ION CHANNEL EXPRESSION AND THE CONTROL OF VENTRICULAR REPOLARIZATION

by

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Ling Xiao

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

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This thesis is dedicated to:

My dearest father, Liuqing Xiao, my dearest mother, Fengzhen Liu, my lovely sister, Yangmei Xiao, and my boyfriend, Xiaobin Luo

for their endless love, consistent support and encouragement, understanding and patience along my way to pursue my PhD.
VENTRICULAR ARRHYTHMIA IS THE MAIN LEADING CAUSE OF SUDDEN CARDIAC DEATH, WHICH REMAINS A MAJOR PUBLIC HEALTH PROBLEM. TO DATE, NO AVAILABLE ANTIARRHYTHMIC DRUG THERAPIES HAVE BEEN PROVEN MORTALITY BENEFIT. ABNORMALITIES IN CARDIAC REPOLARIZATION ARE IMPORTANT CONTRIBUTORS TO THE DEVELOPMENT OF LETHAL VENTRICULAR ARRHYTHMIAS. THE BASIC ION-CHANNEL CONTROL MECHANISMS FOR VENTRICULAR REPOLARIZATION ARE STILL POORLY UNDERSTOOD. THIS THESIS ADDRESSED POTENTIAL MECHANISMS RESPONSIBLE FOR ION CHANNEL REGULATION INVOLVED IN THREE CLINICALLY RELEVANT PARADIGMS OF VENTRICULAR REPOLARIZATION CONTROL.

I first characterized in detail the ionic currents controlling repolarization across the ventricular wall in female/male canine left ventricles. I found intrinsic sex-related differences in overall inward L-type calcium current (I_{Ca,L}) and transmural repolarizing K^+ currents, including the slowly-activated delayed rectifier K^+ current (I_{Ks}) and transient outward potassium current (I_{to}). Offsetting effects of larger female I_{Ks} and I_{Ca,L} explain similar male/female APDs in epicardium and endocardium. However, I found larger I_{Ca,L} with similar repolarizing K^+ currents between female and male in the midmyocardium, which may account for the observed longer female action potential duration (APD) in this specific cell-type. The reduced repolarization reserve in the female midmyocardium explains the female sensitivity to QT-prolonging drugs and Torsades de pointes (TdP).

Chronic tachycardia produces ventricular electrical remodeling, heart failure (HF) and susceptibility to cardiac arrhythmia. I assessed the hypothesis that increased heart rate per se regulates ion channel expression. I found that the I_{to} and its subunit Kv4.3 are directly regulated by increased electrical firing rate. I established the signaling pathways transducing changes in cardiac firing rate into downregulation of Kv4.3 transcription. I determined that Ca^{2+}/calmodulin-dependent CaMKII and calcineurin/NFAT systems play key Ca^{2+}-sensing and signal-transducing roles in rate-dependent I_{to} control, identifying molecular
mechanisms contributing to cardiac excitation-transcription coupling.

I identified a novel mechanism potentially governing repolarization reserve, feedback regulation of ion channel expression. Traditionally, cardiac repolarization reserve refers to functionally-based compensatory increases in repolarizing currents, usually $I_{Ks}$, that minimize changes in APD caused by dysfunction or inhibition of a single $K^+$-current. I found that chronic action potential prolongation, induced by inhibiting the rapidly-activated delayed rectifier $K^+$ current ($I_{Kr}$), increases repolarization reserve through compensatory upregulation of $I_{Ks}$ and the protein levels of its molecular components KvLQT1 and minK. The feedback regulation of KvLQT1 expression is partly mediated by downregulating the inhibitory microRNA (miRNA) miR-133. Impairment of this feedback system could contribute to the occurrence of cardiac arrhythmias in repolarization dysfunction paradigms like congenital and acquired long-QT syndromes.

In conclusion, my findings in this thesis highlight the delicate and complex control of ventricular repolarization through homeostatic regulation of underlying ion-channel function and expression under physiological and pathophysiological conditions.
RESUME GENERALE

L’arythmie ventriculaire est la principale cause principale de mort subite d'origine cardiaque et demeure un problème de santé majeur. À date, aucun traitement médicamenteux antiarythmique a démontré utile dans la prévention de la mort subite. Les anomalies de la répolarisation cardiaque contribuent d’une façon importante à l'apparition d'arythmies ventriculaires létales. Les mécanismes impliqués dans la régulation des canaux ioniques qui contrôlent la répolarisation ventriculaire sont encore mal compris. L'objectif central de cette thèse était de déterminer les mécanismes responsables de la régulation des canaux ioniques dans trois paradigmes cliniquement significative du contrôle de la repolarisation ventriculaire.

Nous avons d'abord caractérisé en détail les courants ioniques en relation avec la répolarisation à travers la paroi ventriculaire chez les ventricules gauches canins selon le sexe. Nous avons noté des différences selon le sexe des animaux dans les courants calciques de type L (I_{CaL}) et l’ensemble des courants potassiques, incluant le « slow delayed rectifier » I_{Ks} et le courant transitoire sortant I_{to}. L’augmentation parallèle de I_{Ks} et I_{Ca} chez les femmes aux niveaux sous épicaudique et endocaudique n’a pas amené à un changement dans la répolarisation. Par contre, au niveau du milieu du myocarde, le I_{CaL} a été augmenté chez les femmes sans changement dans les courants potassiques, ce qui a provoqué une augmentation dans la durée du potentiel d’action (DPA). Le réduction du « repolarization reserve » à ce niveau peut expliquer la sensibilités des femmes aux molécules qui prolongent le DPA et leur prédilection pour les TdPs.

La tachycardie chronique produit un remodelage électrique cardiaque, une insuffisance cardiaque et une susceptibilité à l'arythmie cardiaque. Nous avons évalué l'hypothèse qu’une augmentation de la fréquence cardiaque peut, en soi, affecter l'expression des canaux ioniques. Nous avons constaté que l'expression de l’I_{to} et de sa sous unité sous jacente Kv4.3 sont supprimé une augmentation dans la
fréquence d’activation des cellules cardiaques. Nous avons également établi les voies de signalisation sous jacentes, qui impliquent le CaMKII Ca²⁺/calmoduline-dépendant et le système calcineurine/NFAT, qui ressentent le taux cellulaire de Ca²⁺ et ajustent l’expression de Kv4.3 en conséquence. Il s’agit d’une découverte des mécanismes moléculaires qui contribuent au couplage entre l'excitation cardiaque et la transcription génique.

Nous avons identifié un nouveau mécanisme qui la réserve de la répolarisation (« repolarization reserve »), médié par la régulation de l'expression des canaux ioniques. Traditionnellement, la réserve de la répolarisation cardiaque fait référence à des augmentations fonctionnelles compensatoires dans les courants répolarisants, en particulier Iₖₛ, qui minimisent les changements dans la DPA causés par un dysfonctionnement d'un simple courant potassique. Nous avons alors constaté que la prolongation chronique de la DPA induite par l'inhibition du courant « rapid delayed rectifier » Iₖᵣ de la réserve rapidement activé redresseur retardé courant K⁺ (Iₖᵣ), entraîne une augmentation de la réserve de la répolarisation par une augmentation dans l’expression de l'Iₖₛ qui est réalisé par une augmentation de l’expression des taux de protéines de ses composants moléculaires KVLQT1 et minK. Cette régulation semble produit par une diminution dans l’expression d’une petite molécule ARN inhibitrice, le micro-ARN (miRNA) miR-133. Une déficience de ce système de contrôle peut contribuer à la survenue d'arythmies cardiaques chez les patients atteints d’une dysfonction de répolarisation.

En conclusion, nos résultats dans cette thèse soulignent le contrôle délicat et complexe de la répolarisation ventriculaire par la régulation homéostatique du fonctionnement des canaux ioniques dans les conditions physiologiques et physiopathologiques.
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STATEMENT OF AUTHORSHIP

In accordance with the Faculty of Graduate and Postdoctoral Studies, the candidate has the option of including as part of the thesis the text of original papers already published by learned journals, and original papers submitted or suitable for submission to learned journals. In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense.

The following is a statement regarding the contributions of co-authors and myself to the three published papers included in this thesis.

Paper 1 (Chapter 2)

Dr. Stanley Nattel proposed the initial idea. I designed and completed all the experiments, performed all the data analysis, and wrote the whole manuscript. Liming Zhang and Dr. Han Wei helped me with many aspects of technical assistance in initiating the myocyte isolation and patch-clamp techniques. Liming Zhang involved in part of the $I_{CaL}$ recordings. Dr. Zhiguo Wang provided supervision on data analysis and part of the work, and problem solving in patch-clamp set-up. Dr. Stanley Nattel fully supervised, generated feedback, suggested additional experiments and edited the final version of the manuscript.

Paper 2 (Chapter 3)

Based on the initial idea suggested by Dr. Nattel and my previous work, Dr. Nattel and I proposed the original research plan. I designed and performed all the experiments, analyzed all the data, and wrote the full manuscript. Dr. Pierre Coutu performed part of the calcium transient recordings and data analysis. Louis Villeneuve performed the confocal microscopy imaging. Artak Tadevosyan performed part of the Western blot experiments. Drs. Ange Maguy and Sabrina Le Bouter helped me with technical assistance in Western blot and real-time RT-PCR. Dr. Bruce Allen provided problem solving in membrane protein extraction and in Western blot. Dr. Nattel fully supervised the whole work, clarified the ideas, generated feedback, suggested additional experiments and edited the final version of the manuscript.

Paper 3 (Chapter 4)


Dr. Nattel provided the original ideas. Dr. Nattel, Dr. Wang and Xiaobin Luo suggested the ideas of microRNA work. I designed and carried out all the electrophysiological studies, Western blot and real-time RT-PCR experiments. I analyzed the corresponding data and wrote the manuscript. Dr. Jiening Xiao performed microRNA extraction, quantification and analysis, and wrote the methods of microRNA techniques for the manuscript. Xiaobin Luo performed the cloning experiments, bioinformatic analysis of microRNA binding sites and prepared Figure 8 and online Figure 5&6. Dr. Huixian Lin provided assistance in the microRNA work. Dr. Zhiguo Wang provided supervision in the microRNA related work. Dr. Nattel fully supervised the entire work, had the original idea, generated feedback,
suggested additional experiments and finalized the manuscript for publication.
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<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMO</td>
<td>anti-miRNA antisense inhibitor oligonucleotides</td>
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<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>APs</td>
<td>action potentials</td>
</tr>
<tr>
<td>APD</td>
<td>action potential duration</td>
</tr>
<tr>
<td>APD_{30}</td>
<td>action potential duration at 30% repolarization</td>
</tr>
<tr>
<td>APD_{90}</td>
<td>action potential duration at 90% repolarization</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>atrioventricular</td>
</tr>
<tr>
<td>BCL</td>
<td>basic cycle length</td>
</tr>
<tr>
<td>Bpm</td>
<td>beats per minute</td>
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<tr>
<td>[Ca]_{i}</td>
<td>intracellular concentration</td>
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<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>Cav</td>
<td>voltage-gated Ca^{2+} channel</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calcium-calmodulin dependent protein kinase II</td>
</tr>
<tr>
<td>CFTR</td>
<td>The cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<tr>
<td>CHF</td>
<td>congestive heart failure</td>
</tr>
<tr>
<td>CL</td>
<td>cycle length</td>
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<tr>
<td>DAD</td>
<td>delayed afterdepolarization</td>
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DHT  
dihydrotestosterone

EAD  
early-afterdepolarization

EC coupling  
Excitation-contraction coupling

Endo  
endocardial/endocardium

eNOS  
endothelial nitric oxide synthase

Epi  
epicardial/epicardium

ER  
Estrogen receptor

ET coupling  
Excitation-transcriptional coupling

HDAC  
histone deacetylase

HPTXs  
Heteropoda toxins

HERG  
human ether-a-go-go-related gene

HF  
Heart failure

HRE  
Hormone response element

$I_{CaL}$  
inward L-type calcium current

$I_{CaT}$  
inward T-type calcium current

$I_{Cl,Ca}$  
Ca$^{2+}$-dependent outward chloride current

$I_{Cl,PKA}$  
protein kinase A-dependent chloride current

$I_{Cl,Swell}$  
swelling-induced chloride current

$I_{f}$  
pacemaker current

$I_{HERG}$  
HERG current

$I_{K1}$  
inward rectifier potassium current

$I_{KACH}$  
acetylcholine-activated potassium current

$I_{KATP}$  
ATP-sensitive potassium current

$I_{Kr}$  
rapidly activated delayed rectifier potassium current

$I_{Ks}$  
slowly activated delayed rectifier potassium current

$I_{Kur}$  
ultra-rapidly activating delayed-rectifier K$^{+}$-current

$I_{Na}$  
inward sodium current

$I_{NCX}$  
sodium calcium exchange current

$IP_3$  
inositol 1,4,5-triphosphate

$IP_3R$  
inositol 1,4,5-triphosphate receptor
I_p  Na⁺/K⁺ ATPase pump generated outward current
I_o  transient outward potassium current
I-V  current to voltage curve
JLN  Jervell-Lange-Neilsen syndrome
JT interval QTc interval-QRSc interval
Kv  voltage-gated K⁺ channel
KChAP  K⁺ channel accessory protein
KChIP2  K⁺ channel interacting protein 2
LQTS  long QT syndrome
LV  left ventricle
M cell  Masonic midmyocardial Moe cell
MEF2  myocyte enhancer factor 2;
Mid  midmyocardium
MiRNA  microRNA
MLCK  myosin light chain kinase
MVT  monomorphic ventricular tachycardia
Nav  voltage-gated Na⁺ channel
[Na]ᵢ  intracellular Na⁺ concentration
NCX  sodium-calcium exchanger
NFAT  nuclear factor of activated T cells
NO  Nitric Oxide
ORCH  Orchiectomized
OVX  Ovariectomized
PI3K  phosphatidylinositol 3-kinase
PKA  protein kinase A
PKC  protein kinase C
PLB  Phospholamban
PLC  phospholipase C
PR  progesterone receptor
PVT  polymorphic ventricular tachycardia
QTc  heart rate corrected QT interval
RAAS  the renin-angiotensin-aldosterone system
RV    right ventricle
RyR2  ryanodine receptor 2
SA    sinoatrial
SCD   sudden cardiac death
SERCA SR Ca\(^{2+}\)-ATPase
SR    sarcoplasmic reticulum
SUR   sulfonylurea receptor
TA    Triggered activity
TDR   transmural dispersion of repolarization
Tdp   Torsades de pointes
TNF-α tumor necrosis factor-α
TTX   tetrodotoxin
3’-UTR 3’-untranslated region
\(V_{1/2}\) half voltage of maximum voltage-dependent kinetics
VT    ventricular tachycardia
VF    ventricular fibrillation
Chapter 1. Introduction
1. Cardiac repolarization

1.1 Cardiac action potential

The cardiac action potential (AP) is the key determinant of cardiac electrical activity and the normal function of the heart. Figure 1A is a schematic representation of a cardiac ventricular action potential and the principal currents involved in its various phases (1).

![Schematic cardiac action potential](image)

**Figure 1.** Schematic cardiac action potential with the various phases and principal corresponding ionic currents indicated. The horizontal lines at the bottom indicate schematically the portions of the action potential during which each current flows. NCX, Na⁺-Ca²⁺ exchanger. (Adapted from Figure 1 of reference 1 with permission)
The cardiac AP reflects a sequence of changes in potential difference across the cardiac cell membrane as a function of time. It is measured by inserting a microelectrode into the cell through the plasma membrane and represents the voltage differences between the cell interior and the cell exterior. Unlike the action potentials (APs) in skeletal muscle and nerve, which last only a few milliseconds, cardiac action potentials last much longer, over 200 milliseconds. A typical cardiac AP in the working myocardium (atria and ventricles) and specialized ventricular conducting tissue Purkinje fibers is composed of 5 phases (Figure 1): the upstroke or depolarization phase 0, a brief early repolarization phase 1, the plateau phase 2, repolarization phase 3 and the resting phase 4. These complex characteristics are determined by the opening and closing of ion channels and transporters and their resulting ionic currents in the heart’s plasma membrane.

The depolarization phase 0 is largely determined by fast opening inward sodium currents ($I_{Na}$). Activation of $I_{Na}$ rapidly depolarizes the cell membrane to more positive potentials, leading to the activation of a number of other ionic currents. The initial early repolarization phase 1 results from inactivation of $I_{Na}$, activation of the transient outward potassium ($I_{to}$) and the outward chloride current ($I_{Cl}$) and the ultra-rapidly activating delayed-rectifier K⁺-current ($I_{Kur}$) in the atria. Phase 0 and phase 1 together are also referred as the “spike”; phase 1 displays a prominent $I_{to}$ mediated notch; and these result in the classic “spike and dome” configuration of cardiac APs. The plateau phase 2 is the most distinctive characteristics of the cardiac AP and accounts for the long cardiac AP as compared to that in nerve or skeletal muscle. In phase 2, the membrane potential stabilizes at ~+20 mV for ventricles(2), which is mainly due to Ca²⁺ influx through the L-type calcium channel and a balance
between inward (such as late \( I_{Na} \) and inward L-type calcium current (\( I_{CaL} \)) and outward currents (such as rapidly or slowly activated delayed rectifier potassium currents-\( I_{Kr} \) or \( I_{Ks} \)). The rapid repolarization phase 3 is dominated by several potassium currents such as \( I_{Kr} \), \( I_{Ks} \), \( I_{Kur} \) and the inward rectifier potassium current (\( I_{K1} \) that terminate the AP. Finally, the membrane potential returns to diastolic or resting level (phase 4, around -80 to -90 mV in the ventricle), which is largely maintained by \( I_{K1} \). Activation of acetylcholine-activated inward rectifier potassium current (\( I_{KAC} \)) can also stabilize the resting membrane potential in the atria. In the working myocardium, the sodium/calcium exchange current (\( I_{NCX} \)) can also generate an inward current at the end of repolarization and therefore may contribute to action potential duration (APD).

1.2 Regional cardiac action potentials

The characteristics of cardiac action potentials vary from region to region as shown in Figure 2 (3). The distinctive features of regional AP waveforms account for their specialized electrophysiological roles in the heart.
Figure 2. Electrical activity in the myocardium. Top: schematic of a human heart with illustration of typical action potential waveforms recorded in different regions. Bottom: schematic of a surface electrocardiogram of one heart beat. RV, right ventricle; LV, left ventricle. (Adapted from Figure 1 of reference 3 with permission)

The cardiac impulse is initiated by spontaneous regular firing from a group of pacemaker cells located in the sinoatrial (SA) node (Figure 2). APs of the SA node are small in amplitude, and have a slow upstroke in phase 0 and a spontaneous diastolic depolarization in phase 4 operated mainly by $I_{CaL}$ and pacemaker current ($I_o$), generating propagated APs to initiate contraction in all regions of the heart. The electrical impulse from the SA node first spreads to and fires the atria, causing atrial contraction. Atrial APs are similar in morphology to APs from ventricles and Purkinje fibers (Figure 1 & 2), but they are shorter in duration than the latter. APD is shorter in the left atrium than in the right, which might due to a larger $I_{Kr}$ in the left atrial
cells. After passing through the atria, the electrical impulse arrives at the atrioventricular (AV) node. The AV node is the only conducting pathway between the atria and the ventricles. Importantly, there is a conduction delay of the electrical impulse in the AV node to provide time for the ventricles to fill before they contract. This AV conduction delay is due to the small particular structure and gap junction properties of the AV node, and its AP characteristics. Similar to the SA node, APs in the AV node have a spontaneous diastolic depolarization and a slow upstroke depolarization due to the slowly activated I_{CaL} and a lack of functioning I_{Na}. After a conduction delay in the AV node, the electrical impulse spreads via the His-Purkinje fiber ventricular conducting system to activate the ventricles. APs in the Purkinje fiber have a very high rate of phase 0 depolarization, which favors rapid conduction. APD is very long in His-Purkinje fibers, which helps to provide a safety factor to prevent reentry of the electrical impulse from the ventricle. The ventricle is the main pumping part of the heart. It is very crucial for cardiac function and abnormalities in the ventricle can cause life-threatening arrhythmias. The typical ventricular AP is similar to that in Figure 1. However, the ventricle contains intrinsic transmural AP heterogeneity that is functionally very important for synchronizing ventricular excitation and optimizing contraction.

The sequential activation of these regions generate time dependent electrical potential differences on the body surface as recorded by electrocardiogram (ECG), shown in Figure 2 bottom. For example, P wave corresponds to atrial activation, the QRS to the depolarization of ventricular tissue and the QT interval, the time between initiation of depolarization and end of repolarization of the ventricle.
1.3 Ventricular transmural repolarization

Ventricular myocardium is not a homogeneous tissue at the cellular level. It contains at least three distinct cell types: epicardial, mid-myocardial (or M cell) and endocardial (6;7). Besides their anatomical localization, these three ventricular cell types are distinguished from each other by their different AP properties (Figure 3), with two particularly important aspects: 1) AP morphology, 2) APD. Epicardial and mid-myocardial APs possess a prominent “spike and dome” morphology that is not seen in endocardium. This difference is due to much larger Ito mediated phase 1 repolarization in epicardial and mid-myocardial myocytes than in endocardium (8;9). Epicardial APD is known to be shorter than endocardial APD, partly due to larger Ito in epicardium. M cells have the longest APD and a steeper APD-rate relationship than the other cell types (10-12). The distinct M cell AP properties partly result from smaller IKs, larger late INa (a small but sustained inward Na\(^+\) current) and INCX in this cell type than in epicardial and endocardial cells (13-15). The intrinsic transmural AP heterogeneity in the ventricle results in transmural dispersion of repolarization (TDR). Normal ventricular TDR has been found in humans (16) and in a wide variety of animals from rodent (mouse (17), rat (18) and guinea pig (19)) to larger mammals such as rabbit (20), pig (21) and dog (11;22). The transmural gradient of ventricular repolarization contributes importantly to the inscription of the T wave (587;588). Changes of APs in any region of the ventricle can alter the normal physiological TDR and predispose to arrhythmias.
Figure 3. Examples of transmembrane action potentials recorded under different frequencies (0.5, 1 and 2 Hz) from epicardial (Epi), M cell, and endocardial (Endo) cells isolated from canine left ventricle. (Modified from Figure 1 of reference (22) and used with permission)

2. Ion channels underlying ventricular repolarization

Transmural differences in ventricular repolarization are determined by underlying ion channel and ion transporter expression levels and resulting inward and outward ionic current balances. Ion currents especially known to be transmurally heterogeneous include $I_{io}$, $I_{ks}$, late $I_{Na}$ and $I_{NCX}$ etc. (8). Normal electrical and mechanical activity of the ventricular myocardium requires delicately orchestrated
gating of these ion channels. Changes in ion channels or transporters will alter ventricular repolarization and are potentially detrimental to the proper function of the ventricles.

2.1 Na\(^+\) channels

Voltage-gated Na\(^+\) channels are the most abundantly expressed ion channels in the heart (23). There are two kinetic components to Na\(^+\) currents in the ventricle: fast \(I_{Na}\) and late \(I_{Na}\). They play very important roles in cardiac electrophysiology. Voltage-gated Na\(^+\) channels generate inward currents that are responsible for the initial depolarization of cardiac APs, the conduction of excitation and the rapid electrical pulse propagation, and they also play an important role in governing APD. They are clinically important therapeutic target of a variety of drugs such as class I antiarrhythmic drugs (24) with local anesthetic properties (25). Gain- and loss-of-function gene mutations in Na\(^+\) channels are linked to congenital long QT syndrome (LQTS) and the Brugada syndrome respectively (26).

2.1.1 \(I_{Na}\)

\(I_{Na}\) activates and inactivates very rapidly (27). \(I_{Na}\) opens within a fraction of a millisecond through conformational changes in underlying channel proteins, generating very large inward current due to the movement of Na\(^+\) ions down their electrochemical gradient. This resulting rapid and brief influx of Na\(^+\) causes very rapid depolarization of cardiac cellular membrane to positive potential, with a rate of 100-500 V/sec (28). This depolarization triggers the activation of voltage-gated Ca\(^{2+}\) and K\(^+\) channels. Within milliseconds after activation, \(I_{Na}\) inactivates voltage and time
dependently, greatly decreasing conductance of Na\(^+\) channels. \(I_{\text{Na}}\) inactivation contains two kinetically different components: initial fast inactivation and slow inactivation (29). Fast inactivation of \(I_{\text{Na}}\) contributes to phase 1 repolarization. The slowly inactivating component of \(I_{\text{Na}}\) can last over several hundred milliseconds (30), shows slow recovery (31), and is part of the late inward Na\(^+\) current during the plateau phase 2 of ventricular AP (32).

### 2.1.2 Late \(I_{\text{Na}}\)

In the ventricle, there is a small but sustained inward Na\(^+\) current activating at depolarized potentials during the AP plateau, the so called “late” \(I_{\text{Na}}\). Late \(I_{\text{Na}}\) may result from a time-invariant Na\(^+\) “window current” (the overlap of \(I_{\text{Na}}\) activation and inactivation voltage-dependent curves (33)) or steady-state or persistent \(I_{\text{Na}}\) and the slowly inactivated portion of \(I_{\text{Na}}\) (34) during a prolonged depolarization as the AP plateau. This sustained late \(I_{\text{Na}}\) is small compared to peak \(I_{\text{Na}}\) (less than 0.5\% of peak (35)), but importantly, it helps to maintain the AP plateau. Tetrodotoxin (TTX) shortens dog Purkinje fiber APD at concentrations not affecting the upstroke or fast \(I_{\text{Na}}\) (36). Recently, late \(I_{\text{Na}}\) has been found to be transmurally heterogeneously distributed (15;37) in the ventricle, with M cell displaying larger late \(I_{\text{Na}}\) density and greater APD abbreviation by TTX than epicardial or endocardial ventricular cells (15), which may contribute to governing the longer APD in M cell, normal TDR and the development of ventricular arrhythmias (26).

### 2.1.3 Molecular nature of Na\(^+\) channels

Functional cardiac Na\(^+\) channels are macromolecular complexes composed of
a pore-forming α-subunit and one or more auxiliary β-subunits (38;39). At least eleven genes (SCN1a-SCN11a) have been cloned from different species, encoding ten homologous isoforms of Na⁺ channel α-subunits (Na,1.1-1.9, Na,2.1) (40), among which Na,1.5 (SCN5a) is the predominant cardiac Na⁺ channel α-subunit. Na,1.5 contains approximately 2016 amino acids and has four structural domains (I-IV), each consisting of six transmembrane-spanning segments, S1-S6, along with intracellular N and C termini. The four structural domains together form the Na⁺-selective channel pore. The P-loops (intramembrane loops between transmembrane segments S5 and S6) determine Na⁺-selectivity; the S4 segments form the “voltage sensor” responsible for voltage-dependent gating; the intracellular loops between domains are sites for biochemical modulation, drug binding and, in particular, the II–III linker is important for channel inactivation (41). Na,1.5 subunits localize to the intercalated disks of ventricular cardiomyocytes, likely in relation to their role in AP propagation (42). Na⁺ channel β-subunits assemble with α-subunits to form the heteroligomeric and mature glycosylated (260-280 kDa) channel protein (43). Four Na⁺ channel β-subunits (β1-β4), encoded by SCN1b-SCN4b, have been identified and are all found to be expressed in the heart (44;590). They all possess only a single transmembrane domain, a small intracellular C-terminal region and a large extracellular N-terminal domain with an immunoglobulin-like fold. Coexpression of β-subunits with Na⁺ channel α-subunits modifies Na,1.5 cell surface expression as well as its current amplitude and kinetics (45). There is heterogeneous transmural expression of Na,1.5 and associated β3 subunits in the ventricle: both are more highly expressed in endocardium than in epicardium (46;47). The molecular basis responsible for transmural late I Na heterogeneity is not clear. Gain-of-function mutations in SCN5a
have been linked to LQTS3 (48) and loss-of-function mutations of SCN5a are linked to the Brugada syndrome (49); both are highly arrhythmogenic and lethal ventricular diseases.

2.2 Ca^{2+} channels

Cardiac Ca^{2+} channels are a critical contributor to cardiac conduction, APs and most importantly, excitation-contraction (EC) coupling in working myocardium (50). There are mainly two types of Ca^{2+} channels in the heart, the T-type (transient-opening or tiny conductance) and L-type (long-lasting or large conductance) Ca^{2+} channels, or I_{CaT} and I_{CaL} for their functional currents (51). T-type Ca^{2+} channels are mainly expressed in atrium (52) and Purkinje fibers (53), also in some pacemaker cells (54), and in neonatal or hypertrophic ventricular myocytes (55-57), but are none or very lowly expressed in adult ventricular myocytes (58-61). L-type Ca^{2+} channels are present in all regions of the heart. I_{CaL} is the main route of Ca^{2+} entry into cardiac cells, and the main trigger for calcium-related contraction. I_{CaL} also plays roles in early afterdepolarization (EAD) related arrhythmogenesis (62). Therefore, in this section, only the L-type Ca^{2+} channel is discussed.

2.2.1 I_{Ca,L}

Rapid depolarization of cardiac cell membranes by I_{Na} activates I_{CaL}, a large inward current carried by Ca^{2+} down its electrochemical gradient. Activation of I_{CaL} is both voltage- and time-dependent. I_{CaL} activates more slowly than I_{Na}, taking a few milliseconds to reach maximum current amplitude. I_{CaL} starts to activate at a membrane potential of ~40 mV and peaks around 0-10 mV (50). Therefore, it is
active at AP plateau potentials and helps maintain phase 2 of the ventricular AP. Importantly, Ca\textsuperscript{2+} influx through activation of I\textsubscript{CaL} triggers Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) via Ca\textsuperscript{2+}-release channels, also called type 2 ryanodine receptors (RyR2), causing a rapid increase in intracellular Ca\textsuperscript{2+} concentration [Ca\textsubscript{i}]. This allows Ca\textsuperscript{2+} to bind to the myofilaments and initiate cardiac contraction (63). Inactivation of I\textsubscript{CaL} is not only voltage- and time-dependent, but also intracellular Ca\textsuperscript{2+}-dependent (64). Half-maximum voltage (V\textsubscript{1/2}) of I\textsubscript{CaL} inactivation is around -45 mV and I\textsubscript{CaL} availability decreases to almost zero around -15 mV (50). Overlap of activation and inactivation voltage-dependent curves allows for “window current” at around -28 mV, which is involved in the mechanism for EAD generation (65). Inactivation is strongly affected by local [Ca\textsubscript{i}], in that higher [Ca\textsubscript{i}] induces rapid inactivation; recovery from inactivation of I\textsubscript{CaL} is also accelerated with increased [Ca\textsubscript{i}] or with decreased membrane potential (50). There is an interesting, yet functionally unclear, biophysical property of I\textsubscript{CaL}: Ca\textsuperscript{2+}-dependent facilitation, i.e., increased pacing frequency increases current amplitude and slows inactivation (66). Our understanding of the transmural distribution of cardiac I\textsubscript{CaL} is controversial. I\textsubscript{CaL} has been found to be homogeneous across the left ventricular wall in dogs (22;67) and male rabbits (68), or to be larger in female rabbit epicardium (69) and canine endocardium (70). Recently, I\textsubscript{CaL} was also reported to show apex-base heterogeneity in adult female rabbit ventricles (71).

2.2.2 Molecular basis of L-type Ca\textsuperscript{2+} channels

Cardiac L-type Ca\textsuperscript{2+} channels are formed by a group of subunits, including a pore-forming \(\alpha\)-subunit (\(\alpha\)1) and at least two accessory subunits \(\alpha\)2\(\delta\) and \(\beta\) (72).
Structurally similar to the Na\(^+\) channel, the L-type Ca\(^{2+}\) channel \(\alpha\)-subunit contains four homologous domains (I-IV), each of which has six transmembrane-spanning segments (S1-S6), an S4 voltage sensor, the Ca\(^{2+}\) selective pore (loops between S5 and S6), and channel gating and binding sites for modulation or drugs (73). There are four subfamilies of voltage-dependent Ca\(^{2+}\) channel (Cav) pore-forming \(\alpha1\) subunits, Cav1-Cav4; each subfamily has several family members or splice variants (74). Cav1.2 or \(\alpha1C\) is the underlying pore-forming \(\alpha1\) subunit of the cardiac L-type Ca\(^{2+}\) channel, which is a member of the Cav1 subfamily and is encoded by \textit{CACNA1C}. Cav1.2 is very sensitive to block by dihydropyridines (DHP) (75). Recently it was reported that gain-of-function mutations in \textit{CACNA1C} underlie the Timothy Syndrome that causes significant QT prolongation, cardiac arrhythmias and sudden death (76;77). Cav\(\beta\)2 is the most prominent Cav \(\beta\) subunits in the cardiac L-type Ca\(^{2+}\) holochannel (78). Cav\(\beta\)2 is a cytosolic protein that binds to \(\alpha1C\) via an interacting region between the DI and DII linker, increasing \(\alpha1C\) surface expression and \(I_{CaL}\) amplitude, and modifying \(I_{CaL}\) kinetics (79). Besides Cav\(\beta\)2, functional cardiac L-type Ca\(^{2+}\) channel complexes also need another accessory subunit, Cav\(\alpha2\delta\) (80), which is a transmembrane accessory subunit. Co-expression of Cav\(\alpha2\delta\) with \(\alpha1C\) can increase \(I_{CaL}\) amplitude and accelerate current activation and inactivation (81;82). There is very limited information regarding the transmural expression of Cav1.2 or Cav\(\beta\)2 and Cav\(\alpha2\delta\) in the left ventricle, except that Gaborit et. al. found higher Cav1.2 transcript expression in epicardium than in endocardium of human left ventricle (83).

### 2.3 K\(^+\) channels

Cardiac K\(^+\) channels can be classified into three main categories: voltage-gated
(Kv) (I\textsubscript{to}, I\textsubscript{Kr} and I\textsubscript{Ks}), inward rectifiers (I\textsubscript{K1}, I\textsubscript{KATP}, etc.) and background K\textsuperscript{+} channels (TASK-1, TWIK-1/2) (84). The voltage-gated K\textsuperscript{+} channels provide the primary repolarizing currents determining AP repolarization. They are largely responsible for AP differences in development, between atrium and ventricle, in different regions of the heart and of the transmural ventricular walls, and between normal and diseased myocardium (84).

2.3.1 Transient outward K\textsuperscript{+} channel, I\textsubscript{to}

The transient outward potassium current I\textsubscript{to} activates and inactivates rapidly on membrane depolarization to potentials positive to approximately \(-30\) mV, is responsible for early phase 1 repolarization and influences the plateau phase 2 of ventricular APs (85). Both activation and inactivation of I\textsubscript{to} are voltage-dependent with half voltage of activation (V\textsubscript{1/2}) around +6 to +20 mV and inactivation V\textsubscript{1/2} around -35 mV in the ventricle (86;87). The activation time constant is on the order of milliseconds; I\textsubscript{to} inactivation time constants range from tens to hundreds of milliseconds (88). According to its recovery kinetics, I\textsubscript{to} is also distinguished as I\textsubscript{to,fast} and I\textsubscript{to,slow} (89). Recovery time constants are on the order of \(~20-100\) ms for I\textsubscript{to,fast} and range from \(~100-1000\) ms for I\textsubscript{to,slow} (90-93). I\textsubscript{to,fast} can also be distinguished from I\textsubscript{to,slow} by different sensitivity to the spider K\textsuperscript{+} channel toxins Heteropoda toxins (HPTXs), which blocks I\textsubscript{to,fast} but not I\textsubscript{to,slow} in nanomolar concentrations (94). I\textsubscript{to} can be blocked by 4-aminopyridine (4-AP) with IC\textsubscript{50} \(~0.2\) mM in rat or \(~0.5\) mM in dog ventricles (86;95).

