid ventricular pacing was performed at 200 to 240 bpm continuously for 2-3 weeks to induce heart failure. Rapid pacing was stopped and a 5 week recovery was allowed.

8.5. **Acute induction of myocardial infarction**

An acute testing protocol was scheduled 5 weeks after completion of the rapid-pacing protocol to induce arrhythmias. After baseline EP testing, dogs underwent induction of transient (2-minute) cardiac ischemia induced by inflating an angioplasty balloon placed within the left circumflex coronary artery. Complete occlusion of the proximal left circumflex coronary artery was confirmed angiographically.

9. **Statistical analysis**

All pooled data are presented as mean ± S.E.M. The effects of dynamic clamp simulation in a same cell before and after were analyzed using paired, two-tail Student's t-tests. mRNA and protein expression levels in RVOT were analyzed using one-way ANOVA. Other statistical analysis was performed using unpaired Student’s t-tests. Probability values of < 0.05 were considered as significant.
Chapter III: Results

1. Effect of simulated $I_{to}$ on guinea pig and canine action potential morphology and duration

1.1. Background and rationale

The transient outward potassium current ($I_{to}$) is the dominant repolarizing current and is responsible for phase 1 repolarization of the action potential (AP) in the heart. In many cardiac diseases, such as heart failure, one of the most characteristic electrophysiological changes is the downregulation of $I_{to}$ (Kaab et al., 1996; Tsuji et al., 2000; Li et al., 2002). It was proposed that $I_{to}$ downregulation contributes to the APD prolongation (Beuckelmann et al., 1993; Kaab et al., 1996), which is believed to predispose the heart to afterdepolarization and reentrant arrhythmias (Tomaselli et al., 1994). However, the influence of $I_{to}$ on action potential duration (APD) in large animals is still controversial (See Introduction, page 32).

In an earlier study (Sun & Wang, 2005), our laboratory has shown that while $I_{to}$ determined the depth of the phase 1 notch in canine ventricular cardiomyocytes, the relationship between $I_{to}$ and APD is bi-phasic: below a certain threshold, $I_{to}$ slightly increases the APD; above the threshold, $I_{to}$ significantly shortens APD.

Interestingly, these results in canine contrast previous studies in guinea pig (Hoppe et al., 1999; 2000). Guinea pigs are unusual in that they lack a native $I_{to}$ current in the heart. In the studies by Hoppe et al., exogenous Kv4.3 current was
introduced into guinea pig ventricular myocytes by either gene transfer or cell fusion techniques. Introduction of such an \( I_{to} \)-type current, instead of producing a phase 1 notch, progressively suppressed the AP plateau and shortened the APD over the same \( I_{to} \) density range which we have tested in canine. The significant difference between the results in canine and guinea pig raises the possibility that guinea pig cardiomyocytes are unique in their electrophysiological response to an exogenous \( I_{to} \).

To test this possibility and to further understand the species-dependent role of \( I_{to} \) in shaping the AP, in the first part of my thesis research, \( I_{to} \) was simulated in guinea pig ventricular cells using the dynamic clamp technique and the effects of this simulation on the AP morphology and duration were studied.

A notable limitation of our earlier study (Sun & Wang, 2005) is the use of the whole-cell patch clamp technique and intracellular \( \mathrm{Ca}^{2+} \) buffer, which can alter \( \mathrm{Ca}^{2+} \) intracellular handling and affect the AP properties. In the present study, a perforated patch clamp technique was used, which only allows singly charged ions to pass through the cell membrane. As doubly charged ions, such as \( \mathrm{Ca}^{2+} \), cannot pass through, this preserves the physiological \( \mathrm{Ca}^{2+} \) handling. We carried out simulation studies of \( I_{to} \) in canine endocardial myocytes using the perforated patch clamp technique to both verify our earlier results and to allow for side-by-side comparison of canine and guinea pig ventricular cells.

1.2. Electrophysiological properties of guinea pig ventricular myocytes

As the first step, basic electrical properties of guinea pig LV cardiomyocyte were characterized. Figure 10A shows the representative AP curves at the firing rate of 1Hz and 3Hz. Notably, the AP lacked phase 1 repolarization, and had a waveform similar to that in canine endocardial ventricular myocytes. The average \( \text{APD}_{90} \) at 1Hz was
Figure 10

**Figure 10. Electrical properties of guinea pig ventricular myocytes.**

A, Representative action potential traces recorded from guinea pig ventricular myocytes at 1 Hz and 3 Hz. B, $I_{to}$ is not present in guinea pig ventricular cells, but was readily detectable in canine epicardial cells (inset). Cells were depolarized to voltages ranging from -30 to +50 mV in 10 mV increments, from a holding potential of -70 mV at a frequency of 0.1 Hz. Notice the difference in current scales. C, $I_K$ recorded from guinea pig ventricular myocytes. Currents were in response to depolarizing steps ranging from -30 to +50 mV in 10 mV increments, from a holding potential of -40 mV. Tail currents were recorded at -40 mV.
196.4 ± 12.1 ms (n = 18), and was abbreviated to 137.4 ± 9.9 ms at 3 Hz (n = 6) (Fig. 10A). Consistent with their lack of a phase 1 notch, whole-cell voltage clamp recordings showed that there is no native I_{to} current in guinea pig ventricular myocytes (Fig. 10B). By comparison, in response to the same voltage clamp protocol and under the same recording conditions, a robust I_{to} was evident in canine epicardial myocytes (Fig. 10B, inset). Repolarizing currents in guinea pig ventricular cells were dominated by a large delayed rectifier current (I_{K}) (Fig. 10C).

1.3. Mathematical modeling of the ventricular I_{to}

I_{to} is absent in guinea pig ventricular myocytes (Fig. 10B). The dynamic clamp technique allows us to introduce an artificial voltage-dependent I_{to} conductance to this blank background. We developed formulations of I_{to} based on voltage clamp data from canine epicardial ventricular myocytes (Sun & Wang, 2005) and a published canine ventricular I_{to} model (Dumaine et al., 1999). We define I_{to} as a quick activation, rapidly and fully inactivation current. Figure 11A shows the simulated I_{to} waveform in response to a voltage-clamp protocol. The waveform closely resembles the native current (Fig. 10B, inset). Figure 11B left shows the peak conductance of the model and time constant of the activation (m) gate of the model I_{to} at various voltages. Steady-state inactivation and time constants of the inactivation gate of the model I_{to} are shown in Fig. 11B right.

1.4. Dynamic clamp simulation of I_{to} in guinea pig ventricular cells

To understand the role of I_{to} in shaping the AP in guinea pig ventricular cells, we introduced a canine LV epicardial I_{to} in guinea pig myocytes using the dynamic clamp. APs were triggered at 1 Hz and recorded at steady state. The use of the
Figure 11. Mathematical modeling of canine epicardial transient outward current (I_{to}).

A, Waveform of the model I_{to} in response to voltage steps ranging from −60 to 50 mV from a holding voltage of −80 mV. B, Peak conductance (g_{to}) and time constant of activation (m) gate of the model I_{to} at various voltages (left) and steady-state and time constant of inactivation (h) gate at various voltages (right). g_{to}, peak conductance of simulated I_{to}; Vm, membrane potential; a.u., arbitrary unit.
perforated patch clamp presented a challenge for the implementation of the dynamic clamp technique. We only studied cells with a stable series resistance of <7 MΩ, which was carefully monitored and fully compensated. The dynamic clamp output (Fig. 1A, inset) and the voltage traces were without oscillation or distortion. As shown in Figures 1A&B, simulation of a typical canine epicardial-sized I_{to} produced a distinct phase 1 notch and a "spike-and-dome" AP configuration in guinea pig ventricular cells. Interestingly, this simulation did not significantly affect the APD (Fig. 1A).

We examined the density-dependent effect of simulated I_{to} on the AP. Over a wide density range, simulated I_{to} produced a distinct phase 1 notch and a spike-and-dome AP waveform (Fig. 1B). Low densities of I_{to} did not significantly affect the APD (Fig. 1B, left). As the I_{to} density was further increased, the APD was moderately prolonged (Fig. 1B, left, bold line) before reaching a threshold. At this threshold point, the AP alternated between one with a deep notch and a markedly prolonged APD and all-or-none repolarization (Fig. 1B, right, bold lines). Any simulated I_{to} larger than the threshold produced a brief, spike-like AP (Fig. 1B, right). Figure 1C shows the I_{to} density-APD_{90} relationships for the 18 guinea pig cells we studied, and the average data are shown in Fig. 1D. When the simulated I_{to} was 21.9 ± 5.3 and 27.3 ± 6.2 pA/pF, APD_{90} was not significantly changed (0.98 ± 0.05 and 1.00 ± 0.04 times the control value, n = 16 and 14 cells, respectively; P > 0.2). Prolongation of APD was observed with 33.2 ± 5.5 and 39.4 ± 5.6 pA/pF of I_{to}, which resulted in APD_{90} values of 1.06 ± 0.05 and 1.18 ± 0.06 times control, respectively (n = 18 cells for both, P < 0.01). Larger I_{to} produced dramatic shortening of the APD: APD_{90} ratio was reduced to 0.21 ± 0.11 with a simulated I_{to} of 43.9 ± 5.9 pA/pF (n = 11 cells, P < 0.001).
Figure 12
Figure 12. Dynamic clamp simulation of $I_{to}$ in guinea pig ventricular myocytes.

A, APs recorded at 1 Hz from a guinea pig ventricular myocyte under control conditions (dash line) and with the simulation of a canine epicardial $I_{to}$ (25 pA/pF), using the dynamic clamp technique (solid line). Bottom trace is the current output of the dynamic clamp. Inset shows the current output on a larger scale. B, APs recorded from a guinea pig ventricular myocyte with the simulation of incremental densities of $I_{to}$. The “threshold” phenomenon with $I_{to} = 48$ is shown in the right panel indicated by bold lines. C, APD ($APD_{90}$, expressed as the ratio over the control) versus $I_{to}$ density relationships collected from 18 guinea pig ventricular myocytes. The average data are shown in D. Individual traces were aligned such that the last points before “all-or-none repolarization” fell into one group. Vertical and horizontal error bars are ± S.E.M. of the $APD_{90}$ ratio and $I_{to}$ density, respectively. In Fig. 11D, asterisks indicate a statistical significance of $P < 0.01$ in a paired Student’s $t$ test. Scale bars for the action potentials are 100 ms and 40 mV.
1.5. Dynamic clamp simulation of \( I_{to} \) in canine endocardial myocytes

In our earlier study (Sun & Wang, 2005), the dynamic clamp technique was implemented with the conventional whole-cell patch clamp. The use of \( Ca^{2+} \) buffer in those recordings could have potentially affected the functional behavior of \( I_{to} \). Therefore, to both allow for comparison between guinea pig and canine ventricular cells and to verify our earlier \( I_{to} \) simulation results in canine ventricular cells, we performed dynamic clamp simulations using the perforated patch clamp technique in this study. Under this condition, simulation of \( I_{to} \) in canine endocardial cells generated AP waveforms that were remarkably similar to those recorded from canine myocyte using microelectrode recordings (Litovsky & Antzelevitch, 1990; Liu et al., 1993; Lukas & Antzelevitch, 1993). The overall effect of simulated \( I_{to} \) on canine endocardial APD was similar to that in guinea pig cells (Figs 12 & 13), and qualitatively similar to our earlier results (Sun & Wang, 2005; Fig. 3B). Low levels of \( I_{to} \) had little effect on the APD; further increases in \( I_{to} \) density progressively and moderately prolonged the APD before collapsing the plateau and markedly shortening the APD (Fig. 13). When the simulated \( I_{to} \) was 22.8 ± 1.7 and 28.6 ± 2.0 pA/pF, the APD\(_{90}\) ratios of the control were 1.02 ± 0.01 and 1.05 ± 0.01, respectively (n = 8 and 5, \( P > 0.02 \) and < 0.01, respectively). APD\(_{90}\) was further prolonged to 1.12 ± 0.02 and 1.19 ± 0.03 times the control, with a simulated \( I_{to} \) of 33.2 ± 1.5 and 40.2 ±1.9 pA/pF, but was shortened to 19 ± 0.3% of the control with an \( I_{to} \) of 44.0 ± 2.0 pA/pF (n = 7, \( P < 0.01 \) for all groups).

1.6. Effect of different \( I_{to} \) model formulation

The dynamic clamp has the advantage of working with real, living cells compared to purely computational simulation. However, it still involves simulation
Figure 13. Dynamic clamp simulation of $I_{lo}$ in canine endocardial ventricular myocytes.

A, APs recorded from a canine endocardial myocyte with various densities of simulated $I_{lo}$. For the left panel, $I_{lo}$ density ranged from 5.6 to 33 pA/pF, in increments of about 5.6 pA/pF. B, Average APD$_{90}$ ratio vs. $I_{lo}$ density relationship for canine endocardial cells. Data are averaged and plotted as described in Figure 12 from 7 cells. Asterisks indicate a statistical significance of $P < 0.01$ in a paired Student’s $t$ test. Scale bars for the APs are 200 ms and 40 mV.
of artificial conductances. The density-dependent effects of $I_{to}$ on APD we observed above could be the result of a particular formulation of $I_{to}$. To determine whether our $I_{to}$ simulation results are unique to the particular formulations of our canine epicardial $I_{to}$ model or instead represent a true $I_{to}$ effect, we performed simulations of an atrial $I_{to}$ model (Ramirez et al., 2000) in guinea pig ventricular cells, and compared its effects with those of our ventricular $I_{to}$ model. The two models used in the study are entirely unrelated and described by different equations. The $I_{to}$ conductances described by the two models have different activation rates, conductance-voltage relationships, and steady-state inactivation properties. These differences resulted in noticeably different waveforms of dynamic clamp $I_{to}$ current output when simulated in the same guinea pig ventricular cell (Fig. 14 A & C, insets). Nevertheless, the effects of the two $I_{to}$ models on AP morphology and duration were remarkably similar (Fig. 14 A & C), as were the relationships between $I_{to}$ conductance and $APD_{90}$ (Fig. 14 B & D). Similar experiments were performed in a total of 5 guinea pig cells and in 3 canine endocardial myocytes. These findings demonstrate that our simulation results are a representation of the true $I_{to}$ functional property and not limited to our particular $I_{to}$ formulation.

1.7. Effect of pacing rate on the simulation study

As shown in Figure 10A, the length of AP is pacing rate dependent. Guinea pigs have a heart rate of over 200 beats per minute, i.e. ~ 3 Hz. Our above experiments were all performed at 1 Hz. At a higher and more physiological rate of 3 Hz, the cumulative activation of $I_K$ would favor repolarization of the AP, and the effect of $I_{to}$ on AP morphology may differ from that observed at 1 Hz. We therefore performed simulation studies of $I_{to}$ at pacing rates of 1 and 3 Hz. As shown in Figure 15, the
Figure 14. Simulations of different mathematical $I_{to}$ models in guinea pig ventricular cells.

