Repolarization abnormalities and afterdepolarizations in a canine model of sudden cardiac death.

Arun Sridhar\textsuperscript{1,3}, Yoshinori Nishijima\textsuperscript{1,4}, Dmitry Terentyev\textsuperscript{1,3}, Radmila Terentyeva\textsuperscript{1,3}, Rebecca Uelmen\textsuperscript{5}, Monica Kukielka\textsuperscript{3}, Ingrid M. Bonilla\textsuperscript{4}, Gail A. Robertson\textsuperscript{5}, Sandor Györke\textsuperscript{1,4}, George E. Billman\textsuperscript{1,3}, Cynthia A. Carnes\textsuperscript{1,4}.

\textsuperscript{1}Davis Heart and Lung Research Institute, \textsuperscript{2}Biophysics Program, \textsuperscript{3}Department of Physiology and Cell Biology, \textsuperscript{4}College of Pharmacy, The Ohio State University, Columbus, OH, USA, 43210, \textsuperscript{5}Department of Physiology, University of Wisconsin, Madison, WI, USA 53706.

Running head: Ventricular Repolarization and Sudden Death

Correspondence:
Cynthia A. Carnes, Pharm.D., Ph.D.
College of Pharmacy
500 W. 12\textsuperscript{th} Avenue
Columbus, OH 43210
PH 614-292-1715
FAX 614-292-1335
Email: carnes.4@osu.edu
ABSTRACT
Ventricular tachyarrhythmias are the most common cause of sudden cardiac death (SCD); a healed myocardial infarction increases the risk of SCD. We determined the contribution of specific repolarization abnormalities to ventricular tachyarrhythmias in a post-infarction model of SCD. Methods: We used a post-infarction canine model of SCD, where an exercise and ischemia test was used to stratify animals as either susceptible (VF+) or resistant (VF-) to sustained ventricular tachyarrhythmias. Results: No changes in global left ventricular contractility or volumes occurred after infarction. At 8-10 weeks post-MI, myocytes were isolated from the LV mid-myocardial wall and studied. In the VF+ animals, myocyte action potential (AP) prolongation occurred at 50% and 90% repolarization ($p<0.05$) and was associated with increased variability of AP duration and afterdepolarizations. Multiple repolarizing $K^+$ currents ($I_{Kr}$, $I_{to}$) and inward $I_{K1}$ were also reduced ($p<0.05$) in myocytes from VF+ animals compared to control, non-infarcted dogs. In contrast, only $I_{to}$ was reduced in VF- myocytes compared to controls ($p<0.05$). While afterdepolarizations were not elicited at baseline in myocytes from VF- animals, afterdepolarizations were consistently elicited after the addition of an $I_{Kr}$ blocker. Conclusions: A loss of repolarization reserve via reductions in multiple repolarizing currents in the VF+ myocytes, leads to action potential prolongation, repolarization instability, and afterdepolarizations in myocytes from animals susceptible to sudden cardiac death. These abnormalities may provide a substrate for initiation of post-MI ventricular tachyarrhythmias.

Keywords: Potassium currents, repolarization reserve; myocardial infarction
INTRODUCTION

Sudden Cardiac Death (SCD) is a major cause of cardiovascular mortality in the United States, accounting for ~500,000 deaths annually. Ambulatory ECG recordings have established that the vast majority (>80%) of these deaths result from tachyarrhythmias that culminate in ventricular fibrillation (VF)(1; 5; 19; 21). Post-mortem examinations indicate that scar tissue due to a previous myocardial infarction (MI) is present in ~1/3 of SCD subjects(51). It has been estimated that up to 80% of SCD results from myocardial ischemia or its sequelae(40).