The density and kinetics of I\textsubscript{to} have significant species and regional differences. I\textsubscript{to} is an important repolarizing current shaping ventricular APs in most species,
except for the guinea pig in which \( I_{to} \) is undetectable and phase 1 early repolarization is absent (96-98). Both \( I_{to,fast} \) and \( I_{to,slow} \) have been reported in human, ferret, mouse and rat (99-104). In rabbit ventricular myocytes, \( I_{to,slow} \) is the prominent transient outward \( K^+ \) current and \( I_{to,fast} \) is not detected (105;106). In dog ventricle, \( I_{to,fast} \) is the major transient outward \( K^+ \) current determining phase 1 of the AP (107;108). The transmural distribution of \( I_{to} \) is strikingly different across the ventricle. \( I_{to} \) density is significantly higher in epicardium and mid-myocardium than in endocardium, and mediates the prominent phase 1 and notch morphology of APs in Epi and M cells (109). Transmural heterogeneity of \( I_{to} \) has been documented in human, dog, rabbit, ferret, feline, rat and mouse (108;110-116). \( I_{to} \) can also influence the overall APD via its interaction with \( I_{cal} \) and delayed rectifier currents. \( I_{to} \) block can prolong or shorten ventricular repolarization depending on secondary changes in other currents, and by affecting plateau duration and voltage \( I_{to} \) can modulate \( Ca^{2+} \) entry and EC coupling (117;118).

Kv4.2, Kv4.3 and Kv1.4 are pore forming \( \alpha \)-subunits underlying cardiac \( I_{to} \) that belong to Kv channel Shaker-related subfamilies (Shaker Kv1.x, Shab Kv2.x, Shaw Kv3.x and Shal Kv4.x) (119;120). Kv channel\( \alpha \)-subunits are like individual six transmembrane-spanning domains found in Nav and Cav that coassemble as tetramers to form functional \( K^+ \) selective Kv channels (121). Each Kv channel \( \alpha \)-subunit contains six transmembrane-spanning segments (S1 to S6) with cytoplasmic N- and C-termini. The S1 to S4 segments are responsible for voltage-sensing, whereas the S5, S6 segments and the P-loop (linker between S5 and S6) are critical for forming the channel pore. For Kv1.x and Kv4.x \( \alpha \)-subunits, most evidence suggests that they can coassemble as heterotetramers within the same subfamily (within Kv1.x or within
Kv4.x) to form functional channels (122;123). According to their biophysical and pharmacological properties, it is now known that Kv4.2 and Kv4.3 (encoded by KCND2 or KCND3 genes) α-subunits, generate the rapidly activating, inactivating and recovery transient outward K⁺ current that can be blocked by HPTXs and 4-AP, underlie \( I_{\text{to,fast}} \). Kv1.4 (encoded by KCNA4) produces transient outward K⁺ currents that display rapid activation and inactivation, very slow recovery kinetics and block by 4-AP (but not HPTXs) which underlies \( I_{\text{to,slow}} \) (102;119;124-126). In rodents, Kv4.2 is the major α-subunit for \( I_{\text{to,fast}} \); however in larger mammals such as dog and man, Kv4.3 is the primary α-subunit contributing to \( I_{\text{to,fast}} \) (127). One or more of these three α-subunits are expressed in the ventricles from various species including human, but not in guinea pig (97;127;128). The expression levels of Kv4.2, Kv4.3 and Kv1.4 vary in different species and regions of the heart, which contributes to the diverse species and regional distribution of \( I_{\text{to}} \) (127;129). In dog and human ventricles, Kv4.3 expression is somewhat larger in epicardium (Epi) than in endocardium (Endo), paralleling the transmural gradient of \( I_{\text{to,fast}} \) (130;131). Kv1.4 mRNA levels are around 16% that of Kv4.3 mRNA in canine left ventricle, corresponding to little \( I_{\text{to,slow}} \) seen in dog (132). In ferret heart, Kv4.2 protein levels are more highly expressed in LV Epi than in LV Endo, Kv4.3 protein distributes evenly across the LV wall and Kv1.4 protein is expressed more strongly in LV Endo than in LV Epi, contributing to the transmural distribution of \( I_{\text{to}} \) (100). In rat and mouse hearts, there is a similar transmural heterogeneity of Kv4.2 expression (LV Epi > LV Endo) that contributes to the transmural gradient of \( I_{\text{to,fast}} \) in rodent LV (101;102;133-136).

Native functional cardiac \( I_{\text{to}} \) requires both pore-forming α-subunits and regulatory accessory subunits. Accessory subunits that can interact with cardiac
Kv1.4, Kv4.2 and Kv4.3 and importantly regulate α-subunit function are Kvβ1 and Kvβ2 (both are encoded by \( \text{KCNAB} \) genes), KChAP (\( \text{K}^+ \) channel accessory protein, encoded by \( \text{PLASS} \) gene) and KChIP2 (\( \text{K}^+ \) channel interacting protein 2, encoded by \( \text{KCNIP2} \)) (119;137-139). Kvβ1 and Kvβ2 are cytosolic proteins. They interact with \( I_{\text{to}} \) α-subunits at the N-terminals to increase Kv1.4, Kv4.2 and Kv4.3 cell surface expression, accelerate Kv1.4 activation and inactivation kinetics and slow recovery from inactivation of Kv1.4 (119;140-144). KChAP belongs to the family of transcription factor-binding proteins, and functions as a chaperone protein and has been shown to increase Kv4.3 channel cell membrane expression without affecting channel kinetics (137;145). KChIP2 is the main cardiac isoform of KChIPs that belong to a family of four EF-hand motif-containing Ca\(^{2+}\)-binding proteins including recoverin, neuronal calcium sensor-1, etc. (146). KChIP2 is a cytosolic protein and specifically interacts with Kv4 α-subunits at the N-terminal (147). Coassembly of KChIP2 and Kv4 α-subunits increases Kv4.2 and Kv4.3 cell surface expression, slows current inactivation, speeds recovery kinetics and shifts the voltage dependence of activation (147;148). The mRNA and protein expression of KChIP2 is homogeneous across the ventricular wall of mouse and rat hearts (149;150). In dogs and humans, there is a transmural gradient of KChIP2 across the ventricular wall; mRNA and protein expression of KChIP2 is higher in epicardium and midmyocardium than in endocardium (130;151;152). Knockout of KChIP2 leads complete loss of \( I_{\text{to}} \) and prolongation of AP, and confers susceptibility to ventricular tachycardia in mouse heart (586).

### 2.3.2 Delayed rectifier \( \text{K}^+ \) channels, \( I_{\text{Kr}} \) and \( I_{\text{Ks}} \)
Compared to the above mentioned cardiac ionic currents, the delayed rectifier K⁺ currents activate more slowly; therefore, they were designated “delayed” currents. Rapidly (I_{Kr}) and slowly activating (I_{Ks}) delayed rectifier K⁺ currents are very important outward currents responsible for phase 3 repolarization of APs in most areas of the heart, notably so in the ventricles (153). I_{Ks} has been implicated in rate-dependent APD shortening (154). Alterations in I_{Kr} and/or I_{Ks} due to genetic or pathological causes can significantly influence ventricular repolarization and function (155;156).

I_{Kr} activates faster and at more negative potentials than I_{Ks}: activation V_{1/2} is around -20 mV for I_{Kr} and +24 mV for I_{Ks}; (157) time constant is in the order of several tens or hundreds of milliseconds for I_{Kr} and of seconds for I_{Ks} (158). The I-V (current–voltage) relation of I_{Ks} is linear as it is non-inactivating (159). However, I_{Kr} inactivates more rapidly than it activates at positive membrane potentials, yielding a non-linear bell-like I-V relation with peak currents at around 0 to 10 mV, which accounts for its typical inward rectification (157;159;160). Deactivation kinetics of I_{Kr} vary among different species, but are slower than for I_{Ks} (161). Pharmacological properties also distinguish between I_{Kr} and I_{Ks}. I_{Kr} is selectively blocked by the methanesulfonanilide class III antiarrhythmic agents such as E-4031, dofetilide, and D-sotalol (157;162). I_{Kr} can also be blocked by the class 1A drug quinidine, the class 1B drug mexiletine and the class 1C flecainide (163). I_{Ks} is selectively blocked by chromanol 293B (164), the benzodiazepine L-735,821 (165), the diuretic indapamide (166) and the new chromanol derivative and potent I_{Ks} blocker HMR 1556 (167).

Both I_{Kr} and I_{Ks} are expressed in guinea pig, rabbit, dog and human ventricles and are the prominent repolarizing currents in these species (168-171). In adult rodent
ventricles, however, their densities are very low or undetectable (172;173).

The pore-forming $\alpha$-subunits underlying $I_{Kr}$ and $I_{Ks}$ are HERG (human ether-a-go-go-related gene, encoded by $KCNH2$) and KvLQT1 (encoded by $KCNQ1$) Kv channel subfamilies (177). As Kv channels, the basic structures of HERG and KvLQT1 are similar to $I_{io}$ $\alpha$-subunit structures described in 2.3.1. Four KvLQT1 proteins coassemble as homotetramers to form a functional channel (178). It doesn’t form heterotetramers with other $KCNQ$ family members (600). An N-terminal truncated isoform 2 of KvLQT1 splice variants is also present in the human heart, which has dominant-negative effects on the full length KvLQT1 (isoform 1) and the level of its expression can affect the amplitude of KvLQT1 current (179). KvLQT1 tetramer interacts with its main accessory subunit minK (minimal $K^+$-channel protein, encoded by the $KCNE1$ gene) to form functional cardiac $I_{Ks}$ (183;184). Coexpression of minK with KvLQT1 increases KvLQT1 current amplitude, slows activation, abolishes inactivation, negatively shifts voltage dependence and increases KvLQT1 channel unitary conductance (183;185-187). MinK belongs to the KCNE $\beta$-subunit family that has five members encoded by $KCNE1$-$5$ genes and are all expressed in the human heart with $KCNE1$ is the predominant one (601). All KCNE subunits are small single-transmembrane proteins with an extracellular N-terminus and an intracellular C-terminus (602). In heterologously expressed systems, KvLQT1 or $KCNQ1$ channels can associate with all $KCNE1$-$5$ proteins, resulting in a $\beta$-subunit-specific change of the current characteristics (602). KCNE3 mutation is recently found to be associated with LQTS (603).

HERG channels are also homotetramers (177). Current conducted by HERG is largely similar to native cardiac $I_{Kr}$ in terms of inward rectification and sensitivity to
methanesulfonanilide drugs, but is different in its slower activation and deactivation kinetics than cardiac $I_{Kr}$ (604). Splice variants of HERG (N- or C-terminal truncation isoforms) have been reported to be present in the heart and coexpression of these isoforms with the main isoform (HERG1) alters HERG channel gating or expression (180;181). Native $I_{Kr}$ might result from homomultimers or heteromultimers of HERG1 with these splice variants. MiRP1 (MinK-related peptide 1, encoded by the $KCNE2$ gene) can interact with HERG to produce currents close to native $I_{Kr}$ therefore was considered to be a functionally important accessory subunit underlying cardiac $I_{Kr}$ (182). However, there is some controversy in MiRP1 as an major $\beta$-subunit for cardiac $I_{Kr}$ since Weerapura et al found that the current resulted from coexpression of MiRP1 and HERG couldn't recapitulate native $I_{Kr}$ (605). Coexpression of MiRP1 with HERG decreases peak HERG current ($I_{HERG}$), slows activation, hyperpolarizingly or positively shifts activation $V_{1/2}$, increases the rate of inward HERG channel deactivation but not the more physiological outward deactivation (182;605). Besides MiRP1, HERG is found to be able to associate with minK that affects HERG trafficking (189;606;607).

Inherited mutations in HERG (LQT2) or MiRP1 (LQT6) causing defects in $I_{Kr}$ and in KvLQT1 (LQT1) or minK (LQT5) causing defects in $I_{Ks}$ are all associated with congenital long QT syndromes (178;190;191).

A transmural gradient of $I_{Ks}$ density is present in canine LV, as Epi and Endo cells have larger $I_{Ks}$ than M cells, contributing to longer APD in M cells (168). The protein level of KvLQT1 is found to be higher in Epi than in Mid whereas MinK protein level is less in Epi than in Mid in both canine and human ventricle (192;193). The mRNA level of KvLQT1 isoform 1 is reported to be homogeneously expressed among Epi,
M and Endo of human ventricle, however its isoform2 (a dominant negative splice variant) is found to be more abundant in Mid, and is suggested to be responsible for the less $I_{Ks}$ density in M cell than other two layers (179). Apico-basal different distribution of KvLQT1 and MinK is also observed in canine and human ventricles as higher apical expressions of both subunits (194). $I_{Kr}$ density has been reported to be comparable among the three layers (168), but has also been reported to be larger in Epi than in Endo of canine LV, possibly contributing to shorter APDs in Epi (174). In canine and human LV, HERG expression has been found to be greater in Epi than in Endo or in Mid (174;192). In rabbit ventricles, $I_{Ks}$ density has been found to be greater in Epi than in Endo and $I_{Kr}$ density similar between the two layers (175). $I_{Kr}$ and $I_{Ks}$ densities are smaller in guinea pig ventricular Endo cells than in Epi and midmyocardial cells (176).

2.3.3 Inward rectifier K$^+$ channel, $I_{K1}$

The most important function of cardiac inward rectifier K$^+$ current ($I_{K1}$) is to maintain the cellular resting membrane potential. It also contributes to terminal repolarization. $I_{K1}$ easily conducts inward current. It carries outward current at potentials more positive than the K$^+$ equilibrium potential (-90 mV); however, its outward conductance decreases at progressively depolarized membrane potentials, reflecting "inward rectification" (195). $I_{K1}$ rectification is attributed to block of the channel pore by intracellular Mg$^{2+}$, Ca$^{2+}$ and polyamines (putrescine, spermidine and spermine) (196). $I_{K1}$ can be blocked by extracellular Ba$^{2+}$ and intracellular Cs$^+$ (197). $I_{K1}$ is more prominent in the ventricle compared to atrium and is absent in nodal cells (198-202). It shows no transmural differences (203).
There are seven known pore-forming α-subunit subfamilies for inward rectifier K⁺ channels (Kir1 to 7) (122). Kir channel pore-forming α-subunits contain only two transmembrane segments (M1 and M2) connected by a pore-forming P-loop (H5), and cytoplasmic N- and C-termini, which are similar to the S5, S6 and P-loop regions of Kᵥ channels (204). Functional Kir channels are also tetramers of Kir α-subunits. Heterotetramers containing coassembled Kir2.1, Kir2.2, Kir2.3 and Kir2.4 channels are believed to underlie functional cardiac Iᵥ (205-209), whereas Kir3.1/3.4 coassembled heterotetramers are known to form cardiac muscarinic receptor activated Iᵥ, and Kir6.2 are pore-forming α-subunits of I(var) (599). Kir2.1 is quantitatively the most important subunit underlying Iᵥ. Mutations in the Kir2.1 encoding gene KCNJ2 have been linked to Anderson Syndrome, an inherited disorder that is often life-threatening due to ventricular tachyarrhythmias (210). Kir2.1 and Kir2.3 are expressed at higher levels in the ventricle than in the atrium (200-202).

2.4 Other channels and transporters

Besides the above-mentioned voltage-gated Na⁺, Ca²⁺ and K⁺ channels that are important to ventricular repolarization, some other ionic currents, transporters and exchangers play important roles in the AP and may also have transmural differences affecting TDR under normal or pathological conditions.

2.4.1 Na/Ca exchanger (NCX)

The cardiac Na⁺-Ca²⁺ exchanger (NCX) plays a pivotal role in cardiac EC coupling, being the main pathway for Ca²⁺ extrusion (211). [Ca]i is dynamically regulated during each heart beat as reflected by the [Ca]i transient (212). After
ICaL-induced SR Ca\(^{2+}\) release and myocyte contraction, it is essential for cytosolic [Ca]\(_i\) to decline, allowing Ca\(^{2+}\) dissociation from the myofilaments. [Ca]\(_i\) decreases because of Ca\(^{2+}\) reuptake by SR Ca\(^{2+}\)-ATPase (SERCA) and Ca\(^{2+}\) extrusion, predominantly via NCX (213). NCX is an electrogenic membrane ion counter-transporter. It has two modes of ion transport: forward (Ca\(^{2+}\) efflux) or reverse (Ca\(^{2+}\) influx) mode. Generally, it exchanges one Ca\(^{2+}\) ion for three Na\(^{+}\) ions, moving one net positive charge in the direction of Na\(^{+}\) transport (214). The direction and activity of NCX depends on the internal and external concentration of both Na\(^{+}\) and Ca\(^{2+}\), as well as on the membrane potential. High [Ca]\(_i\) favors forward mode (inward NCX current I\(_{NCX}\)) whereas positive membrane potential and high [Na]\(_i\) favor reverse mode (outward I\(_{NCX}\)) (214;215). I\(_{NCX}\) can influence the ventricular AP, with the inward I\(_{NCX}\) activating during the plateau and at the end of repolarization, tending to maintain the plateau and prolong APD (213). I\(_{NCX}\) plays a role in EADs and delayed afterdepolarizations (DADs) under pathological conditions (216;217). NCX1.1 is the main cardiac isoform of NCX protein (218;219). NCX expression is homogeneous across the left ventricle (220). However, I\(_{NCX}\) shows significant functional transmural heterogeneity. Zygmunt et. al. found that I\(_{NCX}\) densities are larger in canine midmyocardium than in Epi or Endo, potentially contributing to longer APD in M cells (221). Xiong et. al. reported transmural I\(_{NCX}\), with Epi>M cell>Endo function in normal ventricles that is disrupted in heart failure (222).

### 2.4.2 Na\(^{+}\)/K\(^{+}\) ATPase

The cardiac Mg\(^{2+}\)-activated Na\(^{+}\)/K\(^{+}\) ATPase is responsible for maintaining physiological Na\(^{+}\) and K\(^{+}\) concentration gradients in cardiomyocytes, essential for
normal cardiac electrical activity. Na⁺/K⁺ ATPase is a sarcolemmal ATP-dependent enzyme transporter that transports three intracellular Na⁺ ions to the extracellular compartment and moves two extracellular K⁺ ions into the cell; therefore, it is electrogenic, producing a small outward current $I_P$ (223). It functions to remove intracellular Na⁺ to restore the intracellular Na⁺ concentration ([Na]ᵢ) level after $I_{Na}$ induced rapid depolarization. It is sensitive to [Na] and is also voltage-dependent (224). Inhibition of the Na⁺/K⁺ ATPase pump prolongs APD (225;226), indicating that it contributes to cardiac AP control. Transmural differences in Na⁺/K⁺ ATPase pump activity have been reported recently. The outward current $I_P$ due to Na⁺/K⁺ ATPase pump function has a transmural gradient under experimental conditions: Epi < M cells < Endo, which may be due to the transmural gradient of [Na]ᵢ: Epi < M cell < Endo (227). The Na⁺/K⁺ ATPase pump α-subunit (α₁ isoform) is homogeneously expressed across the rabbit left ventricle (228).

2.4.3 $I_{Cl}$

There are at least three types of chloride currents that have been found in the ventricle: protein kinase A (PKA)-dependent Cl⁻ current ($I_{Cl,PKA}$), Ca²⁺-dependent Cl⁻ current ($I_{Cl,Ca}$) and a swelling-induced Cl⁻ current ($I_{Cl,Swell}$) (229). They are usually outward currents carried by the inward movement of Cl⁻ and are controlled by different mechanisms. Activation of chloride currents can shorten APD (230). $I_{Cl,Ca}$ is activated by an increase of [Ca]ᵢ due to Ca²⁺ entry through $I_{Ca,L}$ and Ca²⁺ induced Ca²⁺ release from the SR, and is believed to contribute to phase 1 early repolarization and the generation of DADs (231;232). $I_{Cl,Ca}$ is similar between Epi and Endo (233). $I_{Cl,PKA}$ has been shown to be more abundant in epicardial than endocardial ventricular
cells in guinea pig and rabbit (234;235). Several Cl− channel gene families are identified in the heart (592). \textit{CLCA-1}, \textit{TMEM16} and \textit{Bestrophin} are all candidate genes for I\textsubscript{Cl,CA} (593;594). \textit{CFTR} (the cystic fibrosis transmembrane conductance regulator) is responsible for I\textsubscript{CLPKA} (595). \textit{ClC-3} (a member of the ClC voltage-gated Cl− channel superfamily) is responsible for I\textsubscript{CL,swell} (596).

\textbf{2.4.4 I\textsubscript{KATP}}

ATP-sensitive K\textsuperscript{+} current (I\textsubscript{KATP}) is a non-voltage-gated weak inward rectifier K\textsuperscript{+} current (236). I\textsubscript{KATP} is carried by Kir6.x channels, and its permeability is suppressed by intracellular ATP but enhanced by nucleotide diphosphates such as ADP (236;237). It is a functionally important current believed to link cellular metabolism and membrane potential. Under normal physiological conditions, I\textsubscript{KATP} seems not to contribute to ventricular repolarization, since it is not active at physiological concentrations of intracellular ATP (238). However, under conditions of metabolic stress like hypoxia and ischemia, I\textsubscript{KATP} is activated and shortens APD while increasing heterogeneities in ventricular repolarization, thereby creating a substrate for re-entrant arrhythmias (239). I\textsubscript{KATP} activity is transmurally heterogeneous with epicardial I\textsubscript{KATP} displaying greater ATP sensitivity, causing greater APD shortening in Epi under ischemia (240;241). Cardiac sarcolemmal I\textsubscript{KATP} channels are formed by the heteromeric assembly of pore-forming Kir6.2 and regulatory sulfonlurea receptors SURx (ATP-binding cassette proteins) such as SUR1 or SUR2A in the heart (237). Transmural distribution of Kir6.2 or SUR1 transcript levels has been observed in human ventricle (591). Kir6.2 transcript is lower in Endo than in Epi or Mid, SUR1 transcript is more abundant in Mid than in Endo (591).
3. Mechanisms of ventricular arrhythmias due to impaired ventricular repolarization

3.1 Overview

Ventricular tachyarrhythmias (VTs: ventricular ectopic rhythms faster than 100 bpm) and/or ventricular fibrillation (VF: chaotic ventricular firing with no cardiac output) are contributors to sudden cardiac death (SCD), of which the incidence is as high as 0.1% of the world population annually (242). Alterations in ventricular ion channels and/or ion transporters due to disease-induced electrical remodeling or genetic channelopathies can lead to impaired ventricular repolarization (e.g., excessive prolongation or abbreviation of ventricular APD) and cause lethal ventricular tachyarrhythmias (243-248). VT can be classified as monomorphic (MVT) or polymorphic ventricular tachycardia (PVT) (249). PVTs, such as Torsades de Pointes (TdP), are potentially lethal VTs that can degenerate into VF and lead to SCD (244;250;251). The most important underlying mechanisms associated with VTs are triggered activity such as early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs), and reentry (245;252;253). In general, VTs are initiated by spontaneous firing(s) at one or several sites in the ventricle due to triggered activity or abnormal automaticity, and are maintained by reentry often involving abnormal TDR.

3.2 Cellular mechanisms

Afterdepolarizations can give rise to triggered depolarizations causing premature systoles and sustained VTs (254). Afterdepolarizations are abnormal depolarizations
that appear during or after normal repolarization. There are two types of afterdepolarizations: early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs) as shown in Figure 4. Both EADs and DADs are capable of initiating and maintaining cardiac arrhythmias.

![Figure 4. Afterdepolarizations. A, early afterdepolarizations (EADs). B, delayed afterdepolarizations (DADs). TA, triggered activity. Adapted from Figure 2 of reference 242 and used with permission.]

### 3.2.1 EADs

EADs can occur during plateau phase 2 (potentials generally between -10 and -30 mV) or repolarization phase 3 (potential between -30 and -60 mV) (254;255). EADs are favored by prolonged APs caused by abnormalities in the ionic currents responsible for ventricular repolarization. Experimental and mathematical modeling studies suggest that EADs occurring at phase 2 result from reactivation of $I_{Ca,L}$ after a prolonged AP plateau (256-258). The $I_{Ca,L}$ window current falls in the AP plateau voltage range where both $I_{Ca,L}$ activation and inactivation can overlap as discussed in Section 2.2. A prolonged AP plateau provides enough time for $I_{Ca,L}$ to recover from voltage- and calcium-dependent inactivation, leading to $I_{Ca,L}$ reactivation, increased
inward current that can further depolarize cardiac membrane potential, and therefore leads to EAD formation (259;260). Intracellular Ca$^{2+}$ overload and Ca$^{2+}$ release from the SR are not essential for phase 2 EADs (257). EADs that arises at more negative phase 3 voltages may be caused by reactivation of I$_{Na}$. EADs can result from excessive AP prolongation due to opening of late I$_{Na}$ and delayed I$_{Na}$ inactivation by pharmacological interventions such as veratridine or ATXII (sea anemone toxin), or SCN5A gene mutations seen in LQT3 (245;261-263). TTX but not the I$_{CaL}$ blocker Cd$^{2+}$ completely eliminated veratridine induced EADs in guinea pig LV myocytes (264). Inward Na$^+$ current carried by NCX may also be involved in phase 3 EADs (265).

3.2.2 DADs

DADs happen after repolarization is completed, during phase 4. The occurrence of DADs is associated with spontaneous diastolic SR Ca$^{2+}$ release, and increased transient inward currents (267-269). Intracellular Ca$^{2+}$ overload as seen with digoxin toxicity can cause diastolic SR Ca$^{2+}$ release, which can also result from leaky SR Ca$^{2+}$ release channels (ryanodine receptors). The transient inward currents for DAD generation result from Ca$^{2+}$-dependent opening of non-specific cation channels, or high [Ca$^{2+}$]i activated inward I$_{NCX}$ (270-272). A DAD can be subthreshold, or can reach threshold potential and result in an AP, producing extrasystoles as shown in Figure 4B (273).

3.3 Reentry

As shown in Figure 5, the electrical impulse normally dies out after normal
activation of the heart.

**Figure 5. Normal and reentrant impulse propagation.** 1) Unidirectional block. 2) Multiple pathways. 3) Slowing of conduction to allow reentry into previously refractory tissue. Arrows are directions of electrical impulse propagation

Reentry arises when a propagating electrical impulse is blocked in one of two potential conducting pathways (unidirectional block), followed by successful conduction in one direction with failure in the opposite direction (Figure 5, 1)). Propagation from the alternative conducting pathway (Figure 5, 3)) reenters the previously refractory region (Figure 5, 1)), reactivates this region, and then returns to the previous conducting pathway, forming a reentrant circuit (252;253). When the cycle length (CL) is longer than the longest refractory period in the conducting pathway, the reentrant circuit is able to fire repetitively (252;253;274). Factors favoring reentry are premature impulses (that initiate reentry), refractoriness heterogeneity (providing the basis for unidirectional block, e.g. increased TDR), and slow conduction (e.g. caused by abnormalities in I_{Na} or connexins), allowing enough time for the previously refractory pathway to recover by the time the returning
impulse reaches the previously blocked region (252;275;276). The refractory period, the period of time during an AP that a new AP can’t be re-initiated, is a key determinant of reentry (252;253;277). Abnormalities in cardiac ion channels and/or in structure of ventricular muscle can produce a substrate for reentry (276;278). Reentry can occur in a single circuit, producing MVT, or multiple unstable reentrant circuits simultaneously, producing more irregular activity such as PVT or VF (252;278). During VF, uncoordinated regional contraction caused by multiple unstable reentry reduce cardiac output to near zero, causing SCD (279).

3.4 Transmural dispersion of repolarization (TDR) and ventricular arrhythmias

Augmented TDR is an important substrate for maintenance of ventricular reentry, facilitating unidirectional block (253;277). Ventricular APs are spatially heterogeneous, with variation produced by regional differences in the densities of membrane ion channels setting the physiologic TDR. The ion channel properties of the M cell (less $I_{Ks}$ more late $I_{Na}$ and $I_{NCX}$ than Epi and Endo (8)) result in a decrease in net repolarizing current, which accounts for the longer and steeper rate-dependent APD in M cells. Reduced repolarizing current sensitizes the M cell to conditions that further reduce net repolarizing current, such as congenital or acquired LQTS (280-283). Disproportionate M cell APD prolongation under repolarization stress increases TDR, which can forms zones of increased refractoriness in the M cell region that are responsible for conduction block and self-sustained intramural re-entrant circuits underlying TdP (284;589). i.e., the M cell contributes to the maintenance of TdP. The M cell also more readily develops EADs or DADs than Epi or Endo upon exposure to $I_{Kr}$ blockers or Ca$^{2+}$ overload, contributing to the genesis of
TdP (285;286). The transmural heterogeneity of ventricular repolarization is responsible for the T wave on the surface ECG and the $T_{\text{peak}}$ (corresponding to repolarization of Epi) to $T_{\text{end}}$ (repolarization of M cells) interval may provide a clinically useful measure of TDR and arrhythmic risk (282;287-289).

### 3.5 Torsades de Pointes

Torsades de Pointes (TdP), a form of polymorphic ventricular tachycardia, was first described by Dessertenne in 1966 as a characteristic twisting of QRS complexes around the isoelectric ECG baseline (Figure 6) (292). Average ventricular rates during TdP range from 150 beats per minute (bpm) to 250 bpm (293). TdP is an uncommon lethal ventricular arrhythmia with higher prevalence in women than in men (294;295). It is associated with a fall in arterial blood pressure, inducing syncope, or can deteriorate into VF, leading to SCD.

![Figure 6. Example of self limiting TdP (A) or TdP leading to VF (B). (Adapted from reference (296) Figure 5A and used with permission)](image)
TdP is closely linked to abnormal ventricular repolarization and marked prolongation of the QT interval (244;297). The underlying mechanisms for TdP involve EADs and increased TDR (244;298;299). A variety of conditions can cause excessive QT prolongation and predispose the heart to TdP incidence as listed in Table 1. (300).

### Table 1: Factors associated with an increased tendency toward TdP.

<table>
<thead>
<tr>
<th>Factor</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital LQTS</td>
<td>Hypomagnesemia</td>
</tr>
<tr>
<td>Acquired LQTS</td>
<td>Hypokalemia</td>
</tr>
<tr>
<td>Class IA antiarrhythmics</td>
<td>Hypocalcemia</td>
</tr>
<tr>
<td>Class III antiarrhythmics</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>Non-cardiac drugs</td>
<td>Acidosis</td>
</tr>
<tr>
<td>Female gender</td>
<td>Subarachnoid hemorrhage</td>
</tr>
<tr>
<td>Heart failure</td>
<td>Etc.</td>
</tr>
<tr>
<td>Left ventricular hypertrophy</td>
<td></td>
</tr>
<tr>
<td>Bradycardia</td>
<td></td>
</tr>
</tbody>
</table>

4. **Paradigms of impaired ventricular repolarization**

Prolongation of the QT interval results from the prolongation of ventricular repolarization, which is accomplished by either a reduced outward K⁺ current ($I_{Kr}$, $I_{Ks}$, $I_{K1}$ or $I_{Ko}$) or increased inward currents ($I_{CaL}$, late $I_{Na}$). Impaired ventricular repolarization is observed in diverse settings including congenital and drug-induced LQTS or with cardiac diseases like congestive heart failure (HF). Traditionally, in humans rate corrected QT interval (QTc) values in excess of 440 ms are considered prolonged; but in woman, QTc values up to 460 ms can still be normal because of baseline gender differences (301).
4.1 LQTS

Long QT syndrome (LQTS) is characterized by an excessively prolonged QT interval on ECG with clinical manifestation of recurrent syncope or SCD due to TdP (302-304). LQTS is either inherited (most commonly caused by mutations of ion channels responsible for cardiac repolarization) or acquired (due to drugs or other factors that inhibit cardiac $K^+$ channels) (305).

4.1.1 Congenital LQTS

Since Keating and colleagues linked the congenital LQTS to mutations in the genes encoding ion channel proteins in 1995 and 1996 (306), there are now at least ten autosomal dominant genotypes of congenital LQTS, as listed in Table 2 (244;284). Two autosomal recessive genotypes of LQTS, the Jervell-Lange-Neilsen syndrome, are caused by mutations in genes encoding $I_{Ks}$ subunits (307). The prevalence of congenital LQTS is estimated to be around 1/10000 in the population (one gene carrier in 10,000 population) or 1/2500 in live births (303;307). All the LQTS genes identified so far encode cardiac ion channel subunits or proteins involved in ionic channel function (Table 2). LQT1, LQT2 and LQT3 are the most prevalent forms of LQTS (307;308). LQT1 is caused by a mutation in $KCNQ1$, encoding $I_{Ks}$, and makes up about 40-55% of cases of congenital LQTS (309). LQT2 is due to mutations of the HERG ($KCNH2$) gene, leading to reduced $I_{Kr}$ and make up about 35-45% of congenital LQTS (310). LQT3 is the third most prevalent LQTS, making up around 10% of cases; and is due to mutations in the $SCN5A$ gene, causing delayed $I_{Na}$ inactivation and increased late $I_{Na}$ (311).
Table 2: Congenital LQTS

<table>
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<tr>
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<th>Gene</th>
<th>Locus</th>
<th>Classification</th>
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<tr>
<td>IKs</td>
<td>KCNE1, minK</td>
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<td>LQTS-JLN2</td>
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<tr>
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<td>17q23</td>
<td>LQT7 (Anderson-Tawil syndrome)</td>
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<tr>
<td>INa</td>
<td>SCN5A, Navl.5</td>
<td>21q22</td>
<td>LQT5</td>
</tr>
<tr>
<td>IKr</td>
<td>KCHN2, HERG</td>
<td>7q35</td>
<td>LQT2</td>
</tr>
<tr>
<td>IKs</td>
<td>KCNQ1, KvLQT1</td>
<td>11p15</td>
<td>LQT1</td>
</tr>
</tbody>
</table>

LQT1-10 are autosomal dominant without deafness (also termed Romano-Ward syndrome). JLN: Jervell-Lange-Neilsen syndrome (autosomal recessive with deafness) arises in individuals who inherit abnormal alleles from both parents, and is much rarer. (Adapted from Table 1 of reference (284) and used with permission)

The autosomal dominant LQT1-10 syndromes are often associated with episodes of syncope and/or SCD due to TdP, commonly occurring with adrenergic stimulation or emotional stress; whereas the autosomal recessive Jervell-Lange-Neilsen syndrome usually includes deafness, QT prolongation and SCD in childhood (307).

4.1.2 Acquired or drug induced LQTS

Acquired LQTS is caused by drugs or other factors (e.g. hypokalemia, hypomagnesemia, hypocalcemia) that prolong APD, often with co-existing risk factors (312;313). Drug induced LQTS is the most common form of acquired LQTS, and is a major concern for clinical practice and drug development (Table 3) (251;314).
Almost all drugs that cause acquired LQTS block $I_{Kr}$ (250). HERG has unique high-affinity binding sites at aromatic residues close to the pore region, and lack two proline residues in this area, making it uniquely susceptible to drug block (317). Blockade of HERG trafficking to the membrane can also cause acquired LQTS (318). Interaction of HERG with the membrane protein KCR1 ($K^+$ channel regulator protein 1, a putative 12 transmembrane domain protein that modulate hERG) can also modify the sensitivity of HERG to blockade by dofetilide, sotalol and quinidine (319). Enhancing Na entry into cells during the plateau has been suggested to be the mechanism of ibutilide-induced repolarization delay, but the drug also blocks $I_{Kr}$ (320;321). Clinically, drug induced TdPs are often associated with underlying risk.
factors, e.g., female gender, acquired heart diseases like HF or hypertrophy, hypokalemia, hypomagnesemia, bradycardia, atrial fibrillation and subclinical congenital LQTS (313;322). These conditions reduce repolarization reserve increasing vulnerability to drug induced QT prolongation, TdP and SCD (323).

4.1.3 Gender differences in LQTS

There is a 2-3 times higher incidence of drug-induced TdPs and greater drug-induced QT prolongation in women than in men (324-334). The risk of cardiac events is also higher in women than in men with LQT1 and LQT2 (335). The underlying mechanisms remain unclear. Women have longer baseline QTc intervals than men after puberty (329-335). Gender differences in LQTS may due to underlying gender differences in cardiac repolarization. A more detailed literature review of the control of cardiac repolarization by gender will be discussed in 5.1.