APs recorded from a guinea pig ventricular myocyte with various levels of $I_{to}$ simulated based on our canine ventricular $I_{to}$ model (A) and a canine atrial $I_{to}$ model (C) (Ramirez et al., 2000). The insets show the current output of the dynamic clamp. The APD$_{90}$ ratio vs. $I_{to}$ conductance relationships for A and C are shown in B and D. Scale bars for the APs are 100 ms and 40 mV.
influences of \( I_{to} \) on AP are generally similar at both pacing rates, except a higher density of \( I_{to} \) was needed at 3 Hz to produce the same notch depth, or to achieve “all-or-none repolarization” (Fig. 15C). In a representative cell shown in Figure 15, the \( I_{to} \) density for “all-or-none repolarization” is 40 pA/pF at 1 Hz compared to 45 pA/pF at 3 Hz. This shift of the \( I_{to} \) density-\( APD_{90} \) curve at 3 Hz towards a higher \( I_{to} \) density probably reflects a lower degree of \( I_{to} \) recovery from inactivation at a higher pacing frequency.

1.8. Effect of sustained outward current on APD

Our dynamic clamp simulation results differ significantly from those using the gene transfer or cell fusion approaches to introduce Kv4.3 currents in the guinea pig ventricular cells (Hoppe et al., 1999, 2000). In those studies, a sustained outward current existed along with a transient \( I_{to} \)-like current. This can be explained by the presence of slowly inactivating components when Kv4.3 channels are expressed alone (Greenstein et al., 2000). We speculated that this is the major cause of the remarkable suppression of APD in their studies. A previous computer modeling study suggested that the introduction of such a non-inactivating current contributed to the shortening of the APD by the exogenous Kv4.3 current (Liu & Antzelevitch, 1995). To test this in guinea pig ventricular myocytes, we modified our \( I_{to} \) model to include a non-inactvation component. As shown in Figure 16, again, simulation of a fully inactivating \( I_{to} \) had the typical bi-phasic effect on AP waveform and duration in guinea pig cells (Fig. 16A). In the same cells, simulation of an \( I_{to} \) with a 10% to 16% sustained component (a fraction similar to those in Hoppe et al’s studies) progressively and significantly suppressed the plateau and shortened the APD over the entire density range we tested (Fig. 16B). With the presence of this sustained
Figure 15. Simulation of $I_{to}$ in guinea pig ventricular myocytes at different pacing rates.

AP traces from a guinea pig ventricular cell with incremental densities of simulated canine epicardial $I_{to}$ at pacing rates of 1 Hz (A) and 3 Hz (B). C, APD$_{90}$ vs. $I_{to}$ density relationships at 1 and 3 Hz for the same cell. Scale bars for the APs are 100 ms and 40 mV.
component, $I_{to}$ shortens the APD continuously (Fig. 16B) and its density had a monotonic inverse relationship with $APD_{90}$, instead of the bi-phasic relationship observed when the simulated current fully inactivated (Fig. 16C). Similar results were obtained in a total of 4 guinea pig cells. Results shown in Figures 16B & C reproduced the observations in Hoppe et al.’s studies, suggesting that the presence of a sustained outward current accounted for the monotonic shortening of APD by $I_{to}$ in these earlier studies.

1.9. Summary and conclusions

In above studies, simulated $I_{to}$ conductance was introduced in guinea pig and canine endocardial ventricular myocytes to understand the role of $I_{to}$ in regulating the ventricular AP morphology and duration. The effects of simulated $I_{to}$ in both types of cells were complex and biphasic, separated by a clear density threshold of 40 pA/pF. Below this threshold, simulated $I_{to}$ resulted in a distinct phase 1 notch and had little effect on or moderately prolonged the APD. $I_{to}$ above the threshold resulted in all-or-none repolarization and precipitously reduced the APD. These effects of $I_{to}$ are not linked to the particular mathematical formulations of the $I_{to}$ model or a specific stimulation rate. We conclude that 1) contrary to previous gene transfer studies involving the Kv4.3 current, the response of guinea pig ventricular myocytes to a fully inactivating $I_{to}$ is similar to that of canine ventricular cells and 2) in animals such as dogs that have a broad cardiac AP, $I_{to}$ does not play a major role in setting the APD.
Figure 16. Effect of a sustained component in $I_{to}$ on action potential shape and duration.

A, APs from a guinea pig ventricular cell with simulation of incremental densities of $I_{to}$ from 8.8 to 60 pA/pF, in increments of ~ 8.8 pA/pF. B, APs from the same cell with various densities of simulated $I_{to}$ that had a non-inactivating (sustained) component. Total simulated $I_{to}$ density ranged from 8.9 to 44 pA/pF, in 8.9 pA/pF increments. The sustained component was 16% of the total peak current when elicited by a voltage step to +40 mV from a holding potential of -80 mV. C, $APD_{90}$ ratio vs. $I_{to}$ density relationships from the same cell with simulations of $I_{to}$ that had various fractions of sustained component ($I_s$). Scale bars for the APs are 100 ms and 40 mV.
2. Role of I\text{to} in regulating contractility in canine ventricular myocytes

2.1. Background and rationale

The above studies elucidate the role of I\text{to} in regulating AP morphology and duration in ventricular myocytes. Recent studies suggested that I\text{to} also affects the mechanical function of the cardiomyocytes via its influence on excitation-contraction (EC) coupling (Sah et al., Introduction page 35). The exact nature of such influence, however, is less clear. It was suggested that I\text{to} enhances cardiac EC coupling by generating early AP repolarization. Along this line, it has been shown that rapid phase 1 AP repolarization increases the peak magnitude of the L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca-L}) influx elicited by the AP (Greestein et al., 2000; Sah et al., 2002; Cordeiro et al., 2004). The higher I\textsubscript{Ca-L} influx in turn leads to an increase in the temporal synchronization of sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release (Sah et al., 2002; Harris et al., 2005). Based on these findings, it is proposed that downregulation of I\text{to} is a major contributor to the impaired EC coupling and myocyte contraction in HF (Sah et al., 2003). However, other studies have yielded contrary results. In canine ventricular myocytes, it was shown that the magnitude of I\textsubscript{Ca-L} influx is actually smaller rather than bigger when activated by an AP waveform that does not have a prominent phase 1 notch when compared to an AP waveform that does (Zygmunt et al., 1997; Banyasz et al., 2003), suggesting that I\text{to} may induce larger sarcolemmal Ca\textsuperscript{2+} influx.

The above studies highlight the lack of knowledge on the role of I\text{to} in regulating EC coupling activity. To help delineate this role, in the following studies, myocyte mechanical measurements were combined with dynamic clamp simulation as well as
intracellular Ca\(^{2+}\) measurement to elucidate the correlation between I\(_{\text{to}}\) density and myocyte mechanics. Also, the effect of AP phase-1 repolarization on I\(_{\text{Ca-L}}\) influx was also quantitatively examined using AP voltage clamp.

2.2. Electrical and mechanical heterogeneity across the LV wall

The transmural electrical heterogeneity in the left ventricle (LV) of large mammals has been well documented (Antzelevitch & Fish, 2001, review). As shown in Figure 17A, AP in canine LV epicardial cells was characterized by a “spike-and-dome” waveform with a prominent phase 1 notch, while the notch was shallow or almost absent in endocardial cells. This transmural difference in AP morphology is the result of a marked transmural gradient in I\(_{\text{to}}\) density (Fig. 17B). I\(_{\text{to}}\) was significantly higher in epicardial myocytes than that in the endocardial myocytes. In our recordings, the average I\(_{\text{to}}\) density was 21.23 ± 3.01 pA/pF at +40 mV in LV epicardial cells (n = 15) and was 3.01 ± 0.32 pA/pF in endocardial cells (n = 12). Parallel to the transmural electrical heterogeneity, heterogeneous mechanical function in canine LV epi- and endocardial cells was observed (Fig. 17C). Under field stimulation, endocardial cells exhibited a significantly stronger cell shortening (Fractional shortening (FS): 9.527 ± 0.238%; n = 43 cells), which was 67% higher than that in epicardial myocytes (5.672 ± 0.215%; n = 43 cells; \(P<0.01\)) (Fig. 17C, right). In accordance, intracellular Ca\(^{2+}\) signal measurement showed a higher Ca\(^{2+}\) transient amplitude in endocardial than in epicardial cells (Fig. 17D), with an average ΔF/F\(_0\) of 1.45 ± 0.18 (n = 20) in endo- and 0.84 ± 0.10 (n = 17) in epicardial cells (\(P<0.01\)). These data delineated a fundamental difference of epi- and endocardial myocytes in their electrical properties, mechanical function, and Ca\(^{2+}\) homeostasis, and provided a possibility that the myocyte mechanical heterogeneity may correlate
Figure 17

A

ENDO

EPI

200 ms

40 mV

C

FS=4%

1 sec

D

$\Delta F/F_0 = 0.5$

$\Delta F/F_0$

Endo  Epi

Endo  Epi

B

$I_b$ (pA/μF)

-60  -30   0   30   60

Vm (mV)

EPI  ENDO

FS (%)

0  2  4  6  8  10

Endo  Epi

**

**
Figure 17. Electrical and mechanical heterogeneity in canine LV cardiomyocytes.

A, Representative AP traces from endo- (left) and epicardial cells (right). B, Current-voltage relationships of I_{to} current in endo- (n=13) and epicardial cells (n=12). C, Representative traces of cell shortening of endo- (left) and epicardial myocytes (middle) elicited by field stimulation, and the average fractional shortening (FS) in these myocyte types (n = 46 myocytes from 3 hearts for both endo- and epicardial myocytes). D, Representative traces of Ca^{2+} transient from endo- (left) and epicardial myocytes (middle) elicited by field stimulation, as well as their average transient amplitude (ΔF/F0). N = 17 and 20 for epi- and endocardial myocytes from 3 hearts. Values = Mean ± S.E.M. **: P<0.01 in an unpaired t-test.
to the transmural $I_{to}$ difference.

2.3. Effect of simulated $I_{to}$ on endocardial myocyte contraction

We next used the dynamic clamp to determine the effect of $I_{to}$ on myocyte mechanical property, taking advantage of the near-absence of native $I_{to}$ in endocardial myocytes. Endocardial myocytes were recorded under perforated patch configuration. An artificial $I_{to}$ conductance was “inserted” in endocardial myocytes, and its impact on AP and contraction were simultaneously recorded. This simulated $I_{to}$ has been described above (page 84-85). AP and cell shortening were recorded simultaneously at steady-state at a pacing rate of 1 Hz. A representative experiment is shown in Figure 18A. Introduction of an epicardial-level of $I_{to}$ (22 pA/pF) generated a prominent phase 1 notch. The membrane potential in phase 1 notch is lowered from an average of $13.34 \pm 2.73$ to $-15.73 \pm 2.27$ mV. More interestingly, this $I_{to}$ simulation significantly reduced cell shortening. This inhibitory effect reversed within several stimuli after the $I_{to}$ simulation was withdrawn (Fig. 18B). Quantitatively, the epicardial-level $I_{to}$ caused a significant suppression in cell contractility by 19%, from a FS of $8.01 \pm 0.84$% in control to $6.48 \pm 0.67$% in the presence of $I_{to}$ (n=12, $P < 0.01$). In addition, the maximum rate of cell lengthening ($+dL/dt$) was significantly reduced after $I_{to}$ “addition” (from $61.64 \pm 7.15$ µm/s to $47.96 \pm 4.99$ µm/s, $P < 0.01$, Fig. 18D left), so was the maximum rate of relaxation (from $58.42 \pm 8.30$ µm/s to $42.09 \pm 5.59$ µm/s, $P < 0.01$, Fig. 18D right).

2.4. $I_{to}$ density dependent effect on contractility

The density-dependent effect of $I_{to}$ on cell contractility was studied. A range of $I_{to}$ was introduced in an endocardial cell using the dynamic clamp. As shown in
Figure 18. Effects of I_{to} simulation on mechanical properties of canine LV endocardial myocytes.

A, Representative APs (upper) and concurrent cell shortenings (lower) recorded simultaneously from a canine LV endocardial myocyte under control condition (black line) and with the simulation of a canine epicardial I_{to} (22 pA/pF) using the dynamic clamp (red line). The myocyte was stimulated with 2 ms just-threshold depolarizing steps at 1Hz. B, Contractility of the same endocardial myocyte as in A, as the myocyte myocyte was continuously paced and with I_{to} simulation turned “on” and “off”. C-D, Average fractional shortening (C), rate of myocyte contraction (+dL/dt; D left), and rate of relaxation (~dL/dt; D right) from 12 endocardial myocytes without and with I_{to} simulation. Values = Mean ± S.E.M. **: P<0.01 vs control in a paired t-test.
Figure 19A, when $I_{to}$ density was increased, phase 1 repolarization became more and more prominent and the AP duration slightly lengthened, until the threshold for all-or-none repolarization was reached. This is accompanied by suppression of myocyte contractility (Fig. 19 A & B). Figure 19B shows that during the time course as we “turned on” and then “turned off” $I_{to}$ simulation, the peak value of cell shortening gradually went down and recovered. Interestingly, although increasing amount of $I_{to}$ progressively deepened the notch, and caused an almost linear relationship between $I_{to}$ density and notch voltage, the effect of $I_{to}$ on cell shortening was more stair-like (Fig. 19C). Low densities of $I_{to}$ (<9 pA/pF) only had a minor influence on contractility. Contractility was significantly suppressed only when the simulated $I_{to}$ was increased to near-epicardial level (~20 pA/pF) and the notch voltage dropped below 0 mV (Fig. 19C). When $I_{to}$ was increased beyond the threshold for all-or-none repolarization, the AP became triangular and myocyte contraction almost completely stopped.

2.5. Effect of simulated $I_{to}$ on endocardial Ca$^{2+}$ transient

The inhibitory effect of simulated $I_{to}$ on myocyte contractility is most likely mediated by alteration of intracellular Ca$^{2+}$ dynamics. To test it, endocardial myocytes were loaded with fluo-4 AM, and Ca$^{2+}$ transients were measured along with $I_{to}$ “insertion”. As shown in Figure 20A, simulation of an epicardial-level $I_{to}$ suppressed the amplitude of Ca$^{2+}$ transient. Peak amplitude of Ca$^{2+}$ transient ($\Delta F/F_0$) was decreased by 11%, from $1.10 \pm 0.13$ to $0.98 \pm 0.11$ ($P<0.05$; Fig. 20B). This mirrors the results on cell shortening. Interestingly, the rising rate of Ca$^{2+}$ transient, an indication of SR Ca$^{2+}$ release synchronization, was notably faster under $I_{to}$ simulation (Fig. 20A, inset). The maximum rising rate of $d[Ca^{2+}]/dt$ was $17.0 \pm 3.2$ with $I_{to}$ simulation and $13.17 \pm 2.1$ under control ($P<0.05$; Fig. 20C). This observation
Figure 19. Density dependent effect of simulated $I_{to}$ on endocardial myocyte contraction.

A, APs (upper) and cell shortenings (lower) simultaneously recorded from a canine endocardial myocyte with various densities of $I_{to}$ simulation. B, Time course of cell shortening with various densities of $I_{to}$ simulation (as indicated by the colored lines) in the same endocardial myocyte as in A. C, Contractility and AP phase-1 notch voltage under various simulated $I_{to}$ densities.
Figure 20. Effect of simulated $I_{to}$ on $\text{Ca}^{2+}$ transient in endocardial myocytes.