4.2 Heart failure

Heart failure (HF) is a syndrome resulting from impaired pump function that interferes with the ability of the heart to provide sufficient blood and oxygen for the body. HF is the leading cause of death worldwide with an annual mortality rate of 10% (336). SCD accounts for almost 50% of total HF-related mortality, and often results from VTs including TdP (337). Prolongation of ventricular APD is a consistent finding in HF patients and experimental models (338-341). Increased transmural APD heterogeneity is observed, with preferential APD prolongation in M cells (342).

Expression of a variety of ion channels are altered in HF, contributing to abnormal ventricular repolarization (343). Inward late $I_{Na}$ is increased and
inactivation is slowed in HF (344;345). $\mathbf{I_{CaL}}$ is largely unchanged in ventricles of HF patients and experimental animals (340;346;347). However, reduced $\mathbf{I_{CaL}}$ has also been reported in human HF (348). $\mathbf{I_{NCX}}$ is upregulated, which may compensate for impaired cytosolic $\mathbf{Ca^{2+}}$-removal due to reduced SERCA function (343;349-353). Increased $\mathbf{I_{NCX}}$ is greater in Mid and Endo from HF animal hearts (349). $\mathbf{Ca^{2+}}$ handling is significantly altered in HF. SR $\mathbf{Ca^{2+}}$ stores are reduced and diastolic $[\mathbf{Ca^{2+}}]_i$ is increased, contributing to impaired contractile function (354). In contrast to the upregulation of inward currents (late $\mathbf{I_{Na}}$ and $\mathbf{I_{NCX}}$), outward $\mathbf{K^+}$ currents are commonly downregulated in HF. A reduction in $\mathbf{I_{to}}$ is one of the most consistent findings in HF, both in patients and in experimental-HF animal hearts (340;355-357). HF-related $\mathbf{I_{to}}$ reduction can be explained by downregulation of Kv4.3 at the mRNA and protein levels (358-362). The reduction of $\mathbf{I_{to}}$ and Kv4.3 expression is observed in both Epi and Endo of HF-animal hearts (340;359;360). Decreased expression of $\beta$-subunit KChIP2 also occurs and may contribute to $\mathbf{I_{to}}$ downregulation (361;363). $\mathbf{I_{Kr}}$ and its $\alpha$-subunit HERG are reported to be unchanged in most studies (340;356;359). However, reduced $\mathbf{I_{Ks}}$ has been observed in diseased human right ventricle and in tachypacing-induced HF-animal models (340;356;359;361;364). Downregulation of $\mathbf{KvLQT1}$ and minK in HF likely contributes to reduced $\mathbf{I_{Ks}}$ (359). $\mathbf{I_{K1}}$ reduction is found in ventricular cardiomyocytes from terminally-failing human hearts and in several HF-animal models, and can be explained by downregulation of Kir2.1 (340;353;355;356;361;365).

The mechanisms responsible for ion-channel remodeling in HF are complicated and remain unclear. HF also involves neurohumoral (sympathetic and renin-angiotensin) systems designated to restore blood pressure, increase heart rate,
increase cardiac output and improve cardiac contractility (246;337). Activation of the sympathetic nervous and the renin-angiotensin-aldosterone (RAAS) systems can also alter cardiac electrophysiology (246). Sustained β-adrenergic stimulation down-regulates $I_{Ks}$ and $I_{K1}$ (366). Angiotensin II and aldosterone can prolong ventricular APD by enhancing late $I_{Na}$ and $I_{CaL}$ and decreasing $I_{to}$ and $I_{K1}$ (367-373).

Arrhythmogenic mechanisms in HF include EADs (due to excessive APD prolongation), DADs (due to abnormal SR function and diastolic [Ca$^{2+}$]i elevation) and TDR alteration, all of which have been reported in failing ventricles (340-342;353). The net repolarizing current reduction in HF decreases repolarization reserve and may lead to exaggerated repolarization abnormalities under circumstances that further affect ventricular repolarization, such as with congenital LQTS, electrolyte disturbances, QT-prolonging drugs or bradycardia.

5. **Control of ventricular repolarization**

5.1 **Gender**

More than eighty years ago, Bazett first reported sex-related differences in the ECG (374). Since then, it has long been known that women have 2-6% longer QTc intervals than men (375;376). This gender difference in QTc interval didn’t arouse much attention until the mid-1990s, when many clinical studies reported a striking gender difference in the occurrence of the life-threatening arrhythmia TdP, with women having a much higher incidence of TdP than men after the use of antiarrhythmic drugs (377). Gender differences in TdP occurrence are associated with greater QT prolongation in women than men after administration of drugs that prolong the QT interval, including not only class Ia or class III antiarrhythmic drugs...
but also a variety of QT-prolonging agents with I_{Kr}-blocking effects (378;379). The risk of cardiac events is also higher in women than men with congenital LQT1 and LQT2 (380). Risk factors predisposing to TdP, such as hypokalemia, hypomagnesaemia, digoxin, bradycardic responses to drugs, or hypothyroidism, or circulating serum levels of drugs cannot account for sex-differences in risk of drug-induced TdP (381-385). Therefore, intrinsic sex-related differences in ventricular repolarization may account for gender differences in drug-induced QT prolongation and TdP.

The mechanisms related to the control of ventricular repolarization by gender have been investigated in human and animal models such as rabbit, mouse, rat or guinea pig (386). Besides a longer QTc, women have much steeper rate-dependence of QT intervals (387-389). Gender differences in transmural heterogeneity of ventricular repolarization have been reported. Men have a longer T_{peak} to T_{end} interval, but the rate-dependent increase in transmural repolarization heterogeneity and the baseline TDR is greater in women (390-393). Similar gender differences in the rate dependence of QT interval, drug-induced QT prolongation and arrhythmia are found in rabbit hearts (379;394-396). Pham et al. reported shorter male endocardial APD_{30} (action potential duration at 30% repolarization) but similar male-female Epi-Endo APD_{90} (action potential duration at 90% repolarization) in isolated rabbit LV myocytes (397). Greater I_{Kr} blockade-induced APD prolongation, Epi-Endo APD dispersion and a higher incidence of EADs are present in female rabbit left ventricles (397;398). Larger epicardial I_{Ca,L}, smaller I_{Kr} and lower density of the outward part of I_{K1} in female rabbit ventricular cells may account for gender differences in rabbit ventricular repolarization (399-401). In mouse hearts, ventricular APDs are longer in
females than in males (402;403). However, the baseline QT interval is similar between female and male mice, but female mice have more PVT induced by halothane (a volatile anesthetics that prolongs QT interval) (404). Lower $I_{K_{ur}}$ (ultra-rapid delayed rectifier $K^+$ current) density and lower expression levels of its $\alpha$-subunit Kv1.5 in female mice ventricles may underlie longer female ventricular APDs (405). $I_{K_{ur}}$ is a voltage-dependent $K^+$ current participating in cardiac repolarization (406-408). It is mainly expressed in the atrium of human or dog hearts, but is also present in mouse and rat ventricle (406;409-412). Results from studies using guinea pig or rat hearts are quite controversial. There is some evidence of shorter APDs in male guinea pig cardiomyocytes (413). Brouillette et. al., however, found no sex-related differences in guinea pig ventricular repolarization (414). In rat hearts, there is no baseline gender difference in ventricular repolarization (415) but there may be smaller outward $K^+$ currents in male versus female rat ventricle (416).

Gender differences in ventricular repolarization are likely mediated, at least in part, by sex hormones, especially testosterone (379;417). After puberty, QTc intervals shorten in men. This observation, combined with the evidence that JT intervals (interval between end of QRS and end of T wave, reflecting ventricular repolarization (418)) are longer in orchiectomized (ORCH) than non-orchiectomized men, suggests the importance of androgens in sex-related repolarization differences (419;420). Results from animal studies indicate a protective role of testosterone in ventricular repolarization. ORCH male rabbits or mice have prolonged QT intervals and APDs and correspondingly, decreased $I_{K_{ur}}$ and Kv1.5 expression are found in ORCH mouse ventricle (397;421). Similar results are reported in male C57BL/6 mice, a strain of mice with low background levels of testosterone (422). Androgen treatment of ORCH
male rabbits or male C57BL/6 mice with DHT (dihydrotestosterone) shortens QT intervals and APDs, increasing $I_{K1}$ and $I_{Kr}$ in DHT-treated rabbits or $I_{Kur}$ and $Kv1.5$ in DHT-treated male C57BL/6 mice (423;424). DHT treatment also reduces drug-induced QT prolongation and APD in normal or OVX (ovariectomized) female rabbits (425;426). The EAD incidence is also diminished in DHT-treated normal female rabbit hearts exposed to dofetilide (426). Effects of female sex hormones (estrogen and progesterone) on cardiac repolarization are more complex and have been less studied. Studies in humans suggest that the ratio of estrogen to progesterone levels may contribute to the higher incidence of drug-induced TdP in women (427;428). Compared to normal female rabbits, OVX female rabbits have similar QT intervals and APDs but reduced dofetilide-induced APD prolongation and EAD occurrence, and absence of Epi-Endo $I_{Ca,L}$ gradient (397;399). Chronic 17$\beta$-estradiol-treated OVX female rabbits have greater E4031-induced APD prolongation and EAD than DHT- or placebo-treated groups (429). However, both chronic 17$\beta$-estradiol and DHT treatments prolong QT and APD$_{90}$, decrease mRNA level of Kv1.5 and mink, and increase epicardial $I_{Ca,L}$ conductance in OVX female rabbit hearts (399;426;430).

5.2 Heart rate and ventricular repolarization

The relationship of heart rate to QT interval is well appreciated (431). However, chronic alterations in heart rate can cause electrical remodeling and are associated with potentially lethal arrhythmias in both research and clinical settings (432;433). Both bradycardia and tachycardia can induce electrical remodeling, resulting in ventricular repolarization abnormalities and predisposition to lethal tachyarrhythmias.
5.2.1 Bradycardia

Normal resting heart rate is about 70 bpm in men and 75 bpm in women (389). Bradycardia is defined as heart rate less than 60 bpm, and can result from SA or AV nodal dysfunction (433). Complete heart block (CHB) is associated with significant bradycardic remodeling. Patients with CHB exhibit prolonged QT intervals and increased risk of TdP (435). Bradycardia due to experimental CHB causes QT interval and APD prolongation associated with EADs and susceptibility to TdP and SCD. Reduction of Ikr and IKs and downregulation of ERG, KvLQT1 and minK expression are prominent features of bradycardia-induced electrical remodeling in CHB-canine or rabbit hearts (434;436-441).

5.2.2 Tachycardia

Tachycardia is defined as a cardiac rhythm producing a ventricular rate greater than 100 bpm. Prolonged tachycardia has been well-recognized clinically to induce cardiomyopathy, impair left ventricular function and cause congestive HF (442-444). Chronic ventricular tachycardia has been widely used as an experimental model of low output biventricular failure (445-447). Ventricular tachycardia-induced electrical remodeling causes prolongation of APD associated with downregulation of several repolarizing potassium currents including Ito, IK1 and IKs, and upregulation of NCX function or expression (432;448). The most consistent findings are downregulation of Ito, decreased mRNA and protein levels of Kv4.3 (434;449-453).
5.3 Repolarization Reserve

The concept of “repolarization reserve” was first brought forward by Dan Roden in 1998 to describe mechanisms of drug-induced TdP and its clinical variability (455). Repolarization reserve is a specific presentation of the more general concept that physiological redundancies in biological systems protect against extreme perturbations (456). Normal cardiac repolarization includes multiple repolarizing ionic currents to provide a safety reserve. When there is impairment in one repolarizing current due to acquired diseases, genetic defects or pharmacological blockade, this above is insufficient to elicit marked QT prolongation and TdP occurrence since other normal repolarizing currents can compensate. However, if there are subclinical deficiencies in the repolarization process, e.g., congenital dysfunction of a K⁺ current, or repolarizing current abnormalities due to clinical factors like heart failure or hypokalemia, repolarization reserve is compromised and the superimposition of K⁺ channel block can produce exaggerated APD and QT prolongation and lead to life-threatening TdP (457). Repolarization reserve compensation can result from functional augmentation of outward K⁺ currents (458;459). A well studied example is the evidence that I_{Ks} is the major source of repolarization reserve that protects against TdP during I_{Kr} block (460;461). I_{Ks} blockade by its selective antagonists (chromanol 293B or HMR-1556) produces little effects on human, rabbit or canine ventricular APDs (462-464). Inhibition of I_{Kr} by dofetilide increases human ventricular APD as expected but it is further prolonged by addition of HMR-1556 (465). When APD is prolonged, I_{Ks} density increases, restricting exaggerated APD prolongation (466). The augmented I_{Ks} density during longer APDs is related to the kinetic properties of I_{Ks} (slow activation,
non-inactivation and fast deactivation in human and dog): the longer the APD, the larger the current (467-469). However, other mechanisms of compensation in repolarization reserve may exist. In transgenic mice lacking $I_{\text{to,fast}}$, $I_{\text{to,slow}}$ is increased due to upregulation of Kv1.4 expression; and in mice lacking $I_{K_{\text{slow1}}}$, the expression of Kv2.1 is upregulated to increase $I_{K_{\text{slow2}}}$ (470-472). Therefore, compensation may also result from increased repolarizing ion channel expression. Increased inward currents (late $I_{\text{Na}}$ or $I_{\text{Ca,L}}$) can also reduce repolarization reserve, sensitizing the ventricle to excessive QT prolongation and lethal arrhythmias.

6. Regulation of ventricular ion-channel expression

6.1 Homeostatic regulation

The quantities of various types of ion channels expressed in any given cardiomyocyte are generally determined by developmental regulation or homeostatic regulation (473). Homeostatic regulation is an important mechanism controlling ion channel expression in the adult heart (473;474). It refers to how a single cardiac myocyte can monitor and maintain a stable electrophysiological phenotype under physiological conditions, as well as provide certain plasticity for the adult cardiac cell in response to genetic, pathological, or pharmacological insults (473). Rosati et al. have provided an idealized and simplified model to understand the potential mechanisms of homeostatic regulation for ion channel expression.
As seen in Figure 7, functional ion channels on cardiac membranes are expressed through a complicated biosynthetic/trafficking/insertion/degradation system from initial gene transcription to final cellular surface phenotype. Regulation can happen at multiple levels to control ion channel expression under physiological circumstances or pathophysiological stimuli. Alterations in any step of the regulatory system of an ion channel protein may lead to changes at downstream levels trying to maintain a stable final phenotype. Changes in electrophysiological phenotype of cell-surface ion channels can be fed back into the biosynthetic pathway through cognate or compensatory feedback pathways to maintain the phenotype within limits appropriate to the function of a particular cell type and the physiological stresses on that cell (473).
6.2 Regulation of ventricular ion channel expression by sex hormones

Sex hormones can regulation gene transcription through intracellular receptors (475). Binding of gonadal hormones to their receptors converts the latter into transcription factors, which can bind to the hormone response element (HRE) of target genes and regulate transcription. Androgen receptors (ARs), estrogen receptors (ERs) and progesterone receptors (PRs) are all found in cardiac muscle (476-480). Currently, there is very little information concerning the transcriptional regulation of cardiac ion channel genes by sex hormones and the role of modulation of gene expression in the gender differences in ventricular repolarization. The cardiac \( CACNA1C \) gene encoding \( I_{Ca,L} \) \( \alpha \)-subunit \( \alpha1C \) has been found to contain HRE and the mRNA expression of \( \alpha1C \) is upregulated by testosterone or 17\( \beta \)-estradiol (481-483). Kv4.3 expression and trafficking have been reported to be inhibited by oestrogen in the myometrium of the pregnant rat uterus (484). The mRNA expression of \( \text{minK} \) is expressed in an oestrogen-dependent way in the rat uterus (485). Sex hormones can also acutely regulate cardiac ion channel activity through non-genomic pathways involving nitric oxide via the PI3K/AKt/eNOS signaling (486). Reduced \( I_{Ca,L} \) in the rodent ventricular myocyte has been consistently reported after acute application of testosterone or 17\( \beta \)-estradiol (487).

6.3 Calcium signaling pathways in cardiac ion channel regulation

\( \text{Ca}^{2+} \) plays an essential role in cardiac excitation-contraction (EC) coupling (488). \( \text{Ca}^{2+} \) can also act as an intracellular messenger, and \( \text{Ca}^{2+} \)-activated signaling pathways have important regulatory effects on EC coupling and ion channel function, and may modulate gene expression (e.g. through excitation-transcription (ET) coupling) in
hypertrophy and heart failure (489).

6.3.1 Intracellular Ca\textsuperscript{2+} signaling pathways in cardiac ventricular myocytes

Figure 8 illustrates Ca\textsuperscript{2+} signaling in cardiomyocytes. Ca\textsuperscript{2+} enters cardiomyocytes through I_{Ca,L} on a heartbeat-to-heartbeat basis (490). Two important Ca\textsuperscript{2+}-activated protein targets in cardiac myocytes include the myofilament protein troponin C and calmodulin (CaM). Troponin C is the central player in contractile machinery in cardiac muscle (491;492). Ca\textsuperscript{2+} entry activates ryanodine receptors (RyRs), sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release channels, to induce Ca release from SR stores, resulting in elevation of [Ca\textsubscript{i}] that activates myofilaments (troponin C) to initiate cardiac contraction (EC coupling). [Ca\textsubscript{i}] then decreases quickly through SR Ca\textsuperscript{2+} reuptake via SERCA and Ca\textsuperscript{2+} extrusion via NCX, allowing relaxation (488).

CaM is a ubiquitous Ca\textsuperscript{2+} sensor and a critical signaling messenger that can deliver intracellular Ca\textsuperscript{2+} signals (490;493). CaM is activated by the binding of four Ca\textsuperscript{2+} ions to its two EF-hands (494). Activated CaM (Ca\textsuperscript{2+}-CaM) has three main protein targets in cardiac ventricular cells: Ca\textsuperscript{2+}-CaM-dependent protein kinase II (CaMKII), calcineurin (protein phosphatase 2B), and myosin light chain kinase (MLCK, less important to EC coupling in cardiac muscle than in smooth muscle) (489). Ca\textsuperscript{2+}-CaM binding to the regulatory domain of CaMKII can lead to the activation and auto-phosphorylation of CaMKII on its Thr-286 residue, which keeps it in an activated (Ca\textsuperscript{2+}-independent) state after [Ca\textsuperscript{2+}], declines (495;496). CaMKII can translate the frequency of Ca\textsuperscript{2+} spikes into distinct levels of kinase activity as increasingly active states as activation frequency increases (497). Therefore, CaMKII has the ability to transduce both the frequency and amplitude of intracellular Ca\textsuperscript{2+}
transients into activity levels of CaMKII-dependent proteins, and thereby modulate downstream regulatory effects on gene transcription in cardiomyocytes (490;498-500). CaMKII can phosphorylate and regulate the function of ion channels, proteins involved in EC coupling (e.g., PLB, RyR2), and transcription factors in response to Ca^{2+} signals (501). Ca^{2+}-CaM dependent CaMKII pathways that have been shown to regulate gene transcription in the heart is the Ca^{2+}-CaM-CaMKII-HDAC-MEF2 pathway (490;501;502). CREB-dependent gene regulation might be potentially modulated by Ca^{2+}-CaM-CaMKII.

**Figure 8.** Calcium signaling in ventricular myocytes. Black arrows (solid or dashed lines) indicate Ca^{2+}-dependent signaling to EC coupling and ion channels. Red arrows indicate Ca-dependent excitation-transcriptional (ET) coupling. CaM, calmodulin; CaMKII, calcium-calmodulin dependent protein kinase II; CREB, cAMP response element binding; CRE, cAMP response element; CBP, CREB-binding protein; HDAC, histone deacetylase; MEF2, myocyte enhancer factor 2; NFAT, nuclear factor of activated T cells; PLB, phospholamban. (Adapted from Figure 8 of reference (490) with modification, used with permission)
CaMKII phosphorylates histone deacetylase (HDAC), leading to the dissociation of HDAC from HDAC-MEF2 complex, allowing its nuclear export, thereby releasing myocyte enhancer factor 2 (MEF2) from HDAC suppression and promoting MEF2-driven transcription as in the hypertrophic heart (503, 504). In neurons or other cell types, Ca$^{2+}$-CaM can translocate into the nucleus and activate nuclear CaMKII that phosphorylates a ubiquitous transcription factor CREB (cAMP response element binding protein) at the Ser 142 site, which prevents CREB dimerization and recruitment of CREB binding protein (CBP), negatively regulating CREB promoted transcription (505-507). CREB can also be phosphorylated by PKA, PKC or CaMKII at Ser 133, which increases dimerization and binding, and promotes transcription of several cardiac genes such as Kv1.5, phospholamban, c-fos etc (508-511, 597).

Therefore, it is possible that phosphorylation of CREB by CaMKII at different sites can potentially regulate its transcriptional activities in the heart. However, some recent evidence showed cardiac nuclear CaMKII isn’t involved in regulating CREB-dependent transcription (598). Ca$^{2+}$-CaM also activates phosphatase 2B (calcineurin), which can regulate RyR2 function and dephosphorylate the transcription factor NFAT (nuclear factor of activated T cells) (489, 512). Dephosphorylation of NFAT allows for NFAT nuclear import, permitting cooperative NFAT interaction with the transcription factor GATA4 to activate hypertrophic genes or repress ion channel gene transcription (513-516). Other transcription factors that can be activated by Ca$^{2+}$ signaling include NF-kB, JNK and Elk-1 (517, 518).

Intracellular Ca$^{2+}$ levels and signaling are also affected by neurohumoral stimulation such as adrenergic, endothelin-1 or angiotensin II activated pathways (502, 519). Activation of α- or β-adrenergic signaling can increase intracellular Ca$^{2+}$.
levels via their regulatory effects on I_{CaL} via G protein-activated PKA or local Ca^{2+} release from IP_{3}R via G protein-activated PLC/IP_{3}/PKC (520;521).

6.3.2 Regulation of cardiac ion-channel function by calcium signaling

Cardiac Ca^{2+} signaling acutely regulates the function of several ion currents such as I_{CaL}, I_{NCX}, I_{Na}, I_{to} and I_{Ks}. Inactivation of I_{CaL} is calcium-dependent and is mediated by CaM (522). Elevated local [Ca\textsubscript{i}] due to Ca^{2+} entry through I_{CaL} and/or SR Ca^{2+} release causes Ca^{2+} binding to CaM that binds with the C-terminus of α1C, resulting in accelerated inactivation (523;524). Cardiac I_{CaL} exhibits CaMKII-dependent facilitation over a time scale of seconds that results in increased current amplitude and slowing of inactivation (525-527). Regulation of I_{CaL} by Ca^{2+} signaling may protect cardiomyocytes against intracellular Ca^{2+} overload and offset reduced I_{CaL} availability due to increased frequency (528;529). Increased [Ca\textsubscript{i}] activates NCX by binding to its allosteric regulatory site and promoting Ca^{2+} extrusion, reducing intracellular Ca^{2+} overload (530;531). Ca^{2+}/CaM increases the slow component of I_{Na} inactivation (532). CaMKII associates with and phosphorylates the sodium channel α-subunit in rabbit and rat ventricular myocytes, altering I_{Na} gating to reduce availability at high heart rates, and enhancing late I_{Na}, which may contribute to arrhythmogenesis in HF (533). CaMKII acutely modulates I_{to} kinetics by slowing I_{to} inactivation and enhancing recovery from inactivation in human atrial and rat ventricular cells through phosphorylation of Kv4.2 and Kv4.3 (534;535). Elevated [Ca^{2+}]\textsubscript{i} can increase I_{Ks} and CaM is a constitutive regulator of KCNQ1, serving as an Ca^{2+} sensor (536;537). Ca^{2+}-CaM binding facilitates KCNQ1 assembly and affects I_{Ks} gating by preventing inactivation, mediating Ca^{2+}-dependent increase of I_{Ks} and
shifting $I_{Ks}$ voltage-dependent activation to more negative potentials (538).

6.3.3 Regulation of cardiac ion-channel expression by calcium activated pathways

Ca$^{2+}$/CaM-related signaling through CaMKII and calcineurin are involved in transcriptional regulation and hypertrophic signaling in the heart (518;539). Ca$^{2+}$ signaling can also regulate cardiac ion channel gene expression. Many studies show that the calcineurin/NFAT pathway represses expression of several ion channel genes. Reduced mRNA and protein levels of Kv1.5, Kv2.1, Kv4.2 and Kv4.3, and increased NFAT activity are seen with chronic β-adrenergic stimulation, expression of constitutively activated NFATc3, or in myocardial infarctions, and are absent following inhibition of calcineurin or NFATc3 knockout (540). Higher [Ca], and calcineurin/NFATc3 activity in Endo than in Epi, is associated with lower endocardial Kv4.2 expression in mouse ventricles (515). Overexpression of calcineurin reduces cardiomyocyte Kv1.5 and Kv2.1 expression in transgenic mice (541). In neonatal rat ventricular cells, overexpression of calcineurin upregulates Kv4.2 expression (542). In a pacing-induced long-term cardiac memory canine model, reduced Kv4.3 and KChIP2 mRNA and protein expression are associated with decreased CREB levels modulated by calcium and angiotensin II, and with reduced CRE binding to the Kv4.3 promoter (509;510). Knockdown of CREB by adenoviral anti-sense reduces $I_{so}$ in canine LV epicardial cells (509). Angiotensin II can induce α1C ($I_{CaL}$) expression via Ser 133 phosphorylation of CREB through PKC/reactive oxygen species pathways in HL-1 cells (543). cAMP and KCl-induced depolarization upregulates Kv1.5 transcription via CREB binding in atrial cells (544). Transgenic expression of a
CaMKII inhibitory peptide (AC3-I) led to increased $I_{CaL}$, $I_{to}$ and $I_{K1}$ expression and shortening of APD in the mouse ventricle (545).

6.4 Regulation of ion channel expression by microRNAs

6.4.1 Role of microRNAs in gene expression: post-transcriptional regulation

MicroRNAs (miRNAs) were recently found to be important regulators of gene expression in plants and animals, playing critical roles in development and physiology (546-548). Mature miRNAs are families of 21-25 nucleotide small endogenous non-coding RNAs that can repress gene expression at the post-transcriptional level (549;550). In animals, most miRNAs bind to their target genes at the 3′-untranslated region (3′-UTR) with imprecise complementarity, and repress the translation of target mRNAs through unknown mechanisms that preserve the stability of mRNA targets (551-554). If the complementarity between miRNAs and target mRNAs are strong, an miRNA will likely induce degradation of target mRNA; if there is more than one miRNA binding site in a target gene, the effect is greater (555-557). Recently, Vasudevan et al. reported that miRNAs can also function as potential translation enhancers under certain conditions (558). As abundant RNA species, miRNAs are predicted to be able to regulate ~30% of protein-coding genes (559).

6.4.2 Cardiac microRNAs

The expression of mammalian miRNAs is tissue specific (560;561). To date, miRNAs that are highly expressed in the heart include $miR-1$, $miR-133$, $miR-126$, $miR-30c$, $miR-26a$, let-7, $miR-143$, $miR-181$ and $miR-195$, among which $miR-1$ and
miR-133 are muscle specific (561-563). The expression of cardiac miRNAs is also disease-dependent, and a variety of studies have suggested that cardiac miRNAs play critical roles in pathological processes of adult hearts, including cardiac hypertrophy, heart failure, cardiomyopathy, angiogenesis and arrhythmogenesis (564-571).

6.4.3 Control of cardiac ion channel expression by microRNAs

Bioinformatic analyses predict that genes encoding cardiac ion-channel molecular components are potential targets for a large number of known cardiac miRNAs (572). However, direct experimental data are rare. In vitro studies have shown that miR-1 can repress the protein translation of GJA1 (encoding connexin 43 (Cx43), a gap junction protein), KCNJ2, KCNE1, HCN2 and HCN4, and that miR-133 represses KCNQ1, KCNH2 and HCN2 translation, without altering the mRNA levels of these genes (573-577). Effects of miR-1 and miR-133 on these mRNAs can be inhibited by anti-miRNA antisense inhibitor oligonucleotides (AMO). Regionally determined expression levels of miR-1 or miR-133 contribute to the regionally heterogeneous protein of KvLQT1 and minK in the ventricle, despite homogeneous mRNA levels (578-580). Greater miR-1 or miR-133 expression is observed in parallel with downregulated Kir2.1 and Cx43 proteins in infracted rat hearts, reduced ERG protein in diabetic rabbit hearts, or decreased HCN2/HCN4 protein expression in hypertrophic cardiomyocytes (581-584).
7. Basis for hypotheses tested:

Sudden cardiac death due to lethal ventricular arrhythmias from many different causes remains a leading cause of death. Our understanding of normal and pathological ventricular electrophysiology has expanded greatly from the cellular to molecular level. However, standard antiarrhythmic drug therapy has failed to reduce and in some instances has increased the incidence of SCD (585). A better understanding of the mechanisms responsible for regulation of ion channel expression and function is still a challenge. The central goal of the work described in the following chapters is to explore the basic mechanisms of ion channel regulation in the control of ventricular repolarization under three key clinically relevant conditions:

1) Cellular mechanisms of gender differences in ventricular repolarization.

I hypothesized that gender differences in QT interval and drug-induced QT prolongation and TdP susceptibility are linked with sex-related differences in ventricular ionic currents and repolarization. Given the complex nature of transmural heterogeneity of ventricular ion channel function and repolarization, the assessment of male/female differences for a broad range of ionic currents across the ventricular wall was necessary to address this hypothesis.

2) Control of ion channel expression by heart rate.

Sustained heart rate abnormalities such as tachycardia can produce ventricular electrical remodeling, heart failure and susceptibility to cardiac arrhythmia. These are generally considered to be consequences of altered ventricular function and the heart failure state. The possibility that heart rate per se regulates ion channel expression has not been investigated. I hypothesized that increases in cardiomyocyte activation rate might alter ion channel expression directly. Heart rate changes in vivo causes
alterations in neurohormones and cardiac hemodynamics that might have effects unrelated to the direct role of heart rate. Therefore, an isolated-cell model was needed to investigate the potential effect of heart rate per se on ion channel expression as well as the mechanisms underlying any changes observed.

3) Feedback remodeling of cardiac K⁺ current expression: a novel potential mechanism for control of repolarization reserve.

   Repolarization reserve usually involves functionally-based compensatory increases in repolarizing currents that minimize changes in APD caused by dysfunction or inhibition of a single K⁺-current, an important mechanism explaining clinical risk factors for TdP. I hypothesized that repolarization reserve also involves compensatory expression changes in cardiac ion channels by feedback regulation to maintain repolarization in response to sustained dysfunction of a K⁺-current.
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Chapter 2. Cellular mechanisms of gender differences in cardiac ventricular repolarization
I first studied the cellular mechanisms of gender differences in ventricular repolarization. Women are clinically well known to be more susceptible to drug-induced QT prolongation and TdP. Available experimental data to address the intrinsic cellular mechanisms responsible for male/female differences in QT interval and drug-induced QT prolongation were discrepant as discussed in section 5.1 Chapter 1. This is not surprising due to the existence of species-dependent differences in cardiac repolarization, and the complex nature of the ventricle itself that is usually not carefully evaluated in these gender-related studies. Therefore, the underlying ionic mechanisms of gender-dependent ventricular repolarization differences remain poorly understood. The dog heart is electrophysiologically more similar to the human heart in terms of transmural ion channel distribution and repolarization heterogeneity. Therefore, we investigated sex-related differences in transmural ventricular repolarization on a broad range of ionic currents across the ventricular wall in the canine heart.
Sex-based transmural differences in cardiac repolarization
and ionic-current properties in canine left ventricles

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Short title: Gender and cardiac transmural ion current heterogeneity

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Abstract
Female gender is associated with longer electrocardiographic QT intervals and increased proarrhythmic risks of QT-prolonging drugs. This study examined the hypothesis that sex differences in repolarization may be associated with differential transmural ion-current distribution. Whole-cell patch-clamp and current-clamp were used to study ionic currents and action potentials (APs) in isolated canine left ventricular cells from epicardium, midmyocardium and endocardium. No sex differences in AP duration (APD) were found in cells from epicardium versus endocardium. In midmyocardium, APD was significantly longer in female dogs (e.g., at 1 Hz, female vs. male: 288±21 vs. 237±8 ms; \( P<0.05 \)), resulting in greater transmural APD heterogeneity in females. No sex differences in \( I_{K1} \) were observed. \( I_{to} \) densities in epicardium and midmyocardium also showed no sex differences. In endocardium, female dogs had significantly smaller \( I_{to} \) (e.g. at +30 mV, female vs. male: 2.5±0.2 vs. 3.5±0.3 pA/pF; \( P<0.05 \)). \( I_{Kr} \) density and activation voltage-dependence showed no sex differences. Female dogs had significantly larger \( I_{ks} \) in epicardium and endocardium (e.g. at +40 mV; tail densities, female vs. male; epicardium: 1.3±0.1 vs. 0.8±0.1 pA/pF; \( P<0.001 \); endocardium: 1.2±0.1 vs. 0.7±0.1 pA/pF; \( P<0.05 \)), but there were no sex differences in midmyocardial \( I_{Kr} \). Female dogs had larger \( I_{Ca,L} \) densities in all layers than male dogs (e.g. at -20 mV, female vs. male, epicardium: -4.2±0.4 vs. -3.2±0.2 pA/pF; midmyocardium: -4.5±0.5 vs. -3.3±0.3 pA/pF; endocardium: -4.5±0.4 vs. -3.2±0.3 pA/pF; \( P<0.05 \) for each). We conclude that there are sex-based transmural differences in ionic currents which may underlie sex differences in transmural cardiac repolarization.
Key Words: sex difference, transmural dispersion of repolarization, ion-channels, action potential
Introduction

Women have a longer rate-corrected QT interval (QTc) than men (4, 24), and compared to men, women respond differently to QT-prolonging drugs (class IA and III antiarrhythmic drugs, antianginal drugs, antihistamines, antibiotics and antimalarials, etc.) and have an increased risk of acquired Torsades de Pointes (TdP) arrhythmias (11, 22). The mechanisms underlying these sex differences have been investigated in human subjects and in animal models (mouse, guinea pig and rabbit) (14, 20, 28, 32, 34), but are still incompletely defined.

The crucial importance of transmural heterogeneity in action potential and ionic current properties is well recognized (2, 27). Female adult mice (32, 34), guinea pigs (14) and rabbits (10) have longer ventricular action potential durations (APDs) than their male counterparts. Ionic mechanisms that may account for these differences have been reported, including differences in transient (I_{to}) and sustained (I_{sus}) outward K^{+}-current (32), ultrarapid delayed-rectifier current, I_{Kur} (34), inward-rectifier current, I_{K1}, delayed-rectifier K^{+}-current, I_{K} (14, 20), rapid delayed-rectifier current, I_{Kr} (20), and L-type Ca^{2+}-current, I_{Ca,L} (14, 25). Sex hormone manipulations on rabbits (10, 13, 19, 25, 26) or mice (7) can affect QT intervals, APDs, ionic currents and arrhythmia incidence. Early afterdepolarizations (EADs) and increased transmural dispersion of repolarization (TDR) have been proposed to underlie TdP (3). Previous studies suggest that female rabbit hearts have a greater susceptibility to EADs (21). Transmural ion-channel differences are known to be important in TdP susceptibility (2, 27).
However, we were unable to identify studies that assess male-female differences for a broad range of ionic currents across the ventricular wall. This study was designed to assess transmural cardiac repolarization and ion-channel function in female and male adult canine left ventricular myocytes, with a view to elucidating male-female differences.
METHODS

Cell Preparation

All animal care and handling procedures followed the Guidelines of the Canadian Council for Animal Care. Adult mongrel dogs of both sexes (female 26.4±5.2 kg, n=67; male 26.9±5.9 kg, n=68; P=NS) were anesthetized with pentobarbital (30 mg/kg iv) and ventilated with room air. A left lateral thoracotomy was performed, and hearts were quickly excised and immersed in oxygenated Tyrode solution at room temperature. The transmural free wall (~3 × 5 cm) of the lateral left ventricle, which was irrigated by a coronary artery branching from the left circumflex coronary artery, was quickly dissected and the artery was cannulated. Cell isolation was performed as previously described (36) by perfusion with a solution containing collagenase (120-150 U/ml, Worthington type II). When the tissue was well-digested, small tissue blocks were removed from the epicardial (Epi) surface (1-1.5 mm thick), midmyocardial (Mid) layer (2-5 mm thick) and endocardial (Endo) surface (1-1.5 mm thick). Cells were dispersed by gentle trituration with a Pasteur pipette, and were kept in a high-K⁺ storage solution (see solutions) at 4°C.