A, Representative APs (upper) and concurrent $\text{Ca}^{2+}$ transient traces (lower) simultaneously recorded in an endocardial myocyte under control (black line) and with $I_{to}$ simulation (red line). Inset shows rise of $\text{Ca}^{2+}$ level on a larger scale. B, Average effect of $I_{to}$ simulation on the amplitude of $\text{Ca}^{2+}$ transient ($\Delta F/F_0$) from 6 endocardial myocytes. The rising rates of intracellular $\text{Ca}^{2+}$ signal under control and with $I_{to}$ simulation are shown in C. Values = Mean ± S.E.M. *: $P<0.05$ vs control in a paired $t$-test.
agrees with previous finding that phase-1 repolarization increases SR Ca$^{2+}$ release synchronization (Harris et al., 2005).

2.6. Effect of I$_{to}$ subtraction on epicardial cell shortening

As a complementary experiment, we next examined the effect of subtraction, or “blockade” of the native I$_{to}$ on contractility in epicardial myocytes. To do so, an artificial I$_{to}$ of inward polarity was introduced using the dynamic clamp, and its amplitude adjusted such that the epicardial AP notch was reduced or eliminated (Fig. 21A). The resulting AP waveform resembled that of the endocardial myocytes. Importantly, such “blockade” significantly enhanced cell shortening in epicardial myocytes (Fig. 21A & B). When I$_{to}$ subtraction was withdrawn, myocyte contractility completely returned to the control level (Fig. 21B). Interestingly, the time course of the effect of I$_{to}$ “blockade” on cell shortening followed two patterns. For most myocytes (5 out of 8), enhancement of contractility and its reversal were both near-instantaneous (Fig. 21B left). In the second pattern, on both the way up and down, contractility overshot then regressed to steady state (Fig. 21B right). Quantitatively, for the 8 epicardial myocytes we studied, FS was 6.5 ± 0.8% under control and was increased to 8.1 ± 0.7% under I$_{to}$ subtraction (Fig. 21C left, $P < 0.05$). I$_{to}$ subtraction also increased the contraction kinetics of epicardial myocytes (Fig. 21C). Maximum +dL/dt was increased from 57.1 ± 11.7 to 78.3 ± 13.4 µm/s ($P < 0.05$), and maximum -dL/dt from 44.2 ± 13.5 to 61.6 ± 14.5 µm/s ($P < 0.05$).

2.7. Influence of I$_{to}$ on the I$_{Ca,L}$ profile under AP voltage clamp configuration in canine ventricular myocytes

Above studies demonstrated an inhibitory effect of I$_{to}$ on cell shortening and
Figure 21. Effect of $I_{to}$ “subtraction” on mechanical properties of epicardial myocytes.

A, APs (upper) and corresponding cell shortenings (lower) simultaneously recorded from a canine epicardial myocyte without (black line) or with $I_{to}$ “blockade” using the dynamic clamp (red line). B, Time course of fractional cell shortening under $I_{to}$ subtraction (indicated by the red line) from two typical epicardial myocytes. C, Average data from 8 epicardial myocytes showing the effect of $I_{to}$ “blockade” on myocyte fractional shortening (left), rate of contraction (middle), and rate of relaxation (right). Values = Mean ± S.E.M. *: $P<0.05$ vs control in a paired $t$-test.
Ca^{2+} transient in ventricular cardiomyocytes. Previous studies have shown that $I_{\text{to}}$, by shaping the AP phase 1, influences $I_{\text{Ca,L}}$ profile (Greenstein et al. 2000; Banyasz et al., 2003). We hypothesized that the suppression effect of $I_{\text{to}}$ on Ca^{2+} dynamics is mediated by the inhibition of $I_{\text{Ca,L}}$, leading to a reduced sarcolemmal Ca^{2+} influx and decreased intracellular Ca^{2+} transient. To test this hypothesis, $I_{\text{Ca,L}}$ was measured in the same cell imposed with different pre-recorded AP waveforms. These AP waveforms were pre-recorded in a canine LV endocardial myocyte when a broad range of simulated $I_{\text{to}}$ was introduced in the cell using the dynamic clamp (Fig. 2 A&B upper panel). The only altered factor that induced the changes in these AP waveforms is $I_{\text{to}}$. To eliminate contaminations from other ionic conductance and to minimize the residual capacitive artifacts, $I_{\text{Ca,L}}$ in our study was defined as the nifedipine ($5 \mu$M) -sensitive current. $I_{\text{Ca,L}}$ was recorded using these voltage waveforms from both epicardial and endocardial myocytes with near identical results.

As shown in Figure 22B, all AP waveforms triggered a fast and initial activation of $I_{\text{Ca,L}}$. The amplitude of this first spike was slightly increased and then decreased with increasing $I_{\text{to}}$ (Fig. 22C). The peak $I_{\text{Ca,L}}$ triggered by an “all-or-none repolarization” AP waveform was remarkably small and was only ~20% of that in the control. In the AP waveforms where a prominent notch exists, the $I_{\text{Ca,L}}$ decayed quickly following the early repolarization and the $I_{\text{Ca,L}}$ developed a 2nd spike. The 2nd $I_{\text{Ca,L}}$ spike existed during the formation of the AP “dome”. The amplitude of the 2nd peak correlated inversely with the notch depth (Fig. 22C). To evaluate the $I_{\text{Ca,L}}$-mediated sarcolemmal Ca^{2+} influx, the integration of $I_{\text{Ca,L}}$ trace was calculated and was presented as a function of $I_{\text{to}}$ density. The initial Ca^{2+} influx, defined as the integration of the $I_{\text{Ca,L}}$ curve before the deepest point of the phase 1 notch, was only slightly increase at low density of $I_{\text{to}}$ simulation, and then gradually decrease with an
almost linear relationship to the $I_{to}$ density (Fig. 22D). Endocardial level of $I_{to}$ corresponds to the control curve and the epicardial $I_{to}$ corresponds to the 4th curve (20 pA/pF). Compared to the endocardial, the epicardial $I_{to}$ resulted in a significantly smaller initial Ca$^{2+}$ influx to the cytosol, possibly contributed to a smaller SR Ca$^{2+}$ release and hence cell contractility. Interestingly, with the exception of all-or-none repolarization, the total Ca$^{2+}$ influx elicited by various AP waveforms remained nearly constant despite the dramatically different $I_{Ca-L}$ dynamics, and the $I_{to}$ versus total Ca$^{2+}$ influx relationship traced closely with the $I_{to}$-AP duration relationship (Fig. 22E), implying that the total Ca$^{2+}$ influx correlates tightly with AP duration.

2.8. Summary and conclusions

In this study, we investigated the role of $I_{to}$ in regulating the mechanical function of canine ventricular myocytes using the dynamic clamp. We show that introduction of an artificial epicardial-level $I_{to}$ suppressed contractility and Ca$^{2+}$ transient amplitude in canine LV endocardial myocytes, where the native $I_{to}$ is near absent; conversely, “blockade” of native $I_{to}$ in epicardial myocytes increased contractility. The inhibitory effect of $I_{to}$ on myocyte mechanics is likely mediated by the suppression of $I_{Ca-L}$, particularly early $I_{Ca-L}$ influx, by phase-1 AP repolarization. We conclude that under non-diseased conditions, epicardial-level of $I_{to}$ has a negative, rather than positive effect on myocyte contractile function.
**Figure 22. Influence of I_{to} on the I_{Ca,L} profile under AP voltage clamp configuration in canine ventricular myocytes.**

A, A representative I_{Ca,L} trace (lower) recorded in response to a pre-recorded canine LV endocardial AP waveform (upper). All the I_{Ca,L} current traces were defined as the nifedipine (5 \mu M)-sensitive difference current. Resting membrane potential was set to -75 mV. B, Representative I_{Ca,L} traces in response to AP waveforms with various densities of simulated I_{to}. Insets show the current peak on a larger scale. C, The first I_{Ca,L} peak amplitude, the second I_{Ca,L} peak amplitude, and the depth of phase 1 notch as a function of I_{to} density. D, Relationship of I_{to} density vs. initial Ca^{2+} influx, expressed as ratio over control and calculated as the integral of the I_{Ca,L} traces before the deepest point of phase-1 notch (inset, 18 ms from AP upstroke). E, Relationships of I_{to} density vs. total Ca^{2+} influx and AP duration (APD_{90}), both expressed as ratios over control. Data in C – E are averages from 4 myocytes and expressed as ± S.E.M.
3. Contribution of $I_{\text{to}}$ to APD prolongation in canine failing ventricular cardiomyocytes

3.1. Background and rationale

Heart failure (HF) is the end phase of a number of cardiovascular diseases. It is characterized by cellular electrical and mechanical abnormalities and these abnormalities result in the rhythmic and contractile dysfunctions. One of the most consistent and significant electrical changes in failing hearts is the prolongation of AP (Tomasselli et al., 1994). The prolonged APD is believed to predispose the heart to afterdepolarization and/or reentrant arrhythmias (Carmeliet, 2006). Another hallmark electrical change in the failing cardiomyocytes is the downregulation of $I_{\text{to}}$ (Kaab et al., 1996; Tsuji et al., 2000; Beuckelmann et al., 1993; Li et al., 2004). The role of $I_{\text{to}}$ downregulation in electrical and mechanical abnormalities, however, is not fully understood. The above studies in this thesis have shown that physiological $I_{\text{to}}$ is not a key determinant of APD but may be a negative regulator of EC coupling and cell contractility. In this section, a preliminary study was performed to study the functional role of $I_{\text{to}}$ under failing settings. The specific aims included: 1) To evaluate the electrical and contractile changes in ventricular myocytes from a canine HF model. 2) To elucidate the contribution of $I_{\text{to}}$ downregulation to the abnormal AP profile in failing myocytes. Different densities of $I_{\text{to}}$ were simulated to restore the normal $I_{\text{to}}$ amplitude in failing ventricular myocytes using the dynamic clamp technique. The effect of such simulations on AP morphology and duration were assessed.
3.2. Characterization of electrical and mechanical properties of ventricular cardiomyocytes from a canine HF model

We utilized a canine HF model that was generated by Dr. John Lopshire at the Indiana University Clarion Cardiovascular Center. As shown in Figure 9 (Method section, page 79), HF was induced by embolization followed by quick pacing. The average ejection fraction was significantly decreased after induction of failure, from 64% ± 5% in control to 18% ± 4% in HF (P<0.002). Symptoms and other parameters also demonstrated that HF was successfully induced in this model (Table 3).

Failing cardiomyocytes are characterized by significant electrical remodeling. We first evaluated APs in myocytes from a HF model and compared them with APs from control animals (without IM induction and high rate pacing treatments). As shown in Figure 23A, the AP phase 1 notch was notably shallower in HF than in normal epicardial myocytes. Also, the duration of APs was significantly prolonged, with an average APD₉₀ of 445.4 ± 30.2 ms in midmyocardial cells from failing hearts (n = 8 from 2 hearts, P<0.01), while the average APD₉₀ in normal cells was only 225.3 ± 5.7 ms in our recordings (Fig. 23B). Associated with the shallow phase 1 notch was the downregulation of I_{to}. As shown in Figure 23C, I_{to} was remarkably reduced in epicardial myocytes from the failing heart compared to that in normal cells. The average peak level of I_{to} density at +40 mV was decreased by 80%, from 18.3 ± 1.5 pF/pA (n = 13 from 2 hearts) to 3.5 ± 0.3 pF/pA (n = 10 from 2 hearts, P < 0.005, Fig. 23D).

We next characterized the contractile properties in isolated myocytes from this HF model. As shown in Figure 24, cell shortening was significantly reduced in HF midmyocardial cells compared to the control cells. The average FS was 4.93 ± 0.22 % in the normal cells and was 3.71 ± 0.22% in the cells from failing hearts (P<0.05).
Table 3. Parameters at study entry and after induction of HF in canines

<table>
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<th>Parameter</th>
<th>Study Entry</th>
<th>After Induction</th>
<th>P value</th>
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<tr>
<td>Systolic BP (mmHg)</td>
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<td>95±5</td>
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<td>Diastolic BP (mmHg)</td>
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<td>118±19</td>
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<td>Weight (Kg)</td>
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<tr>
<td>QT interval (ms)</td>
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<td>258±29</td>
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<tr>
<td>Ejection Fraction (%)</td>
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<td>18±4</td>
<td>0.002</td>
</tr>
<tr>
<td>Fractional Shortening</td>
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</tr>
<tr>
<td>LVES diameter (cm)</td>
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<tr>
<td>B-type Natriuretic peptide (BNP) (pg/ml)</td>
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<td>972±415</td>
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Figure 23. Electrical remodeling in ventricular cardiomyocytes from a canine HF model.

A, Representative AP traces recorded from a normal LV epicardial myocyte and from a failing heart. B, Average APD<sub>90</sub> in these two groups of cells. C, I<sub>t0</sub> recorded in LV epicardial cells from normal and failing hearts activated by voltage steps from a holding potential of −70 mV to voltages ranging from −30 to +50 mV, in 10 mV increments at 10 s intervals. D, Current-voltage relationships of I<sub>t0</sub> current in epicardial myocytes from control (n=13 from 2 hearts) and from failing hearts (n=10 from 2 hearts). Error bars are ± S.E.M. ** P<0.01.
**Figure 24.** Attenuated myocyte contractility in a canine HF model.

A, Representative cell shortening waveform in midmyocardial cells from control (left) and failing hearts (right). Cells were paced at 1Hz. Average fractional shortening (panel B), maximum rate of cell contraction (+dL/dt, panel C), and maximum rate of relaxation (-dL/dt, panel D) analyzed from control (n=76 from 2 hearts) and failing midmyocardial cells (n=24 from 2 hearts). Values = Mean ± S.E.M. *: *P*<0.05 vs control.
The maximum rate of contraction (+dL/dt) and relaxation (-dL/dt) were also reduced in failing cells, from $47.8 \pm 3.0$ in normal heart to $37.1 \pm 4.7 \mu m/s$ in failing and from $34.9 \pm 3.3$ in normal to $30.9 \pm 4.9 \mu m/s$ in failing, respectively. However, these differences did not reach statistical significance.

Myocytes functions from a same failing heart are different due to cell-to-cell variation. While some cells developed more severe electrical and mechanical dysfunctions in a failing heart, some cells did not. All above data in single myocytes were acquired from 2 normal or 2 failing hearts. Although some of the parameters have reached statistical significance between normal cells and cells from the failing hearts, we expected that as we continuing our experiments and obtaining more animal numbers, the final results would be more accurate.