Solutions

The standard Tyrode solution contained (in mM) NaCl 136, KCl 5.4, MgCl₂ 1, CaCl₂ 1, NaH₂PO₄ 0.33, HEPES 5 and dextrose 10 (pH 7.35 with NaOH). The high-K⁺ storage solution contained (in mM) KCl 20, KH₂PO₄ 10,
dextrose 10, mannitol 40, L-glutamic acid 70, β-OH-butyric acid 10, taurine 20, EGTA 10 and 0.1% BSA (pH 7.3 with KOH). The standard pipette solution used in most experiments contained (in mM) K-aspartate 110, KCl 20, MgCl₂ 1, MgATP 5, GTP 0.1, HEPES 10, Na-phosphocreatine 5, EGTA 5 (for current recording) or 0.025 (for action potential (AP) recording), with pH adjusted to 7.3 with KOH.

For AP recordings, external solutions contained 2 mM CaCl₂. For K⁺-current recordings, atropine (1 μM) and CdCl₂ (200 μM) or nimodipine (5 μM, for I_K) were added to external solutions to eliminate muscarinic K⁺ currents and to block Ca²⁺-currents. Na⁺ current contamination was avoided by using a holding potential (HP) of -50 mV or by substitution of equimolar Tris-HCl for external NaCl. For currents other than transient outward current (Ito), 1 mM 4-AP was used to block Ito. Inward rectifier current (I_K₁) was studied as 1 mM Ba²⁺-sensitive current. For studies of rapid delayed rectifier current (I_Kr) and slow delayed rectifier current (I_Ks), chromanol 293B (50 μM) was added to record I_Kr (6) and E4031 (5 μM) was used to record I_Ks, after verification that chromanol 293B-resistant tail current was strongly blocked by E-4031 and E-4031-resistant current was blocked by 50 μM chromanol 293B.

For L-type calcium current (I_{Ca,L}) studies, the external solution contained (in mM) tetraethylammonium chloride (TEA-Cl) 136, CsCl 5.4, CaCl₂ 2, MgCl₂ 0.8, HEPES 10 and dextrose 10 (pH 7.4 with CsOH). Niflumic acid (50 μM) was added to inhibit I_{Cl,Ca}. The pipette solution contained (mM) CsCl 20, Cs-aspartate 110, MgCl₂ 1, MgATP 5, GTP 0.1, Na₂Phosphocreatine 5, EGTA 10 and HEPES 10 (pH 7.2 with CsOH).
Data Acquisition and Analysis

The whole-cell patch-clamp technique was applied to record ionic currents and APs at 36°C, as previously described in detail (35, 36). Ionic currents were recorded in the voltage-clamp mode, and APs were recorded in current-clamp mode. Compensated series resistance and capacitive time constants (τs) averaged 2.3±0.1 MΩ and 294±10 μs. Junction potentials (~10 mV) were corrected for AP recordings only. Leakage compensation was not used. Cell capacitance averaged: epicardium, 118.1±3.4 pF in female (n=87) and 126.4±3.9 pF in male cells (n=86), \( P=NS \); midmyocardium, 123.2±3.1 pF in female (n=88) and 131.6±3.2 pF in male cells (n=93), \( P=NS \); endocardium, 113±2.9 pF in female (n=77) and 116.7±3.4 pF in male cells (n=76), \( P=NS \). Currents are expressed in terms of density (normalized to capacitance).

To obtain QT-interval data, 14 female dogs (weight 25 ± 5 kg) and 14 male dogs (weight 26 ± 4 kg) were studied in vivo. On study days, dogs were anesthetized with acepromazine (0.07 mg/kg IV), ketamine (5.3 mg/kg IV), valium (6.25 mg/kg IV), and isoflurane (2%) and were mechanically ventilated with air. Radiofrequency ablation of the AV node was performed in order to study the QT interval over a range of controlled basic cycle lengths (BCLs). Body temperature was maintained at 37°C. A median sternotomy was performed and a bipolar Teflon-coated stainless-steel electrode was hooked into the right
ventricular free walls for stimulation. A programmable stimulator was used to deliver 2-ms twice-threshold pulses and surface ECGs were recorded at BCLs of 300, 400, 600 and 1000 ms. QT interval was measured from lead 2 and the mean of 3 QT interval measurements at each BCL for each dog was used for analysis.

Nonlinear least-square curve-fitting algorithms were performed for curve fitting. Unpaired Student’s \( t \)-tests were used for comparisons between female and male groups. \( P<0.05 \) was taken to indicate statistical significance and group data are expressed as mean\( \pm \)SEM.

**RESULTS**

*Action Potentials*

Examples of APs recorded in isolated cells from various layers of female and male canine left ventricles (at 1 and 0.5 Hz) are shown in Fig. 1A, B and C. In both female and male left ventricular cardiomyocytes, APs showed a prominent phase 1 and “spike-and-dome” configuration, with an intervening notch, in epicardial (Fig. 1A) and midmyocardial (Fig. 1B) cells. Endocardial cells showed more limited phase 1 repolarization and virtually no notch (Fig. 1C). APD was consistently longer in midmyocardial cells than in epicardial and endocardial cells. This pattern of transmural AP heterogeneity is consistent with the results of previous studies (2, 27).

APD was similar for female and male canine left ventricular cardiomyocytes in both epicardium and endocardium (Fig. 1D, 1 Hz). In
midmyocardium, female dogs had significantly longer APDs compared to male dogs (Fig. 1D). For example, at 1 Hz, APD to 90% repolarization (APD_{90}) averaged 292±17 ms in females (n=11) vs. 235±7 ms in males (n=21, P<0.05), whereas APD to 50% repolarization (APD_{50}) was 249±19 ms in females (n=11) vs. 186±6 ms in males (n=21, P<0.05). In agreement with the APD data, QT interval at matched cycle lengths was significantly larger in female versus male dogs (Fig. 1E, n=14,14 for male and female dogs respectively).

No sex differences were observed in resting membrane potentials. Resting potentials averaged -78.4±0.7 mV (female, n=20) vs. -79.9±0.7 mV (male, n=19) in epicardium, -80.0±0.6 mV (female, n=15) vs. -81.3±0.6 mV (male, n=21) in midmyocardium, -79.8±0.8 mV (female, n=28) vs. -81.2±0.6 mV (male, n=20) in endocardium (P=NS for all male-female comparisons).

\[ I_{K1} \]

\[ I_{K1} \] was studied as 1 mM barium-sensitive current. Figure 2A shows representative \[ I_{K1} \] recordings from female and male epicardial cells. \[ I_{K1} \] density was similar between female and male dogs for epicardium, midmyocardium and endocardium as shown in Fig. 2B, C and D respectively.

\[ I_{to} \]

A typical transmural gradient in \[ I_{to} \] was present in both female and male left ventricles (Fig. 3A, C and E). Mean \[ I_{to} \] density was similar for epicardial and
midmyocardial cells in female and male dogs (Fig. 3B and D). In endocardial cells, mean $I_{to}$ density was significantly larger in male dogs than in female dogs (Fig. 3F), with mean current density at +30 mV averaging 2.5±0.2 pA/pF (female, $n=24$ cells) and 3.5±0.3 pA/pF (male, $n=28$ cells, $P<0.05$). No sex differences in the form of the $I_{to}$-V relations were found for all transmural levels.

The voltage dependence of $I_{to}$ inactivation was studied with a two-pulse protocol, providing the results illustrated in the left panels of Fig. 4. Inactivation and activation $V_{1/2}$ and slope factors were not significantly different between cells from males vs. females for any myocardial layer (Table 1). $I_{to}$ inactivation kinetics were well-fitted by biexponential relations, and inactivation time constants were similar between female and male left ventricular cells (Fig. 4, B, E, and H). $I_{to}$ reactivation was assessed with the two-pulse protocol shown in Fig. 4C. Reactivation kinetics were well-fitted by biexponential relations (Fig. 4, C, F, and I), with no apparent dependency from sex difference. A detailed presentation of $I_{to}$ recovery kinetics for each layer is shown in Table 1, and indicates no significant between-sex differences. $I_{to}$ frequency dependence, as determined by steady-state current at 0.1, 0.5, 1, 2 and 5 Hz upon 100 ms pulses from -80 to +50 mV, also showed no sex differences within any regions of the left ventricle (data not shown).

$I_K$

Figure 5A shows representative recordings of chromanol 293B (50
µM)-resistant $I_{Kr}$ in epicardial cells from female and male dogs. $I_{Kr}$ activated with half-activation voltages of -1.9 (male) and +2.0 mV (female, $n=8$ cells/group, $P=\text{NS}$), based on tail-currents at -40 mV following 4 s depolarizations to various test voltages. There were no significant sex differences in $I_{Kr}$ tail-current density (Fig. 5, C, D, and E) and activation voltage-dependencies in any regions. The kinetics of $I_{Kr}$ showed no differences between male and female dogs. Activation was well-fitted by biexponential kinetics. Detailed activation time-constant data upon depolarization to +40 mV are provided in Table 2 and indicate no significant sex differences for any layer. Deactivation was similarly biexponential, and detailed results obtained upon repolarizing from +40 to -40 mV (Table 2) show no significant sex-based differences.

Representative recordings of E4031 (5 µM)-resistant $I_{Ks}$ from epicardial cells of female and male dogs are shown in Fig. 6A. $I_{Ks}$ activation voltage-dependence was assessed by normalizing tail-current amplitudes (obtained with the pulse protocol shown in Fig. 6A) to tail-current amplitude following depolarization to +70 mV. There were no differences in activation voltage-dependence between female and male dogs in any regions, as illustrated by the mean data for epicardial cells shown in Fig. 6B. A transmural $I_{Ks}$ gradient has been implicated in the important and well-known transmural APD gradient (2, 27). $I_{Ks}$ showed a greater transmural density gradient in female than in male dogs (Fig. 6, C, D, and E). The $I_{Ks}$ density was larger in female than in male dog cells in both epicardium (Fig. 6C) and endocardium (Fig. 6D, e.g., at +40 mV,
female vs. male \(I_{ks}\) tail-current density averaged: Epi, 1.3±0.1 pA/pF vs. 0.8±0.1 pA/pF, \(n=25\) (female), 20 (male) cells, \(P<0.001\); Endo: 1.1±0.2 pA/pF vs. 0.7±0.1 pA/pF, \(n=16\) cells/group, \(P<0.05\). By contrast, \(I_{ks}\) density in midmyocardial cells was comparable for both female (0.5±0.05 pA/pF, \(n=21\) cells) and male (0.4±0.02 pA/pF, \(n=24\), \(P=NS\)) cells. As in the case of \(I_{kr}\), the kinetics of \(I_{ks}\) were similar for male and female dogs. For example, upon depolarization to +40 mV, \(I_{ks}\) activation was monoexponential and showed no sex-dependent differences (Table 3). Upon repolarization from +40 to -30 mV, deactivation was biexponential, and once again there were no significant sex differences (Table 3).

**L-type Ca\(^{2+}\) Current**

Examples of representative \(I_{Ca,L}\) recordings from epicardial cells are shown in Fig. 7A (female) and B (male). No transmural \(I_{Ca,L}\) density gradient was observed in either female or male left ventricles. Female dogs had significantly larger \(I_{Ca,L}\) density than male dogs for each transmural level (Fig. 7, C, E, and G). For example, at +20 mV, in epicardium, \(I_{Ca,L}\) density averaged -4.2±0.4 pA/pF in females and -3.2±0.2 pA/pF in male cells respectively \((P<0.05, n=15\) cells/group\); in midmyocardium, mean \(I_{Ca,L}\) density was -4.5±0.5 pA/pF in females and -3.3±0.3 pA/pF in males \((P<0.05, n=15\) (female), 18 (male) cells\); in endocardium, current density was -4.5±0.4 pA/pF in females and -3.2±0.3 pA/pF in males \((P<0.05, n=16\) (female) or 8 (male) cells/group\). The normalized \(I_{Ca,L}\) I-V relations were similar for female and male results in all regions (Fig. 7, D, F, and
ICa,L inactivation was fitted by monoexponential relations and showed no sex differences, as illustrated by the mean data for midmyocardium shown in Fig. 8A. The voltage-dependence of ICa,L activation and inactivation was evaluated as illustrated in Fig. 8B, and showed no sex-dependent differences. Activation voltage-dependence was assessed according to the relation ITP=aTPGmax(VTP-VR), with VR obtained from a linear fit to the ascending portion of the I-V relation. There were no significant sex-based differences in mean activation or inactivation V1/2s or slope factors in any layer (Table 4). Figure 8C shows ICa,L reactivation kinetics at holding potential of -80 mV in midmyocardium. Reactivation Time constants of reactivation were similar between cells from female and male (dogs in any layer (Table 4). Similarly, no sex differences in ICa,L frequency-dependence were found. Figure 8D shows mean data for midmyocardium. Similar data were obtained for epicardium and endocardium.

DISCUSSION

In the present study, we analyzed in detail transmural ionic current function in left ventricular cardiomyocytes from male versus female dogs. We found male-female differences in 3 ionic-current systems: Ito, IKs and ICa,L. For Ito and IKs, the male-female differences varied transmurally, in a way that may have functional significance. In addition, we observed statistically-significant longer APDs in females for M-cells only, increasing transmural APD heterogeneity.
Relation to Previous Findings Regarding Gender-related Ionic Current Differences in the Literature

A variety of differences in ionic current properties have been described between male and female animals, and there are numerous discrepancies in the literature, possibly related to interspecies and inter-strain differences. Trepanier-Boulay et al. showed similar $I_{\text{to}}$ and reduced $I_{\text{Kur}}$ in female mice (32), whereas Wu and Anderson reported that female mice have smaller $I_{\text{to}}$ and larger sustained depolarization-induced outward current (which has a major contribution from $I_{\text{Kur}}$) (34). In contrast to Trepanier-Boulay et al., who observed longer APDs and smaller $I_{\text{to}}$ in female mice (32), Brunet et al. could not identify sex differences in $K^+$-currents and ventricular repolarization in mice (8). Previous studies on rabbit hearts pointed to smaller $I_{\text{Kr}}$ in female rabbits, associated with longer QT intervals (20). We found no sex differences in $I_{\text{Kr}}$ at any level of the canine left ventricle. Liu et al. also reported smaller outward (but not inward) $I_{\text{K1}}$ in female rabbits (20), whereas James et al. observed smaller inward (but not outward) $I_{\text{K1}}$ in female guinea pigs (14). We did not observe any sex-dependent $I_{\text{K1}}$ differences in dogs. Unlike our observation of larger $I_{\text{Ca,L}}$ in females, Trepanier-Boulay et al. did not note an $I_{\text{Ca,L}}$ difference between male and female mice (32). We were unable to identify previous studies of sex-dependent differences in $I_{\text{KS}}$. 
**Potential Relevance to Gender Differences in Electrophysiology**

We noted rather complex sex-based differences in ionic currents across the canine ventricular wall. A smaller $I_{to}$ was found in females only in the endocardial layer. Females had larger $I_{Ca,L}$ across the ventricular wall, but $I_{Ks}$ was larger in females only in epicardium and endocardium, but not midmyocardium. These differences would be expected to cause transmurally-based differences in APD, which we observed. The absence of significant male-female APD differences in endocardium and epicardium could be due to offsetting differences in inward $I_{Ca,L}$ and outward $I_{Ks}$. The larger female $I_{Ca,L}$ in the face of similar $I_{Ks}$ in midmyocardium could account for the larger midmyocardial APD in females. The larger APD observed in female midmyocardium relative to male, in the face of similar endocardial and epicardial APD, increased the transmural dispersion of repolarization in females vs. males. Despite smaller $I_{to}$ in female endocardium, we did not observe any associated APD differences. The lack of appreciable impact of the endocardial male-female $I_{to}$ difference in overall APD may have been due to the fact that $I_{to}$ flows primarily during the very early phases of the AP and inactivates well before the onset of phase 3. Variations in $I_{to}$ over the range that we observed (3-6 pA/pF) had no effect on canine endocardial APD in a recently-published study using an elegant dynamic clamp technique (31).

The presence of significant sex-based differences in cardiac repolarization is well recognized. The QT interval is longer in women (4, 24), and women clearly have an increased sensitivity to drug-induced QT-interval prolongation and TdP (5,
11, 16, 17, 22). The basis for these differences remains poorly understood. The present results point to complex male-female differences in transmural ion-channel function. The transmural distribution of cardiac ion channels is complex, and this complex distribution plays a key role in cardiac electrophysiology (1, 2, 27, 29). There is evidence for greater repolarization heterogeneity in women compared to men (30). Pham et al. observed greater transmural repolarization heterogeneity in female dogs upon exposure to $I_{Kr}$ blockers (25). Despite the importance of transmural ion-channel function in repolarization heterogeneity and arrhythmias, we were unable to find detailed studies of ion-current properties in female compared to male subjects. Pham et al. found somewhat larger $I_{Ca}$ densities in the epicardium of female vs. male rabbits, in accordance with our results, but no substantial endocardial differences (23). Midmyocardial cells were not studied, nor were other ionic currents. Greater midmyocardial APD has been attributed to a variety of factors, including smaller $I_{Ks}$ density in the midmyocardium (1, 12, 18). Consistent with this notion, we observed smaller and similar $I_{Ks}$ in midmyocardial vs. endocardial or epicardial cells for both male and female dogs. Because $I_{Ks}$ was larger in female than male dogs in epicardium and endocardium, female dogs had a larger transmural $I_{Ks}$ gradient compared to male, potentially contributing to longer midmyocardial APDs and a larger repolarization gradient in females. These in turn may contribute to increased QT intervals and greater risks of TdP.

Sex differences in currents governing transmural repolarization might be
expected to produce differences in QT interval and T wave morphology, as well as in arrhythmia susceptibility. Women do have longer corrected QT intervals than men (4,24). Young men have larger T-wave offset dispersion than young and old women, whereas women have greater T-wave complexity following exercise and with autonomic blockade (33). Susceptibility to drug-induced TdP is clearly greater in women than men (11,22). Women might be less prone to reentrant arrhythmias for which M cell repolarization is limiting because of longer APDs; however, the enhanced transmural repolarization gradient we observed could promote reentry in women by favoring the establishment of unidirectional at the M cell border. This complex area clearly requires further study and analysis.

The principle arrhythmic risk known to be enhanced in women is drug-induced long QT syndrome, almost uniformly by \( I_{Kr} \) blocking drugs. Our data suggest reduced repolarization reserve in the M cell layer, since unlike the other 2 transmural layers, the larger \( I_{Ca,L} \) in females was not offset by larger \( I_{Ks} \). The notion of repolarization reserve involves an ability of the heart to minimize the effects of agents impairing repolarization, in particular \( I_{Kr} \) blocking drugs, by enhancing outward current carried by other channels, in particular \( I_{Ks} \) (28). When repolarization reserve is reduced, as in female M cells in the present study, the effect of \( I_{Kr} \) blockers would be expected to be enhanced, leading to excessive delay and destabilization of M cell repolarization and potentially early afterdepolarizations, transmural reentry, and TdP.
Potential Limitations

We performed detailed studies of a wide range of $K^+$-currents and of L-type $Ca^{2+}$-currents at 3 transmural levels of male and female canine myocardium. This is, to our knowledge, a much more broad and detailed comparison than in previous comparisons between male and female cardiomyocytes in the literature. However, other relevant currents and transport systems that could contribute to male-female differences, such as various $Cl^-$-currents, the $Na^+$-current system, the $Na^+\cdotCa^{2+}$-exchanger and $Na^+\cdotK^+$-ATPase were not assessed and should be in future studies. In addition, we compared ionic currents transmurally at one ventricular site, but AP properties are known to differ among cardiac regions with the potential for distinct transmural properties in different right and left ventricular areas (8, 9, 15, 29, 33). This issue, too, would be worthy of attention in further studies. It would be interesting to quantify potential differences between males and females in the transmural expression of subunits like Kir2.1-2.3, Kv4.3, Kv4.2, Kv1.4, KChIP2, Cav1.2, Kv1.5, ERG, minK, and KvLQT1, in order to define potential molecular bases for the current differences we observed. In addition, it would be interesting to study regulatory differences (e.g. in protein kinase A and C phosphorylation, phosphatase-mediated dephosphorylation) and differences in membrane trafficking of ion-channel subunits. However, the studies required would be extensive and go well beyond the scope of the present manuscript.
ACKNOWLEDGMENTS

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REFERENCES


18. **Liu DW, and Antzelveitch C.** Characteristics of the delayed rectifier current (I_{Kr} and I_{Ks}) in canine ventricular epicardial, midmyocardial, and


<table>
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<th>Region</th>
<th>Gender</th>
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<th>Slope (mV)</th>
<th>N</th>
<th>$V_{1/2}$ (mV)</th>
<th>Slope (mV)</th>
<th>N</th>
<th>$\tau_{fast}$ (ms)</th>
<th>$\tau_{slow}$ (ms)</th>
<th>N</th>
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<tr>
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<td>Female</td>
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<td>8.2±2.0</td>
<td>12.6±0.7</td>
<td>6</td>
<td>12.7±5.0</td>
<td>103±17.0</td>
<td>6</td>
</tr>
<tr>
<td></td>
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<td>12.1±0.7</td>
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<td>11.7±2.6</td>
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<td>11.8±4.1</td>
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<td>7.3±3.0</td>
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<td>6</td>
<td>19.6±1.0</td>
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There were no statistically significant differences between male and female results for corresponding regions.
<table>
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<td></td>
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<td>112±37</td>
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<td>109±38</td>
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There were no statistically significant differences between male and female results for corresponding regions.
**Table 3: $I_{\text{Ks}}$ Kinetics**

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<td>120±11 780±218 10</td>
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<tr>
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<td>1106±77 11</td>
<td>90±15 544±198 10</td>
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<tr>
<td>Mid</td>
<td>Female</td>
<td>1034±90 11</td>
<td>72±15 518±179 8</td>
</tr>
<tr>
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<td>1106±107 11</td>
<td>99±22 534±130 8</td>
</tr>
<tr>
<td>Endo</td>
<td>Female</td>
<td>1128±78 11</td>
<td>105±28 629±309 6</td>
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<tr>
<td></td>
<td>Male</td>
<td>1138±118 11</td>
<td>80±13 317±47 7</td>
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There were no statistically significant differences between male and female results for corresponding regions.
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<th>Activation</th>
<th>Recovery</th>
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<td>Slope (mV)</td>
<td>N</td>
</tr>
<tr>
<td>Epi</td>
<td>Female</td>
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<td>-5.8±0.3</td>
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<tr>
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<td>7</td>
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<tr>
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<td>6</td>
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<tr>
<td></td>
<td>Male</td>
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<td>-6.2±0.5</td>
<td>6</td>
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<tr>
<td>Endo</td>
<td>Female</td>
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<tr>
<td></td>
<td>Male</td>
<td>-43.3±0.7</td>
<td>-7.0±0.3</td>
<td>7</td>
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There were no statistically significant differences between male and female results for corresponding regions.
Figure legends

Figure 1. A, B and C, Typical AP recordings from female (left) and male (right) canine left ventricular myocytes in epicardium (A), midmyocardium (B) and endocardium (C) at 1 and 0.5 Hz. D, Mean±SEM of APD_{90} (at 90% repolarization) at 1 Hz in female (epicardium, n=17; midmyocardium, n=11; endocardium, n=16) and male (epicardium, n=16; midmyocardium, n=21; endocardium, n=16) transmural ventricular cells. E, mean±SEM QT interval over a range of BCLs in male vs female dogs. *P<0.05, **P<0.01, female vs. male.

Figure 2. A, Representative 1 mM Ba^{2+}-sensitive IK_{1} recordings from female and male epicardium cells by using the voltage protocol in the inset at 0.1 Hz. B, C and D, Mean±SEM IK_{1} density in female and male epicardium, midmyocardium and endocardium. TP, test potential.

Figure 3. A, C and E, Typical I_{to} recordings from female (left) and male (right) canine epicardium (A), midmyocardium (C) and endocardium (E) cells. I_{to} was obtained with 100-ms test pulses at 0.1 Hz (voltage protocol in inset). B, D and F, Mean±SEM I_{to} density from female and male epicardium, midmyocardium and endocardium, respectively. * P<0.05, ** P<0.01, female vs. male. TP, test potential.

Figure 4. I_{to} voltage dependence and kinetics. A (endocardium), D
(midmyocardium) and G (epicardium), $I_{so}$ inactivation and activation voltage dependence. Inactivation was evaluated with 1000-ms prepulses followed by a 200-ms test pulse to +50mV (at 0.1 Hz). Activation voltage dependence was analyzed from data obtained with the protocol in Fig. 3 ($I_{TP}=\alpha_{TP}G_{max}(V_{TP}-V_R)$). Data are mean±SEM ($n=6$ cells/group, inactivation; $n=6$ cells/group, activation); curves are best-fit Boltzmann relations. B (endocardium), E (midmyocardium) and H (epicardium), Mean±SEM inactivation $\tau_s$ ($n=10$ cells/group). C (endocardium), F (midmyocardium) and I (epicardium), $I_{so}$ reactivation time course evaluated by ratio of current ($I_2$) during a 100-ms test pulse ($P_2$, identical to $P_1$) to current ($I_1$) during a conditioning pulse ($P_1$) with varying $P_1$ to $P_2$ interval (HP = -80 mV, step to +50 mV at 0.07 Hz). Data are mean±SEM ($n=6$ cells/group); Curves are biexponential fits.

Figure 5. A, Representative chromanol 293B (50 $\mu$M)-resistant $I_{Kr}$ recordings in female (Left) and male (Right) epicardium cells. B, Mean±SEM normalized $I_{Kr}$ tail currents ($n=8$ cells/group, from epicardium) and best-fit Boltzmann relations. C, D and E, Mean±SEM $I_{Kr}$ density voltage relations in female and male epicardium (C), midmyocardium (D) and endocardium (E) cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$, female vs. male.
Figure 6. A, Representative E-4031 (5 µM)-resistant $I_{Ks}$ recordings in female (Left) and male (Right) epicardium cells, with 4-second depolarizing pulses (0.1 Hz) and 2-second repolarizations to -40 mV. B, Mean±SEM normalized $I_{Ks}$ tail currents ($n=5$ cells/group, from epicardium) and best-fit Boltzmann relations. C (epicardium), D (midmyocardium) and E (endocardium), Mean±SEM $I_{Ks}$ density voltage relations in female and male left ventricular cells.

Figure 7. Typical recordings of $I_{Ca,L}$ from epicardium of canine left ventricles in female (A) and male (B) dogs, obtained with 250-ms pulses (0.1 Hz) and a HP at -50 mV. C (epicardium), E (midmyocardium) and G (endocardium), Mean±SEM $I_{Ca,L}$ current density recorded by the protocol shown in A between female and male dogs. D (epicardium), F (midmyocardium) and H (endocardium), $I_{Ca,L}$ I-V relations (mean ± SEM, current was normalized to maximum current at +20 mV for each cell). *$P<0.05$, female vs. male.

Figure 8. $I_{Ca,L}$ kinetics and voltage dependence from epicardium (mean±SEM). Similar results were obtained in all regions. A, Mean±SEM $I_{Ca,L}$ inactivation time constant ($n=10$ cells/group), obtained with the protocol in Fig. 7A. B, Voltage dependence of $I_{Ca,L}$ inactivation and activation. Steady-state inactivation was assessed with 1000-ms conditioning pulses followed by a 300-ms
test-pulse to +10 mV (0.1 Hz). Activation was assessed from data obtained with the protocol in Fig. 7A (with \( I_{TP}=a_{TP}G_{max}(V_{TP}-V_R) \)). Data are mean±SEM (\( n=6 \) cells/group); curves are best-fit Boltzmann relations. C, \( I_{Ca,L} \) reactivation time course, studied with paired 100-ms pulses delivered with varying interpulse intervals at 0.1 Hz. Curves are monoexponential fits (\( n=6 \) cells/group). D, \( I_{Ca,L} \) frequency dependence, determined from the ratio of current during the 15\(^{th} \) pulse to current during the first pulse of a train of 100-ms depolarizations from -80 mV to +10 mV at frequencies indicated (\( n=6 \) cells/group).
Figure 1

A  Female  Male

B  M

C  Endo

D  APD_{90} (ms)

E  QT Interval (ms)

- Female
- Male

1 Hz  0.5 Hz

20 mV  100 ms

1 Hz  0.5 Hz

1 Hz  0.5 Hz

1 Hz  0.5 Hz
Figure 4

Endo

(A) Normalized Current vs. TP (mV)

(B) Time Constant (ms) vs. TP (mV)

(C) Time Constant (ms) vs. P1-P2 Interval (ms)

Mid

(D) Normalized Current vs. TP (mV)

(E) Time Constant (ms) vs. TP (mV)

(F) Time Constant (ms) vs. P1-P2 Interval (ms)

Epi

(G) Normalized Current vs. TP (mV)

(H) Time Constant (ms) vs. TP (mV)

(I) Time Constant (ms) vs. P1-P2 Interval (ms)
Figure 8

A

Time Constant (ms)

- Female
- Male

-10 0 10 20 30 40 50
TP (mV)

B

Normalized Current

- Female
- Male

-100 -80 -60 -40 -20 0 20 40 60
TP (mV)

C

\( \frac{I_2}{I_1} \)

- Female
- Male

0 0.2 0.4 0.6 0.8 1.0
P_1-P_2 Interval (ms)

D

\( \log \frac{1}{f_1} \)

- Female
- Male

0 1 2 3 4 5 6
Frequency (Hz)
Chapter 3. Mechanisms underlying rate-dependent remodeling of transient outward potassium current in canine ventricular myocytes
I then studied the mechanisms underlying regulation of ion channel expression in relation to another clinical relevant condition. Downregulation of $I_{to}$ and its pore-forming $\alpha$-subunit Kv4.3 expression in the ventricle is the most consistent finding in chronic ventricular tachycardia induced failing animal heart or failing human hearts. Mechanisms underlying $I_{to}$ and Kv4.3 remodeling in tachycardia induced HF ventricles are not clear. The studies in this Chapter were designed to, first, test the hypothesis that increased heart rate *per se* regulates ion channel expression (specifically, $I_{to}$) by applying a novel *in vitro* adult ventricular cell pacing model; second, identify underlying signaling pathways involved in this potential rate-dependent regulation of $I_{to}$. The candidate signaling pathways that might contribute to rate-dependent $I_{to}$ remodeling, is the intracellular Ca/CaM activated CaMKII or calcineurin/NFAT signaling, since there are beat-to-beat oscillations in $[Ca]_i$ and potential heartbeat-to-heartbeat response of Ca signaling over a longer time scale that may regulate gene expression. We used different pharmacological tools to investigate roles of Ca signaling in tachypacing induced $I_{to}$ remodeling.
Mechanisms Underlying Rate-dependent Remodeling of
Transient Outward Potassium Current in Canine
Ventricular Myocytes

Ling Xiao, Pierre Coutu, Louis R. Villeneuve, Artavazd Tadevosyan,
Ange Maguy, Sabrina Le Bouter, Bruce G. Allen, Stanley Nattel

Short title: Tachycardia induced I_to downregulation
Word count: 5998 (not counting Word count, subject codes and short title)
Subject codes: [5], [132], [152]

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Abstract—Transient outward K⁺-current (I_{to}) downregulation following sustained tachycardia in vivo is usually attributed to tachycardiomypathy. This study assessed potential direct rate-regulation of cardiac I_{to} and underlying mechanisms. Cultured adult canine left-ventricular cardiomyocytes (37°C) were paced continuously at 1 or 3 Hz for 24 hours. I_{to} was recorded with whole-cell patch-clamp. 3-Hz pacing reduced I_{to} by 44% (P<0.01). Kv4.3 mRNA and protein expression were significantly reduced (by ~30% and ~40% respectively) in 3-Hz paced cells relative to 1-Hz cells, but KChIP2 expression was unchanged. Prevention of Ca^{2+}-loading with nimodipine or calmodulin inhibition with W-7, A-7 or W-13 eliminated 3-Hz pacing-induced I_{to}-downregulation, whereas downregulation was preserved in the presence of valsartan. Inhibition of Ca^{2+}/calmodulin-dependent protein kinase-II (CaMKII) with KN93, or calcineurin with cyclosporine-A, also prevented I_{to}-downregulation. CaMKII-mediated phospholamban phosphorylation at threonine-17 was increased in 3-Hz paced cells, compatible with enhanced CaMKII activity, with functional significance suggested by acceleration of the Ca^{2+},i-transient decay time-constant (Indo 1-AM microfluorescence). Total phospholamban expression was unchanged, as was expression of Na⁺,Ca^{2+}-exchange and sarcoplasmic reticulum Ca^{2+}-ATPase proteins. Nuclear localization of the calcineurin-regulated nuclear factor of activated T-cells (NFAT) c3 was increased in 3-Hz paced cells compared to 1-Hz (immunohistochemistry, immunoblot). INCA-6 inhibition of NFAT prevented I_{to} reduction in 3-Hz paced cells. Calcineurin-activity increased after 6 hours of 3-Hz pacing. CaMKII-inhibition prevented calcineurin-activation and NFATc3 nuclear-translocation with 3-Hz pacing. We conclude that tachycardia downregulates I_{to}-expression, with the Ca^{2+}/calmodulin-dependent CaMKII and calcineurin/NFAT
systems playing key $\text{Ca}^{2+}$-sensing and signal-transducing roles in rate-dependent $I_{\text{to}}$ control.

**Key Words:** potassium channels, calcium, calmodulin, remodeling, arrhythmias
Introduction

Sudden cardiac death due to ventricular tachycardia or fibrillation is an important contributor to mortality in congestive heart failure (CHF) patients. Rapid heart-rhythms can impair cardiac function and patients with “tachycardiomyopathy” are at risk of sudden cardiac death. Chronic ventricular tachypacing (VTP) in experimental animals produces a dilated cardiomyopathy that mimics clinical tachycardiomyopathy and is often used as an experimental model to study CHF-related cardiac remodeling. Changes in cardiac ion-channel transport are an important component of this remodeling and extensive evidence suggests that these ion-transport changes are a crucial contributor to the pathogenesis of CHF-related ventricular tachyarrhythmias and sudden death. Among the most ubiquitous changes are alterations in the transient-outward $K^+$-current ($I_{to}$), which play potentially important roles in repolarization abnormalities, cardiac dysfunction and arrhythmogenesis. Although CHF itself can cause $I_{to}$-downregulation, the possibility that rapid cardiomyocyte rate per se can alter $I_{to}$ function has not been examined. This possibility cannot be examined directly in vivo, because sustained tachycardia causes a CHF syndrome, with major attendant hemodynamic, neurohumoral and autonomic nervous system alterations, making it impossible to discern the role of heart rate per se. In the present study, we used a model of paced adult canine ventricular cardiomyocytes to determine: 1) whether increases in firing-rate alter $I_{to}$; and if so, 2) the signaling systems involved.
Methods

An expanded Materials and Methods section is available in the On-line Data Supplement.

In Vitro Cellular-Pacing Model

Animal-care procedures followed National Institutes of Health guidelines. Adult male mongrel dogs (20-37 kg, n=114) were anesthetized with pentobarbital (30 mg/kg IV). Epicardial cardiomyocytes were isolated as previously described. After isolation, cells were kept in medium-199 and centrifuged (500 rpm, 1 minute, 25°C). Cell-pellets were re-suspended and plated on laminin-coated glass coverslips (for electrophysiology or immunohistochemistry) or 4-well rectangular petri dishes (Western blot or real-time RT-PCR). After 4-hour preincubation, cells were divided into groups for parallel study in each experimental protocol and were electrically paced with 5-ms pulses at 1 or 3 Hz (1-Hz and 3-Hz paced cells respectively) for 24 hours, unless otherwise indicated. After pacing, cells were kept in a high-K⁺ storage solution at 4°C for electrophysiological studies, or fast-frozen at -80°C for biochemical studies.