### 3.3. Simulation of I_{to} in failing ventricular cardiomyocytes

To elucidate the role of $I_{to}$ in regulating the AP profile in HF, we performed the dynamic clamp to simulate different densities of $I_{to}$ in the failing epicardial myocytes. In our previous study, we found that $I_{to}$ and APD have a bi-phasic relationship in control cardiomyocytes (Sun & Wang, 2005; also see Result Fig. 12&13). In failing cells, we observed two types of a relationship between $I_{to}$ and APD. The first type was similar with our observations in the normal cells: low density of $I_{to}$ had small or modest effects on the APD, until $I_{to}$ reached a threshold point where $I_{to}$ resulted in a deep phase 1 notch and a delayed repolarization phase. Any $I_{to}$ that was larger than this threshold collapsed APs to the “all-or-none repolarization” and significantly shortened the APD (Fig. 25A). In the second type, increasing $I_{to}$ density gradually decreased the length of AP, i.e., $I_{to}$ and APD had a close-to-linear relationship (Fig. 25B). The correlation coefficient for linear regression is 0.9625.
Our current experimental data are still preliminary. Due to limited availability of HF animals and the difficulty in performing the dynamic clamp in failing cells, we have not been able to obtain enough data to draw any solid conclusions regarding the relationship between $I_{to}$ and APD in failing settings. This issue will continue to be studied by our laboratory in the future.
Figure 25. Dynamic clamp simulation of I_{to} in canine HF ventricular myocytes.

A left & B left, Representative AP waveforms in canine HF midmyocardial myocytes with the simulation of I_{to} using the dynamic clamp technique. A right & B right, APD_{90} (expressed as the ratio over control) versus I_{to} relationships. In panel B right, the correlation coefficient for linear regression is represented as R^2.
4. The possible ionic mechanism for the electrical and mechanical dysfunction in Brugada syndrome

4.1. Background and rationale

Brugada syndrome is a ventricular arrhythmia that is believed to be responsible for 4% - 12% of all sudden cardiac deaths in those patients who have no obvious structural abnormalities (Benito et al., 2008). Up to 30% of the Brugada syndrome patients have been linked to loss-of-function mutations in the cardiac Na\(^+\) channel-encoding gene, SCN5A. The ionic mechanism of the syndrome has been proposed as an imbalance between the inward Na\(^+\) current (I\(_{\text{Na}}\)) and the transient outward K\(^+\) current (I\(_{\text{to}}\)) (Yan & Antzelevitch, 1999). It was believed that the reduced I\(_{\text{Na}}\) combined with a large I\(_{\text{to}}\) in the right ventricular (RV) epicardial myocytes results in the depression or loss of the AP dome and formation of all-or-none repolarization in the epicardium, but not in the endocardium. This heterogeneous loss of the AP dome predisposes the ventricle to the development of phase 2 reentrant extrasystoles, precipitating ventricular arrhythmias. Evidence supporting this model comes from studies using in vitro ventricular wedge preparations, where terfenadine and verapamil were used to induce a Brugada syndrome-like electrical phenotype, and that 4-AP corrected the abnormal pseudo-ECG (Yan & Antzelevitch, 1999; Fish & Antzelevitch 2008). These works provide the foundation for our current understanding of the mechanism underlying Brugada syndrome. Limitations of these studies include their reliance on non-selective channel blockers, such as terfenadine, to reduce I\(_{\text{Na}}\) and induce a Brugada phenotype, and non-selective K\(^+\) blockers, such as 4-AP, to elucidate to role of I\(_{\text{to}}\). In particular, terfenadine is a potent blocker of the
L-type Ca$^{2+}$ channel and the delayed rectifier K$^+$ channels (Ming & Nord, 1995; Liu et al., 1997) in addition to $I_{Na}$.

In addition, although Brugada syndrome is defined as an electrical disorder of the heart (Antzelevitch et al., 2002), several scholars have argued that Brugada syndrome is only a form of arrhythmogenic right ventricular cardiomyopathy/dysplasia (Martini et al., 1993; Naccarella, 1993; Ohe, 1996). Along with this argument were reports of structural and wall motion abnormalities found in patients with Brugada syndrome (Frustaci et al., 2005; Papavassiliu et al., 2004; Takagi et al., 2001, 2003; Huang et al. 2007). Although it was hypothesized that these contractile abnormalities seen in Brugada patients can be explained by the primary electrical dysfunction (Antzelevitch, 2002), this hypothesis is not supported by any experimental evidence, which represents an important gap in our current understanding of this clinical entity.

This section of my thesis study was designed to test this hypothesis. Also, a quantitative interplay between reduced Na$^+$ current and $I_{to}$ in the development of “all-or-none repolarization” at the cellular level has been lacking and was elucidated in the current study. Low doses of tetrodotoxin (TTX) were used to partially repress $I_{Na}$ and to mimic the reduced $I_{Na}$ in Brugada syndrome for no genetic Na$^+$ channel knockdown animal model is available.

In addition, the arrhythmic events reported in the Brugada patients were mostly initiated at the right ventricular outflow tract (RVOT) (Shimada, 1996; Ogawa, 2001; Takagi et al., 2001; Papavassiliu et al., 2004; Coronel, 2005; Yokokawa, 2006). Previous studies have shown that the phase 1 notch is deepest in RVOT compared to other areas of RV, suggesting that RVOT has a high tendency for “all-or-none repolarization” to occur (Kurita et al., 2002; Morita et al., 2007). In this section, the
possible molecular basis for the unique electrical property of RVOT was examined.

4.2. Characterization of action potential morphology and $I_{to}$ density in canine RV myocytes

One of the aims of our study is to understand the combined effects of a reduced $I_{Na}$ and a physiological $I_{to}$ on AP alterations in the Brugada setting. As the first step, APs and $I_{to}$ in canine RV epi-, endo-, and LV epicardial cells were characterized. As shown in Figure 26A, APs of RV epicardial myocytes were characterized by a prominent “spike-and-dome” waveform. The phase 1 notch in this cell type was more profound than in the LV epicardial cells, while the notch was almost unnoticeable in the RV endocardial myocytes. The different depths of the AP notch correlate well with the $I_{to}$ density. RV epicardial cells had a robust $I_{to}$ current with an average level of $26.71 \pm 2.93 \text{ pA/pF (n=7)}$ at $+40 \text{mV}$. The $I_{to}$ level was much lower in LV epicardial cells ($19.99 \pm 3.07 \text{ pA/pF}$), and was only $4.77 \pm 0.69 \text{ pA/pF}$ in RV endocardial cells ($n=9$). Previous literature consistently reported that there is no transmural difference in $I_{Na}$ current (Antzelevitch & Fish, 2001; Zygmunt et al., 2001).

4.3. $I_{Na}$ reduction by TTX alters repolarization of AP in canine RV epicardial myocytes

To mimic $I_{Na}$ reduction caused by genetic mutations in the Brugada syndrome, low doses of TTX (1 or 3 µM) were used to partially block the cardiac $I_{Na}$. These dosages corresponded to 50% and 75% $I_{Na}$ reduction, respectively (Sakakibara et al., 1992). As shown in Figure 27A left, 1 µM TTX resulted in a tenuously deepened phase 1 notch in canine RV epicardial cells. Also, the time course of repolarization in AP was slightly delayed, resulting in a modest prolongation of AP. In some cells where
Figure 26. Heterogeneity in AP and $I_{to}$ in canine left and right ventricular epicardial and endocardial myocytes.

A, Representative AP traces recorded from canine RV epicardial (Epi) cells, endocardial (Endo) cells, and LV epicardial cells. B, Representative $I_{to}$ recorded from RV epicardial cells activated by voltage steps from a holding potential of $-70$ mV to voltages ranging from $-30$ to $+50$ mV, in $10$ mV increments at $10$ s intervals. C, Average peak amplitudes of $I_{to}$ at the holding potential of $+40$ mV in the RV epi-, endo-, and LV epicardial cells. Error bars are ± S.E.M.
the notch was the deepest, 1 µM TTX collapsed the APs, resulting in “all-or-none repolarization” and a short APD (Fig. 27A, right). With a higher concentration of TTX (3 µM), APs in most cells collapsed (Fig. 27B). These data suggested that $I_{Na}$ reduction significantly affected myocyte repolarization and altered AP morphology.

4.4. Effect of TTX on $I_{to}$ threshold for “all-or-none repolarization”

To quantitatively investigate how TTX influences the repolarization process and the interplay between $I_{Na}$ blockade and the early repolarization current $I_{to}$, we next used the dynamic clamp technique to introduce a various densities of $I_{to}$ in cells while $I_{Na}$ current was reduced by TTX. RV endocardial cells were chosen to perform this experiment for their small native $I_{to}$. In previous studies, we have shown that below a certain density threshold, $I_{to}$ deepened the phase 1 notch but did not or only slightly increased APD; higher-than-threshold $I_{to}$ resulted in “all-or-none repolarization” and significantly shortened the APD (see Fig. 13, page 90). As shown in Figure 27, this relationship between $I_{to}$ density and AP morphology was qualitatively unchanged in the presence of TTX. However, the threshold for $I_{to}$-induced all-or-none repolarization was significantly reduced under TTX (Figure 27C, right). Increasing concentrations of TTX progressively shifted the $I_{to}$ density-APD relationship curve to the left (Figure 27D). The average threshold for “all-or-none repolarization” was decreased from $42.4 \pm 3.8$ pA/pF in control to $24.3 \pm 2.6$ 8 pA/pF with 3 µM TTX. The native $I_{to}$ value in RV epicardial myocytes is $26.71 \pm 2.93$ pA/pF at +40 mV (indicated in the APD$_{90}$–$I_{to}$ density plot, Fig. 27D). This $I_{to}$ is clearly lower than the threshold for collapsing AP in control; however, this $I_{to}$ is within the range to collapse AP with 1 or 3 µM TTX. These results indicated that $I_{Na}$ inhibition predisposes the RV epicardial myocytes to abnormal “all-or-none repolarization” AP morphology.
Figure 27. The threshold for $I_{to}$-induced “all-or-none repolarization” was reduced in TTX-induced Brugada syndrome settings.

Representative APs recorded from two canine RV endocardial cells under control conditions (dash line) and with the treatment of 1 µM (A) or 3 µM (B) TTX (solid line). Cells were all paced at 1Hz. C, APs recorded from an endocardial cell with the simulation of various densities of $I_{to}$ under control (left) or with 3 µM TTX (right). D, The average data of $APD_{90}$ (expressed as the ratio over control) versus $I_{to}$ density with or without TTX treatments. The solid line with a filled square indicates the average $I_{to}$ densities in canine RV epicardial cells.
4.5. Effects of $I_{Na}$ reduction on cell mechanics and $Ca^{2+}$ transient

The above experiments have demonstrated that $I_{Na}$ reduction produced abnormal electrical phenotypes that are believed to happen in single cardiomyocytes of Brugada syndrome patients. We next studied how these electrical alterations affect cell mechanical functions. Cell shortening or $Ca^{2+}$ transients were simultaneously measured as we performed the dynamic clamp technique and AP recording. 1 µM TTX, while deepening the phase 1 notch and prolonging APD, attenuated the cell shortening and delayed the relaxation (Fig. 28A, left), and suppressed peak amplitude and delayed the rise of the $Ca^{2+}$ transient (Figure 28A, right). In myocytes where TTX resulted in an “all-or-none repolarization” (triangular AP), the myocytes contraction and the $Ca^{2+}$ transient were almost completely inhibited (Fig. 28B). Quantitatively, under 1 µM TTX and in RV epicardial myocytes where a spike-and-dome AP morphology was preserved, the fractional shortening was decreased by 28% (from 6.84 ± 0.80% to 4.89 ± 0.60%; $P<0.01$), and the fractional shortening was only 1.42 ± 0.30% with triangular AP. The maximum rate of contraction (+dL/dt) and relaxation (-dL/dt) were reduced from 95.25 ± 13.89 to 57.08 ± 8.05 µm/s ($P<0.01$) and from 76.67 ± 7.60 to 54.08 ± 5.41 µm/s ($P<0.05$), respectively (Fig. 28C).

4.6. $I_{to}$ is critical for TTX-induced repolarization and contractile alterations

In contrast to the findings in RV epicardial myocytes, 1 µM TTX or up to 3 µM TTX only slightly affected AP morphology and did not cause all-or-none repolarization in LV epicardial and RV endocardial cells, where the native $I_{to}$ is smaller (Figure 29A, upper panel). TTX also did not significantly affect contraction
Figure 28

Figure 28. TTX inhibited myocyte contractility and Ca\(^{2+}\) transient in RV epicardial cells.

Representative cell shortening (left) and Ca\(^{2+}\) transient curve (right) simultaneously recorded with AP as 1 μM TTX (red line) produced “spike-and-dome” (A) or triangular (B) AP waveforms in canine RV epicardial cells. Upper panel, AP profile. Lower panel, cell shortening (left) or Ca\(^{2+}\) transient (right) measurement. Cells were paced at 1Hz. C, Average fractional shortening, rate of cell contraction (+dL/dt), and rate of relaxation (-dL/dt) with TTX. Values = Mean ± S.E.M. *: P<0.05, **: P<0.01 vs control.
in these cells (Figure 29A, lower panel). It suggested that I_{Na} reduction alone was not enough to induce the “all-or-none repolarization” and attenuated cell shortening; a coexistence of high levels of I_{to} density was required.

We next used a comparably high concentration of TTX (5 µM) to produce a high degree of I_{Na} reduction in RV epicardial cells. As expected, TTX collapsed the AP and resulted in marked suppression of cell shortening (Fig. 29B, left). Interestingly, “subtraction” or “blockade” the native I_{to} by simulation of a negative I_{to} in inward polarity by the dynamic clamp was able to recover the “spike-and-dome” AP waveform. Moreover, it also restored cell contraction to nearly the control level (Fig. 29B, right). This result further demonstrated that the electrical and contractile changes in RV epicardial cells upon I_{Na} reduction depend critically on the presence of a large I_{to}.

4.7. Computational model of the RV cardiac myocyte

A modified Hund-Rudy canine ventricular myocyte model (Hund & Rudy, 2004) was used to further examine the influence of I_{Na} and I_{to} on AP morphology. Modifications include addition of Ca^{2+} compartments in the submembrane and T-tubule/SR regions, and adjustment of the conductance of I_{to} to reflect that found in RV epicardial myocytes. As shown in Figure 30, modeling-generated APs and the effects of Na^{+} conductance (G_{Na}) reduction agreed well with our experimental data. The APs showed an increasing duration as G_{Na} was decreased until the AP collapsed at approximately 50% G_{Na} (Fig. 30 A&B). Interestingly, this bi-phasic effect of G_{Na} reduction on APD is nearly identical to the relationship between increasing G_{to} and APD (Fig. 30B, 30C; also see Fig. 27D). The model showed that decreasing G_{Na} progressively shifted the G_{to}-APD relationship curve to the left and lowered the
Figure 29. $I_{\text{to}}$ facilitates the effect of TTX.

A, Representative cell shortening simultaneously recorded with AP under control condition (black line) or with 3 μM TTX (red line) in canine RV endo- (left) or LV epicardial cells (right). B, Left, AP and cell shortening recorded from a canine RV epicardial cell under control conditions and with 5 μM TTX. Right, $I_{\text{to}}$ “subtraction” using the dynamic clamp in the same cell. Inset, dynamic clamp current output. Upper panel, AP profile. Lower panel, cell shortening measurement.
threshold for $I_{to}$-induced all-or-none repolarization (Fig. 3O C), reproducing our experimental observations shown in Figure 27D.