Electrophysiology

I_{to} was recorded at 36±0.5°C (for details see on-line Methods). Currents are expressed as current-densities (normalized to cell capacitance). Resting potentials, compensated series-resistances, cell-capacitances and cell-dimensions were similar for 1-Hz and 3-Hz groups (on-line Table 1). Correction for liquid junction-potentials
(which averaged ~10 mV) was applied only for resting-potential and reversal-potential values.

**Real-time PCR**

Total RNA was extracted from 1-Hz and 3-Hz paced cells with TRIzol™. Real-time reverse transcription (RT)-quantitative polymerase chain reaction was performed with Taqman assays for Kv4.3, Kv1.4 and KChIP2 with 18S ribosomal-RNA as the internal control.

**Immunoblotting**

Membrane-protein fractions were isolated from 1-Hz and 3-Hz paced cells incubated without (CTL) or with KN93 or KN92. Proteins were separated on 8% or 12% SDS-PAGE gels and transferred to PVDF membranes. Blots were probed with primary antibodies against GAPDH (internal control for protein-loading), Kv4.3, KChIP2, phospholamban phosphorylated at threonine-17, total phospholamban, calcineurin, Na⁺,Ca²⁺-exchanger (NCX), ryanodine-receptor (RyR2), sarcoplasmic-reticulum (SR) Ca²⁺-ATPase (SERCA2a), NFATc3 and NFATc4.

**Confocal Microscopy**

After 24-hour pacing, cells were washed with culture-medium, then fixed with 2%-paraformaldehyde and thrice-washed (5 minutes each) with phosphate-buffered saline (PBS). After blocking and permeabilization (2%-normal donkey serum, NDS, and normal goat serum, NGS, along with 0.2% Triton X-100 for 1 hour), cells were incubated overnight at 4°C with primary antibodies for nuclear factor of activated T-
cells (NFAT) c3 (1:200, mouse monoclonal) and NFATc4 (1:100, rabbit polyclonal) in PBS containing 1%-NDS and 1%-NGS and 0.05%-Triton, followed by 3 washes and secondary antibody (donkey-anti-mouse Alexa-547 and goat-anti-rabbit Alexa-488) incubation. Cells were then treated with RNaseA (100-µg/mL) and incubated with ToPro3 (1-µmol/L, for nuclear-contour definition). Confocal microscopy was performed with a Zeiss LSM-510 system. Images were deconvolved using measured point-spread functions. Nuclear and cytosolic NFATc3 and NFATc4 staining densities were determined as the sum of the pixels within each region normalized to region-area. Measurements were repeated in 5 Z-stacks showing the maximum nuclear area in each cell.

**Calcineurin-activity**

Paced-cell samples were collected after 6 hours of 1-Hz, 3-Hz CTL, 3-Hz+KN93 or 3-Hz+KN92 (culture media containing 1-µmol/L KN93 or KN92) pacing, based on preliminary studies showing peak activity after 6-hour 3-Hz pacing. Calcineurin-activity was assessed with the Calcineurin Cellular Activity Assay Kit (Calbiochem).

**Data Acquisition and Analysis**

Clampfit 6.0 (Axon) and GraphPad Prism 3.0 were used for data-analysis; curve-fitting was performed with nonlinear least-square algorithms. Group comparisons were performed with paired or unpaired Student *t*-tests or repeated-measures ANOVA with Bonferroni-corrected *t*-tests or Dunnett’s tests. A two-tailed *P*<0.05 indicated statistical significance; group data are expressed as mean±SEM.
Results

**Rapid Rates Downregulate $I_{to}$ and Kv4.3**

Representative $I_{to}$-recordings from cells paced at 1 Hz (to mimic normal resting heart rates) and 3 Hz (to mimic tachycardia) are shown in Figure 1A. Cells paced at 3 Hz showed smaller $I_{to}$ with otherwise similar morphology versus 1-Hz paced cells and had significantly-smaller $I_{to}$-densities over a wide range of voltages, with an ~45% reduction (Figure 1B). $I_{to}$ inactivation-kinetics were well-fitted by biexponential relations, with inactivation time-constants not different between 1-Hz and 3-Hz cells (Figure 1C). The voltage-dependence of $I_{to}$ inactivation was studied with a two-pulse protocol (Figure 1D inset). Boltzmann-relation fits showed no differences between 1-Hz and 3-Hz: $V_{1/2}$s and slope factors averaged -39.9±2.8 mV and -3.8±0.1 mV respectively in 1-Hz (n=6) and -39.8±1.8 mV and -4.1±0.1 mV in 3-Hz cells (n=7, $P=NS$ versus 1-Hz). $I_{to}$ activation voltage-dependence was assessed based on the relation $I_v = I_{max}(V-V_r)(G_v/G_{max})$, where $I_v$ and $G_v$ are current and conductance at voltage $V$; $I_{max}$ and $G_{max}$ are maximum current and conductance and $V_r$ is the reversal potential. $V_r$ was determined by analyzing tail-currents after 2.2-ms depolarizations to +50 mV and averaged -75.9±0.5 mV in 1-Hz (n=5) and -75.4±2.3 mV in 3-Hz cells (n=5; $P=NS$). $I_{to}$ activation $V_{1/2}$ averaged 8.9±0.6 mV in 1-Hz (n=9) and 9.7±1.5 mV in 3-Hz cells (n=8, $P=NS$). $I_{to}$ reactivation (2-pulse protocol, Figure 1E) was well-fitted by biexponential relations. Recovery time-constants ($\tau$s) averaged 30±2 ms ($\tau$-fast) and 130±21 ms ($\tau$-slow) in 1-Hz (n=7) and 29±3 ($\tau$-fast) and 149±12 ms ($\tau$-slow) in 3-Hz cells (n=8, $P=NS$ versus 1-Hz). $I_{to}$ frequency-dependence based on steady-state currents at 0.1, 0.5, 1, 2 and 5 Hz (100-ms pulses...
from -80 to +50 mV) was not different between 1-Hz and 3-Hz cells (on-line Figure 1). To assess $I_{\text{to}}$-downregulation in vivo, we tachypaced 4 dogs at 240 bpm for 24 hours and compared $I_{\text{to}}$ on freshly-isolated cardiomyocytes from tachypaced and control dogs. The results (on-line Figure 2) show significant decreases in $I_{\text{to}}$, consistent with in-vitro observations.

To address the potential mechanisms underlying $I_{\text{to}}$-downregulation, we first assessed mRNA and protein expression of potential underlying subunits: Kv4.3, Kv1.4 and KChIP2. 3-Hz pacing significantly downregulated Kv4.3 mRNA (Figure 2A). Kv1.4 and KChIP2 mRNA-expression was unaffected by 3-Hz pacing. Figure 2B shows examples of Kv4.3, KChIP2 and GAPDH immunoblots (top) and overall mean±S.E.M. protein-expression (bottom). Consistent with mRNA results, 3-Hz pacing downregulated Kv4.3 protein-expression by ~40%, whereas KChIP2 protein-expression was unchanged.

These data indicate that rapid firing-rates reduce $I_{\text{to}}$-density through downregulation of Kv4.3 mRNA and protein. We next determined whether $I_{\text{to}}$-downregulation requires cardiomyocyte mechanical activity and associated metabolic demands or whether electrical activity is sufficient for downregulation. Blebbistatin (5-µmol/L), an excitation-contraction uncoupler with minimal direct electrophysiological actions, was added to the culture medium during 24-hour pacing at 1- and 3-Hz. Ca$^{2+}$-transient activity in the absence of cell-shortening confirmed cell-capture during electromechanical uncoupling, as previously described. On-line Figure 3A shows $I_{\text{to}}$ recordings from cells studied in parallel with 1-Hz and 3-Hz pacing, with and without blebbistatin, which failed to prevent rate-induced $I_{\text{to}}$-downregulation (on-line Figure 3B). We then addressed the possibility that the
results of 3-Hz stimulation could be due to direct effects of larger total durations of electrical field-stimulation. In parallel experiments, we subjected cells to 1- and 3-Hz pacing with 3-ms stimuli, as well as to 1-Hz stimulation with 9-ms stimuli (to provide the same total field-stimulation duration as 3-Hz 3-ms stimuli), keeping stimulus-intensities constant. As shown in on-line Figure 4, 1-Hz stimulation with 9-ms pulses failed to reproduce the effects of 3-Hz 3-ms stimuli. We next determined whether downregulation of $I_{to}$ requires angiotensin-II receptor (ATR) stimulation. Cells were subjected to 24 hours of 1- or 3-Hz pacing in the presence of the type-1 ATR-antagonist valsartan (1-µmol/L). Valsartan failed to alter $I_{to}$ rate-regulation (on-line Figures 5A and B). We then turned to investigate candidate Ca$^{2+}$-dependent signal-transduction mechanisms for rate-dependent $I_{to}$ regulation.

Role of Ca$^{2+}$-Entry and Calmodulin

Intracellular [Ca$^{2+}$] and Ca$^{2+}$-binding to calmodulin are dynamic, changing on a beat-to-beat basis in cardiomyocytes. If [Ca$^{2+}$]$_{i}$-changes are important in mediating the $I_{to}$ frequency-response, suppressing activation-related Ca$^{2+}$-entry through I$_{CaL}$ should prevent rate-related $I_{to}$-downregulation. Figures 3A and B show $I_{to}$ recorded from cells cultured during 1-Hz or 3-Hz pacing, in the presence of nimodipine (0.5-µmol/L, which decreased I$_{CaL}$ by ~75%, on-line Figure 6) or matching vehicle (CTL). The $I_{to}$-suppressant effect of 3-Hz pacing was eliminated by I$_{CaL}$-blockade (Figure 3C). We then incubated cells with W-7 (1-µmol/L, to inhibit calmodulin) or vehicle, and repeated these studies with 2 other calmodulin-antagonists, A-7 (at 1- and 5-µmol/L) and W-13 (40-µmol/L), along with its inactive analog W-12 (40-µmol/L), because of potential concerns about the efficacy and specificity of W-7. Calmodulin-inhibition
prevented $I_{to}$-downregulation in 3-Hz cells (Figure 3D, on-line Figure 7). Thus, $Ca^{2+}$-dependent calmodulin function is implicated in rate-dependent $I_{to}$-downregulation.

**Role of CaMKII**

High-frequency activation of $Ca^{2+}$-transients increases CaMKII-activity. To determine whether CaMKII-activity is increased by 3-Hz pacing, we determined CaMKII-mediated Thr-17 phosphorylation of phospholamban in cells cultured with vehicle (CTL), KN93 (1-$\mu$mol/L, a CaMKII inhibitor) or equivalent concentrations of the inactive analog KN92, after preliminary experiments showed that 1-$\mu$mol/L KN93 has no effect on $I_{CaL}$ (on-line Figure 8). CaMKII-phosphorylated phospholamban expression was significantly increased in 3-Hz paced cells (Figure 4A; for expanded Western blots see on-line Figure 9A), although total phospholamban expression was unaltered. For cells paced at 1- or 3-Hz in the presence of KN93 to inhibit CaMKII activity, there were no differences in Thr-17 PLB-phosphorylation (Figures 4C and D). Cells paced at 3-Hz in the presence of KN92 showed increased CaMKII phospholamban-phosphorylation similar to controls (Figures 4E and F).

To obtain functional evidence for CaMKII-activation, we studied the potential effect of CaMKII-hyperphosphorylation of phospholamban, which should enhance the rate of removal of cytosolic $Ca^{2+}$ via SR $Ca^{2+}$-uptake (by removing phospholamban inhibition of SR $Ca^{2+}$-ATPase function). $Ca^{2+}$-transients were recorded with Indo-1 AM as previously described. $Ca^{2+}$-transients recorded at a 1000-ms cycle-length from 1- and 3-Hz paced cells are shown in Figure 4G. The $Ca^{2+}$-transient decay time-constant was significantly decreased in 3-Hz paced cells (Figure 4H: time-constants averaged 345±35 ms (n=6) in 3-Hz cells versus 454±30
ms in 1-Hz cells (n=8), \( P=0.037 \). Differences in Ca\(^{2+}\)-transient decay-time could also be due to alterations in other Ca\(^{2+}\)-handling proteins. The expression of other important Ca\(^{2+}\)-handling proteins was assessed (on-line Figure 10) and showed no effect of 3-Hz pacing.

To determine whether increased CaMKII activity contributes to the \( I_{\text{to}} \)-suppressing effect of 3-Hz pacing, \( I_{\text{to}} \) was recorded from cells exposed to vehicle (CTL), KN93 (1-\( \mu \)mol/L) or KN92 (1-\( \mu \)mol/L) during 24-hour 1- or 3-Hz pacing. Figures 5A and B show original recordings of \( I_{\text{to}} \) upon depolarization to +40 mV from 1- and 3-Hz paced cells. CaMKII-inhibition by KN93 prevented 3-Hz pacing-induced \( I_{\text{to}} \) reduction, but had no effect on \( I_{\text{to}} \) in 1-Hz paced cells (Figure 5C). KN92 had no protective effect on \( I_{\text{to}} \)-downregulation. We then studied the effects of CaMKII-inhibition on Kv4.3 and KChIP2 protein-expression (Figure 5D; expanded Western-blots in on-line Figure 9B). Whereas in the presence of CaMKII-inhibition with KN93, Kv4.3 protein-expression was not reduced in 3-Hz cells, 3-Hz cells incubated in KN92 continued to show significant Kv4.3-downregulation. KChIP2 expression was unaltered in the presence of KN93, excluding non-specific effects on protein-expression.

**Role of Calcineurin/NFAT System**

Ca\(^{2+}\)/calmodulin also activates calcineurin, a protein-phosphatase that alters gene-expression by dephosphorylating the transcription factor NFAT. We first compared calcineurin-activity in 1- and 3-Hz paced cells at 6 hours after pacing initiation. As shown in Figure 6A, calcineurin-activity was increased >2-fold in 3-Hz paced cells. Calcineurin protein-expression was not altered after 6 hours of 3-Hz pacing (Figure
6B; expanded Western-blots in on-line Figure 9C), consistent with the notion that calcineurin was functionally activated by increased Ca\(^{2+}\)-entry in 3-Hz cells. To test for the potential role of calcineurin in I\(_{to}\)-downregulation, I\(_{to}\) changes were studied in 1- and 3-Hz paced cells incubated with cyclosporine-A (1-\(\mu\)g/mL, ~0.8-\(\mu\)mol/L) or vehicle (CTL) during pacing (I\(_{to}\)-recordings shown in Figure 6C). Cyclosporine-A prevented 3-Hz pacing-induced I\(_{to}\)-reduction (Figure 6D), supporting the importance of calcineurin in I\(_{to}\)-downregulation.

To assess the potential role of the calcineurin downstream mediators NFAT c3 and c4, their cellular localization was studied by confocal microscopy. Deconvolved images of NFATc3 (red) and c4 (green) staining are shown in Figure 7A. Figure 7B shows relative nuclear/cytosolic signal ratios. Examples of ToPro3 co-localization used to identify the nuclear region are shown in on-line Figure 11. The NFATc3 nuclear/cytosolic staining-ratio was significantly increased in 3-Hz cells, compatible with nuclear relocalization. In order to assess nuclear NFAT-localization with an independent method, we performed immunoblots on purified nuclear extracts. The results confirmed increased nuclear NFATc3-localization in 3-Hz paced cells (on-line Figure 12). We then applied a cell-permeable NFAT inhibitor, INCA-6, to study the functional importance of NFAT in the I\(_{to}\)-response.\(^{14}\) Cells were incubated with vehicle (CTL) or INCA-6 (5-\(\mu\)mol/L) during 24-hour pacing at 1- or 3-Hz. I\(_{to}\)-recordings at +40 mV are shown in Figure 7C and mean data in Figure 7D. INCA-6 prevented I\(_{to}\)-downregulation in 3-Hz cells, supporting the importance of NFAT as a mediator.

Our results point to the participation of both CaMKII and calcineurin systems as mediators of Ca\(^{2+}\)/calmodulin-effects. Previous studies suggest potential cross-talk
between CaMKII and calcineurin systems. We wondered whether cross-talk between these systems could be contributing to calcineurin-mediated effects in our model, and assessed the effects of the CaMKII inhibitor KN93 or its inactive analog KN92 on calcineurin-activation by 3-Hz pacing. Calcineurin-activation was suppressed by CaMKII inhibition with KN93 (Figure 6A), but not by KN92, suggesting that intact CaMKII function is needed for 3-Hz pacing-induced enhancement of calcineurin function. Further support for this notion was provided by examining the effects of CaMKII inhibition on nuclear translocation of NFATc3. As shown in Figure 7B, KN93 (but not KN92) prevented NFATc3 nuclear translocation.

**Discussion**

In this study, we found that rapid cardiomyocyte firing decreases $I_{to}$-density through downregulation of $I_{to} \alpha$-subunit (Kv4.3) gene and protein expression. Rate-dependent $I_{to}$-downregulation is mediated by increased $\text{Ca}^{2+}$/calmodulin-activated CaMKII and calcineurin/NFAT signaling. Blockade of these pathways prevents rate-related $I_{to}$-remodeling. A schematic summary of our findings is presented in Figure 8.
Relation to Previous Studies of $I_{to}$-Downregulation in Tachypaced Models

Ventricular tachypacing is frequently used to create in vivo animal models of CHF.\(^1\)\(^4\)\(^\text{,}^5\) $I_{to}$-downregulation is a consistent finding,\(^1\)\(^3\)\(^5\)\(^,\)\(^6\) generally with reductions in $I_{to}$-density unaccompanied by significant changes in biophysical properties. In our in vitro tachypaced cardiomyocyte model, $I_{to}$-density was similarly reduced with no change in voltage-dependence or kinetic properties. As observed for in vivo tachypaced dog\(^1\)\(^7\)\(^,\)\(^8\) or rabbit\(^1\)\(^9\) models, Kv4.3 mRNA and protein were reduced, consistent with transcriptional downregulation.

Previous investigators have provided evidence suggesting that increased heart rate may be involved in cardiac hypertrophic signaling.\(^2\)\(^0\)\(^,\)\(^2\)\(^1\) The absence of changes in cellular capacitance and dimensions in 3-Hz paced cells make significant cellular hypertrophy unlikely in our model. Shortly after the onset of rapid electrical stimulation (15 minutes), angiotensin-II secretion and expression levels increase in cultured neonatal rat cardiomyocytes, returning to baseline after several hours.\(^2\)\(^2\) Incubation of epicardial ventricular myocytes with angiotensin-II decreases $I_{to}$ amplitude and changes its voltage-dependent and kinetic properties.\(^2\)\(^3\) Angiotensin-receptor stimulation was not essential for $I_{to}$-downregulation by 3-Hz pacing in our model, because significant downregulation continued to occur in the presence of AT\(_1\)-receptor blockade with valsartan.

Ca\(_{2+}\)/calmodulin as a Rate Sensor

Intracellular Ca\(_{2+}\)-concentration changes provide key signaling messages in a variety of systems.\(^1\)\(^1\)\(^,\)\(^1\)\(^2\)\(^,\)\(^2\)\(^4\) The role of Ca\(_{2+}\) is particularly important in sensing alterations in the frequency and form of neuronal activity,\(^1\)\(^2\) with Ca\(_{2+}\)-calmodulin binding inducing
CaMKII autophosphorylation and activation. Dynamic fluctuations in calmodulin-bound \( Ca^{2+} \) show both phasic components tracking intracellular \( Ca^{2+} \)-concentration alterations and sustained changes that integrate \( Ca^{2+} \)-concentrations over time.\(^\text{12}\) Inhibition of \( Ca^{2+} \)-entry through L-type \( Ca^{2+} \)-channels suppresses long-term memory effects \textit{in vivo} in dogs\(^\text{25}\) and tachycardia-induced decreases in \( Ca_{v1.2} \) protein expression in HL-1 cells.\(^\text{26}\) The importance of \( Ca^{2+} \)/calmodulin sensing in rate-dependent \( I_{to} \) changes in our system was indicated by the ability of either \( Ca^{2+} \)-channel blockade or calmodulin inhibition to prevent \( I_{to} \)-downregulation.

**Role of CaMKII and Calcineurin Signaling**

\( Ca^{2+} \)-response amplitude and duration are coupled to a variety of downstream regulatory systems including transcription changes, for which NFAT is particularly important.\(^\text{24}\) NFAT is activated by calcineurin dephosphorylation of the NFAT regulatory domain, which is triggered by increased \( Ca^{2+} \)-calmodulin binding.\(^\text{27}\)

\textit{In-vitro} tachystimulation of neonatal rat cardiomyocytes\(^\text{28}\) or atrial tissue slices\(^\text{20}\) activates calcineurin/NFAT signaling. Calcineurin has been reported to alter \( I_{to} \) expression in a number of cardiac systems. Increased extracellular \( Ca^{2+} \)-concentration induces \( I_{to} \)-downregulation in rat ventricular cardiomyocytes due to reduced \( Kv4.2 \) mRNA expression, which is prevented by the calcineurin inhibitors FK506 or cyclosporine-A.\(^\text{29}\) In mice, the transmural \( I_{to} \) gradient is set by calcineurin/NFAT-mediated downregulation of endocardial \( Kv4.2 \) and KChIP2 expression, related to higher intracellular \( Ca^{2+} \)-concentrations in endocardium.\(^\text{30}\) Similarly, \( I_{to} \) and corresponding-subunit mRNA downregulation resulting from acute myocardial infarction in rats is associated with increased NFAT-activity, and is
abolished in NFATc3 knockout mice or by treatment with cyclosporine-A.\textsuperscript{31} Our results agree with these studies showing that calcineurin/NFAT-signaling plays a central role in I\textsubscript{to}-downregulation. In contrast, Gong et al. found that overexpression of constitutively-active calcineurin in neonatal rat cardiomyocytes induces hypertrophy and I\textsubscript{to}-upregulation.\textsuperscript{32} This discrepancy may be due to differences in the cellular environment within which calcineurin-activation occurs.

High-frequency activity in neurons is transduced into CaMKII activation by increased Ca\textsuperscript{2+}-calmodulin binding.\textsuperscript{12} Mice with chronic CaMKII-inhibition show action potential shortening caused by upregulation of both I\textsubscript{to} and the inward-rectifier I\textsubscript{K1}.\textsuperscript{33} This response requires the presence of intact phospholamban and appears to be related to reduced CaMKII-induced phospholamban phosphorylation. There is evidence for interactions between calcineurin and CaMKII signaling effects on cardiac electrophysiology. Khoo et al. showed that CaMKII-signaling is increased in calcineurin overexpressing mice, and that their arrhythmias and left-ventricular dysfunction are improved by CaMKII-inhibitory drugs or in cross-bred calcineurin-overexpressing/CaMKII-inhibited strains.\textsuperscript{34} We found that calcineurin-activation and NFAT-relocalization did not occur in cells that were tachypaced in the presence of a CaMKII-inhibitor. Prior studies have shown CaMKII-phosphorylation of calcineurin at very low Ca\textsuperscript{2+}-concentrations that inhibit calcineurin function.\textsuperscript{15} It is conceivable that CaMKII may modulate calcineurin function by phosphorylating other regulatory proteins, but the biochemical mechanisms involved remain to be established.

\textbf{Novel Findings and Potential Significance}
Although tachycardia-induced cardiomyopathy consistently causes $I_{to}$-downregulation \textit{in vivo},\textsuperscript{3-6} it is impossible in such a system to discriminate frequency-dependent phenomena from secondary changes due to altered hemodynamics, neurohormonal state and the heart failure syndrome. Our study is to our knowledge the first to assess the effect of firing-rate on $I_{to}$ in an adult cardiomyocyte system and to study underlying regulatory mechanisms. We have uncovered a complex system in which Ca\textsuperscript{2+} acts as a frequency sensor that couples via calmodulin to downstream signals that alter $I_{to}$-expression by changing the phosphorylation states of key proteins. Our results add to a growing body of evidence indicating that calcineurin/NFAT-signaling acts to downregulate $I_{to}$ in a variety of physiological contexts including tachycardia, Ca\textsuperscript{2+}-loading caused by increased extracellular Ca\textsuperscript{2+},\textsuperscript{29} subendocardial tissues\textsuperscript{30} and myocardial infarction.\textsuperscript{31} Like Rossow et al,\textsuperscript{30} we found increased calcineurin-activity with unchanged calcineurin expression in a context of Ca\textsuperscript{2+}-loading related $I_{to}$ regulation. In addition, we noted that CaMKII-inhibition prevents calcineurin-activity increases. Tachycardia shortens action potential duration, which would tend to reduce Ca\textsuperscript{2+}-entry as well as the time available for systole and cell contraction. Reductions in $I_{to}$ might offset this effect by raising the plateau level and maintaining contraction strength. In pathological situations, however, $I_{to}$-downregulation could promote arrhythmogenesis, particularly in contexts of abrupt rate slowing and reduced repolarization reserve.

**Potential Limitations**

There are well-recognized transmural differences in $I_{to}$-density and properties.\textsuperscript{30,35} In addition, different regions of the heart vary in electrophysiological and ion-current...
These could affect rate-dependent $I_{to}$ regulation. We performed our studies in cells from the epicardium of canine left ventricles in order to prevent regional and transmural differences from adding uncontrolled variability to the results. A comprehensive study of the mechanisms of rate-dependent regulation of $I_{to}$ in different cardiac regions and transmural layers is beyond the scope of the present study, but the issue merits further investigation. The cell-permeable agents available for CaMKII inhibition, of which KN93 is the most widely used, have imperfect specificity, including potential $I_{Ca,L}$-blocking properties. For this reason, we were particularly careful to verify that the KN93 concentration we used does not block $I_{Ca,L}$. However, we cannot totally exclude the possibility that actions of KN93 other than its CaMKII-inhibiting ability could have contributed to its effects in our system.

Tachycardia *per se* is clearly not the only factor that can alter $I_{to}$ in ventricular-tachypaced *in-vivo* models. In addition to CHF-related neurohumoral activation, hemodynamic and metabolic changes, an altered sequence of ventricular activation can importantly alter cardiac repolarization by affecting $I_{to}$. Yu et al have shown that 2-Hz ventricular-pacing of dog hearts for 3 weeks slows recovery from inactivation, positively shifts inactivation voltage-dependence and reduces conductance. Consistent with our findings, $I_{to}$-conductance decreases were associated with comparable reductions in mRNA-expression; however, $I_{to}$-kinetics and voltage-dependence were not changed in our cells. The discrepancies may be due to differences in tachypacing-rate (2 versus 3-Hz) and duration (24 hours versus 3 weeks), along with the absence of altered activation pattern-related factors in our *in vitro* model.
NFAT is generally associated with upregulation of gene-expression in hypertrophic programs, but our results implicate NFAT in tachycardia-induced $I_{to}/Kv4.3$ downregulation. Recent work has established that NFAT may also selectively repress gene-expression, with important consequences for lymphocyte, adipocyte and activity-dependent skeletal-muscle gene regulation.³⁹

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**Disclosures Section**

None.
References


**Figure Legends**

**Figure 1.** A, Representative $I_{to}$ recordings from 1-Hz (left) and 3-Hz (right) paced cells. $I_{to}$ was obtained with 100-ms test pulses at 0.1-Hz (voltage protocol in the inset, right panel). B, Mean±S.E.M. density-voltage relations for $I_{to}$ in 1-Hz and 3-Hz cells. **$P<0.01$, ***$P<0.001$ versus 1-Hz. TP, test pulse. C, Mean±S.E.M. $I_{to}$ inactivation $\tau$ values (n=8 cells/group). D, Mean±S.E.M. voltage-dependence of $I_{to}$ inactivation and activation. E, $I_{to}$ reactivation time course evaluated by the ratio of current ($I_2$) during a 100-ms test pulse ($P_2$, HP=-80 mV, step to +50 mV at 0.07 Hz) to current ($I_1$) during a conditioning pulse ($P_1$, identical to $P_2$) with varying $P_1$ to $P_2$ interval. Data are mean±S.E.M; curves are biexponential fits.

**Figure 2.** A, Mean±S.E.M. normalized results for Kv4.3 (n=12/group), KChIP2 (n=12/group) and Kv1.4 (n=7/group) real-time PCR obtained with 1-Hz and 3-Hz paced cells. ***$P<0.001$, 3-Hz versus 1-Hz. B, Top, examples of Kv4.3, KChIP2 and GAPDH (performed on the same samples as in the lanes above) Western blots from 1-Hz and 3-Hz paced cells. Kv4.3 and KChIP2 bands were seen at the expected molecular masses (~70 kDa and ~32 kDa respectively). Bottom, mean±S.E.M. Kv4.3 and KChIP2 expression-levels relative to 1-Hz cell values (n=6/group). **$P<0.01$ versus 1-Hz.

**Figure 3.** A & B, Representative $I_{to}$ recordings obtained with 100-ms pulses from a holding potential of -50 mV to +40 mV (at 0.1-Hz) in parallel studies with 1-Hz or 3-Hz control (CTL) cells (panels A and B), 1-Hz or 3-Hz cells incubated in nimodipine
(Nimo, 0.5-μmol/L, panel A), and 1-Hz or 3-Hz cells in W-7 (1-μmol/L, panel B). C, Mean±S.E.M. I_{io} densities at +40 mV from 1-Hz CTL (n=7), 3-Hz CTL (n=8), 1-Hz nimodipine (n=5) and 3-Hz nimodipine (n=8) cells. **P<0.01, 3-Hz nimodipine versus 3-Hz CTL. D, Mean±S.E.M. I_{io} densities at +40 mV from 1-Hz CTL (n=11), 3-Hz CTL (n=8), 1-Hz W-7 (n=8) and 3-Hz W-7 (n=11) cells. *P<0.05, 3-Hz W-7 versus 3-Hz CTL.

**Figure 4.** A, C & E, Representative Western blots of phospholamban phosphorylated by CaMKII at Thr17 (P-PLB-17), total phospholamban (total PLB) and GAPDH from 1-Hz and 3-Hz CTL (A, n=6/group), 1-Hz and 3-Hz KN93 (C, n=3/group), 1-Hz and 3-Hz KN92 (E, n=6/group) paced cells. B, D & F, Mean±S.E.M. expression-levels for P-PLB-17, total PLB and ratio of P-PLB-17 to total PLB (P17/TPLB), following normalization to GAPDH-band intensities on the same lane, expressed relative to values in 1-Hz paced cells. *P<0.05, **P<0.01 versus 1-Hz. G, Representative Ca^{2+}-transients recorded from 1-Hz and 3-Hz paced cells during 1-Hz electrical stimulation. Ca^{2+}-transient decays were fitted by monoexponential relations. H, Mean±S.E.M. decay time-constants. *P<0.05, 1-Hz versus 3-Hz.

**Figure 5.** A & B, Representative I_{io} recordings under CTL, KN93 or KN92 treatment conditions from 1-Hz (A) and 3-Hz (B) paced cells, obtained with the voltage protocol shown in the inset. C, Mean±S.E.M. I_{io} densities at +40 mV for 1-Hz CTL (n=10), KN93 (n=7) and KN92 (n=8) cells and 3-Hz CTL (n=9), KN93 (n=7)
and KN92 (n=8) cells. *P<0.05, 3-Hz KN93 versus 3-Hz CTL. D, Upper panels: examples of Kv4.3, KChIP2 and GAPDH Western blots from KN93 or KN92-treated 1-Hz and 3-Hz paced cells. Bottom panel: Mean±S.E.M. Kv4.3 and KChIP2 expression-levels for KN93 (n=3/group) or KN92 (n=5/group) treated cells, normalized to GAPDH and expressed relative to 1-Hz cell values. *P<0.05 versus 1-Hz.

**Figure 6.** A, Mean±S.E.M. calcineurin-activity in 1-Hz, 3-Hz and 3-Hz paced cells cultured with KN93 or KN92 (n=15/group for 1-Hz and 3-Hz, n=9/group for 3-Hz+93 and 8/group for 3-Hz+92). *P<0.05, **P<0.01 versus 1-Hz. B, Top, examples of calcineurin (~61 kDa) and GAPDH Western blots from 1-Hz, 3-Hz and 3-Hz cells with KN93 (3-Hz+KN93) or KN92 (3-Hz+KN92) treatment during 6-hour pacing. Bottom, mean±S.E.M. calcineurin protein-expression after normalization to GAPDH (n=9/group). C, Examples of I$_{\text{to}}$ recordings in 1-Hz and 3-Hz cells incubated during 24-hour pacing under control (CTL) or Cyclosporin A (CyA, 1-μg/mL) conditions. D, Mean±S.E.M. I$_{\text{to}}$ densities at +40 mV for 1-Hz CTL (n=9) and CyA (n=9), 3-Hz CTL (n=10) and CyA (n=11) cells. ***P<0.001, 3-Hz CyA versus 3-Hz CTL.

**Figure 7.** A, Immunolocalization of NFATc3 and NFATc4 in 1-Hz and 3-Hz paced myocytes. B, Mean±S.E.M. ratios of nuclear/cytosolic NFATc3 and NFATc4 fluorescence intensities expressed relative to 1-Hz values. X/Y=number of cells/experiments for each bar. NFATc3 nuclear/cytosolic ratios increased
significantly with 3-Hz pacing relative to 1-Hz under control and KN92 conditions but not in the presence of KN93. No significant changes in NFATc4 ratios occurred.

C. Examples of $I_{to}$ recordings (same recording protocol as Figure 6) under control (CTL) or INCA-6 (5-μmol/L) treatment conditions in 1-Hz and 3-Hz paced cells.

D. Mean±S.E.M. $I_{to}$ densities at +40 mV for 1-Hz CTL and INCA-6 treated cells (n=10 cells/group), 3-Hz CTL and INCA-6 treated cells (n=9 cells/group). **P<0.01, 3-Hz INCA-6 versus 3-Hz CTL.

**Figure 8.** A schematic representation of our findings. Blockers used to confirm the role of specific components are shown in solid boxes. $I_{to}$ downregulation was prevented by inhibition of Ca$^{2+}$-entry, Ca$^{2+}$/calmodulin-inhibition, CaMKII-inhibition, calcineurin-inhibition and prevention of calcineurin-NFAT interaction, implying roles of the respective pathways. In addition, CaMKII-inhibition prevented calcineurin-activation and NFAT-relocalization, suggesting that CaMKII affects $I_{to}$ via crosstalk with calcineurin.
Figure 1

A 1-Hz

B

$10 \text{ pA/pF}$

25 ms

3-Hz

$70 \text{ mV}$

-50 mV

-40 mV

$T_d$ (N=12)

$3\text{-Hz}$ (N=14)

$I_d$ density (pA/pF)

TP (mV)

C

D

E

Normalized Current

$50 \text{ mV}$

$-80 \text{ mV}$

$100 \text{ ms}$

$P_1$

$P_2$

$I_1/I_2$

$L_1 - L_2$ interval (ms)

mV

ms

$-110$ $-70$ $10$ $50$

$-110$ $-70$ $10$ $50$

$-110$ $-70$ $10$ $50$

$-110$ $-70$ $10$ $50$

$mV$
Figure 4

A  CTL  C  KN93  E  KN92

B  CTL

D  KN93

F  KN92

G

H

[Ca^2+](nM)

Decay time constant (ms)

Time (ms)

(P=PLB-17  Total PLB  GAPDH)

(N=6)  (N=6)

1-Hz  3-Hz

Relative level to 1Hz

P=PLB-17  TPLB  P17/TPLB

P=PLB-17  TPLB  P17/TPLB

P=PLB-17  TPLB  P17/TPLB

1-Hz  3-Hz

*  **
Figure 7

A

1-Hz

3-Hz

nfatc3

nfatc4

B

Nuclear/Cytosolic ratio

P<0.001

P=0.66

P=NS

P=NS

P=NS

P=NS

C

1-Hz CTL

3-Hz INCA-5

3-Hz INCA-6

D

I_o density (pA/pF)

CTL

INCA-6

1-Hz

3-Hz

CTL

INCA-6
Figure 8

Tachycardia

Extracellular space

Sarcolemma

Cytosol

Ca\(^{2+}\)

CaM

CaMKII

Calcineurin

NFATc3/c4

KN93

W-7, A-7, W-13

Nimodipine

INCA-6

CyA

Kv4.3 Transcription

Kv4.3 Protein

\(I_{to}\)
On-line Materials and Methods

**In vitro Cellular Pacing Model**

*Cell Isolation.* All animal care and handling procedures followed the Animal Care Guidelines of the National Institutes of Health and were approved by the animal research ethics committee of the Montreal Heart Institute. Male adult mongrel dogs (20–37 kg) were anesthetized with pentobarbital (30 mg/kg IV) under artificial ventilation. Left lateral thoracotomy was performed, and hearts were quickly excised and immersed in oxygenated Tyrode’s solution at room temperature. The transmural free wall (~30 × 50 mm) of the anterior left ventricle, which contains coronary artery branches from the left circumflex coronary artery, was quickly dissected and the artery was cannulated. Cell isolation was performed by perfusion with Tyrode’s solution containing collagenase (120 U mL⁻¹, Worthington, type II) as previously described.¹ Cells from the epicardial (Epi) surface (1~1.5 mm thick) were taken after digestion and were kept in culture medium for further studies.

*Cell Culture and Pacing.* All procedures for cell culture and pacing were performed under aseptic conditions. After cell isolation, the cell suspension was centrifuged at 500 rpm for 1 minute at 4°C and cell pellets were re-suspended in fresh culture medium. Culture medium contained Medium 199 (GIBCO, Invitrogen Corp., with Earle’s salts, L-glutamine and 2,200 mg/L sodium bicarbonate), Na-penicillin G (100-U/mL) and streptomycin sulfate (100-μg/L), and was supplemented with Insulin-Transferrin-Selenium-X (GIBCO, Invitrogen Corp., containing 0.01-mg/mL insulin, 5.5-μg/mL transferrin, 6.7-ng/mL sodium selenite and 2-μg/mL
ethanolamine). Cells were plated at \(1 \times 10^4\) cells/cm\(^2\) on circular (18-mm diameter) or rectangular (24x55 mm) glass coverslips or 4-well rectangular petri dishes (24x67 mm/well, Nalge Nunc International) pre-coated with laminin (15-\(\mu\)g/mL). Myocytes were maintained in the culture media at 37°C in a humidified, 5% CO\(_2\)-enriched environment in the incubator. After 4 hours, any dead or unattached myocytes were washed off gently with fresh media to leave a homogeneous layer of rod-shaped cells attached to the coverslips or the 4-well petri dish. Cells were divided into 2 groups electrically paced at 1 Hz or 3 Hz respectively for 24 hours in the incubator. In some studies, cells were divided into different groups for both 1-Hz and 3-Hz paced cells, each including CTL (vehicle) or drug (nimodipine, W-7, A-7, W-13, W-12, KN93, KN92, cyclosporine-A or INCA-6 etc.). The electrical pacing system includes C-Pace100™ and C-Dish100™ units (IonOptix Corporation, Milton, MA, USA). The stimulation pulse duration was 5 milliseconds and the pulse voltage was 45\(\pm2\) V. Evidence of capture was examined by microscopic observation at the beginning and before termination (after 24 hours pacing) of stimulation. Capture efficiency was \(~100\%\) for cells attached on laminin-coated coverslips or plates. After 24 hours, cells on coverslips were kept in the high-K\(^+\) storage solution at 4°C for electrophysiological studies. Cells directly on culture plate were scraped off with a cell scraper (SARSTEDT) and were centrifuged at 1000 rpm for 5 minutes at 4°C. Cell pellets were frozen and kept at -80°C immediately for further molecular biology studies.

In vivo tachypacing
Four mongrel dogs (22-26 kg) were subjected to \textit{in vivo} right ventricular tachypacing (240 bpm) for 24 hours as previously described.\textsuperscript{2}

**Electrophysiology**

Whole-cell patch-clamp technique (voltage-clamp mode) was applied for $I_{io}$ recordings at 36±0.5°C. Borosilicate-glass electrodes had tip resistances between 1.5 and 3.0 MΩ when filled. Cell capacitance and series resistance were compensated by ~80% to 90% to minimize the capacitive surge on the voltage recording. Leakage compensation was not used. Cell capacitances were not different between 1-Hz and 3-Hz paced cells (on-line Table 1). Currents are expressed in terms of density.

The standard Tyrode solution contained (in mmol/L) NaCl 136, KCl 5.4, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 1, NaH\textsubscript{2}PO\textsubscript{4} 0.33, HEPES 5 and dextrose 10 (pH 7.35 with NaOH). The high-K\textsuperscript{+} storage solution contained (in mmol/L) KCl 20, KH\textsubscript{2}PO\textsubscript{4} 10, dextrose 10, mannitol 40, L-glutamic acid 70, β-OH-butyric acid 10, taurine 20, EGTA 10 and 0.1% BSA (pH 7.3 with KOH). The standard pipette solution used in most experiments contained (in mmol/L) K-aspartate 110, KCl 20, MgCl\textsubscript{2} 1, MgATP 5, GTP 0.1, HEPES 10, Na-phosphocreatine 5, EGTA 5 (for current recording) or 0.025 (for action potential (AP) recording), with pH adjusted to 7.3 with KOH.

For $I_{io}$ recordings, atropine (1-μmol/L) and CdCl\textsubscript{2} (200-μmol/L) were added in external solutions to eliminate muscarinic K\textsuperscript{+}-currents and to block Ca\textsuperscript{2+}-currents. Na\textsuperscript{+}-current contamination was avoided by using a holding potential (HP) of -50 mV or by substitution of equimolar Tris HCl for external NaCl. For L-type calcium current ($I_{CaL}$) studies, the external solution contained (in mmol/L)
tetraethylammonium chloride (TEA-Cl) 136, CsCl 5.4, CaCl$_2$ 2, MgCl$_2$ 0.8, HEPES 10 and dextrose 10 (pH 7.4 with CsOH). Niflumic acid (50 μmol/L) was added to inhibit $I_{\text{Cl,Ca}}$. The pipette solution contained (mmol/L) CsCl 20, Cs-aspartate 110, MgCl$_2$ 1, MgATP 5, GTP 0.1, Na$_2$Phosphocreatine 5, EGTA 10 and HEPES 10 (pH 7.2 with CsOH).

**Compounds**

W-7, nimodipine, KN93 and KN92 were purchased from Calbiochem. Valsartan was kindly provided by Novartis, East Hanover, NJ. Cyclosporine A (CyA), W-13 and W-12 were purchased from Sigma. A-7 was purchased from e-BioMed GmbH. Drugs were prepared in stock solution with stock concentration being 1-mmol/L for W-7, nimodipine and valsartan, 5-mmol/L for A-7, W-13 and W-12, 25-mmol/L for KN93 and KN92, and 5-mg/mL for CyA. Individual control (CTL) vehicles were individual solvents to dilute the specific drug (DMSO for W-7, nimodipine, KN93 and KN92; H$_2$O for valsartan, A-7, W-13 and W-12; ethanol for CyA).

**Real-time PCR**

Total RNA was extracted from 1-Hz and 3-Hz snap-frozen cell samples with homogenization in TRIzol Reagent (Invitrogen), chloroform extraction and isopropanol precipitation. Genomic DNA was eliminated by incubation in DNase I (0.1-U/μL, 37°C) for 30 minutes, followed by phenol-chloroform acid extraction and gel verification. RNA was quantified spectrophotometrically at a 260-nm wavelength and integrity was confirmed on a denaturing agarose gel. RNA samples were stored in DEPC H$_2$O at -80°C. First-strand cDNA was synthesized by RT with 2 μg of
RNA samples, random primers and MMLV reverse transcriptase (High Capacity cDNA Archive Kit, Applied Biosystems). Real-time PCR was conducted with Stratagene Mx3000P QPCR detection system and was performed with Taqman quantitative assay. Commercially purchased 18s rRNA (Applied Biosystems) was used as the internal control. Primers and probes for real-time PCR reaction are listed in on-line Table 2. Each sample was run in duplicate and PCR products were verified with gel electrophoresis. Kv4.3, Kv1.4 and KChIP2 were normalized to 18s rRNA; internal control data were obtained from the same samples at the same time and there were no differences of 18s rRNA expression between 1-Hz and 3-Hz paced groups.

**Protein Extraction and Western blot**

**Protein extraction:** Membrane protein fractions were isolated with extraction buffer containing 25-mmol/L Tris-HCL (pH 7.4), 5-mmol/L EGTA, 5-mmol/L EDTA, 1-mmol/L Na$_3$VO$_4$, 0.5-mmol/L AEBSF, 1-mmol/L iodoacetamide and β-2-mercaptoethanol, 10-μg/mL aprotinine and leupeptin and 1-μg/mL pepstatin followed by homogenization. After centrifugation at 1000 rpm and 4°C for 5 minutes, the supernatant containing the cell membranes was centrifuged at 100,000 g for 1 hour. Membrane protein pellets were re-suspended in extraction buffer supplemented with 1% triton and stored at -80°C. For calcineurin detection, total cell lysates were extracted according to the manufacturer’s instruction as described in the Calcineurin activity assay kit.

**Cellular fractionation:** The nuclear isolation was done according to a customized version of a previously-described method.$^3$ Briefly, myocyte pellets were powdered
under liquid nitrogen, resuspended in cold PBS and homogenized using a universal Polytron (8500rpm; 2x15s). The total extract was labelled as Fraction A. All the following steps were carried out on ice or at 4°C. Homogenates were centrifuged at 500 x g for 15 minutes and the supernatants, referred to as Fraction B, were diluted 1:1 with buffer A (10 mmol/L K-HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 1 mmol/L DTT, 25 μg/mL leupeptin, 0.2 mmol/L Na₃VO₄), incubated for 10 minutes on ice and recentrifuged at a higher speed (200 x g) for 15 minutes. The resulting supernatant was discarded. The pellet was considered to be crude nuclei (Fraction C), and was resuspended in buffer B (0.3 M K-HEPES, pH 7.9, 1.5 M KCl, 0.03 M MgCl₂, 25 μg/mL leupeptin, 0.2 mmol/L Na₃VO₄), incubated on ice for 10 minutes, and centrifuged for 15 minutes at 2000 x g. The pellet, which is referred to as the enriched nuclear fraction (Fraction D), was resuspended in buffer C (20 mmol/L Na-HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.2 mmol/L EGTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L DTT, 25 μg/mL leupeptin, 0.2 mmol/L Na₃VO₄) and snap frozen with liquid nitrogen and stored at -80°C. As shown in the image below, the respective fractions were loaded on SDS-PAGE gels, and the nuclear fraction showed enrichment of nucleoporin (NP62) and almost undetectable levels of the endoplasmic reticulum marker GRP78. For experiments assessing nuclear localization of NFATc3 and c4, only fractions A, C and D were loaded on the membranes. Nuclear localization was assessed by quantifying total cell-extract (Fraction A) and nuclear-enriched (Fraction D) NFAT band-densities. Nucleoporin62 (NP62) was used as the loading control and the ratio of nuclear to total cell lysate level of NP62 was not different between 1-Hz and 3-Hz conditions.
Immunoblotting: Protein concentration was determined with the Bradford method. 20-40 μg of membrane protein samples, total cell lysates or total cell extract and nuclear extract from 1-Hz or 3-Hz cells was denatured with Laemmlı buffer and was fractionated on 8% or 12% SDS-polyacrylamide gels, then proteins were transferred electrophoretically to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) in 25-mmol/L Tris-base, 192-mmol/L glycine and 20%-ethanol at 0.3 A for 1 hour. Membranes were blocked in 1 X TBS-T with 5% non-fat dry milk for 1 hour and incubated with primary antibodies (goat anti-Kv4.3, 1:500, Santa Cruz; mouse anti-KChIP2, 1:5000, from Dr. Trimmer; rabbit anti-phospholamban [phosphorylated at threonine 17], 1:5000, Badrilla; mouse anti-phospholamban [total], 1 μg/mL, Affinity Bioreagent (ABR); mouse anti-calcineurin, 1:5000, BD Transduction; mouse anti-NCX, 1:2500, ABR; mouse anti-SERCA, 1:2500, ABR;
mouse anti-RyR2, 1:1000, ABR; mouse anti-NFATc3, 1:200, Sigma; rabbit anti-
NFATc4, 1:200, Sigma; rabbit anti-nucleoporin p62, 1:1000 BD Transduction; rabbit
anti-GRP78 BiP, 1:1000, ABcam; mouse anti-annexin, 1:1000, BD Transduction)
overnight at 4°C. After washing and re-blocking, membranes were incubated with
horseradish peroxidase-conjugated goat anti-rabbit, donkey anti-goat or goat anti-
mouse IgG secondary antibody (1:10,000, Jackson Immunolabs or Santa Cruz).
Antibody was detected with Western-Lightening Chemiluminescence Reagent Plus
(Perkin-Elmer Life Sciences). Later, the same membranes were also probed with
anti-GAPDH at room temperature for 2 hours in order to control for protein loading.
Secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG
(1:10,000, Santa Cruz).

Calcineurin-Activity Assay
Calcineurin-activity was assessed using the Calcineurin Cellular Activity Assay Kit
(Calbiochem) according to the manufacturer’s instruction. Briefly, paced cell
samples were collected at 6 hours during 1-Hz, 3-Hz CTL, 3-Hz+KN93 or 3-
Hz+KN92 pacing: 0 hour (just before pacing). Samples were lysed in lysis buffer
(25-mmol/L Tris-HCl (pH 7.5), 0.5-mmol/L DTT, 50-µmol/L EDTA, 50-µmol/L
EGTA and 0.1% NP-40) with protease inhibitors. The cell lysates were incubated
with RII phosphopeptide (750-µmol/L, the well known substrate for calcineurin) in
assay buffer containing 100 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 6-mmol/L
MgCl₂, 0.5-mmol/L CaCl₂, 0.5-mmol/L DTT, 0.025% NP-40 and 500-nmol/L
Okadaic acid with or without 10-mmol/LM EGTA. After 30 minutes at 30°C,
reactions were terminated by adding 100 µL GREEN™ reagent and fluorescence was
measured at 620 nm using a 96-well plate reader (Tecan SPECTRA Rainbow, PAA Ltd.).

**Confocal microscopy**

After 24-hour pacing, the paced cells were washed with fresh culture medium, then fixed with 2%-paraformaldehyde (20 minutes, Sigma-Aldrich) and washed 3 times (5 minutes each) with phosphate buffered saline (PBS). Cells were blocked and permeablized with 2% normal donkey serum (NDS, Jackson) and normal goat serum (NGS, Jackson) and 0.2% Triton X-100 (Sigma) for 1 hour. Cells were then incubated overnight at 4°C with primary antibodies for nuclear factor of activated thymocytes (NFAT) c3 (1:200, mouse monoclonal, Santa Cruz) and NFATc4 (1:100, rabbit polyclonal, Santa Cruz) in PBS containing 1%-NDS and NGS and 0.05%-Triton, followed by 3 washes and secondary antibody (donkey-anti-mouse Alexa-547 and goat-anti-rabbit Alexa-488, Jackson) incubation at room temperature for 1 hour. Cells were then treated with RNaseA (100-µg/mL, Roche) for 25 minutes at 37°C followed by 3 washes, then incubated with ToPro3 (1-µmol/L, for nuclear labeling, emission at 661-nm, Invitrogen) for 45 minutes at room temperature. Confocal microscopy was performed with a Zeiss LSM-510 system. Control experiments omitting primary antibodies revealed absent or very low-level background staining. Images were deconvolved with Huygens Professional software (Scientific Volume Imaging) using measured point spread functions (PSFs). Measured PSFs were acquired with the same parameters as the images of interest. Nuclear/cytoplasmic ratios were analyzed using Zeiss LSM 510 software. For each cell analyzed, the nuclear and cytosolic densities of NFATc3 and NFATc4 staining were determined as
the sum of the pixels within nuclear or cytosolic regions normalized to the corresponding nuclear or cytosolic areas. Measurements were repeated in 5 Z-stacks showing the maximum nuclear area and mean densities were calculated in each cell. ToPro3 staining was used to determine nucleus contours. Results are presented as the ratio of the mean nuclear staining density to the mean cytosolic staining density.

**Data Acquisition and Analysis**

Clampfit 6.0 (Axon) and GraphPad Prism 3.0 were used for data analysis. Nonlinear least-square curve-fitting algorithms were performed for curve fitting. Real-time PCR results were analyzed with MXPro software from Stratagene. Western blot results were analyzed with Quantity One software (Biorad). Statistical comparisons were performed with paired or unpaired student $t$-tests if only 2 group means were compared. When multiple groups were studied simultaneously, group comparisons were performed with ANOVA. If significant differences were indicated by analysis of variance, $t$-tests with Bonferroni’s correction or Dunnett’s tests were used to evaluate differences between individual mean values. A two-tailed $P<0.05$ was taken to indicate statistical significance. Data are expressed as mean±SEM.


Online Table 1

Properties of 1-Hz and 3-Hz paced cells

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<th>RP (mV)</th>
<th>Rs (MΩ)</th>
<th>Capacitance (pF)</th>
<th>Cell dimensions</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Length (µm)</td>
<td>Width (µm)</td>
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<td>4.8±0.3</td>
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<td>-73.5±0.6</td>
<td>5.1±0.3</td>
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</tbody>
</table>

RP=Resting membrane potential, Rs=compensated series resistance value, n=number of cells. *P=*NS.

1-Hz versus 3-Hz groups, for all variables.
## Online Table 2

### Primer and probe information

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<th>Primers and Probes</th>
<th>Sequences</th>
<th>GeneBank#</th>
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<td>F: TCCCCCTGTAATCTTGACGAACCTR: TTCTGCTCAAACATCTGCTCATCT Probe: CCACCATCAAGAACCA</td>
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On-line Figure Legends

On-line Figure 1. $I_{to}$ frequency-dependence, determined from the ratio of current during the 15th pulse to current during the first pulse of a train of 100-ms depolarizations from -80 mV to +50 mV at the frequencies indicated. Data are mean±S.E.M (n=5 cells in 1-Hz, and n=6 cells in 3-Hz conditions).

On-line Figure 2. A, Examples of $I_{to}$ recordings obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from CTL and 24-hour VTP dog cells. B, Mean±S.E.M. $I_{to}$ densities for epicardial myocytes freshly isolated from control (CTL, n=13 cells) and 24-hour VTP (n=19 cells) dogs. *$P<0.05$, **$P<0.01$, CTL versus 24-hour VTP.

On-line Figure 3. A, Examples of $I_{to}$ recordings obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz and 3-Hz paced cells incubated under control (CTL) or blebbistatin (BBST, 5-μmol/L) conditions throughout the pacing period. Cell activation and mechanical uncoupling in the presence of BBST were verified by recording Ca$^{2+}$-transients (Indo-1 AM) and cell-shortening (video-imaging). B, Mean±S.E.M. $I_{to}$ densities at +40 mV of 1-Hz CTL and BBST, 3-Hz CTL and BBST groups, n=8 cells/group. *$P<0.05$, 1 Hz versus 3 Hz.

On-line Figure 4. A, Representative $I_{to}$ recordings obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz (3 ms),
1-Hz (9 ms) and 3-Hz (3 ms) paced cells. **P<0.01, 3-Hz (3 ms) versus 1-Hz (3 ms) & 1-Hz (9 ms).

**On-line Figure 5.** A, Examples of $I_{to}$ recordings obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz and 3-Hz paced cells incubated in the absence (CTL) or presence (Val) of 1-μmol/L valsartan (Val) throughout the pacing period. B, Mean±S.E.M. $I_{to}$ densities for 1- (n=10 cells) and 3-Hz (n=9 cells) CTL, 1- (n=8 cells) and 3-Hz (n=10 cells) Val cells, with the recording protocol used in panel A. **P<0.01, 1-Hz versus 3-Hz cells.

**On-line Figure 6.** Acute effects of 0.5-μmol/L nimodipine on $I_{CaL}$. A & B, $I_{CaL}$ recorded during the last pulse at 1 or 3 Hz before (CTL, A) and 5 minutes after addition of nimodipine (0.5 μmol/L, B). Currents were recorded with 10 consecutive 100-ms pulses from a holding potential of -50 mV to a depolarizing potential of +10 mV, with a 1 Hz or 3 Hz pulse frequency. C, Mean±S.E.M. percentage decrease of $I_{CaL}$ density by 0.5-μmol/L nimodipine (n=3 cells). D, Mean±S.E.M. $I_{CaL}$ densities under each condition (n=3 cells). *P<0.05 by one-way ANOVA, CTL versus nimodipine.

**On-line Figure 7.** A, Mean±S.E.M. $I_{to}$ densities obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz and 3-Hz paced cells incubated under control (CTL) or A-7 (1- or 5-
μmol/L) conditions throughout the pacing period. n=7-11 cells/group for 1-Hz, n=8-9 cells/group for 3-Hz. *P<0.05, 1-Hz versus 3-Hz. B, Mean±S.E.M. I_{io} densities obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz and 3-Hz paced cells incubated under control (CTL), W-13 (40-μmol/L) or its inactive congener W-12 (40-μmol/L) throughout the pacing period. n=6-7 cells/group for 1-Hz, n=6-7 cells/group for 3-Hz. *P<0.05, 1-Hz versus 3-Hz.

On-line Figure 8. Acute effects of KN93 and KN92 on I_{CaL}. Currents were recorded with 10 consecutive 100-ms pulses from a holding potential of -50 mV to a depolarizing potential of +10 mV, with a 1 Hz or 3 Hz pulse frequency. A, Top: I_{CaL} recorded during the last pulse at 3 Hz before (CTL) and 5 minutes after addition of KN93 (1 μmol/L), and 5 minutes after subsequent addition of nimodipine (0.5 μmol/L) as a positive control for each cell. Bottom: Mean±S.E.M. I_{CaL} densities under each condition (n=5 cells). B, Top: I_{CaL} recorded during the last pulse at 3 Hz before (CTL) and after addition of KN92, and 5 minutes after subsequent addition of nimodipine (0.5 μmol/L) as a positive control for each cell. Bottom: Mean±S.E.M. I_{CaL} densities under each condition. (n=4 cells).

On-line Figure 9. A, Expanded presentation of Western blot results shown in Figures 4A, C & E. Full lanes are shown for CaMKII-
phosphorylated phospholamban at the left (along with corresponding GAPDH bands) and corresponding results obtained from the same gels after stripping and re-probing for total phospholamban are shown at the right. **B**, Expanded presentation of gels for Western blot results shown in Figure 5D for Kv4.3 and KChIP2 from KN93 and KN92 treated cells. **C**, Expanded presentation of gels for Western blot results shown in Figure 6B for calcineurin. For all proteins, prominent bands were seen at the expected molecular masses.

**On-line Figure 10.** **A**, Examples of sodium-calcium exchanger (NCX, ~120 kDa), ryanodine receptor (RyR2, ~520 kDa), sarcoplasmic endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a, ~110 kDa, abbreviated as SERCA in figure) and GAPDH Western blots from 1-Hz and 3-Hz paced cells. **B**, Mean±S.E.M. NCX, RyR2 and SERCA band intensities after normalization to GAPDH-band intensities (n=4 blots each for all proteins, each blot being from 2 hearts, total of 8 hearts/group).

**On-line Figure 11.** Examples of immunolocalization of NFATc3 (red), NFATc4 (green), ToPro3 (blue) and their merged co-localization in 1- and 3-Hz paced myocytes.

**On-line Figure 12.** **A&B**, Top, examples of NFATc3 (A, ~130kDa) and NFATc4 (B, ~160 kDa) Western blots from 1-Hz and 3-Hz paced-cell total extracts and nuclear extracts. Bottom, mean±S.E.M. NFATc3
and NFATc4 nuclear-enriched (fraction D) and nuclear-deficient (fraction B) band-intensities normalized to total cell-lysate (fraction A) band-intensity (n=4 blots for each protein; each blot was produced from cells exposed to 1- and 3-Hz pacing from 2 hearts each to provide enough protein from the nuclear fraction, thus providing a total of 8 hearts/observation).
Online Figure III

A

BBST = blebistatin
CTL = Control

1-Hz CTL
1-Hz BBST
3-Hz CTL
3-Hz BBST

Time (ms)

B

1-Hz CTL
1-Hz BBST
3-Hz CTL
3-Hz BBST

pA/pF
Online Figure VIII
Online Figure IX

A

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C

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Online Figure XII

A

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NFATc3

Normalized to total lysate fraction A

Fraction B (nuclear-deficient)
Fraction D (nuclear-enriched)

1-Hz (N=4) 3-Hz (N=4)

* P<0.05

B

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NFATc4

Normalized to total lysate fraction A

Fraction B (nuclear-deficient)
Fraction D (nuclear-enriched)
Chapter 4. Feedback remodeling of cardiac potassium current expression: a novel potential mechanisms for control of repolarization reserve
Finally, I studied the regulation of ion channel expression at the level of feedback control of ventricular repolarization. Abnormal ventricular repolarization can lead to potentially life-threatening ventricular arrhythmias such as TdP. Co-existing clinical risk factors increase the propensity to develop TdP, which can be partly explained by impaired “repolarization reserve” in ventricular repolarization. Repolarization reserve usually involves functionally-based compensatory increases in repolarizing currents that minimize changes in APD caused by dysfunction or inhibition of a single K⁺-current, commonly seen between I_{Kr} and I_{Ks}. However, it is not known if repolarization reserve also involves compensatory expression of ion channels by feedback regulation to control AP in response to sustained dysfunction of a K⁺-current. With the useful in vitro cellular model described in chapter 3, we tested our hypothesis that sustained inhibition of I_{Kr} by its specific blocker dofetilide, can induce remodeling of ion-channel expression.
Feedback remodeling of cardiac potassium current expression:
a novel potential mechanism for control of repolarization reserve

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Huixian Lin PhD, Zhiguo Wang PhD, Stanley Nattel MD

Short Title: Feedback control of K+ current and repolarization

Subject Codes: [5], [132], [152]

Word Count: 5,996 (not counting Short title, Subject Codes and Word Count)

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Abstract

**Background**—Inhibition of individual K⁺-currents causes functionally-based compensatory increases in other K⁺-currents that minimize changes in action potential (AP) duration (APD), a phenomenon known as “repolarization reserve”. The possibility that sustained K⁺-channel inhibition may induce remodeling of ion-current expression has not been tested. Accordingly, we assessed the effects of sustained inhibition of one K⁺-current on various cardiac ionic currents. **Methods and Results**—Adult canine left-ventricular cardiomyocytes were incubated in primary culture and paced at a physiological rate (1 Hz) for 24 hrs in the presence or absence of the highly-selective rapid delayed-rectifier K⁺-current (I\textsubscript{Kr}) blocker dofetilide (5-nmol/L). Chronic dofetilide exposure led to shortened APD and increased repolarization reserve (manifested as a reduced APD-prolonging response to I\textsubscript{Kr} blockade). These repolarization changes were accompanied by increased slow delayed-rectifier (I\textsubscript{Ks}) density, whereas I\textsubscript{Kr}, transient-outward (I\textsubscript{to}), inward-rectifier (I\textsubscript{K1}), L-type Ca\textsuperscript{2⁺} (I\textsubscript{CaL}) and late-Na\textsuperscript{+} (Late-I\textsubscript{Na}) current remained unchanged. The mRNA-expression corresponding to KvLQT1 and minK (real-time PCR) was unchanged, but their protein-expression (Western blot) was increased, suggesting post-transcriptional regulation. To analyze possible mechanisms, we quantified the muscle-specific microRNA subtypes miR-133a and miR-133b, which can post-transcriptionally regulate and repress KvLQT1 protein-expression without affecting mRNA-expression. The expression levels of miR-133a and b were significantly decreased in cells cultured in dofetilide compared with control, possibly accounting for KvLQT1 protein upregulation. **Conclusion**—Chronic reductions in I\textsubscript{Kr} may lead to compensatory upregulation of I\textsubscript{Ks} through post-transcriptional upregulation of
underlying subunits, likely mediated (at least partly) by microRNA changes. These results suggest that feedback control of ion-channel expression may influence repolarization reserve.

**Key Words:** potassium channels – repolarization reserve – long QT syndrome - microRNA

**Introduction**

Cardiac repolarization is a key cellular function, disruption of which leads to potentially lethal ventricular tachyarrhythmias.\(^1,2\) The repolarization process is governed by the interplay of multiple ion-channels. An important recently-developed concept has been the notion that the complex of multiple repolarizing ion-channels provides for “repolarization reserve”, in that dysfunction or inhibition of a K\(^+\)-current causes functionally-based compensatory increases in other K\(^+\)-currents that minimize changes in action potential (AP) duration (APD).\(^1,3\) Impairments in repolarization reserve predispose the heart to lethal ventricular arrhythmias.\(^1,2\) The basic notion of repolarization reserve is that a delay in the repolarization process due to impaired function of one K\(^+\)-channel causes greater current-carrying capacity of other K\(^+\)-channels, generally by enhancing voltage and time dependent activation. This functionally-based repolarization reserve is most typically seen for rapid and slow delayed-rectifier K\(^+\)-currents (\(I_{Kr}\) and \(I_{Ks}\)), with \(I_{Ks}\) preventing excessive APD prolongation in response to \(I_{Kr}\) reduction.\(^4,6\)

The possibility that APD prolongation by sustained inhibition of a K\(^+\)-current can elicit increased repolarization reserve by affecting cardiac ion-channel expression has not, to our knowledge, been addressed. Cardiac ion-channel expression is a regulated function, although little is known about the potential feedback mechanisms
and their functional consequences. In transgenic mice engineered to lack the rapid transient-outward K⁺-current (I_{tof}), there is evidence for compensatory upregulation of the slow transient-outward component (I_{tos}) carried by Kv1.4 subunits. Similarly, in mice lacking I_{K,slow1} carried by Kv1 subunits, there is an apparent compensatory regulatory response of Kv2.1 subunits to increase I_{K,slow2}. The present study was designed to assess the effects of APD prolongation by highly-selective I_{Kr} inhibition with dofetilide on cardiac repolarization and repolarization reserve, as well as on the expression of other cardiac ionic currents. In order to allow for well-controlled conditions we used an in vitro model of paced canine ventricular cardiomyocytes.

Materials and Methods

Additional details are available in the On-line Materials and Methods section in the Data Supplement.

In-Vitro Cellular Pacing Model

Cell Isolation. All animal care and handling procedures were approved by the animal research ethics committee of the Montreal Heart Institute. Male adult mongrel dogs (21-35 kg) were anesthetized with pentobarbital (30 mg/kg IV) under artificial ventilation. Hearts were excised via left-lateral thoracotomy and immersed in oxygenated Tyrode-solution at room temperature. The transmural free wall (~30×50 mm) of the anterior left ventricle was dissected and the perfusing coronary artery was cannulated. Cell isolation was performed as previously described. Epicardial (Epi)
cells were removed and kept in culture medium (for contents see On-line Data Supplement) for further studies.

**Cell-Culture and Pacing.** Cell-culture and pacing were performed under aseptic conditions as previously described. The cell-suspension was centrifuged at 500 rpm (1 minute, 4°C) and cell pellets were re-suspended in culture medium. Cells were plated at ~1×10^4 cells/cm² on rectangular glass coverslips or 4-well rectangular Petri dishes pre-coated with laminin (15-µg/mL). Cardiomyocytes were maintained at 37°C in a humidified, 5% CO₂-enriched environment. After 4 hours, any dead or unattached myocytes were washed off to leave a homogeneous layer of adherent rod-shaped cells. Cells were divided into 2 groups: CTL (medium without dofetilide) and Dof (culture media containing 5-nmol/L dofetilide). Both CTL and Dof cells were continuously paced (pulse-duration 5 milliseconds, pulse-voltage 43 V) at 1 Hz for 24 hours in the incubator. Capture-efficiency was verified by eye and by video-recording of cell shortening, and was ~100%. After 24 hours, cells on coverslips were kept in high-K⁺ storage solution at 4°C for electrophysiological studies. Cells were scraped off culture plates and centrifuged at 1000 rpm for 5 minutes at 4°C. Cell pellets were fast-frozen in liquid-N₂ and kept at -80°C for subsequent biochemical analyses.

**Electrophysiology**

Whole-cell patch-clamp was applied for ionic-current and AP recording at 36±0.5°C. Ionic currents were recorded with tight-seal patch-clamp in voltage-clamp mode, and
APs in current-clamp mode with perforated-patch techniques. Borosilicate-glass electrodes had tip resistances between 2 and 5 MΩ. Cell-capacitance and series-resistance were compensated by ~80% to 90%. Junction potentials (~10 mV) were corrected for AP recordings only. Cell-capacitance averaged 160.6±5.7 pF in CTL (n=37) and 169.1±6.0 pF in Dof (n=44, P=NS) cells. Currents are expressed as current-density (normalized to cell-capacitance).

Standard Tyrode-solution contained (mmol/L) NaCl 136, KCl 5.4, MgCl₂ 1, CaCl₂ 1, NaH₂PO₄ 0.33, HEPES 5 and dextrose 10 (pH 7.35 with NaOH). The high-K⁺ storage solution contained (mmol/L) KCl 20, KH₂PO₄ 10, dextrose 10, mannitol 40, L-glutamic acid 70, β-OH-butyric acid 10, taurine 20, EGTA 10 and 0.1% BSA (pH 7.3, KOH). Standard pipette solution contained (mmol/L) K-aspartate 110, KCl 20, MgCl₂ 1, MgATP 5, GTP 0.1, HEPES 10, Na-phosphocreatine 5, EGTA 5 (for current recording) or 0.025 (for AP recording); pH 7.3, KOH).

For AP recording, nystatin (60-µg/mL) was back-filled into the pipette tip and external solutions contained 2-mmol/L CaCl₂. For all K⁺-current recordings, atropine (1-µmol/L) and either CdCl₂ (200-µmol/L) or nimodipine (5-µmol/L, for I₉₋₉ and I₉ₛ) were added to external solutions to eliminate muscarinic K⁺-currents and block Ca²⁺-currents. For K⁺-currents other than transient outward current (Iₒ), 1-mmol/L 4-aminopyridine was added. Inward-rectifier current (Iₚ₁₁) was studied as 1-mmol/L Ba²⁺-sensitive current. HMR1566 (1-µmol/L) was added to inhibit I₈ₛ for I₉₋₉-recording and E4031 (5-µmol/L) was used to inhibit I₉₋₉ for I₉ₛ-recording.

For L-type calcium current (I₉₈₉) studies, external solutions contained (mmol/L) tetraethylammonium chloride (TEA-Cl) 136, CsCl 5.4, CaCl₂ 2, MgCl₂ 0.8, HEPES 10 and dextrose 10 (pH 7.4, CsOH). Niflumic acid (50-µmol/L) was added to inhibit
I_{Cl, Ca}. The pipette solution contained (mmol/L) CsCl 20, Cs-aspartate 110, MgCl$_2$ 1,
MgATP 5, GTP 0.1, Na$_2$-phosphocreatine 5, EGTA 10 and HEPES 10 (pH 7.2,
CsOH).

Late-I$_{Na}$ was recorded as described by Maltsev et al.$^{13}$ The pipette-solution
contained (mmol/L): 5 NaCl, 133 CsCl, 2 MgATP, 20 TEA, 10 EGTA, 5 HEPES; pH
7.3 (CsOH). The bath-solution contained (mmol/L): 140 NaCl, 5.4 CsCl, 1.8 CaCl$_2$,
2 MgCl$_2$, 0.002 nifedipine, 5 HEPES; pH 7.3 (NaOH). Late-I$_{Na}$ (current 200-220 ms
after depolarization-onset) was recorded 5 minutes after cell-rupture at room-
temperature, with 2-second voltage-steps (0.2 Hz) from -120 mV to test-potentials
between -70 and +40 mV.