To better show the relationship between $G_{to}$, $G_{Na}$, and the APD, a three dimensional mesh plot was created (Fig. 3O D). The plot shows that both $G_{Na}$ and $G_{to}$ have a bi-phasic influence on APD. There exists a distinct threshold (or a sharp “cliff”), where APD no longer increases as a function of decreasing $G_{Na}$ or increasing $G_{to}$, but where the AP collapses. The degree of $G_{Na}$ reduction that a myocyte can “tolerate” without AP collapse is critically determined by $G_{to}$; this threshold is about 50% $G_{Na}$ reduction with 1.0X $G_{to}$ (i.e., $I_{to}$ level in RV epicardium) and is much higher with lower levels of $I_{to}$ found in other regions of the ventricles.

4.8. $I_{Na}$ conductance affects intracellular Ca$^{2+}$ cycling

We next examined the influence of $G_{Na}$ on Ca$^{2+}$ dynamics using the RV epicardial cell computational model. Compared to full $G_{Na}$, 70% of $G_{Na}$ resulted in a slightly attenuated and delayed [Ca$^{2+}$]$_i$ surge during an AP (Fig. 31 A dash line). [Ca$^{2+}$]$_i$ surge was almost completely diminished at 30% of $G_{Na}$ (Fig. 31 A dotted line). Figure 31 B shows the “dose-response” relation of $G_{Na}$ and [Ca$^{2+}$]$_i$. At 50% $G_{Na}$ there is a precipitous drop in peak Ca$^{2+}$ transient level, coinciding with the collapse of the AP (Fig. 31 B). Prior to this sharp drop, [Ca$^{2+}$]$_i$ remained constant. We further found that the attenuated intracellular Ca$^{2+}$ concentration was caused by a reduction of SR Ca$^{2+}$ release ($I_{rel}$) (Fig. 31 C). SR Ca$^{2+}$ release decreased as $G_{Na}$ was reduced, and there was a continuing reduction in peak release until the release was approximately zero at 50% of $G_{Na}$ (Fig. 31 C&D). As stated in the Introduction, changes of SR Ca$^{2+}$ release can be caused by alterations of $I_{CaL}$ influx and/or SR Ca$^{2+}$ storage. We next studied which factor or whether both factors contribute to the alteration of SR Ca$^{2+}$.
Figure 30. Computational model of the influence of Na$^+$ and I$_{to}$ conductance on RV epicardial AP morphology.

A, Modeled RV epicardial APs at 100%, 70% and 30% $G_{Na}$. B, Modeled RV epicardial APD$_{90}$ as a function of $I_{Na}$ conductance. C, Modeled RV epicardial APD$_{90}$ as a function of $I_{Na}$ conductance under various fractions of $G_{to}$. D, Modeled APD$_{90}$ as a function of both $I_{Na}$ and $I_{to}$ conductance.
release. As the peak \( I_{\text{Ca.L}} \) had a continuously linear relationship with decreasing \( G_{\text{Na}} \), the total \( I_{\text{Ca.L}} \) influx was continuously decreasing but was near zero at 50% \( G_{\text{Na}} \) (Fig. 31D), suggesting that the “all-or-none repolarization” AP caused a smaller \( \text{Ca}^{2+} \) influx and hence suppressed SR \( \text{Ca}^{2+} \) release. The other factor, the SR \( \text{Ca}^{2+} \) storage, represented as junctional sarcoplasmic reticulum (JSR) stores in the model, was slightly increased at 70% \( G_{\text{Na}} \) but was largely depleted at low \( G_{\text{Na}} \) (Fig. 31E), implying that suppressed \( \text{Ca}^{2+} \) storage was also one of the mechanisms for reduced SR \( \text{Ca}^{2+} \) release in the model. To confirm the above modeling data, we performed an AP voltage-clamp experiment. As shown in Figure 31F, as expected, a normal canine AP waveform elicited a robust contraction, whereas an “all-or-none repolarization” AP waveform failed to elicit a contraction. If the AP was successively clamped in a collapsed form and the cell was applied with \( \text{Ca}^{2+} \) channel activator Bay-K8644 (BayK), we observed that the contraction was almost completely recovered after the first stimulation, reasserting that \( I_{\text{Ca,L}} \) played a key role in mediating the effect of TTX. We also observed that although the \( I_{\text{Ca,L}} \) had been adjusted to the normal size of \( I_{\text{Ca,L}} \), by the second stimulus there was substantially reduced contraction, and near steady state there was almost no contraction. Most likely this was caused by the depletion of the SR \( \text{Ca}^{2+} \) stores over successive runs with the “all-or-none repolarization” AP. In this case, BayK evoked a large amount of \( \text{Ca}^{2+} \) entry and induced normal CICR at the first run. However, this gradually depleted the residual SR \( \text{Ca}^{2+} \) storage to the point where it failed to release enough \( \text{Ca}^{2+} \) to induce contractions.
Figure 31

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Figure 31. Modeling the relationship of $I_{Na}$ conductance and intracellular Ca$^{2+}$ modulation.

A, Modeled internal free calcium for 100%, 70% and 30% $I_{Na}$ conductance for the RV EPI. B, Modeled peak internal free Ca$^{2+}$ for the RV EPI as a function of sodium conductance. C, Modeled SR release of calcium for 100%, 70% and 30% sodium conductance for RV EPI. D, Modeled peak SR Ca$^{2+}$ release, L Type Ca$^{2+}$ influx and total L Type Ca$^{2+}$ influx as a function of sodium conductance for RV EPI. E, Modeled JSR Ca$^{2+}$ concentration as a function of sodium conductance. F, TTX application did not significantly affect SR Ca$^{2+}$ storage. Pre-recorded “all-or-none repolarization” AP waveforms were imposed on a canine RV epicardical myocyte (middle). Contractile response to imposed “all-or-none repolarization” (middle lower). Application of 0.5 μM BayK can elicit a robust contraction with the “all-or-none repolarization” waveform (right lower).
4.9. Heterogenous expression of KCND3 and KChIP2 mRNA across the RVOT wall

In Brugada patients, the arrhythmic events are mostly initiated at the RVOT (for more details, see Introduction). Based on our above observations that the imbalance between the depolarizing and repolarizing currents may result in “all-or-none repolarization” in Brugada syndrome, we hypothesized that RVOT epicardial cells have a high tendency to develop “all-or-none repolarization”, causing by high expression of transient outward I\textsubscript{to} channels and/or low expression of cardiac Na\textsuperscript{+} channels. To test this hypothesis, the expression levels of the I\textsubscript{to} channel-encoding genes KCND3 and KCNIP2, and the I\textsubscript{Na} channel-encoding gene SCN5A were measured by quantitative real-time PCR analysis in different regions of canine RVOT, including the endocardium and epicardium. As a comparison, KCND3, KCNIP2, and SCN5A gene expression in canine LV and RV were also tested. As shown in Figures 32&33, RV epicardium had higher KCND3 and KCNIP2 mRNA expression than that of the LV epicardium, consistent with a previous report (Di Diego et al., 1996). The mean KCNIP2 mRNA levels showed significant transmural gradients in all RV-, LV-, and RVOT-free walls. Notably, KCND3 but not KCNIP2 mRNA level in RVOT epicardium was significantly higher than that in the RV epicardium (an average 23\% increase, \( P<0.05 \), \( n = 5 \)). However, the difference between epi- and endocardium in RVOT was not any larger than that in the RV. The SCN5A gene, on the other hand, only had a small transmural difference in LV, RV, and RVOT, with a higher level in the endocardium than in the epicardium (Fig. 34). No significant difference was found in SCN5A gene expression level in RVOT and RV epicardium.
Figure 32. Regional dependent heterogeneity of KCND3 mRNA expression in canine heart.

Real-time PCR analysis showing fold changes in KCND3 mRNA expression detected from LV epi-, endo-, RV epi-, endo-, and RVOT epi-, endo- cardium. Values are mean ± S.E.M. of quadruplicate analyses from 4–5 animals per experimental group.

*P<0.05, **P<0.01 versus RV epi group. n.s., no significance.
Figure 33. Regional dependent heterogeneity of KCNIP2 mRNA expression in canine heart.

Real-time PCR analysis showing fold changes in KCNIP2 mRNA expression detected from LV epi-, endo-, RV epi-, endo-, and RVOT epi-, endocardium. Values are mean ± S.E.M. of quadruplicate analyses from 4–5 animals per experimental group. 

*P<0.05, **P<0.01 versus RV epi group. n.s., no significance.
Figure 34. Regional dependent heterogeneity of *SCN5A* mRNA expression in canine heart.

Real-time PCR analysis showing fold changes in *SCN5A* mRNA expression detected from LV epi-, endo-, RV epi-, endo-, and RVOT epi-, endo- cardium. Values are mean ± S.E.M. of quadruplicate analyses from 4–5 animals per experimental group.

*P* < 0.05, **P** < 0.01 versus RV epi group. n.s.: no significance.
4.10. Transmural expression of \( I_{to} \) channel proteins in RVOT

To further evaluate the heterogeneous expression of the \( I_{to} \) channel proteins KV4.3 and KChIP2 in the RVOT, Western blot analysis was performed with membrane protein homogenates from canine RVOT epi-, mid-, and endocardium, and from RV epi-, mid-, and endocardium. Figure 35A shows a representative Western blot probed with anti-Kv4.3 antibody. A clear gradient expression was shown in the blots (~75 kDa) in both RV and RVOT, with the highest level in the epicardium and the lowest in the endocardium. Figure 36A shows an example of the Western blot probed for KChIP2 (~ 30 kDa). The transmural gradient in KChIP2 protein expression was also observed in the RVOT. These results indicated for the first time that \( I_{to} \) channel proteins Kv4.3 and KChIP2 are heterogeneously expressed across the RVOT wall. However, the average Kv4.3 expression in RVOT epicardium was lower than that in the RV epicardium (Fig. 35C) and KChIP2 expression level in RVOT epicardium had no significant difference with that in the RV epicardium (Fig. 36B). Also, the disparity between KV4.3 and KChIP2 protein levels in epi- and endocardium in RVOT was not any larger than that in the RV. Although there is a possibility that the functional expressions of these ionic currents are higher in RVOT epicardium than in RV epicardium, we concluded that the protein expression levels of \( I_{to} \) channel proteins are the same in canine RVOT and RV.

4.11. Summary and conclusion

In this part of study, we examined the ionic basis of the cellular electrical abnormalities in brugada syndrome. We demonstrate quantitatively that AP repolarization is influenced by an intriguing interplay between \( I_{Na} \) and \( I_{to} \) conductance. We also examined how cellular electrical abnormalities induced by \( I_{Na} \)
reduction impact on myocyte mechanical properties. Our results indicate that the imbalance between depolarizing $I_{Na}$ and repolarizing $I_{to}$ significantly contribute to the cellular electrical alterations under brugada syndrome setting, and these electrical alterations lead to significant alterations of myocyte EC coupling and mechanics. These altered myocyte mechanics may account for the wall motion abnormalities observed in the disease.

We also tested the hypothesis that $I_{to}$ encoding gene and $I_{to}$ proteins have a higher expression in RVOT epicardium than in RV epicardium. Although $I_{to}$ $\alpha$-subunit encoding gene $KCND3$ has a higher expression level in RVOT epicardium than in RV epicardium, our study did not find any differences in $I_{to}$ protein expression levels in these two tissues. Possibilities that the functional expressions of $I_{to}$ are higher in RVOT epicardium than in RV epicardium, however, is still remained and need to be further examined.
Figure 35. Immunoblotting analysis of Kv4.3 proteins across RV and RVOT ventricular wall.

A, Representative blots of Kv4.3 and calsequestrin (CSQ) immunoblotting analysis in RV and RVOT epicardium (epi), midmyocardium (mid), and endocardium tissues (endo). B, A representative blot showing the immunolabeling of anti-Kv4.3 antibody was blocked by the antigen peptide. C, Average ratio of Kv4.3 over CSQ from n=4 animals. Values= Mean ± S.E.M. *P<0.05, ** P<0.01, *** P<0.001, n.s.= no significance versus RV epi group.
Figure 36. Immunoblotting analysis of KChIP2 proteins across RV and RVOT ventricular wall.

A, Representative blots of KChIP2 and calsequestrin (CSQ) immunoblotting analysis in RV and RVOT epicardium (epi), midmyocardium (mid), and endocardium tissues (endo). B, Average ratio of KChIP2 over CSQ from n=4 animals. Values= Mean ± S.E.M. *P<0.05, ** P<0.01, *** P<0.001, n.s.= no significance versus RV epi group.
Chapter IV: Discussion

1. Role of simulated $I_{to}$ in regulating action potential morphology and duration in normal ventricular cardiomyocytes

   The determinant ionic factor(s) for the length of AP in the heart and the ionic basis of the transmural electrical dispersion of the ventricle are not fully understood. To address these issues, in an earlier study from our laboratory (Sun & Wang, 2005), modeled $I_{to}$ current was introduced in canine ventricular myocytes by the dynamic clamp technique. Our laboratory was one of the first groups to apply this technique in cardiovascular electrophysiology research. In that study, they showed that simulation of an epicardial level of $I_{to}$ (20 pA/pF at +40 mV) in endocardial cells, where the native $I_{to}$ is small, reproduced the characteristic “spike-and-dome” epicardial AP waveform, but did not significantly change the endocardial APD. Conversely, subtraction of the native $I_{to}$ in epicardial cells eliminated the phase 1 notch, but did not significantly alter the epicardial APD. They concluded that the transmural $I_{to}$ gradient, while contributing to the transmural differences in phase 1 repolarization, does not play a key role in determining the APD in canine ventricular myocytes.

   This conclusion, however, is partially compromised by the usage of the whole-cell patch clamp technique and Ca$^{2+}$ buffers. The whole-cell patch clamp technique allows for a free flow of ions. In conjunction with the usage of Ca$^{2+}$ buffer, it interferes with the intracellular Ca$^{2+}$ cycling. Previous studies produced conflicting
results in the presence (Beuckelmann et al., 1993; Kääb et al., 1996; Sun & Wang, 2005) and absence of the Ca²⁺ buffer (Zygmunt et al., 1997). In my thesis study, we used perforated patch-clamp recording to preserve physiological intracellular Ca²⁺ handling and reexamined the effect of I_{to} on canine endocardial cells. Under these conditions, simulation of I_{to} in canine endocardial cells generated AP waveforms that were remarkably similar to those recorded from canine epicardial tissue or myocytes using microelectrode recordings (Litovsky & Antzelevitch, 1988; Litovsky & Antzelevitch, 1990; Liu et al., 1993; Lukas & Antzelevitch, 1993). The APD-I_{to} density relationship is qualitatively similar to our previous findings using whole cell recordings (Fig. 4 of Ref. Sun & Wang, 2005) but with a few noticeable differences. The small depression of APD when I_{to} was 30 pA/pF was not found in the current study and was probably an artifact associated with intracellular Ca²⁺ buffering. Instead, progressive APD prolongation was observed over the entire I_{to} density range from 28 pA/pF to the threshold for all-or-none repolarization. Importantly, in our present study, we found that simulation of canine epicardial level of I_{to} (20 pA/pF) did not significantly affect the endocardial APD, supporting our previous conclusion that physiological levels of I_{to} do not play a significant role in regulating the APD in the canine left ventricle.

The second major issue that I addressed in this part of my thesis research is the species dependent effect of I_{to}. Although the AP waveform in guinea pig ventricular cells shares similarities with those of endocardial ventricular myocytes in large animals, such as dog, the underlying ionic current profiles in guinea pig and large animals is significantly different. I_K is more prominent in guinea pig ventricular cells than in the canine ventricle (Liu & Antzelevitch, 1995; Lu et al., 2001), and it is balanced by a significantly larger L-type Ca²⁺ current (I_{Ca-L}) (Heubach et al., 2000;
Wang & Cohen, 2003). Indeed, it was shown that the effect of exogenous Kv4.3 current on AP in guinea pig ventricular cells (Hoppe et al., 1999; Hoppe et al., 2000) is distinct from that of $I_{to}$ in canine ventricular cells (Sun & Wang, 2005). In the present study, we simulate $I_{to}$ conductances in guinea pig and canine ventricular myocytes and have shown that, despite the significant differences in the cellular electrophysiological background of the two myocytes, the effects of a fully inactivating $I_{to}$ on the AP morphology and duration of these two types of myocytes were remarkably similar. Importantly, a distinctive threshold (40 pA/pF) existed that separated the effect of $I_{to}$ into two phases. Below this threshold, increasing amounts of simulated $I_{to}$ generated an increasingly deep phase 1 notch and did not significantly affect the APD at low densities and moderately prolonged the APD at higher densities. $I_{to}$ above the density threshold led to all-or-none repolarization, resulting in triangular APs with dramatically reduced APD. These effects of $I_{to}$ are not linked to the particular mathematical formulations of the $I_{to}$ model or a specific stimulation rate (Fig. 14 &15). In an earlier study using the WRJ canine model, increasing of pacing rates decreased the availability of $I_{to}$, which is believed to contribute to a shallower AP phase 1 notch and a shorter APD (Greenstein et al., 2000). This rate-dependent effect on the length and morphology of the AP has been observed in canine (Liu et al., 1993), as well as in human cardiomyocytes (Nabauer et al., 1996). In our study, as the myocytes were paced at a higher rate, higher $I_{to}$ density was required to cause the collapse of APs. However, a similar relationship between $I_{to}$ and APD was observed. Collectively, our experimental results are in agreement with the predictions of a modeling study of canine and guinea pig ventricular cells (Greenstein et al., 2000), and probably reflect a general role of the $I_{to}$ conductance in regulating the morphology of broad, plateau-possessing cardiac APs.
The same, however, cannot be stated about \( I_{to} \) in canine right ventricular cells. \( I_{to} \) density is significantly higher in the canine right epicardium than in the left (Di Diego et al., 1996; Rosati et al., 2003). In our recordings, the average \( I_{to} \) density in the RV epicardial cells is \( \sim 27 \text{ pA/pF at } +40\text{mV} \). With the assumption that the APD-\( I_{to} \) density relationship shown in Fig. 13 is shared in right ventricular cells, \( I_{to} \) probably moderately prolongs the APD in the right ventricular cells. Therefore, the influence of \( I_{to} \) on APD cannot be described by a single rule, but is rather \( I_{to} \)-density dependent.

\( I_{to} \) is conventionally defined in modeling studies as the fully and rapidly inactivating component of the total outward current. Such a definition is supported by experimental evidence. It is shown that in mouse ventricular cells, \( I_{to} \) inactivates fully and is separate from the non-inactivating outward currents (Xu et al., 1999). In human atrial and mouse ventricular cells, antisense suppression or functional knockout of the Kv4-generated \( I_{to} \) leaves the noninactivating current intact (Barry et al., 1998; Guo et al., 2000; Wang et al., 1999), arguing that in these native systems, \( I_{to} \) and the noninactivating component are carried by channels with different molecular identities. For these reasons, we defined and modeled \( I_{to} \) as a fully inactivating conductance in our simulation studies. With the simulation of even a small sustained conductance, the biphasic APD-\( I_{to} \) relationship in guinea pig cells was dramatically changed to a monotonic inverse relationship, strikingly similar to those reported earlier (Hoppe et al., 1999; Hoppe et al., 2000). Simulation of a sustained conductance also eliminated the notch and suppressed the plateau, closely reproducing the results of Hoppe et al (both 1999 & 2000). These results point to the importance of the ultrarapid \( I_K \)-type sustained current in regulating the AP morphology. The marked effects of the sustained current are not surprising,
considering the small net current during the plateau phase. In canine ventricular cells, \( I_{to} \) inactivation is rapid and near complete (Fig. 10B). In mouse and rats, \( I_{to} \) density is significantly greater than in human and canine cells, with an average of \( \sim 60 \text{pA/pF} \). According to the APD vs. \( I_{to} \) relationship presented in our current and earlier studies (Sun & Wang, 2005), we argued that the large \( I_{to} \) size in smaller animals, such as mouse, is responsible for their brief APs (Sun & Wang, 2005). In addition to a large \( I_{to} \), prominent slowly inactivating and non-inactivating currents are present in the mouse ventricle (Xu et al., 1999). These currents, when combined, can make up over half of the total outward current amplitude (Xu et al., 1999). It is likely that the presence of such slow and sustained currents also contributes importantly to the brief, spike-like AP in the mouse ventricle. In this case, species differentiation in cardiac AP morphology is achieved by changes in both channel expression level and function.

The underlying mechanism of the biphasic APD-\( I_{to} \) relationship probably lies in the interplay between \( I_{to} \) and \( I_{Ca-L} \), as suggested by the modeling study (Greenstein et al., 2000) and our AP-voltage clamp study shown in Figure 22. It is clear from our studies that the voltage trajectory at the end of phase 1 repolarization depends on the potential of the notch. \( I_{to} \), via its regulation of phase 1 notch potential, either allows reactivation of \( I_{Ca-L} \) at the end of phase 1, as manifested by a second spike, and the development of the plateau (or the "dome"), or it turns off \( I_{Ca-L} \) by moving the notch potential below the activation voltage range of \( I_{Ca-L} \) and completely suppresses the plateau. In our experiments, the average notch potential for such a transition between reactivation and deactivation of \( I_{Ca-L} \) was \(-20 \text{mV} \) for guinea pig ventricular cells and \(-24 \text{mV} \) for canine endocardial myocytes. Prolongation of the APD occurs when \( I_{to} \) causes a delay in the reactivation of \( I_{Ca-L} \) and a shift in the 2nd \( I_{Ca-L} \) time
course, as shown in Fig. 22. The delayed I\textsubscript{Ca-L} prolongs the phase 2 plateau, as well as the APD. In Figure 22 D, we clearly showed that the APD vs. I\textsubscript{to} relationship curve is almost completely overlapped with the total Ca\textsuperscript{2+} influx vs. I\textsubscript{to} curve.

Sudden cardiac death, mostly caused by ventricular arrhythmias, is responsible for about half of the mortalities in heart failure patients (Oudit et al., 2001). One of the most characteristic electrophysiological changes in HF is the prolongation of the APD, which is believed to predispose the heart to afterdepolarization and reentrant arrhythmias (Tomaselli et al., 1994). Accompanying the prolongation of APD, downregulation of I\textsubscript{to} is consistently observed in heart failure patients, as well as in animal models (Oudit et al., 2001; Tomaselli et al., 1999; Wickenden et al., 1998). Based on their close correlation, it was proposed that I\textsubscript{to} downregulation is an important contributor to APD prolongation in failing hearts (Kaab et al., 1996; Wickenden et al., 1998). This is likely to be true in smaller animals, where I\textsubscript{to} is much beyond the density threshold for all-or-none repolarization (Sun & Wang, 2005) and is the major determinant of APD. The same conclusion may not apply to large animals, such as humans (Priebe & Beuckelmann, 1998), because the role of I\textsubscript{to} in regulating AP morphology differs significantly between small and large animals. I\textsubscript{to} in the canine (Sun & Wang, 2005) and human (Nabauer et al., 1996) left ventricle is well below the density threshold for all-or-none repolarization, and our APD-I\textsubscript{to} density curves show that within this density range, reduction of I\textsubscript{to}, regardless of the magnitude, will not prolong the APD under physiological conditions. In heart failure, a concerted change in ion channel expression occurs (Wickenden et al., 1999), and the APD-I\textsubscript{to} density relationship reported in our study may be altered as a result of the remodelings of the cellular electrical background. Also, it is possible that
downregulation of other K\(^+\) channels causes a concomitant decrease in the sustained component of the outward current, thereby resulting in APD changes.
2. Role of simulated $I_{to}$ in regulating myocyte mechanical properties in normal ventricular cardiomyocytes

In the second part of my thesis study, the role of $I_{to}$ in regulating the myocyte mechanical function of canine ventricular cardiomyocytes was studied using the dynamic clamp technique. We found that superimposing an epicardial-level of $I_{to}$ suppressed myocyte contractility and Ca$^{2+}$ transient in canine endocardial cells, while a blockade of $I_{to}$ in epicardial cells significantly increased contractility. The inhibitory effect of $I_{to}$ on myocyte mechanics is likely mediated by the suppression of $I_{Ca-L}$, particularly early $I_{Ca-L}$ influx, by phase-1 AP repolarization. We conclude that $I_{to}$ has a negative rather than positive effect on cardiac contractile function.

Previous studies (Laurita et al., 2003; Cordeiro et al., 2004) and our current results show that there is significant transmural heterogeneity of SR Ca$^{2+}$ release and mechanical properties in the canine LV myocardium. Cordeiro et al. and our results both reported an ~1.6-fold increase in cell contractility in canine LV endo- versus epicardial myocytes (Fig. 17C; Cordeiro et al., 2004). The Ca$^{2+}$ transient was also found to be higher in the endocardial cells in our experiment (Fig. 17D). This heterogeneity in mechanics correlates inversely with the transmural $I_{to}$ density gradient. Our simulation studies show that switching the $I_{to}$ conductance between epi- and endocardial myocytes largely reverses the contractility in the two myocyte types (Fig. 18 & 21). This suggests that the transmural $I_{to}$ gradient contributes to the transmural contractile heterogeneity. However, cell shortening in endocardial myocytes is almost double that of the epicardial cells, while simulation of an epicardial $I_{to}$ resulted in only a 19% decrease of cell shortening in endocardial myocytes, suggesting that factors other than $I_{to}$ also contribute to the transmural mechanical heterogeneity in the heart. Other transmural differences in the proteins
involved in the EC coupling process have been reported, and the overall trend favors enhanced EC coupling and contractile function in the endocardium. For example, Hittinger et al. reported that the cardiac ryanodine receptor (RyR2) protein expression was nearly 2-fold higher in endo- than in epicardial myocytes in canine (1999), which may lead to a larger SR release in epi- than encocardial cells. Also, NCX current density and protein expression levels are significantly lower in endo- than in epicardium in canine LV (Xiong et al., 2005), likely leading to a higher intracellular Ca\(^{2+}\) level in endocardial myocytes. A gradient of I\(_{\text{Ca-L}}\) density, the trigger of SR Ca\(^{2+}\) release, was also described across the canine ventricular wall (Wang & Cohen, 2003) with a higher I\(_{\text{Ca-L}}\) density in endocardial myocytes. All above heterogeneities in Ca\(^{2+}\) handling have the tendency to trigger a higher contractility in endocardium. An exception to this general trend is a lower level of SERCA2a expression in canine endo- than epicardium (Laurita et al., 2003). Taken together, these systematic heterogeneities in elements of EC coupling probably result in transmural differences in magnitude and timing of contraction, which are required for optimal mechanical performance of the myocardium.

Our AP-voltage clamp study indicated that the negative effect of I\(_{\text{to}}\) on myocytes contractility and Ca\(^{2+}\) transient is mediated by the effect of the current on AP trajectory and sarcolemmal Ca\(^{2+}\) flux. With increasing I\(_{\text{to}}\) densities, Ca\(^{2+}\) influx during the first I\(_{\text{Ca-L}}\) activation gradually decreased (Figure 21). We chose Ca\(^{2+}\) influx during the first phase of I\(_{\text{Ca-L}}\) activation (“initial Ca\(^{2+}\) influx”) but not the total Ca\(^{2+}\) influx as the indicator of the effective EC coupling gain for two reasons. Firstly, once Ca\(^{2+}\) influx induces activation of RyRs and SR Ca\(^{2+}\) release, the rise of cytoplasmic [Ca\(^{2+}\)]\(_i\) further activates RyRs, forming a positive feedback for SR Ca\(^{2+}\) release. Although a higher rise of cytoplasmic [Ca\(^{2+}\)] leads to a greater SR Ca\(^{2+}\) release (Lukyanenko et al.
1996; Bassani et al. 1995; Song et al. 1997), the initial Ca^{2+}-induced Ca^{2+} releases broadly depleted SR Ca^{2+} reserve and minimized the effect of the 2nd I_{Ca-L} spike. Secondly, as Ca^{2+} activates RyRs, micromolar [Ca^{2+}]_i can also rapidly (within 100 ms) inactivate SR RyRs (Laver et al., 2007). It has been shown that at 0 mV, SR Ca^{2+} release terminated within 40 ms after the onset of an AP (Sham et al., 1998). The same study also showed that after the termination of Ca^{2+} sparks, RyRs underwent a 40 ms refractory period, and re-activating or increasing the open-duration of L-type Ca^{2+} channels failed to trigger a second intracellular [Ca^{2+}] surge, even though over half of the SR Ca^{2+} storage remained available. Based upon the above evidence, the area over the I_{Ca-L} curve before the deepest point of the phase 1 notch (less than 40ms after the onset of AP) was chosen as the indicator of Ca^{2+} influx.

Our results demonstrate that the primary consequence of phase 1 early repolarization is voltage-dependent deactivation of L-type Ca^{2+} channels. Simulation of an epicardial level of I_{to} in endocardial cells, although did not significantly change the peak amplitude of I_{Ca-L}, quickly turned down I_{Ca-L} by deactivating the Ca^{2+} channels and limiting the Ca^{2+} entry, as displayed by the narrow I_{Ca-L} trajectory (Fig. 22). Our results also showed that at the final period of phase 1, Ca^{2+} channels are reactivated as shown by the 2nd I_{Ca-L} spike, which contributes to the development of the “dome”. The driving force and the initiation time of this second activation depend upon the depth and the duration of the notch, presumably because with a deeper and longer phase 1 notch, the Ca^{2+} channels that are ready to be reactivated become larger. This reactivation of I_{Ca-L} may contribute to prolonging the APD. As shown in Figure 22D, when I_{to} is around 30pA/pF, the increase of the total Ca^{2+} influx overlaps with the lift of the APD-I_{to} curve. Therefore, the complex interaction of I_{to} and I_{Ca-L} is
important for the mechanical properties of the cell as well as for the AP morphology and duration.