**Real-time PCR**

Cell-samples were homogenized in TRIzol (Invitrogen) and RNA extracted with
chloroform and isopropanol precipitation. Genomic DNA was eliminated with
DNase I-incubation (0.1 U/µL, 37°C, 30 minutes), followed by phenol-chloroform
acid extraction and gel verification. RNA was quantified spectrophotometrically at
260-nm. RNA-samples were stored in DEPC H$_2$O at -80°C. First-strand cDNA was
synthesized by RT with 2-µg RNA samples, random primers and MMLV reverse
transcriptase. Real-time PCR was conducted with Taqman ($KCNH2$ and $KCNE2$
transcripts) or SYBR green ($KCNQ1$ and $KCNE1$ transcripts). Canine
$\beta$2-microglobulin (Taqman) and 18S rRNA (SYBR green) were used as internal
controls. Primers and probes for real-time PCR are listed in On-line Table 1. Each
sample was run in duplicate and PCR products were verified with gel electrophoresis
or dissociation curves (SYBR green). $KCNH2$ and $KCNE2$ results were normalized
to β2-microglobulin, *KCNQ1* and *KCNE1* to 18S rRNA, results obtained from the same samples at the same time.

**Protein Extraction and Western Blot**

Membrane proteins were isolated with extraction buffer (for contents see On-line Data Supplement), followed by homogenization. After initial centrifugation (1000 rpm, 4°C, 5 minutes), supernatants containing cell-membranes were centrifuged (100,000 g, 1 hour). Pellets were re-suspended in extraction buffer and 1%-Triton, and stored at -80°C. Protein concentration was determined by Bradford assay. Membrane-protein samples (40 μg) were denatured with Laemmli buffer and fractionated on 8% or 12% SDS-polyacrylamide gels, then transferred electrophoretically to Immobilon-P polyvinylidene fluoride (PVDF) membranes in 25-mmol/L Tris-base, 192-mmol/L glycine and 20% ethanol (0.3 A, 1 hour). Membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) and 5% non-fat dry milk, and incubated with primary antibodies (anti-KvLQT1, 1:500, Alomone; anti-minK, 1:2000, from Dr. Jacques Barhanin) overnight at 4°C. After washing and re-blocking, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:10,000, Jackson). Antibody was detected with Western-Lightening Chemiluminescence Reagent Plus. Membranes were also probed with anti-GAPDH for protein loading.

**Rapid Amplification of cDNA Ends (3’ RACE)**
Ambion’s RNA ligase-mediated 3’RACE kit was used to determine 3’-untranslated region (UTR) sequences of canine KCNE1 and KCNQ1. Gene-specific primers (for sequences see On-line Data Supplement) were designed based on canine KCNE1 (GenBank No. XM_544868) and KCNQ1 (GenBank No. XM_540790) cDNAs.

MicroRNA Detection
The TaqMan® MicroRNA Assay (Applied Biosystems) was used in conjunction with real-time PCR with Taqman probes for quantification of miR-133 (miR-133a and miR-133b) and miR-1 transcripts. RNA samples were isolated from cultured cells with mirVana™ miRNA Isolation Kits. Reactions contained TaqMan® MicroRNA Assay sets specific for miR-133a, miR-133b or RNU24 as a positive endogenous control. QRT-PCR was performed on a GeneAmp 5700 thermocycler (40 cycles) after first determining the appropriate cycle threshold (Ct) with the baseline determination feature. Fold-variations in expression between samples were calculated after normalization to RNU24.

Data Acquisition and Analysis
Voltage-protocols were applied at 0.1-Hz unless stated otherwise. Clampfit 6.0 (Axon), GraphPad Prism 3.0 and Quantity one (Bio-rad) were used for data analysis. Nonlinear least-square curve-fitting algorithms were performed for curve fitting. Paired or unpaired Student’s t-tests were applied for single comparisons between groups. Comparisons involving repeated-measures analyses were performed by ANOVA with Bonferroni-adjusted t-tests in the case of statistically-significant intergroup differences. A two-tailed $P<0.05$ was taken to indicate statistical
significance. Group data are expressed as mean±SEM. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**Influence of Chronic Dofetilide Culture on APD**

We selected a dofetilide concentration for cardiomyocyte incubation (5-nmol/L) of the order of the drug’s EC$_{50}$,\textsuperscript{14,15} in order to ensure significant I$_{Kr}$ inhibition and APD prolongation while facilitating dofetilide washout at the end of the culture period. To confirm effective APD prolongation by this dofetilide concentration, we studied its effects on fresh cells and noted an 18±4% prolongation at 1 Hz (On-line Figure 1).

Original AP recordings obtained from CTL and Dof cells after 24 hours in culture are shown in Figure 1A. Resting membrane potential averaged -75.4±1.0 mV in CTL (n=18) and -77.0±0.9 mV in Dof (n=14, $P$=NS) cells. Figure 1B shows mean APD at 90% repolarization (APD$_{90}$) at different frequencies (0.1, 0.5, 1 and 2 Hz). Cells chronically exposed to dofetilide showed significantly shorter APD$_{90}$ compared to parallel controls (Figure 1B). For example, APD$_{90}$ at 1 Hz averaged 300±21 ms in control cells versus 237±17 ms in dofetilide-incubated cells ($P$<0.05, n=18, 14 respectively). These results suggest that chronic dofetilide exposure results in reduced intrinsic APD. We then evaluated the effects of chronic dofetilide exposure on repolarization reserve by measuring APD in CTL and Dof cells before and after superfusion with 5-nmol/L dofetilide. AP recordings from a CTL and Dof cell before and after acute exposure to dofetilide are shown in Figure 1C. Cells cultured in the
presence of dofetilide had reduced APD and a reduced APD response upon re-exposure to the compound, indicating enhanced repolarization reserve. Mean data in Figure 1D confirm the statistically-significantly reduced APD response of Dof cells to I_{Kr} blockade.

**Effects of Chronic Dofetilide Culture on Ionic Currents**

Figure 2A shows examples of I_{Ks} recorded upon stepping to +40 mV in CTL and Dof cells. I_{Ks} step and tail current-densities were significantly increased after chronic dofetilide exposure (Figure 2B). Mean current-densities at +30 mV were 0.83±0.08 (step) and 0.31±0.03 pA/pF (tail) for control cells, compared to 1.17±0.09 (step) and 0.44±0.03 pA/pF (tail) for dofetilide-incubated cells. The voltage-dependence of I_{Ks} activation (Figure 2C, based on Boltzmann fits to tail currents) was not significantly altered: mean half-activation voltages averaged 31.1±7.5 mV for CTL and 28.1±2.6 mV for Dof cells. Similarly, I_{Ks}-kinetics were unchanged (On-line Figure 2A).

Representative I_{Kr} recordings from control and dofetilide-incubated cells are shown in Figure 3A. Chronic dofetilide exposure did not significantly affect I_{Kr} properties following cell isolation and washing. I_{Kr} tail-current density showed no statistically-significant alterations (Figure 3B). For example, mean I_{Kr}-tail density at +10 mV averaged 0.15±0.01 and 0.16±0.02 pA/pF in CTL and Dof cells respectively. The I_{Kr} half-activation voltage (Figure 3C, based on Boltzmann fits to tail-currents) averaged -22.4±4.3 mV for CTL cells and -24.1±2.7 mV for Dof cells (n=8/group, P=NS). I_{Kr}-kinetics were also unchanged (On-line Figure 2B).

Other currents that can contribute to determining ventricular repolarization were also studied. Figures 4A and B show examples of I_{to} recorded from a CTL and a Dof
cardiomyocyte. There were no differences in mean $I_{to}$ densities between CTL and Dof cells (Figure 4C). For example, $I_{to}$ densities at +30 mV averaged 15.9±3.0 pA/pF in CTL and 14.8±1.4 pA/pF in Dof ($P=NS$ vs CTL) cells. Neither activation (Figure 4D) nor inactivation (Figure 4E) kinetics were altered. Figure 5 shows $I_{K1}$ recordings from a CTL (Figure 5A) and a Dof (Figure 5B) cardiomyocyte, along with mean current density-voltage relations (Figure 5C). $I_{K1}$ density was not changed by chronic dofetilide exposure, e.g. $I_{K1}$ density at -120 mV averaged -23.3±1.5 pA/pF in CTL and -26.2±2.3 pA/pF in Dof ($P=NS$ vs CTL) cells. $I_{Ca,L}$ was similar in CTL (Figure 6A) and Dof (Figure 6B) cells. Mean $I_{Ca,L}$ density was unchanged (Figure 6C), e.g. averaging (at +10 mV) -3.2±0.6 pA/pF in CTL and -3.3±0.4 pA/pF in Dof cells. $I_{Ca,L}$ inactivation kinetics were similarly unaffected (Figure 6D). Late-$I_{Na}$ recordings are shown in On-line Figure 3A, with corresponding mean data at -30 mV in On-line Figure 3B. Chronic dofetilide exposure had no significant effect on Late-$I_{Na}$ density.

The ionic-current recording data suggest that chronic dofetilide exposure increases repolarization reserve by upregulating $I_{Ks}$. If this is the case, the response to $I_{Ks}$-inhibition should differ in CTL versus Dof cells. We tested this possibility by studying the APD-response to 100-nmol/L HMR1566, as illustrated in On-line Figure 4. Whereas $I_{Ks}$-inhibition had a small, non-significant effect on CTL cells, HMR1566 significantly delayed repolarization of Dof cells, consistent with $I_{Ks}$-upregulation.

**Expression of Delayed-Rectifier $K^+$-Channel Subunits**
We then addressed the potential molecular basis of $I_{Ks}$ up-regulation in response to dofetilide exposure by assaying mRNA and protein-expression of the principal subunits believed to participate in forming $I_{Kr}$ and $I_{Ks}$. Figure 7A shows mean mRNA-expression values of $I_{Kr}$-related subunits, $KCNH2$ and $KCNE2$ ($P=NS$), and Figure 7B shows mRNA-expression values for $I_{Ks}$-related subunits $KCNQ1$ and $KCNE1$ ($P=NS$), in CTL and Dof cells. Chronic dofetilide incubation did not affect the mRNA-expression of subunits underlying $I_{Kr}$ and $I_{Ks}$. We then analyzed the protein-expression of the $I_{Ks}$-related subunits. The top panels in Figures 7C and D show examples of KvLQT1 and minK bands, detected at ~78 kDa for KvLQT1 and ~20 kDa for minK, with corresponding GAPDH bands from the same lanes (~34 kDa). The lower panels in Figures 7C and D show mean expression levels for KvLQT1 and minK protein normalized to GAPDH. The protein-expression values for both KvLQT1 and minK were significantly increased, by 21±5% and 26±5% respectively, in dofetilide-exposed cells ($P<0.05$ for KvLQT1, $P<0.01$ for minK).

**MicroRNA Expression After Chronic Dofetilide Culture**

Chronic dofetilide exposure increased $I_{Ks}$ subunit protein-expression but not corresponding mRNA levels, suggesting changes in post-transcriptional regulation of the subunits underlying $I_{Ks}$. Recently, it has been found that microRNAs can regulate ion-channel protein-expression and transmural distribution, and may be important in post-transcriptional regulation of ion-channel expression in the heart.\textsuperscript{16-18} MiR-1 and $miR$-133 are muscle-specific microRNAs (miRNAs) that are strongly and specifically expressed in adult cardiac tissues.\textsuperscript{19} Evidence has been presented to show that $miR$-133 can repress KvLQT1 protein-expression without altering mRNA-expression,
whereas miR-1 represses minK protein but not mRNA-expression. In order to assess whether changes in micro-RNA regulation are a candidate to explain I_{Ks} subunit protein-expression alterations caused by chronic dofetilide exposure, miR-133 and miR-1 expression levels were quantified in CTL and Dof cells. Because we were unable to find documented 3’untranslated region (UTR) sequences of canine KCNQ1 (encoding KvLQT1) and KCNE1 (encoding minK) genes, we first performed 3’-RACE experiments on dog cDNAs to obtain the 3’UTR sequences of canine KCNQ1 and KCNE1 genes. The results are shown in On-line Figures 5 and 6. The 3’UTR of canine KCNQ1 that we sequenced is 919 bp (GenBank accession No. EU162137), whereas the corresponding length for KCNE1 is 303 bp (GenBank accession No. EU162136). Complementary binding sites for miR-133 and miR-1 on canine KCNQ1 and KCNE1 3’UTRs were computationally analyzed and are shown in Figure 8 (left panels). We found 3 miR-133 potential binding sites, but no miR-1 binding site, on the 3’UTR sequence of KCNQ1. We found 1 potential miR-1 binding site on the 3’UTR of the canine KCNE1 gene, on which there were no miR-133 binding sites. Both miR-133a and miR-133b are mature forms of the miR-133 family, having the same core miR-133 sequence (underlined below) which is the main functional sequence for miR-133’s effect on target mRNAs (MiR-133a 3’-5’: UGUCGACCAACUUCCCCUGGUU; miR-133b 3’-5’: AUUCGACCAACUUCCCCUGGUU.). The expression levels of miR-133a, miR-133b and miR-1 were quantified by real-time PCR. As shown in Figure 8B, the expression levels of miR-133a and miR-133b were significantly decreased in Dof cells compared with CTL, a change opposite to the alterations in expression of KvLQT1 protein and consistent with the inhibitory effect of miR-133 on KvLQT1 protein-expression. In
contrast, the expression of miR-1 was not different in Dof cells compared with CTL cells (Figure 8D).

**Discussion**

We have shown that continuous exposure to the I_{Kr}-inhibitor dofetilide causes up-regulation of I_{Ks} and its subunit proteins in canine cardiomyocytes activated at physiological rates, without changing several other currents that contribute to APD regulation (I_{Kr}, I_{K1}, I_{to}, I_{Ca,L}, Late-I_{Na}). Associated physiological consequences include reduced APD and increased repolarization reserve.

Drug-induced long QT syndrome (LQTS) is an important clinical problem.\textsuperscript{1,2} Although the occurrence of drug-induced LQTS is unpredictable, the concept of “repolarization reserve”, first proposed by Roden in 1998,\textsuperscript{1} has provided important insights into the underlying determinants. An intrinsic ability of cardiomyocytes to protect themselves from excess APD prolongation depends on the intactness of a variety of functional K\textsuperscript{+}-channels, through which repolarizing current can increase in response to a repolarization-suppressing challenge. The phenomenon of repolarization reserve has been clearly demonstrated in a variety of experimental paradigms, which show that the loss of more than one K\textsuperscript{+}-current produces much more repolarization impairment than expected based on observations of the effects of the loss of individual currents.\textsuperscript{2-6,20} In the present study, we provide evidence for a novel mechanism contributing to the control of repolarization reserve, feedback regulation of ion-channel expression. Sustained reductions in I_{Kr}, which increase APD acutely, lead to enhanced expression of I_{Ks} subunit proteins and increased I_{Ks} density. I_{Ks} acts as a “safety mechanism” for cardiac repolarization, becoming larger
when APD is increased by reduced outward or increased inward current, and preventing excess repolarization-delays.\textsuperscript{4,6} Increased $I_{Ks}$-density resulting from chronic $I_{Kr}$-block will limit the associated APD-prolongation and create increased repolarization reserve to counteract the effects of repolarization-inhibition, limiting the potential risk of TdP. This effect was illustrated by our experiment showed that chronically dofetilide-treated cells showed smaller APD-increases upon $I_{Kr}$-inhibiting (dofetilide) challenge than control cells.

Our observations provide potential new insights into phenomena of clinical relevance. Loss-of-function mutations in the genes encoding $I_{Ks}$ subunits increase the severity and risk of arrhythmias in congenital and acquired long QT syndromes caused by $I_{Kr}$ abnormalities.\textsuperscript{21,22} This observation has generally been attributed to decreased functional ability to increase $I_{Ks}$ in response to reduced $I_{Kr}$, for which there is much experimental evidence. However, our results suggest that an additional contributory mechanism may be a reduced effectiveness of the upregulation of $I_{Ks}$ currents in response to sustained $I_{Kr}$ decreases.

The phenomena we observed in the present study do have precedents. The loss of a $K^+$-current in genetically-engineered mice results in compensatory upregulation of other cardiac $K^+$-channel subunits.\textsuperscript{8-10} A limitation of the available observations in mouse models is the fact that the dominant repolarizing currents in mice are quite different from those in man. The findings of the present study suggest that the principles regulating $K^+$-current expression in mouse models may also apply to $K^+$-current systems much closer to the dominant repolarizing currents in the human heart.

Our results imply the presence of feedback systems capable of responding to changes in cardiac electrical function by inducing adaptive changes in cardiac ion-
channel expression, as recently suggested on the basis of theoretical considerations.\(^7\)

The detailed molecular mechanisms underlying \(I_{Ks}\) upregulation in response to \(I_{Kr}\) inhibition remain to be established. We observed an increase in KvLQT1 and minK protein without a change in their corresponding mRNA, which indicates that there are potential changes in post-transcriptional regulation of the protein-expression of subunits underlying \(I_{Ks}\) that occur with chronic dofetilide incubation. Micro-RNAs can mediate gene regulation at the post-transcriptional level and have been considered to be an important novel component to the mechanisms regulating the expression of genes and their protein products.\(^{23,24}\) Limited studies have been performed to explore the role of micro-RNA in ion-channel subunit-expression regulation. The available information shows that microRNAs can regulate ion-channel protein-expression and transmural distribution, and that they may be important in post-transcriptional regulation of ion-channel expression in the heart.\(^{16-18}\) Luo et al. showed that \textit{miR}-133 can repress KvLQT1 protein-expression without affecting corresponding mRNA-expression, whereas \textit{miR}-1 represses minK protein but not mRNA-expression.\(^{17}\) Our study suggests that repolarization-delaying interventions can alter the regulation of microRNA expression. In order to study microRNA regulation of KvLQT1 and minK expression we had to first clone the 3′UTRs of dog \textit{KCNQ1} and \textit{KCNE1} genes, which had not been reported in the literature. Having done so, we identified 3 putative binding sites for \textit{miR}-133 on the canine \textit{KCNQ1} 3′UTR and 1 putative binding site for \textit{miR}-1 on dog \textit{KCNE1}. Interestingly, we found that the expression levels of the muscle-specific miRNA \textit{miR}-133 were reduced upon chronic dofetilide culture. The 3 \textit{miR}-133 binding sites on dog \textit{KCNQ1} 3′UTR could cooperate with each other to confer enhanced \textit{miR}-133 binding, therefore restricting KvLQT1
protein-expression. When \textit{miR}-133 expression is reduced during chronic dofetilide incubation, reduced \textit{miR}-133 binding might result in higher KvLQT1 protein levels, as observed in the present study.

The mechanisms responsible for reduced \textit{miR}-133 expression upon long-term dofetilide treatment remain unknown, requiring further detailed study out of the scope of the present study. On the other hand, we found \textit{miR}-1 expression to be unchanged in cells chronically exposed to dofetilide. In addition, we found only one putative \textit{miR}-1 binding site on the \textit{KCNE1} 3’UTR, which raises questions about the importance of \textit{miR}-1 in regulating \textit{KCNE1} expression in the dog. The changes that we observed in minK protein-expression thus remain unexplained. Whether the observed higher minK protein-expression is caused by regulation through other, unknown miRNAs is not clear and will be interesting to explore in the future. Xiao et al. have shown that \textit{miR}-133 represses ERG protein-expression in diabetic rabbit hearts.\textsuperscript{16} We didn’t observe any changes in I\textsubscript{Kr} density after chronic dofetilide exposure, as we might have expected if canine ERG expression is regulated by \textit{miR}-133. The potential role of \textit{miR}-133 in determining canine ERG expression is presently unknown and would be interesting to investigate further.

We cannot exclude additional contributors, besides microRNA changes, to I\textsubscript{Ks} increases in dofetilide-exposed cardiac cells. Sustained APD prolongation could increase cellular Ca\textsuperscript{2+}-entry and cellular Ca\textsuperscript{2+}-content.\textsuperscript{25} Calmodulin (CaM) binding to KvLQT1 is necessary for proper channel assembly and for conferring Ca\textsuperscript{2+}-sensitive stimulation of I\textsubscript{Ks}.\textsuperscript{26} Therefore, remodeling of I\textsubscript{Ks} after chronic dofetilide exposure might be partially due to changes in intracellular [Ca\textsuperscript{2+}] and effects mediated by Ca\textsuperscript{2+}/CaM regulation of KvLQT1 protein assembly. In addition, I\textsubscript{Ks}
subunits are subject to important regulation of expression at the level of cellular trafficking and membrane insertion as well as significant functional regulation by cell-phosphorylation machinery.\textsuperscript{27,28} Post-translational regulatory changes may explain why \(I_{Ks}\) increased by \(\sim 40\%\) despite only 20-25\% increases in underlying-subunit protein expression. Changes in intracellular [\(Ca^{2+}\)]\(_i\) could certainly be involved, because increased [\(Ca^{2+}\)]\(_i\) is well-known to enhance \(I_{Ks}\).\textsuperscript{29} Better insights into the mechanisms underlying feedback regulation of \(K^+\)-channel expression might lead to novel strategies for the treatment of patients at risk of long QT syndromes, as well as to potential new insights into the determinants of long QT syndrome occurrence.

We used an \textit{in vitro} system to assess the effects of sustained \(I_{Kr}\) inhibition on repolarization reserve and on the expression of various cardiac ion-channels. Pacing cells \textit{in vitro} allows them to display cardiac electrical activity at rates equivalent to normal heart rates \textit{in vivo}, providing for results under well-controlled conditions that may be more relevant to \textit{in vivo} physiological function than those obtained in quiescent cell systems. Follow-up studies in other relevant systems would be of interest.

We used CdCl\(_2\) to inhibit \(I_{Ca,L}\) when studying \(I_{to}\) and \(I_{K1}\). While Cd\(^{2+}\) effectively inhibits \(I_{Ca,L}\) and prevents \(K^+\)-current contamination, it positively shifts voltage-dependence by neutralizing membrane surface-charges.\textsuperscript{30} However, this effect should apply equally to CTL and Dof cells and would not explain the lack of change in these currents. For \(I_{Kr}\) and \(I_{Ks}\) measurements, which can be affected in complex ways by divalent cations,\textsuperscript{30} we used nimodipine to inhibit \(I_{Ca,L}\).
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Conflict of Interest Disclosures

None.
References


Figure Legends

Figure 1. A, Original action potential (AP) recordings (0.1 Hz) from control and dofetilide-cultured cells. B, Corresponding mean±SEM APD_{90} values. (*P<0.05 vs. CTL, repeated-measures ANOVA/Bonferroni-corrected t-tests) C, Representative AP recordings (0.1 Hz) from control and dofetilide-cultured cells in the absence (Before) and presence (After) of 5-nmol/L dofetilide. D, Percentage-APD_{90} increase by dofetilide (5-nmol/L) in control and dofetilide-cultured cells (n=6 and 5, respectively) at 0.1 Hz. (**P<0.001 vs. CTL, nonpaired t-test).

Figure 2. A, I_{Ks} in control and dofetilide-cultured cells, recorded with a holding potential of -50 mV, depolarizing to +40 mV for 4 s and repolarizing to -30 mV for 2 s. B, I_{Ks} step and tail density-voltage relations from control and dofetilide-cultured cells. *P<0.05 vs. CTL, repeated-measures ANOVA/Bonferroni-corrected t-tests. C, Mean±SEM normalized I_{Ks} tail currents, along with best-fit Boltzmann relation. (No significant differences, repeated-measures ANOVA.)

Figure 3. A, I_{Kr} recorded by holding at -70 mV, depolarizing to +10 mV for 1 s and repolarizing to -40 mV for 2 s. B, I_{Kr} tail-current density-voltage relations. Boltzmann fits are shown. C, Normalized I_{Kr} tail currents with best-fit Boltzmann relation. (Repeated-measures ANOVA showed no significant differences in B and C.)

Figure 4. A, B, I_{to} recorded with a holding potential of -50 mV and depolarizing to various step potentials for 100 ms (0.1 Hz). C, I_{to} density-voltage relations. D, E, Activation and inactivation time-constants (Taus).
(Repeated-measures ANOVA showed no significant differences in C, D and E.)

**Figure 5.** $I_{K1}$ from control (A) and dofetilide-cultured (B) cells, recorded with a holding potential of -40 mV and 300-ms steps to potentials between -120 and -10 mV. $I_{K1}$ was recorded before and after 1-mmol/L Ba$^{2+}$; Ba$^{2+}$-sensitive currents used to assess $I_{K1}$ are shown here. C, Mean±SEM $I_{K1}$ density-voltage relations from control and dofetilide-cultured cells. (Repeated-measures ANOVA showed no significant differences in C.)

**Figure 6.** $I_{Ca,L}$ recordings from control (A) and dofetilide-cultured (B) cells, recorded with a holding potential of -50 mV and 250-ms steps to potentials between -40 and +60 mV (0.1 Hz). C, $I_{Ca,L}$ density-voltage relations in control and dofetilide-cultured cells (n=5 cells/group). D, $I_{Ca,L}$ inactivation-kinetics. (Repeated-measures ANOVA showed no significant differences in C and D.)

**Figure 7.** A, Mean±SEM ERG and KCNE2 mRNA-expression levels after normalization to β2-microglobulin in control and dofetilide-cultured cells (n=5 samples/group). B, Mean±SEM KCNQ1 and KCNE1 mRNA-expression levels after normalization to 18S rRNA in control and dofetilide-cultured cells (n=7 samples/group). C, Top: Representative Western blots for KvLQT1 (~78 kDa) and GAPDH; Bottom: Mean±SEM KvLQT1-band optical density (O.D.) relative to GAPDH-band O.D. in control and dofetilide-cultured cells. n=6 samples/group, *P<0.05 vs. CTL. D, Top: Representative Western blots
for minK (~20 kDa) and GAPDH; Bottom: Mean±SEM minK-band O.D. relative to GAPDH-band O.D. n=6 samples/group, **P<0.01 vs. CTL. (Statistical-analysis by paired t-tests; no significant differences in A and B.)

**Figure 8.** A, The complementarity (bold font and vertical lines) between miR-133 and each of the three target sites in the 3’UTR of KCNQ1. B, Mean±SEM miR-133a and miR-133b gene-expression levels in CTL and Dof cells (n=8 samples/group). Expression levels were all normalized to corresponding CTL. **P<0.01, ***P<0.001, Dof vs. CTL. C, The complementarity between miR-1 and the target site in the 3’UTR of KCNE1. D, Mean±SEM miR-1 gene-expression level in CTL and Dof (n=6 samples/group). Expression levels are all normalized to corresponding CTL. (Statistical-analysis by paired t-tests; no significant differences in D.)
Figure 3

A

B

C

251
Figure 4

A

B

C

D

E

CTL

Dof

 CTL TauFast (n=7)

 Dof TauFast (n=10)

 CTL TauSlow (n=10)

 Dof TauSlow (n=10)

Activation Time constant (ms)

Time (ms)

pA/pF

pA/pF

TP (mV)

pA/pF

TP (mV)

pA/pF

TP (mV)
Figure 5

A

B

C

Time (ms)

Dpf

TP (mV)

Dof
Figure 6
Figure 7

A

B

C

D
Figure 8

A

Mir-133
Binding site 1:
3'-UGUGGACCAACUUCUCCUUGSUU-5'
460-3GACATTGACACCACAGCCCA-481
Binding site 2:
3'-UGUGGACCAACUUCUCCUUGSUU-5'
517-3GACATTGACACCACAGCCCA-538
Binding site 3:
640-3GACATTGACACCACAGCCCA-661

B

CTL (n=8)  Dof (n=8)

** P<0.01, *** P<0.001

mIR-133 level normalized to CTL

miR-133a

miR-133b

C

Mir-1

miR-1 binding site:
3'-AAUGGUAUGAAGAUAUGAUGU-5'
233-3GACATTGACACCACAGCCCA-254

D

CTL (n=6)  Dof (n=6)

mIR-1 level normalized to CTL

CTL

Dof
On-line Materials and Methods

In vitro Cellular Pacing Model

Cell Isolation. All animal care and handling procedures followed the Animal Care Guidelines of the Canadian Council and the National Institutes of Health, and were approved by the animal research ethics committee of the Montreal Heart Institute. Male adult mongrel dogs (21-35 kg) were anesthetized with pentobarbital (30 mg/kg IV) under artificial ventilation. Left lateral thoracotomy was performed, and hearts were quickly excised and immersed in oxygenated Tyrode solution at room temperature. The transmural free wall (~30×50 mm) of the anterior left ventricle, which contained coronary artery branches from the left circumflex coronary artery, was quickly dissected and the artery was cannulated. Cell isolation was performed by perfusion with Tyrode solution containing collagenase (120 U mL⁻¹, Worthington, type II) as previously described.¹ Regional cells from the epicardial (Epi) surface (1-1.5 mm thick) were taken after digestion and kept in culture medium for further studies.

Cell-Culture and Pacing. All procedures for cell-culture and pacing were performed under aseptic conditions as previously described.² After isolation, the cell-suspension was centrifuged at 500 rpm for 1 minute at 4°C and cell pellets were re-suspended in fresh culture medium. Culture medium contained Medium 199 (GIBCO, Invitrogen Corp., with Earle’s salts, L-glutamine and 2,200 mg/L sodium bicarbonate), Na-penicillin G (100 U/mL) and streptomycin sulfate (100 µg/L), and was supplemented with Insulin-Transferrin-Selenium-X (GIBCO, Invitrogen Corp., containing 0.01
mg/mL insulin, 5.5 µg/mL transferrin, 6.7 ng/mL sodium selenite and 2 µg/mL ethanolamine). Cells were plated at ~1×10⁴ cells/cm² on rectangular (24×55 mm) glass coverslips or 4-well rectangular Petri dishes (24×67 mm/well, Nalge Nunc International) pre-coated with laminin (15-µg/mL). Cardiomyocytes were maintained in the culture medium at 37°C in a humidified, 5% CO₂-enriched environment in the incubator. After 4 hours, any dead or unattached myocytes were washed off with fresh media to leave a homogeneous layer of rod-shaped cells attached to the coverslips or the 4-well petri dish. Cells were divided into 2 groups: CTL (medium without dofetilide) and Dof (culture media containing 5-nmol/L dofetilide). Both CTL and Dof cells were continuously electrically paced at 1 Hz for 24 hours in the incubator. The electrical pacing system includes a C-Pace100™ pacing unit and C-Dish100™ stimulation banks (IonOptix Corporation). The stimulus pulse-duration was 5 milliseconds and the pulse-voltage was 43 V. Evidence of capture was obtained by microscopic observation at the beginning of stimulation and before termination (after 24 hour-pacing). Capture-efficiency was verified by eye and by video-recording of cell shortening and was ~100% for cells attached on laminin-coated coverslips or plates. After 24 hours, cells on coverslips were kept in high-K⁺ storage solution at 4°C for electrophysiological studies. Cells were scraped off culture plates with a cell-scaper (Sarstedt) and were centrifuged at 1000 rpm for 5 minutes at 4°C. Cell pellets were fast-frozen in liquid-N₂ and kept at -80°C for subsequent molecular biology studies.

**Electrophysiology**
Whole-cell patch-clamp was applied for ionic-current and AP recording at 36±0.5°C. Ionic currents were recorded with tight-seal patch-clamp in the voltage-clamp mode, and APs in current-clamp mode with perforated-patch techniques. Borosilicate-glass electrodes had tip resistances between 2 and 5 MΩ when filled. Cell-capacitance and series-resistance were compensated by ~80% to 90%. Junction potentials (~10 mV) were corrected for AP recordings only. Leakage compensation was not used. Cell-capacitance averaged 160.6±5.7 pF in CTL (n=37) and 169.1±6.0 pF in Dof (n=44, P=NS) cells. Currents are expressed in terms of density (normalized to cell-capacitance).

The standard Tyrode solution contained (mmol/L) NaCl 136, KCl 5.4, MgCl₂ 1, CaCl₂ 1, NaH₂PO₄ 0.33, HEPES 5 and dextrose 10 (pH 7.35 with NaOH). The high-K⁺ storage solution contained (mmol/L) KCl 20, KH₂PO₄ 10, dextrose 10, mannitol 40, L-glutamic acid 70, β-OH-butyric acid 10, taurine 20, EGTA 10 and 0.1% BSA (pH 7.3 with KOH). The standard pipette solution used in most experiments contained (mmol/L) K-aspartate 110, KCl 20, MgCl₂ 1, MgATP 5, GTP 0.1, HEPES 10, Na-phosphocreatine 5, EGTA 5 (for current recording) or 0.025 (for AP recording), with pH adjusted to 7.3 with KOH.

For AP recording, nystatin (60-µg/mL) was back-filled into the pipette tip and external solutions contained 2-mmol/L CaCl₂. For all K⁺-current recordings, atropine (1 µmol/L) and CdCl₂ (200-µmol/L) or nimodipine (5-µmol/L, for I_{K₁} and I_{K₅}) were added to external solutions to eliminate muscarinic K⁺-currents and to block Ca²⁺-currents. Na⁺-current contamination was avoided by using a holding potential (HP) of -50 mV. For currents other than transient outward current (I_{to}), 1 mmol/L 4-aminopyridine was used to block I_{to}. Inward-rectifier current (I_{K₁}) was studied as
1 mmol/L Ba$^{2+}$-sensitive current. HMR1566 (1-µmol/L) was added to inhibit $I_{Ks}$ for $I_{Kr}$-recording and E4031 (5-µmol/L) was used to inhibit $I_{Kr}$ for $I_{Ks}$-recording.

For L-type calcium current ($I_{Ca,L}$) studies, the external solution contained (mmol/L) tetraethylammonium chloride (TEA-Cl) 136, CsCl 5.4, CaCl$_2$ 2, MgCl$_2$ 0.8, HEPES 10 and dextrose 10 (pH 7.4 with CsOH). Niflumic acid (50 µmol/L) was added to inhibit $I_{Cl, Ca}$. The pipette solution contained (mmol/L) CsCl 20, Cs-aspartate 110, MgCl$_2$ 1, MgATP 5, GTP 0.1, Na$_2$-phosphocreatine 5, EGTA 10 and HEPES 10 (pH 7.2 with CsOH).

Late-$I_{Na}$ was recorded as described by Maltsev et al. The pipette-solution contained (mmol/L): 5 NaCl, 133 CsCl, 2 MgATP, 20 TEA, 10 EGTA, 5 HEPES; pH 7.3 (CsOH). The bath-solution contained (mmol/L): 140 NaCl, 5.4 CsCl, 1.8 CaCl$_2$, 2 MgCl$_2$, 0.002 nifedipine, 5 HEPES; pH 7.3 (NaOH). Late-$I_{Na}$ (current 200-220 ms after depolarization-onset) was recorded 5 minutes after cell-rupture at room-temperature, with 2-second voltage-steps (0.2 Hz) from -120 mV to test-potentials between -70 and +40 mV.

**Real-time PCR**

Total RNA was extracted from CTL and Dof snap-frozen cell-samples subjected to homogenization in TRIzol Reagent (Invitrogen), chloroform extraction and isopropanol precipitation. Genomic DNA was eliminated by incubation in DNase I (0.1 U/µL, 37°C) for 30 minutes, followed by phenol-chloroform acid extraction and gel verification. RNA was quantified spectrophotometrically at a 260-nm wavelength and integrity was confirmed on a denaturing agarose gel. RNA-samples were stored in DEPC H$_2$O at -80°C. First-strand cDNA was synthesized by RT with 2 µg of
RNA samples, random primers and MMLV reverse transcriptase (High Capacity cDNA Archive Kit, Applied Biosystems). Real-time PCR was conducted with a Stratagene Mx3000P QPCR detection system, with Taqman (\(KCNH2\) and \(KCNE2\) transcripts) or SYBR green (\(KCNQ1\) and \(KCNE1\) transcripts) quantitative assay. Canine \(\beta\)2-microglobulin (Taqman) or 18S rRNA (SYBR green) was used as the internal control. Primers and probes for real-time PCR reactions are listed in online Table 1. Each sample was run in duplicate and PCR products were verified with gel electrophoresis or dissociation curves (SYBR green reaction). \(KCNH2\) and \(KCNE2\) results were normalized to \(\beta\)2-microglobulin, \(KCNQ1\) and \(KCNE1\) results to 18S rRNA; internal control data were obtained from the same samples at the same time.