Several studies have used the peak amplitude of I_{Ca-L} as the indicator for EC coupling gain (Sah et al., 2002; Cordeiro et al., 2004). Our results showed that the peak I_{Ca-L} magnitude is about the same in epicardial and endocardial myocytes, which contradicts with several studies which demonstrated that a robust early AP repolarization is accompanied by an increased peak I_{Ca-L} magnitude and Ca^{2+} release (Sah et al., 2002; Cordeiro et al., 2004). We noticed that in their recordings in canine LV epicardial cells, the phase 1 notch voltages are above zero, which is inconsistent with our and other recordings where the notch voltage is around -10 mV (Fig 17A; Sicouri & Antzelevitch, 1991). Our AP clamp experiments show that the relationship between I_{to}/phase-1 repolarization versus I_{Ca-L} peak amplitude has an inverted-J shape; I_{Ca-L} peak amplitude is enhanced under a small I_{to}, but is progressively suppressed with increasing I_{to} > 20 pA/pF, and the highest I_{Ca-L} amplitude occurs under a notch depth of 5 mV (Fig. 22C). This is consistent with the bell-shaped relationship between I_{Ca-L} and membrane potential, with the maximal amplitude between 0 to +10 mV (Beuckelmann & Wier, 1998; Wang & Cohen, 2003; Nakamura et al., 2001). This non-linear relationship between I_{to}/phase-1 repolarization versus I_{Ca-L} amplitude indicates that the presence of a phase-1 notch per se does not necessarily result in higher (or lower) I_{Ca-L}; rather, the I_{Ca-L} amplitude is mainly determined by the degree of phase-1 repolarization. Consequently, different results may be obtained depending upon the exact AP voltage clamp protocol used. This provides a plausible explanation for the contradiction in past AP clamp studies showing that phase-1 repolarization either increase or decrease peak I_{Ca-L} amplitude in cardiac myocytes.
Another possible factor that mediates the effect of $I_{to}$ is NCX current, which allows $Ca^{2+}$ influx during the early phase of AP and $Ca^{2+}$ efflux during the late phase of AP (Leblanc & Hume, 1990). $I_{NCX}$ is mostly driven by electrochemical influences, including Na$^+$ & Ca$^{2+}$ gradients and membrane potential. When membrane potential exceeds the reversal potential of NCX, Ca$^{2+}$ entry (reverse mode of $I_{NCX}$) is favored. Membrane potential is more negative in epicardial cells during the notch, which decreases Ca$^{2+}$ entry and favors Ca$^{2+}$ extrusion. In a simulation study done by Hund & Rudy (2004), compared to an AP profile with notch, it was found that an AP profile without notch was accompanied by a significantly larger $I_{NCX}$ in reverse mode, suggesting that $I_{to}$, by generating phase-1 repolarization, decreases the reverse mode of $I_{NCX}$ and favors Ca$^{2+}$ extrusion. This may suppress the cytosolic and/or SR Ca$^{2+}$ levels over time and may account for the slow phase of contractility change when $I_{to}$ simulation was turned on or off.

Even though Ca$^{2+}$ transient peak amplitude was reduced by $I_{to}$, the rising rate of Ca$^{2+}$ transient was increased in our study (Fig. 20A inset & C). A similar result was also observed in another study (Cordeiro et al., 2004), implying that $I_{to}$ facilities a quicker SR Ca$^{2+}$ release. It has been shown that SR Ca$^{2+}$ release is best synchronized when membrane potential is around +10 mV, and is less so when membrane potential is more positive or more negative (Harris et al., 2005). Based on this, we speculated that as the membrane potential was driven to lower levels by “inserted” $I_{to}$, faster SR Ca$^{2+}$ release could be the result of better synchronization. We did not observe any significant difference of Ca$^{2+}$ transient rising rate in unloaded epicardial and endocardial cells, suggesting that in native cells, the EC coupling is adapted to optimize the best Ca$^{2+}$ release synchronization.
Our study indicates that the transmural gradient of $I_{to}$ contributes to the heterogenous $Ca^{2+}$ dynamics across the ventricular wall. The differential $[Ca^{2+}]_i$ may also play a role in mediating the non-uniform distribution of $I_{to}$ and Kv4.3 expression, forming a negative feedback loop. It was recently found that regional differences in $Ca^{2+}$-driving calcineurin/NFATc3 signaling contribute to the heterogeneous $I_{to}$ function across the LV free wall in mouse (Rossow et al., 2006). In addition, in a variety of pathophysiological contexts, including tachycardia (Perrier et al., 2004) and myocardial infarction (Rossow, 2004), NFATc3 plays an essential role in the signaling pathway leading to $I_{to}$ downregulation. The causes and functions of $I_{to}$ downregulation in the failing cardiomyocytes have been unknown. Our results, combined with the discoveries by Rossow et al., provide a plausible explanation for the cause of $I_{to}$ downregulation in failing hearts. As the contractile function is compromised in failing cells, $Ca^{2+}$ dynamics would be compensatorily upregulated, with $I_{to}$ downregulation as one of the causing factors; the upregulated $Ca^{2+}$ dynamics then further suppress $I_{to}$ expression.

Currently, it is believed that $I_{to}$ downregulation contributes to the impaired EC coupling and myocyte mechanics in HF, and restoration of $I_{to}$ was proposed as a therapeutic approach for improving cardiac mechanical function (Sah et al., 2003). Based on our present results, we speculate that, on the contrary, $I_{to}$ downregulation may serve to increase SR $Ca^{2+}$ release and myocyte contractility in failure. As such, it may represent a compensatory response rather than a contributing factor to the impaired myocyte mechanical properties in HF. It should be noted that under failure, $Ca^{2+}$ and $Na^+$ homeostasis are significantly altered (Beuckelmann et al., 1992; Despa et al., 2002), which in turn alters the functions of ionic conductances such as NCX (Armoundas et al., 2003). This may potentially impact the influence of $I_{to}$ on $Ca^{2+}$.
dynamics and contraction. Therefore, it is of significant interest to expand our study to failure settings.
3. The role of $I_{to}$ in regulating action potential duration of ventricular cardiomyocytes from a canine HF model

As shown in Figure 25, the relationship between $I_{to}$ and APD has two patterns in cardiomyocytes from the canine HF model. In the first pattern, similar to what we have seen in the normal myocytes, $I_{to}$ and APD have a bi-phasic relationship. Before the threshold, increasing $I_{to}$ densities slightly either increased APD or only had a minor influence on APD; above the threshold, $I_{to}$ simulation caused the collapse of AP and significantly shortened APD. In the second pattern, as we increased the simulated $I_{to}$, the APD was progressively abbreviated. Compared to the first pattern, we noticed that the main difference in the second pattern is that it mostly happened in cells where an extended repolarization and a long APD were present. The different length of APs in the different myocytes is probably due to cell-to-cell variation. The long APs represent a more malignant AP morphology in HF, as the long plateau phase predisposes myocytes to EAD and long QT intervals. Also, this variation demonstrates an increased dispersion in APD which will predispose the heart to reentry arrhythmias.

As a complete delineation of the electrical properties of this canine HF model remains unsolved, it is too early to theorize any mechanism that underlies the linear relationship between $I_{to}$ and APD. It has been reported that multiple ionic channels and transporters undergo remodeling in failing human hearts or in experimental HF animal hearts. These remodelings may significantly change the AP trajectory and Ca$^{2+}$ cycling. Importantly, the changes in Ca$^{2+}$ homeostasis can significantly change the role of the $I_{to}$. In a modeling study, Winslow et al. found that $I_{to}$ has a large effect on APD when intracellular Ca$^{2+}$ is buffered, but not when Ca$^{2+}$ cycling is remained uninterrupted (Winslow et al., 1999). In HF, Ca$^{2+}$ cycling undergo significant changes.
For example, NCX is found to be upregulated in HF, presumably to compensate for reduced cytosolic Ca$^{2+}$ removal due to attenuated SERCA function (Nattel et al., 2001; Terracciano et al., 2001). Changes in NCX may affect the repolarization phase, since the NCX carries net inward current, particularly during later AP phases. I$_{CaL}$ is mostly found unaltered in failing cells (Li et al., 2002; Kaab et al., 1996; Tomaselli et al., 1999), but reduced I$_{CaL}$ has also been reported in some studies (Ouadid et al., 1995). By far, all the studies that favor the important role of I$_{to}$ downregulation in APD prolongation use Ca$^{2+}$ buffer (Kaab et al., 1996; Beuckelmann et al., 1993). The interference of intracellular Ca$^{2+}$ cycling undermines their conclusions. Actually, in a guinea pig model with cardiac hypertrophy and HF, in which the I$_{to}$ is absent, the AP is prolonged due to changes in Ca$^{2+}$ cycling proteins, including upregulation of the NCX, downregulation of SERCA and a reduction of Ca$^{2+}$-dependent inactivation of the L-type Ca$^{2+}$ channel (Ahmmed et al., 2000). This study indicates that I$_{to}$ reduction may not, at least not directly, produce large effects on the APD. Recently, studies also show that chronic HF increases I$_{NaL}$ density and slows inactivation kinetics of I$_{NaL}$. As I$_{NaL}$ is a major contributor to the AP plateau (Maltsev et al., 1998), increases of I$_{NaL}$ changes AP trajectory and alters cell Na$^+$ cycling. As Na$^+$ modulates the NCX function, alterations in Na$^+$ cycling may also potentially affect Ca$^{2+}$ cycling.

In addition to the Ca$^{2+}$ that may mediate the APD prolongation, other candidates that underlie the mechanism for APD prolongation include remodeling of several K$^+$ currents. A reduction in I$_{Ks}$ and I$_{Kr}$ is among the most consistent findings in diseased human HF and in HF animal models (Li et al., 2002; Tsuji et al., 2006; Pogwizd et al., 2001; Rose et al., 2005), and this downregulation has been proposed to contribute to APD prolongation.
4. Ionic mechanisms of cellular electrical and mechanical abnormalities in Brugada syndrome

In the fourth part of my thesis study, we examined the ionic basis of the cellular electrical abnormalities in Brugada syndrome using a combination of patch clamp, dynamic clamp simulation, and computational modeling. We demonstrate quantitatively how AP repolarization and morphology are influenced by an intriguing interplay between $I_{Na}$ and $I_{to}$ conductance. We show that the influence of $I_{Na}$ on AP morphology depends critically on the density of $I_{to}$. We also examined how cellular electrical abnormalities induced by $I_{Na}$ reduction impact myocyte mechanical properties. Our results indicate that cellular electrical alterations under the Brugada syndrome setting lead to significant alterations of myocyte excitation-contraction (EC) coupling and mechanics. These altered myocyte mechanics may account for the wall motion abnormalities observed in the disease.

We demonstrate quantitatively that AP morphology is influenced by a complex interplay between $Na^+$ and $I_{to}$ conductance, primarily by their effects on phase-1 repolarization. Our study showed that in RV epicardial cells, where the depth of the phase 1 notch is the deepest compared to that in RV endocardial and LV epicardial myocytes, all-or-none repolarization can be produced with a lower degree of $I_{Na}$ blockade. The depth of the phase 1 notch reflects the cumulative impact of both the inward (depolarization) and outward (repolarization) currents. It has been well documented, in many publications as well as in our studies (Di Diego et al., 1996; Fig. 26, page 123), that $I_{to}$ is highly expressed in RV epicardial myocytes. The main depolarization current $I_{Na}$, however, does not have a different density in epicardial and endocardial myocytes (Barajas-Martinez et al., 2009; Cordeiro et al., 2008; Rosati et al., 2006), and neither did the L-type $I_{Ca-L}$ (Volk et al., 1999; Cordeiro et al.,
We demonstrated that native Ito in RV epicardial myocytes is below the threshold for all-or-none repolarization (Fig 27, page 125). However, with INa reduction by 1 µM TTX (~ 50% INa reduction), this threshold is significantly reduced and a native RV epicardial Ito is in the range of either moderately prolonging AP or causing the collapse of AP, leading to all-or-none repolarization of AP (Fig. 27D, page 125). The average level of Ito in RV epicardial cells is around 30 pA/pF, which is near the threshold for all-or-none repolarization even under control conditions. With reduced Na+ conductance, the effect of Ito on AP repolarization is significantly altered and the proposed repolarization abnormality model for Brugada syndrome was reproduced. By contrast, the level of Ito in LV epicardial myocytes is around 20 pA/pF and is far below the threshold for all-or-none repolarization. AP morphology in LV will not be significantly affected unless the Na+ current is dramatically reduced, as demonstrated in both the experimental study and the modeling study. Also, as a demonstration of the important role of Ito density in the formation of the abnormal AP morphology, the modeling study shows that a moderate (~ 50%) increase in Ito density can push RV epicardial AP beyond the threshold for all-or-none repolarization even under full INa (Fig. 30, page 131). Collectively, our study clearly illustrate that both INa reduction and a high Ito contribute to the AP repolarization and AP morphology alterations.

In our study, different concentrations of TTX were used to selectively block the INa. The TTX dosages used in our study, 1 µM and 3 µM, represent 50% & 75% of INa reduction, respectively, which correlates with the INa reduction found in the Brugada syndrome patients with SCN5A mutations (Smits et al., 2005; Baroudi et al., 2004; Keller et al., 2005). In the Luo–Rudy cell model, membrane excitability is not compromised unless the INa conductance falls below 20% of its maximal value (Shaw
and Rudy, 1997). Our study showed that an even lower $I_{\text{Na}}$ amplitude is enough to induce the depolarizing upstroke. 5 µM TTX, which blocks approximately 85% of the maximum density, is still able to induce the robust depolarization in phase 0 (Fig. 29B). These results indicate that the native $I_{\text{Na}}$ current is large and redundant to assure the initiation of an AP upstroke, and that any cellular AP morphological or mechanical change observed as a consequence of $I_{\text{Na}}$ reduction is not due to the abnormality of AP upstroke, but is a result of an alteration of the subsequent AP voltage trajectory.