**Protein Extraction and Western Blot**

Membrane protein fractions were isolated with extraction buffer containing 25 mmol/L Tris-HCL (PH 7.4), 5 mmol/L EGTA, 5 mmol/L EDTA, 1 mmol/L Na\(_3\)VO\(_4\), 0.5 mmol/L AEBSF, 1 mmol/L iodoacetamide and \(\beta\)-2-mercaptalethonal, 10 \(\mu\)g/mL aprotinin and leupeptin and 1 \(\mu\)g/mL pepstatin, followed by homogenization. After centrifugation (1000 rpm, 4°C, 5 minutes), the supernatant containing the cell membranes was centrifuged at 100,000 g for 1 hour. Membrane protein pellets were re-suspended in extraction buffer supplemented with 1%-Triton and stored at -80°C. Protein concentration was determined with the Bradford method. Membrane-protein samples (40 \(\mu\)g) from CTL or Dof cells were denatured with Laemmli buffer and fractionated on 8% or 12% SDS-polyacrylamide gels, then proteins were transferred electrophoretically to Immobilon-P polyvinylidene fluoride (PVDF) membranes.
(Millipore) in 25-mmol/L Tris-base, 192-mmol/L glycine and 20%-ethanol at 0.3 A for 1 hour. Membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) and 5% non-fat dry milk for 1 hour and incubated with primary antibodies (anti-KvLQT1, 1:500, Alomone; anti-minK, 1:2000, from Dr. Jacques Barhanin) overnight at 4°C. After washing and re-blocking, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:10,000, Jackson). Antibody was detected with Western-Lightening Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences). Later, the same membranes were also probed with anti-GAPDH at room temperature for 2 hours to control for protein loading. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000, Santa Cruz).

**Rapid amplification of cDNA ends (3’ RACE) for KCNQ1 and KCNE1**

The sequences of 3’ untranslated regions (UTR)s of canine KCNE1 and KCNQ1 were determined with Ambion’s RNA ligase-mediated 3’RACE kit according to the manufacture’s instructions. Canine RNA was prepared with TRIZOL using epicardial tissue from dog left ventricles. Gene-specific primers for KCNE1 and KCNQ1 were designed based on canine KCNE1 (GenBank accession No. XM_544868) and KCNQ1 (GenBank accession No. XM_540790) cDNAs. The primers used for 3’RACE were: for KCNE1, first (outer) primer, 5’- TTCTTCACCCTGGGCATT-3’ and second (inner) primer, 5’-CCCAACACACCTTTCCCGA-3’; for KCNQ1, first (outer) primer, 5’-GGAAGCCCTCCCTTCATC-3’ and second (inner) primer, 5’-ACCCTGCCCACC
MicroRNA Detection

The TaqMan® MicroRNA Assay (Applied Biosystems), a quantitative reverse transcription-PCR (qRT-PCR) kit enabling sensitive, rapid quantification of microRNA (miRNA) expression from total RNA samples, was used in conjunction with real-time PCR with Taqman probes for quantification of miR-133 (miR-133a and miR-133b) transcripts. RNA samples were isolated from cultured cells with mirVana™ miRNA Isolation Kits. Reactions contained TaqMan® MicroRNA Assay sets specific for miR-133a, miR-133b or RNU24 as a positive endogenous control. QRT-PCR was performed on a GeneAmp 5700 thermocycler for 40 cycles. We first determined the appropriate cycle threshold (Ct) using the baseline determination feature. Fold-variations in expression of miR-133 between RNA samples were calculated after normalization to RNU24.

Data Acquisition and Analysis

Voltage-protocols were applied at 0.1-Hz unless stated otherwise. Clampfit 6.0 (Axon), Graph Pad Prism 3.0 and Quantity one (Bio-rad) were used for data analysis. Nonlinear least-square curve-fitting algorithms were performed for curve fitting. Paired or unpaired Student’s t-tests were applied for single comparisons between groups. Comparisons involving repeated-measures analyses were performed by ANOVA with Bonferroni-adjusted t-tests in the case of statistically-significant intergroup differences. A two-tailed $P<0.05$ was taken to indicate statistical significance. Group data are expressed as mean±SEM. The authors had full access to
the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.
References


On-line Figure Legends

On-line Figure 1.  A, A representative AP recorded at 1 Hz from a freshly-isolated cardiomyocyte before dofetilide exposure.  B, An AP from the same cell in the presence of 5 nmol/L dofetilide.  C, Mean±SEM APD$_{90}$ before and after dofetilide (n=11 cells).  **$P<0.01$ vs pre-dofetilide (paired $t$-test).

On-line Figure 2.  A, $I_{Ks}$ activation and deactivation time constants (Taus) from CTL and Dof cells. Both activation and deactivation time constants were obtained by biexponential fits to currents recorded with the voltage protocol shown at the top.  B, $I_{Kr}$ activation and deactivation time constants (Taus) from CTL and Dof cells. Both activation and deactivation time constants were obtained by biexponential fit to currents recorded with the protocol shown at the top. (Statistical-analysis by nonpaired $t$-tests. No significant differences.)

On-line Figure 3.  A, Representative examples of Late-$I_{Na}$ from CTL (dark) and Dof (grey) cells, recorded with a 2-s pulse to a test potential of -30 mV from a holding potential of -120 mV.  B, Mean±SEM Late-$I_{Na}$ densities at -30 mV from CTL and Dof cells. (Statistical-analysis in B by nonpaired $t$-test. No significant differences)

On-line Figure 4.  A&B, Representative AP recordings (1 Hz) from control (A) and dofetilide-cultured (B) cells in the absence (Before) and
presence (After) of 100-nmol/L HMR-1556. C, Percentage-
APD$_{90}$ increase by HMR-1556 (100-nmol/L) in control and
dofetilide-cultured cells (n=5 cells/group) at 1 Hz. (*$P<$0.05 vs.
CTL, nonpaired $t$-test).

**On-line Figure 5.** Dog 3’UTR complete sequence for $KCNQ1$. The full-length
3’UTR for dog KCNQ1 is 919 bp. The start site of 3’UTR is
counted as “+1”. Potential miR-133 binding sites are indicated.
Letters in bold indicate complementary nucleotides.

**On-line Figure 6.** Dog 3’UTR complete sequence for KCNE1. The full-length
3’UTR for dog $KCNE1$ is 303 bp. The start site of 3’UTR is
counted as “+1”. Potential miR-1 binding site is indicated.
Letters in bold indicate complementary nucleotides.
## Online Table 1

**Primer and probe information:**

<table>
<thead>
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<th>Primsers and Probes</th>
<th>Sequences</th>
<th>GeneBank#</th>
</tr>
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</table>
| ERG                 | F: GCAAAGTGGAGATCGCCTCTAC  
R: CATCCACCAGCCACAGGAA  
Probe: CAGCTCCCATCTTCC | NM_001003145 |
| MiRPI               | F: CCATCCTGGTGAGGACTGTG  
R: AGTCCTTCTACAATGTACTGTGGTTA  
Probe: CCGTCTCTTGGATTTCC | AY_307952 |
| β 2-microglobulin   | F: CCGTGCGCCTGGAT  
R: CCGGGTGTCCGTGAGTACA  
Probe: CCGTCAGCATCCCCAAA | XM_535458 |
| KvLQT1              | F: ATTCGGCCATGCAGTACTT  
R: TTGATGCGCACCATGAGGT | XM_540790 |
| minK                | F: TCTAAATCCACCAGAGTGAT  
R: AAGAAGCCAAGCACCATGAG | XM_544868 |
| 18S rRNA            | F: CCTGGTTGATCTCCTGCCAGTA  
R: CCGTCGCGCATGTATTAGCTC | AY262732 |
Online Data Suppl. Figure 2

A

B

\( I_{ho} \) time constants at 40 mV (ms)

\[ \text{Activation} \quad \text{Deactivation} \]

\[ \text{CTL (n=8)} \quad \text{DoF (n=9)} \quad \text{CTL (n=8)} \quad \text{DoF (n=8)} \]

\[ \text{CTL (n=7)} \quad \text{DoF (n=7)} \quad \text{CTL (n=7)} \quad \text{DoF (n=7)} \]
Online Data Suppl. Figure 3

A

B

Late I_{Ik} density at -30 mV
Online Data Suppl. Figure 5

Dog KCNQ1 3’UTR complete sequence (cDNA)

5’ (+1) – TGGGGCTTGGGCCCCAGGCGCTGCAAAGGAAAGTGCAGAGATGCCCTGTCTAGCCTGGCC
ACCTCCACCCACAGGGCGACCCACCCCCACCCCCACCTGGGACCTGGGATCTCTCAAAAGGCTCAAGAGAC
CCCTCTTCCGAGCCCCACAGCCCGCCAGGCGACCCACCTGAGAGCGCCAGCAAGGTTGACTA
GAATTGGATTGCTCTGAGGAGGCTCCCTTCTTGTGCTGACCTTTGCCCTCCTACTTTGTATGACGTT
GGGACACAGTGGCAGGCTAGCTCCGCAACCCCCTCCCCCCAAGGGGCGCTGCACTCTTAGGCACCTGTC
ATCCCATGATGGCCCAAGACAGCAACAGGGATGACATGAAGGCCCACCTGGGTGGCAGCCAGGGCTCTCGAG
TGGAAGAACGAGGACAGTCTGGACAGGTTATGGTGACCTACACCCACCCCGCCACCCGCACCTCCTAGAG
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CGCCGCTCAGGGGCGCCAGGCTGTGGGGGGGCACGTCGGCCACCTGCTGGGAGGTCTCA
CTGGGGGTGAACGCTCCCCACTTTGAGCTGGACAGCTTCTCTGACCTGCCACTGCCCCGCAAGCAGCCACC
CTCCACACACAGGCTGAGCCGAGGGGAAGGCTGGTACGCGAGAGGGCGCCGCCC
GTAAGAGGCGCTCCGTCACGATCCCAGCAGAGGGCTCCTCTTGCCGGTCATGTCTCAGAAATCGAAAAT
TTTGTTTAAAAA-AATA (-919) 3’
Online Data Suppl. Figure 6

Dog KCNE1 3'UTR complete sequence (cDNA)

5' (+1) - CCCCACAGTCGGTTAAAACCTGGGACAATCTTACTTGGCAATCTGATTTTCTAAATCAGTGCC
CTTTATATTCTTTATTTGATAGGATGTAATGGGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
Chapter 5. General Discussion
This dissertation is manuscript-based, including three published articles. The results, the potential significance and limitations of these studies have been discussed in detail in each manuscript. Thus, the General Discussion will mainly focus on the novel findings of our studies and future research directions.

5.1 Summary of novel findings and overall significance

5.1.1 Characterization of cellular mechanisms underlying gender differences in cardiac repolarization in the dog heart

I have conducted the first relatively scale and comprehensive analysis of sex-based ionic current function and ventricular repolarization in all three electrophysiologically distinct and functionally important transmural layers of the left ventricle in adult dogs (chapter 2). My results significantly help to understand the clinical observation of longer baseline QTc and greater drug-induced QT-prolongation/risk of TdP in women (1-3).

There are numerous discrepancies in measures of sex-related differences in baseline ventricular repolarization and ionic current properties among experimental studies (4-11). This is possibly due to interspecies and interstrain differences. Most of the animal used in these studies are rodents (i.e. mice, guinea pigs, rats) or rabbits. The ionic currents underlying ventricular repolarization in these species vary and differ from those of humans (12). Another possibility is that, even though transmural differences in ventricular repolarization have been well appreciated as a fundamental feature of the ventricle in most species (13;14), these studies are usually performed on unspecified ventricular cells or limited regions of the ventricle. For example, Pham et al did not find male/female APD differences in rabbit right ventricular endocardium and epicardium (5). In my study, I also found similar male/female APDs in LV epicardium and endocardium. However, I found longer female APD in the midmyocardium that likely participates in TdP. A common observation in experimental studies is the greater prolongation of female repolarization (versus
male) upon exposure to $I_{Kr}$ blockers. It has been suggested that the nature of the processes underlying repolarization (i.e., ionic basis of repolarization, dispersion of repolarization), rather than the baseline duration of ventricular repolarization itself, determines the sex-related differences in drug-induced pro-arrhythmia (15). We investigated the ionic basis of gender-related transmural repolarization differences in the left ventricle of the dog heart, which is electrophysiologically similar to human (12; 16).

My results demonstrated that the longer rate-dependent QT interval in female dogs is primarily due to intrinsic male-female differences in transmural ventricular repolarization (17). This finding is in agreement with merging observations from clinical studies that besides gender differences in baseline QTc, there are also potential sex-related differences in the heterogeneity of ventricular repolarization or TDR (18;19). My results presented the first direct cellular evidence that gender-dependent differences in transmural repolarization are mainly due to the longer M cell APDs in females in the setting of similar male/female APDs in Epi and Endo. M cells are known to play important roles in the development of TdP due to their unique physiological and pharmacological properties (20-22). Drugs that block $I_{Kr}$ or $I_{Ks}$, or increase late $I_{Na}$, can preferentially induce greater APD in M cells than in Epi and Endo cells, thereby augmenting TDR (23). Using transmural optical imaging in canine LV wedge preparations, Akar et al provided direct evidence that increased TDR, due to disproportionate M cell APD prolongation, forms zones of increased refractoriness in the M cell region responsible for conduction block and self-sustained intramural re-entrant circuits underlying TdP (22). My findings that females have longer M cell APDs but similar Epi and Endo APDs compared to males suggest that women may develop greater enhancement of TDR upon exposure to QT-prolonging drugs and may be prone to develop re-entry due to unidirectional block in the M cell region.

My results show that the ionic currents across the canine ventricular wall have rather complex sex-based differences. I found male/female differences in
three ionic-current systems: \( I_{lo}, I_{Ks}, \) and \( I_{Ca,L} \). \( I_{lo} \) is smaller in females only in the endocardium. \( I_{Ca,L} \) is larger in females across the transmural ventricular free wall. Pham et al found somewhat larger \( I_{Ca,L} \) densities in the epicardium of female rabbits in accordance with our results, but no substantial endocardial differences (24). I found that \( I_{Ks} \) was larger in females only in the epicardium and endocardium, but similar to male \( I_{Ks} \) in the midmyocardium, indicating a larger transmural \( I_{Ks} \) gradient in females. I was unable to identify previous studies of sex-dependent differences in \( I_{Ks} \). The larger inward \( I_{Ca,L} \) and outward \( I_{Ks} \) in females may offset each other in Endo and Epi, accounting for the absence of significant male/female differences in these regions. However, larger \( I_{Ca,L} \) but similar \( I_{Ks} \) in the midmyocardium may account for longer midmyocardial APD in female dogs, contributing to a greater repolarization gradient. More importantly, my results suggest that females may have reduced repolarization reserve in the M-cell layer since larger inward \( I_{Ca,L} \) tends to delay repolarization. Therefore, \( I_{Ks} \)-blockers can induce greater M-cell repolarization delay, EADs and transmural re-entry in females, which in turn may contribute to increased QT intervals and greater risks of TdP (25).

5.1.2 Rate per se regulates ion channel expression

My studies in Chapter 3 of this thesis have assessed, for the first time in the literature, the direct effect of cardiac firing rate on ion channel expression. My results provide the first evidence that increased electrical activation rates \( \text{per se} \) can regulate the expression of cardiac \( I_{lo} \) (26). More importantly, I have established a well-defined signaling pathway for rate-dependent regulation of \( \text{Kv4.3} \) expression in ventricular myocytes as discussed in 5.1.3. These findings contribute to the emerging field of cardiac excitation-transcription (ET) coupling.

Chronic increases in heart rate are well-known to cause electrical remodelling in the ventricle, impairing cardiac function and promoting lethal cardiac arrhythmias (27;28). Almost all documented studies from human or animal HF models consistently found that chronic tachycardia decreases \( I_{lo} \), partly due to downregulation of \( \text{Kv4.3} \) transcript and protein levels (27;31-33). \( I_{lo} \) is the major
determinant of phase-1 repolarization of the ventricular AP, which plays a role in excitation-contraction coupling through its influences on AP plateau voltage to affect $I_{CaL}$. Unlike in rodents, in which loss of $I_{to}$ prolongs overall APD and can improve cardiac contractility, in failing canine or human hearts, reduced $I_{to}$ and phase-1 of the AP are associated with impaired myocyte contractility and decreased efficiency of excitation-contraction coupling (89;90). Changes in hemodynamics, neurohumoral state or autonomic nervous system function are associated with chronic tachycardia (28). Many factors involved in these changes during HF, such as increased angiotensin II, aldosterone, endothelin and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) etc., are known to downregulate $I_{to}$ (28;91-93). Using an in vitro ventricular cell culture-pacing model, I demonstrated that increased heart rate per se suppresses $I_{to}$ through transcriptional regulation of Kv4.3 expression. Atrial ionic but not structural remodeling in tachypacing-induced HF dogs recovers after long-term removal of tachypacing (94). Even though no obvious HF-related ventricular tissue remodeling is observed after 24 hour ventricular tachypacing (95), I found significant $I_{to}$ reduction in epicardial cells at this early stage. My results argue that rate per se is a major contributor to in vivo tachycardia induced electrical remodelling and that such changes are quite dynamic.

My findings show that chronic changes in electrical activation rate initiate ET coupling processes to translate increased heart rate into reduced Kv4.3 levels. In addition to demonstrating the phenomenon, I defined the signaling mechanisms that translate altered cardiac electrical activity into regulation of ion channel gene transcription through the application of a series of multidisciplinary and integrative approaches. I found that this excitation-transcription system involves the activation of Ca-CaM-CaMKII-Calcineurin-NFAT signaling. A schematic diagram to illustrate the signaling pathways involved in rate-dependent regulation of $I_{to}$ expression is shown in Figure 1. My findings add to the growing recognition that activation of NFATc3 may be a general mechanistic point of convergence among many stimuli that regulate expression of voltage-gated ion channels in the
cardiovascular system (48). Activation of NFATc3 downregulates $I_{o}$ in mouse ventricular myocytes, contributing to normal mouse $I_{o}$ transmural gradient, the loss of $I_{o}$ gradient after long-term β-adrenergic stimulation, and myocardial infarction related $K^{+}$ current remodeling (45;47;96). In smooth muscle, NFATc3 activation decreases the expression of several ion channel subunits (97;98) Calcineurin/NFAT activation is seen in atrial fibrillation and downregulates $I_{Ca,L}$ and Cav1.2 expression in tachypaced atrial myocytes (35;81) All these results suggest that activation of calcineurin/NFAT plays essential roles in electrical remodeling under pathological conditions. Blunting calcineurin/NFAT activation may be an interesting and promising therapeutic target in cardiac arrhythmia therapy. Clinical use of calcineurin inhibitors is efficient in suppressing lymphocyte function and inducing immunosuppression (99). It remains to be determined whether cardioselective calcineurin inhibitors can be developed. However, there is also evidence that NFAT can upregulate Kv4.2 in neonatal rat ventricular myocytes (100). Therefore, a better understanding of the molecular determinants of NFAT-dependent regulation of ion channel expression is needed.
Figure 1. A schematic diagram of signalling cascades involved in the regulation of $I_{to}$ by tachypacing. Tachypacing reduced $I_{to}$ through increased frequency Ca$^{2+}$ entry via APs, which induced larger Ca$^{2+}$/calmodulin activity and resulted in increased CaMKII and calcineurin activities, along with NFATc3 nuclear translocation that downregulated Kv4.3 transcription. (Adapted from Figure 8 of Chapter 3, section 2).

5.1.3 Ca$^{2+}$ signaling in rate-dependent remodeling of $I_{to}$ expression

The demonstration that activation of the Ca-CaM-CaMKII-Calcineurin-NFAT signaling pathway is the underlying molecular mechanisms coupling rate-dependent changes with downregulation of Kv4.3 transcription helps in understanding how ion-channel expression and function are regulated under physiological and pathophysiological cardiac conditions. Ca$^{2+}$ couples via calmodulin to downstream signals that regulate $I_{to}$ expression by altering the phosphorylation states of key proteins. Blocking Ca$^{2+}$ entry through $I_{Cal}$ by nimodipine, or inhibiting calmodulin activity by different available blockers (W-7,
A-7, and W-13) with different affinities to calmodulin (37;38) prevent tachypacing
induced reduction of I\textsubscript{o}. Ca\textsuperscript{2+} sensing of alterations in frequency has been shown
to be very important in regulating neuronal activity by integrating sustained
changes of Ca\textsuperscript{2+} and Ca\textsuperscript{2+} activated calmodulin into CaMKII activation (39). In
cardiac myocytes, increased electrical firing rate can increase global cytosolic
Ca\textsuperscript{2+} (81;101). There is evidence for the involvement of Ca\textsuperscript{2+} entry in regulating
ion channel expression (I\textsubscript{Ca,L} and Cav1.2) in \textit{in vivo} paced dogs and in HL-1 cells
(40;41). Calcium thus plays pivotal roles in both cardiac excitation-contraction
(EC) and excitation-transcription (ET) coupling. Disturbances in Ca homeostasis
due to impaired Ca handling systems such as hyperphosphorylated RyR2 or
reduced SERCA are hallmarks of HF (102). In the cardiac cell, there are two
major intracellular Ca\textsuperscript{2+} pools relying on localized receptors and macromolecular
complexes. The SR Ca store is controlled by I\textsubscript{Ca,L}, RyR2 and SERCA. It
participates importantly in EC coupling and in controlling Ca fluctuations that
contribute to Ca-CaM-calcineurin-NFAT dependent ET coupling (102).
Intranuclear Ca\textsuperscript{2+} mobilization via the nuclear envelope is controlled by inositol
1,4,5-trisphosphate receptors (IP\textsubscript{3}Rs) that trigger local Ca release to regulate
hypertrophic gene transcription through the Ca\textsuperscript{2+}-CaM-CaMKII-HDAC pathway
(102;103). IP\textsubscript{3}R-dependent nuclear Ca\textsuperscript{2+} release and ET coupling is activated by
membrane receptors that couple to phospholipase C (PLC), which is altered in
heart failure (103). Recent evidence showed that IP\textsubscript{3}R-regulated ET coupling may
not be activated by the global Ca\textsuperscript{2+} transients that cause contraction at each heart
beat (104). These results indicate a need for further work to address the details of
intranuclear events involved in Kv4.3 transcription through
Ca\textsuperscript{2+}-CaM-calcineurin-NFAT signaling associated with ventricular tachycardia.

We have demonstrated that Ca\textsuperscript{2+}/calmodulin activation of CaMKII plays a
role in rate-dependent I\textsubscript{o} and Kv4.3 downregulation. We found that tachypacing
elevated CaMKII activity because one of its target proteins (PLB) displayed
increased CaMKII-phosphorylation upon cardiomyocyte tachypacing. This
could result from CaMKII activation by increased Ca\textsuperscript{2+}/calmodulin binding during
sustained increase of firing rate as seen by De Koninck et al in neurons (39). Inhibition of CaMKII activity by KN93 prevented reduction of I_{to} and Kv4.3 protein in our tachypaced cells. Chronic inhibition of CaMKII in mice caused a decrease in APD and increase of I_{to} and I_{K1} with reduced CaMKII phosphorylated PLB (42), consistent with our findings. Our results fit with the increasing recognition that CaMKII plays important roles in cardiac arrhythmia (43;44).

There are two major isoforms of CaMKII in cardiomyocytes that display compartmentalized localization (105). CaMKIIδ_B is the nuclear isoform and CaMKIIδ_C is the cytosolic isoform. CaMKIIδ_B is activated by nuclear Ca release and regulates HDAC/MEF2 dependent transcription but has little direct effects on SR Ca^{2+} handling (106). CaMKIIδ_C is an important SR Ca^{2+} handling regulator but can also regulate gene transcription, via downstream signaling effectors that are not clear but may also involve HDAC/MEF2 (106). My results provide an additional novel finding about CaMKII-dependent transcription regulation. I observed that CaMKII inhibition prevents calcineurin activity increases in tachypaced ventricular myocytes, indicating that there is crosstalk between CaMKII and calcineurin signaling, and that CaMKII can potentially modulate calcineurin function in tachypaced cardiomyocytes. In calcineurin-overexpressing mice, CaMKII signaling is increased. CaMKII inhibition improves arrhythmias and left ventricular dysfunction in calcineurin-overexpressing mice (49). Together, these data suggest that cardiac CaMKII may regulate gene expression by modulating calcineurin/NFAT function. CaMKII phosphorylates calcineurin and at low Ca^{2+} levels inhibits calcineurin activity (50). KN93 can inhibit both isoforms of CaMKII (107). Further work is needed to better define the interactions between these 2 important Ca^{2+}-dependent signaling systems involving CaMKII and calcineurin.

5.1.4 Demonstration of a novel mechanism for control of repolarization reserve

Since its first conceptualization by Roden in 1998, the concept of repolarization reserve has greatly helped cardiovascular scientists and
cardiologists in understanding electrophysiological mechanisms underlying drug induced QT-prolongation and associated lethal ventricular arrhythmias-TdP, as well as in appreciating the intriguing clinical problem of variability in response to QT-prolonging drugs or LQTS-associated mutations (51-53). I have advanced this concept by identifying a novel potential layer of repolarization reserve, feedback regulation of ion channel expression (54). My findings in Chapter 4 are the first demonstration that sustained APD prolongation by potassium channel inhibition can induce remodeling of ionic current expression to increase repolarization reserve in cardiac ventricular myocyte.

The general understanding of repolarization reserve involves functional increases in potassium currents by enhanced voltage and time-dependent activation in response to dysfunction or inhibition of a K\(^+\)-current (52;55;56). This functional level of repolarization reserve is very important in order for cardiomyocytes to respond instantly to impaired function of a single K\(^+\)-current, limiting excessive and potentially lethal APD prolongation (52). The underlying biophysical properties of K\(^+\)-currents critically determine the functional compensatory component of repolarization reserve. This mechanism has been well established with regards to I\(_{Ks}\) when facing I\(_{Kr}\) inhibition (56-59). It was suggested in the recently proposed homeostatic regulation theory of cardiac ion channel expression that the number of specific cardiac ion channels on the cell membrane can be regulated in response to changing physiological conditions (28;60). I found that, upon sustained APD prolongation induced by I\(_{Kr}\) inhibition, I\(_{Ks}\) density is increased without alterations in its voltage-dependence or kinetic properties. The change in I\(_{Ks}\) density is associated with upregulation of KvLQT1 and minK protein expression. My results suggest that ventricular repolarization reserve can be controlled at the level of repolarizing channel-protein membrane-expression. Compensatory upregulation of alternative repolarizing K\(^+\) channel subunits due to loss of a K\(^+\) channel has been shown in transgenic models (64;65). However, the major repolarizing K\(^+\) currents in mice are not the same as in larger mammals such as dogs and humans. My finding that long-term I\(_{Kr}\)
inhibition preferentially upregulates I_{KS}, KvLQT1 and minK expression to increase repolarization reserve supports the growing awareness of the important role of I_{KS} as a source of repolarization reserve protecting the heart against TdP during pathological APD prolongation by I_{Kr} blockers (52;58;59;65;66). More importantly, my results suggest a new mechanism that could potentially contribute to the risk of arrhythmias in congenital or acquired LQTS caused by I_{Kr} abnormalities in the presence of loss-of-function mutations in KvLQT1 or minK (LQT1 or LQT5) (67;68), the inability to upregulate I_{KS} expression in response to sustained I_{Kr} reduction.

The molecular mechanisms underlying the feedback control of I_{KS} upregulation in response to sustained I_{Kr} inhibition clearly involved post-transcriptional regulation of the protein expression of I_{KS} subunits, since I found that the mRNA expression of KvLQT1 and minK was unchanged, but their protein level was significantly increased. Recently, it has been established that the muscle specific microRNAs (miRNAs) miR-133 and miR-1 can repress the translation of ion channel genes involving in cardiac automaticity (HCN2/HCN4), conduction (GJA1, encoding connexin 43), and repolarization (KCNQ1, KCNE1, KCNH2 and KCNJ2) without affecting their transcript levels (69;70;71;84). Interestingly, we found that the level of miR-133 was significantly reduced upon long-term dofetilide treatment. The mechanisms responsible for reduced miR-133 after sustained I_{Kr} decrease are still unknown, but these results have potentially important implications. Though available experimental studies are very limited, the important role of cardiac miRNAs in regulating cardiac ion channels under normal or abnormal physiological conditions is being increasingly recognized (72). The regional distribution of miR-133 and miR-1 corresponds to the heterogeneous protein expression of KvLQT1 and minK, implying a potential role in the regulation of transmural repolarization (69). Increased expression of miR-133 in diabetic rabbit hearts contributes to the downregulation of ERG protein implicated in reduced I_{Kr}, QT prolongation and arrhythmogenesis in diabetic cardiomyopathy (70;86). Changed expression of miR-1 or/and miR-133 in myocardial-infarcted
hearts or hypertrophic cardiomyocytes alters the expression of GJA1, KCNJ2 or HCN2/HCN4, and thus conduction slowing and enhanced automaticity (71;84). Very recent evidence shows that increased miR-1 can dysregulate [Ca\textsuperscript{2+}] homeostasis and promote arrhythmogenesis (87).

My finding that miR-133 expression is altered by repolarization-delaying interventions provides a new potential role for miRNAs in the control of cardiac electrophysiology and arrhythmogenesis. Decreased miR-133 expression upon chronic Ik\textsubscript{s}-inhibition corresponded to upregulated KvLQT1 protein levels. Since the 3′UTR of the canine KCNQ1 gene contains three miR-133 putative binding sites, the reduced miR-133 expression during long-term dofetilide incubation could result in decreased miR-133 binding to its targeted KCNQ1 gene, releasing its translational inhibition of KvLQT1 protein and increasing I\textsubscript{Ks}. My results therefore implicate cardiac miRNA miR-133 in feedback regulation of I\textsubscript{Ks} expression and control of cardiac repolarization reserve. My results also suggest that abnormal regulation of miRNAs may impair repolarization reserve. Overexpressed miR-133 may lead to QT prolongation through repression of ERG protein in diabetic hearts (70;86). However, increased miR-133 can also reduce repolarization reserve in diabetic patients by downregulating KvLQT1, increasing the risk of generating lethal ventricular arrhythmia (69;88).

5.2 Directions for future research

5.2.1 Molecular mechanisms of sex-related differences in transmural ion-channels

I have demonstrated in detail that gender differences in transmural ion-channel function underlie gender-related differences in cardiac repolarization. It would be interesting to quantify potential differences between males and females in the transmural expression of subunits like Kir2.1–2.3, Kv4.3, Kv4.2, Kv1.4, KChIP2, Cav1.2, Kv1.5, ERG, minK, and KvLQT1 to define potential molecular bases for the current differences I observed. It is well recognized that sex hormones have significant impact on the expression of cardiac ion channels
and ventricular repolarization (15,73,74). However, the underlying mechanisms responsible for the regulation of cardiac ion-channel expression by sex hormones are not clear. Very limited studies have been done. Testosterone or 17β-estradiol has been found to upregulate the mRNA level of Cav1.2 (75;76). Kv4.3 expression and trafficking can be inhibited by oestrogen in the myometrium of the pregnant rat uterus (77). MinK is expressed in an oestrogen-dependent way in the rat uterus (78). I have found sex-dependent differences in I_{to}, I_{Ks} and I_{CaL} in adult cardiac ventricular myocytes. Given the complexity of sex hormones and their receptor function as transcription factors to regulate gene expression, it will be interesting to know how sex hormones regulate the expression of cardiac ion-channel genes encoding for I_{to}, I_{Ks} and I_{CaL}. In previous work, our lab cloned the promoter regions of human cardiac CACNA1C (gene for Cav1.2) and KCNE1 (gene for minK) (79;80). Transcriptional regulation of these genes by sex hormones can be studied in in vitro systems to unravel the mechanisms of sex-dependent regulation of cardiac ion channel expression. In addition, it would be interesting to study regulatory differences (e.g., in protein kinase A and C phosphorylation and phosphatase-mediated dephosphorylation) and differences in membrane trafficking of ion-channel subunits. It is also interesting to investigate the role of microRNAs in regulation of ion channel expression by the control of gender.

5.2.2 Regional differences in response to changes in firing rate of cardiac ion channels

I have demonstrated rate-dependent regulation of I_{to} and the underlying molecular mechanisms in adult canine ventricular epicardial cells, as shown in Chapter 3. We identified Ca^{2+}/CaM/CaMKII/Calcineurin/NFATc3 signalling as critically involved in rate-dependent I_{to} regulation. However, in preliminary studies (81), I have noted that tachypacing induced I_{to} downregulation is not present in endocardial cells. Endocardial I_{to} is much smaller compared to epicardial I_{to}. Ca^{2+}/Calcineurin/NFAT signalling is more active in the endocardium and has been implicated in transcriptional control of the I_{to} transmural gradient in
the mouse heart (45). It will be interesting to study the mechanisms contributing to epi/endo differences in rate-dependent regulation of \( I_{Ko} \). The answers may help to understand the molecular mechanisms of regionally different responses upon stressful stimuli.

Previously, I also studied rate-dependent regulation of \( I_{CaL} \) in ventricular myocytes (81) by using the same in vitro pacing model, and found no change. A recently published paper from our lab reported that in vitro tachypacing decreases \( I_{CaL} \) in atrial myocytes and that Ca\(^{2+}\)/CaM/Calcineurin/NFATc3/4 is essential in this regulation (81). There are therefore clear atrial-ventricular differences in response. It will be interesting to investigate the mechanisms underlying differences between atrial and ventricular \( I_{CaL} \) responses to changes in heart rate since it is well known that decreased atrial \( I_{CaL} \) is common in patients with atrial fibrillation and atrial tachycardia animal models, whereas unchanged \( I_{CaL} \) is commonly observed in CHF (27).

5.2.3 Mechanisms involved in feedback regulation of \( I_{Ks} \) by chronic \( I_{Kr} \) blockade

I found that sustained APD prolongation due to \( I_{Kr} \) inhibition upregulated \( I_{Ks} \) and KvLQT1 and minK expression partly through posttranscriptional regulation by microRNAs. Based on my results in Chapter 4, we have proposed that miRNA \( \text{miR}-133 \) can be regulated by repolarization-delaying interventions. MiRNAs are now recognized as important regulators for the expression of a variety of genes in the heart (82; 83). Growing evidence shows that miRNAs play important roles in cardiac repolarization and cardiac arrhythmias (72). It will be interesting to evaluate the regulation of \( \text{miR}-133 \) by sustained APD prolongation because \( \text{miR}-133 \) has been shown to regulate many ion channel genes including KvLQT1 (69;70;84). We observed 40% \( I_{Ks} \) density increase but only 20-25% increase in KvLQT1 and minK protein levels. These differences indicate that there may also be posttranslational regulation of \( I_{Ks} \). Sustained APD prolongation could increase cellular Ca\(^{2+}\) entry and cellular Ca\(^{2+}\) content (27). Ca\(^{2+}\) activated calmodulin binding to KvLQT1 is required for proper channel assembly (85). The role of
Ca^{2+}/calmodulin or its downstream signalling partners in regulation of I_{KS} under sustained I_{Kr} inhibition would be interesting to investigate. Our finding of feedback control of repolarization reserve suggests that long-term administration of I_{Kr} blockers may limit their APD-prolonging effects, an idea that would be interesting to study in other relevant systems such as whole-animal models or human subjects to determine the potential functional importance.

### 5.3 General conclusions

My findings in this thesis highlight the delicate and complex control of ventricular repolarization. Basal intrinsic sex-based transmural ionic current differences critically determine gender differences in transmural repolarization that contribute to greater drug-induced QT prolongation and TdP in women. Increased heart rate, *per se*, is an important regulator of ion channel expression with the Ca^{2+}/calmodulin-dependent CaMKII and calcineurin/NFAT systems playing key Ca^{2+}-sensing and signal-transducing roles in rate-dependent I_{to} control, adding heart rate changes to the list of contributors to ion-channel regulation. In addition, rate-related I_{to} downregulation may contribute to tachycardia-induced ventricular dysfunction via the role of I_{to} in controlling plateau height and Ca^{2+} entry. Chronic action potential prolongation can cause compensatory upregulation of potassium currents, possibly mediated (at least in part) by microRNA changes, adding the regulation of ion channel expression to the potential mechanisms governing repolarization reserve. Function and malfunction of this feedback system could contribute to factors governing the occurrence of cardiac arrhythmias in repolarization dysfunction paradigms like congenital and acquired long-QT syndromes.
5.4 References


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