Brugada syndrome is described as a primarily electrical disease (Antzelevitch et al., 2005). However, many have suggested that it represents a subpopulation of arrhythmogenic cardiomyopathy of the right ventricle (Martini et al., 1993; Naccarella, 1993; Ohe, 1996). After decades of clinical and basic research, the mechanism of Brugada syndrome now falls into 2 viewpoints in terms of its relationship with cardiac structural abnormalities: 1) the structural rather than the electrical abnormalities are the arrhythmogenic substrate; 2) the disease is purely electrical and the structural abnormalities seen in the patients are secondary or coincident. Our present study, in support of the 2nd theory, provided evidence that purely electrical changes are enough to cause the contractile dysfunctions observed in the RV in Brugada syndrome. We showed that in normal canine RV epicardial cells where the native $I_{\text{o}}$ density is high, partial inhibition of $I_{\text{Na}}$ (to mimic a Brugada-setting) induced a delay of repolarization or all-or-none repolarization. The all-or-none AP waveform did not induce normal $Ca^{2+}$ dynamics and failed to elicit a contraction (Fig. 28). This contractile dysfunction is only a consequence of an electrical change. A simple blockade of $I_{\text{o}}$ density restored the normal contraction and $Ca^{2+}$ transient (Fig. 29).
Our study shows that all-or-none repolarization results in pronounced suppression of both myocyte contraction and Ca\(^{2+}\) transient amplitude. In myocytes where AP morphology is not significantly altered by TTX, myocyte contraction is not affected (Fig. 29), suggesting that the observed changes in myocyte mechanics are not due to impaired AP upstroke. Both the simulation and experimental results indicate that a key contributing factor to the suppressed Ca\(^{2+}\) dynamics is a decrease in sarcolemmal Ca\(^{2+}\) influx. As shown and discussed in Result Section 2 (Fig. 22; page 110), all-or-none repolarization is associated with a significantly decreased amplitude of I\(_{\text{Ca-L}}\) and Ca\(^{2+}\) influx. In RV epicardial cells, due to the I\(_{\text{Na}}\) reduction and a large native I\(_{\text{to}}\), accentuation of phase-1 repolarization quickly deactivates the I\(_{\text{Ca-L}}\), and the resulting all-or-none AP trajectory prohibits the reactivation of I\(_{\text{Ca-L}}\). As G\(_{\text{Na}}\) decreases and G\(_{\text{to}}\) increases, I\(_{\text{Ca-L}}\) influx is gradually decreased until there is not sufficient Ca\(^{2+}\) influx to activate RyR and trigger CICR from the SR. Our study also shows that decreased SR Ca\(^{2+}\) content probably becomes a second contributor to the suppressed RV epicardial mechanics under all-or-none repolarization (Fig. 31E). Our experimental study confirmed this by showing that when the I\(_{\text{Ca-L}}\) was brought back to the control level, myocyte contractility was still significantly inhibited (Fig. 31F).

Decreased SR Ca\(^{2+}\) content is likely the result of reduced total Ca\(^{2+}\) entry upon AP collapse, as well as abbreviated AP duration that has been shown to favor the inward NCX and Ca\(^{2+}\) extrusion (Shattock & Bers, 1989) and inhibit SR Ca\(^{2+}\) reloading (Bassani et al., 2004).

Even though this all-or-none repolarization AP morphology causes abnormal contraction in canine myocytes, it represents the native AP waveform and performs perfectly in small animals. Why this AP waveform can induce normal contraction in rat or mouse but not in canine cardiomyocytes? It has been demonstrated that the
intrinsic excitation-contraction (EC) coupling in small animals is different from that of large animals (Bers, 2002). For the normal canine AP morphology, the Ca$^{2+}$ channel deactivates slowly and produces higher Ca$^{2+}$ entry and is able to maintain normal contraction in the large mammals. In contrast, an all-or-none repolarization morphology produces significantly less Ca$^{2+}$ influx at short AP durations and only works well with fast heart beats (Rosati et al., 2008).

In Brugada syndrome, the arrhythmias mostly originate from the RVOT and the abnormal ST segment elevations are mostly observed in the RVOT (Shimada, 1996; Ogawa, 2001; Takagi et al., 2001; Papavassiliu et al., 2004; Coronel, 2005; Yokokawa, 2006). The molecular mechanism for this pro-arrhythmic property in RVOT is still unclear. Morita et al. showed that RVOT has a deeper phase 1 notch at baseline than the RV does in canine heart wedge preparations (2007). A $KCNE3$ mutation that increases $I_{to}$ density has been linked to Brugada syndrome (Delpon et al., 2008).

Based on these facts and on our above observations, we hypothesize that RVOT epicardial myocytes have a higher-than-RV $I_{to}$ and this $I_{to}$ predisposes the cells to all-or-none repolarization. Our results from quantitative immunoblotting, however, do not support our hypothesis. Although we observed a significantly higher $KCND3$ mRNA expression level in RVOT than in RV epicardium, the Kv4.3 protein in RVOT epicardium is about the same as that in the RV epicardium. Possibilities still remain that, due to the post-translational modification, the functional expression of $I_{to}$ is not linearly correlated to the expression of channel proteins. It will be of interest to test the functional expression of $I_{to}$ in RVOT in the future. We are uncertain of the reason for the discrepancy between the protein and mRNA measurements in RVOT, but we noticed that in the real-time study, the $KCND3$ mRNA level had a significant but only ~23% increase in RVOT comparable to in RV, which is commonly considered to be
within the range that could be produced by systematic errors. Also, the immunoblotting technique is powerful enough to detect any differences of at least 50% change in protein level, but it is not powerful enough to quantify small protein differences. Nevertheless, to our knowledge, we are the first group to demonstrate a significant transmural expression of the Kv4.3 and KChIP2 proteins in the RVOT ventricular wall. Our results molecularly confirmed the existence of a potent I\textsubscript{to} electrical heterogeneity in the RVOT free wall, and provided a molecular basis for the arrhythmogenesis in the RVOT in Brugada syndrome. Also, in support of a previous study by Zicha et al. (2004) but in a disapproval of Rosati et al.’s study (2001), our immunoblotting results in RV and LV demonstrate that both the α-subunit (Kv4.3) and the β-subunit (KChIP2) of I\textsubscript{to} channels contribute to the heterogeneous expression of this channel.

At the cellular level, this study quantitatively demonstrates the interplay between I\textsubscript{Na} and I\textsubscript{to} in terms of their impact on the early repolarization phase and how they contribute to the development of the electrical phenotype of Brugada syndrome. It provides a basis for modulating these conductances quantitatively, using either pharmacological or gene transfer methods, as a future therapeutic strategy for Brugada syndrome. In addition, it provides a possible explanation for the contractile dysfunctions observed in the patients of Brugada syndrome and suggests that the wall motion abnormalities in these patients are not necessarily caused by the structural abnormalities.
5. Potential limitations

1) The main technique used in this thesis study, the dynamic clamp technique, has its limitations similar to any experimental techniques. Although using the dynamic clamp makes it possible to study the electrical effects of a specific ionic current, it cannot elucidate the signal conduction effect of the ionic current. This technique artificially produces current output through the patch pipette rather than through real ion channels. Therefore, the local concentration of ions carrying the current may be different from the real situation. This limitation, however, is negligible when the specific current does not involve any secondary signal impacts, and it is even beneficial if the isolated electrical effect of the current is preferred.

   Second, the dynamic clamp calculates the current that would be generated based upon the real-time measurements of the membrane potential. Errors can be produced due to the latency between acquiring membrane voltage and applying the current based upon the voltage. To minimize this latency, our dynamic clamp setup has a PC solely dedicated to the dynamic clamp simulation to ensure a high calculation speed, and it has an update rate of about 5 kHz. Therefore, this latency in our system is small and negligible.

   A third limitation of the dynamic clamp is that it relies on a mathematical description of the conductance of interests. In order to rule out the possible influence of a specific mathematical equation, two different $I_{to}$ equations were used in our study (Fig. 14, page 92). Both $I_{to}$ equations produced similar effects, demonstrating that our observation is not $I_{to}$ equation-dependent but a universal phenomenon.

2) All of the studies in this dissertation were performed in in vitro cell models. The results from isolated myocytes may not always apply to the situation in vivo. It
was found in a simulation study that the electrotonic loadings from adjacent cells lower the AP upstroke at the tissue level (Decker et al., 2009). This could potentially change the driving force for $I_{to}$ activation and alter the function of $I_{to}$ in tissues. Therefore, to definitively answer the role of $I_{to}$ in modulating heart function, *ex vivo* or even *in vivo* studies would be of importance to pursue.

3) In our Brugada syndrome study, a pharmacological method was used to produce $I_{Na}$ reduction in isolated cardiomyocytes to mimic the Brugada setting. This is different from the condition that happens in the clinical Brugada syndrome setting. A more ideal method would be to create an animal model in large animals, such as canine, with $SCN5A$ gene mutations. Currently, however, the technique of generating transgenic, large non-human animal models of disease is still premature. An alternative method would be knocking-down genetically the cardiac $I_{Na}$ in single cells. This method, however, usually requires at least 48 hours for the cultured cells to fully express the desired effects. Studies have shown that primary cardiomyocytes undergo great dedifferentiation, and AP morphology as well as $I_{to}$ density are significantly altered by day 4 after cell culture (Mitcheson et al., 1998). Therefore, the above alternative methods are unpractical at this stage, and to pharmacologically knockdown the native $I_{Na}$ represents a more feasible method for our study.
6. Future directions

This dissertation addressed the role of the transient outward current (I_{to}) in regulating myocyte electrical as well as mechanical functions, and its potential contribution to arrhythmogenesis. This body of work opens up new tracts of interesting questions and possibilities as briefly outlined below, which may further complete our understanding of the role of I_{to} in cardiac physiology and pathophysiology:

1) The effects of I_{to} on APD and cell contractility in normal myocytes have been illustrated in this dissertation. It is of importance to continue our study in heart failure myocytes. The intracellular environments are significantly different in failing myocytes compared to control cells, and HF animal models can recapitulate more closely the clinical features of heart failure. As shown in Results part 3, we have started basal characterization of ventricular cardiomyocytes from a canine HF model. However, at the time the study was limited by the availability of experimental animals, and this needs to be further pursued.

2) We demonstrated that I_{to}, by changing AP trajectory, plays a crucial role in regulating the deactivation and reactivation of I_{CaL}, and significantly affects sarcolemmal Ca^{2+} influx and intracellular Ca^{2+} cycling. The NCX, as a sarcolemmal Ca^{2+} transporter, also modulates Ca^{2+} concentration and Ca^{2+} cycling during EC coupling. Therefore, it will be of great interest to examine the effect of I_{to} on the I_{NCX}.

3) Our data demonstrated that I_{to} has a negative inotropic effect in isolated cardiomyocytes; however, whether such an effect is also true in heart tissues still
needs to be further tested. A recent computational study showed that the peak upstroke voltage of AP is smaller in electronically coupled heart tissues than in single myocytes (Decker et al., 2009). This may cause a smaller $I_{to}$ current and less driving force for $I_{Ca-L}$. With the recently developed $I_{to}$ activator NS5806 (Calloe et al., 2009; a & b), it will be interesting and feasible to test the effects of $I_{to}$ in $ex$ $vivo$ heart preparations.

4) The right ventricular outflow tract (RVOT) is a widely recognized arrhythmogenic starting area in Brugada syndrome (Shimada, 1996; Ogawa, 2001; Takagi et al., 2001; Papavassiliu et al., 2004; Coronel, 2005; Yokokawa, 2006). Using optical mapping, Morita et al. found a deeper phase 1 notch in the RVOT epicardium than in the RV epicardium (Morita et al., 2007). When they used the Na$^+$ channel blocker pilscainide and the K$^+$ channel opener pinacidil on tissue preparations, the notches in both RVOT and RV were deepened, with a larger increase in RVOT. Based on our results that a higher $I_{to}$ density predisposes cardiomyocytes to be closer to the all-or-none repolarization AP, we hypothesized that a higher than RV epicardial $I_{to}$ exists in the RVOT and this $I_{to}$ predisposes this region to arrhythmias. As shown in Results Part 4, we examined the $I_{to}$-related protein expression levels in RVOT and did not find significant differences when compared to those in RV. Possibilities still remained that, due to the post-translational modification, the functional expression of $I_{to}$ is not lineally correlated to the expression of channel proteins. A more direct evaluation of $I_{to}$ expression would be to measure the $I_{to}$ current using the electrophysiology method, and this will further test the above hypothesis.
7. Summary and significance

Cardiac arrhythmias are one of the major healthcare burdens in the US. According to CDC statistical data (Morbidity and Mortality Weekly Report; 2003), more than four million Americans suffer from arrhythmias, the disorders subsequent to organic diseases, such as heart failure, or caused by genetic mutations, such as in Brugada syndrome. Many antiarrhythmic drugs and electronic devices have been developed to restore normal rhythm. However, currently available approaches are ineffective and paradoxically, many antiarrhythmic drugs can also cause severe lethal arrhythmias. These represent a significant gap in our understanding of the cardiac electrophysiology and cardiac ionic currents. One of the most notable examples is the transient outward current ($I_{to}$). Although it is well known that this current is heterogeneously expressed at various regions in the heart, and that it is responsible for shaping the phase 1 early repolarization, the overall function of this current is unclear. This thesis provides important insight into this ionic current. We demonstrated that one of the main functions of $I_{to}$-mediated early repolarization is to modulate the deactivation and reactivation of L-type Ca$^{2+}$ channels. By doing so, the density of $I_{to}$ determines the amount of sarcolemmal Ca$^{2+}$ influx and subsequently modulates the myocytes contractility. This proved our hypothesis that $I_{to}$ plays a critical role in regulating cardiac $I_{Ca-L}$ and EC coupling. We also demonstrated that with different expression across the ventricular wall, $I_{to}$ induces different basal levels of intracellular Ca$^{2+}$-induced Ca$^{2+}$ release, and partially contributes to the transmural mechanical heterogeneity.
This dissertation has also advanced our understanding of the function of I_{to} downregulation in heart failure. Heart failure is one of the most malignant diseases. Nearly 5 million people suffer from HF in the United States (Tomaselli and Zipes, 2004). Of the deaths in HF, up to 50% are sudden and unexpected (Akar and Rosenbaum, 2003; Janse, 2004), and almost all of the patients suffer with significant reduction of basal cardiac contractility (Mason et al., 1970). Previously, I_{to} downregulation was believed to be responsible for a prolonged APD and to predispose the failing myocytes to early afterdepolarizations (EAD) and a bigger dispersion of repolarization. Both will lead to arrhythmias through triggered activity or reentrant mechanisms, respectively. Therefore, gene transfers of various K^+ channels have been proposed as therapeutic targets for heart failure (Kaprielian et al., 2002). Further, I_{to} downregulation was considered detrimental to cell contractility, and restoration of I_{to} was proposed as a therapeutic approach for improving cardiac mechanical function (Sah et al., 2003).

To the contrary, the evidence presented in this dissertation indicates that the main consequence of this I_{to} downregulation is not leading to prolonged APD and arrhythmias; instead, it may represent a compensatory effect for the repressed Ca^{2+} dynamics and cell contractility. This approved our hypothesis that I_{to} is not a significant modulator to the APD. Our results strongly challenge the rationality of restoration of I_{to} as a therapeutic strategy for heart failure. Such concept, however, needs to be further confirmed under the heart failure settings and in whole organ level.

This dissertation study improves our understanding of the molecular mechanisms of Brugada syndrome, a right ventricular arrhythmia disease that was not acknowledged until recently. It is believed that this disease is responsible for 4% -
12% of all cardiac sudden death and up to 20% of sudden cardiac death in patients without concomitant cardiomyopathy (Benito et al., 2008). Our study approved our hypothesis and demonstrated that a high level of $I_{to}$ predisposes RV epicardial myocytes to abnormal AP waveforms which is believed to cause the reentrant arrhythmias in Brugada syndrome patients. Based on this, we herein suggest that suppressing $I_{to}$ to reduce the phase 1 notch may represent a new therapeutic strategy for this disease entity. Our study also demonstrated that, at the cellular level, the mechanical abnormalities observed in Brugada patients can be caused by electrical abnormalities, and are not necessarily associated with structural disorder, in agreement with our hypothesis. It supports the notion that any structural or mechanical disorder observed in the patients is just a secondary or coincident event of the primary electrical disorder, and this clarification will benefit our future development in diagnosis and treatment strategies for Brugada syndrome.
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Appendix: Publications