Ventricular arrhythmias often occur when there is an underlying electrophysiological substrate; alterations in repolarizing potassium currents are known to contribute to arrhythmogenesis(32). Specifically, alterations in $I_{K1}$ (inward rectifier $K^+$ current), and the repolarizing $K^+$ currents: $I_{to}$ (transient outward current), and/or the delayed rectifier $K^+$ currents ($I_{Kr}$ and $I_{Ks}$), are arrhythmogenic. The specific abnormalities in repolarization which predispose to arrhythmias in the setting of a healed MI have not been well defined. Numerous studies have identified electrophysiologic abnormalities in the hours to days following MI in canine models(13; 34; 35). At 5 and 14 days post-infarction, there is shortening of the action potential duration (APD) in the epicardial peri-infarct border zone(10). Specifically, post-MI decrements in $I_{to}$ occur at 5 and 14 days post infarction in the peri-infarct epicardial border zone (EBZ); however, at 2 months post-infarction, $I_{to}$ reportedly returns to control values(13). Jiang et al reported a reduction in both $I_{Kr}$ and $I_{Ks}$ in myocytes isolated from the EBZ of 5 day post-MI canine hearts(25). Notably in these previous studies, stratification for, and the occurrence of, lethal
Arrhythmias were not described, and the specific mechanisms predisposing to post-MI SCD during recurrent ischemia remain poorly defined.

In this study, we utilized a well-characterized, highly reproducible post-MI canine model of sudden cardiac death (6; 41). In this model, after recovery from an MI (3-4 weeks), the animals are risk stratified for susceptibility to sustained ventricular tachyarrhythmias. Arrhythmia susceptibility is assessed by a sub-maximal exercise plus ischemia test to stratify animals as either susceptible or resistant to sustained ventricular tachyarrhythmias; thus, the model simulates post-MI patients who may or may not have residual susceptibility to lethal ventricular arrhythmias during recurrent ischemia or adrenergic activation. Furthermore there is a significant increase in spontaneous ventricular arrhythmias and sudden death in animals stratified as arrhythmia susceptible by the exercise plus ischemia test (6). During arrhythmia testing, susceptible animals have a longer QT interval and abnormal T wave morphology compared to the animals resistant to induction of ventricular arrhythmias (6). While there is ample in vivo data in this model, the cellular electrophysiologic mechanisms of arrhythmia susceptibility have not been defined in this model. In this study, we tested the hypothesis that susceptibility to ventricular tachyarrhythmias results from alterations in repolarization. Our results suggest that downregulation of repolarizing K⁺ currents and afterdepolarizations provide a substrate for initiation of ventricular arrhythmias following myocardial infarction.
MATERIALS AND METHODS

All procedures were approved by The Ohio State University Institutional Animal Care and Use Committee and conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Sixteen mongrel dogs (M/F; 2-3 years of age) had a surgically induced myocardial infarction by occlusion of the left anterior descending coronary artery, as previously described (6). Two-D, B- and M–mode echocardiograms were performed in a subset of dogs (n=5) under butorphanol sedation (0.5 mg kg\(^{-1}\), IM) at baseline and 8 weeks following surgery. In the same subset, 24 hour ambulatory ECG was recorded by Holter monitor. Ten age-matched dogs served as controls (i.e., dogs without myocardial infarction).

In vivo Preparation:

A left ventricular anterior infarction was induced by occlusion of the left anterior descending coronary artery, as previously described (6; 8; 41). At the time of surgery, a pulsed Doppler flow transducer and vascular occluder were placed on the left circumflex artery. After recovery, arrhythmia susceptibility was determined using a combined exercise with ischemia test, where animals exercised on a treadmill to a target heart rate (~210 beats/min or 70% of the maximum heart rate). During the final minute of exercise, the circumflex artery was occluded via the implanted occluder; the occlusion was maintained for one additional minute after the cessation of exercise (6). This method reproducibly induced VF in approximately 60% of the animals, which were classified as “VF+”; the remainder of the animals were classified as “VF-“.

Myocyte Isolation:
Myocytes were isolated 8-10 weeks post-infarction from the antero-lateral left ventricular mid-myocardial wall. To eliminate the possibility of any effects due to acute ischemia during susceptibility testing, a minimum of 5 days elapsed between the final arrhythmia testing and myocyte isolation. For myocyte isolation, dogs were anesthetized by intravenous injection of pentobarbital sodium (Dosage: 120 mg kg\(^{-1}\) for the first 4.5 kilograms and 60 mg kg\(^{-1}\) for every 4.5 kilograms thereafter); after achieving anesthesia the hearts were rapidly excised and perfused with cold cardioplegic solution (containing 5% Glucose, 0.1% Mannitol, 22.4 mM NaHCO\(_3\), 30 mM KCl) injected into the coronary ostia. Following the washout of blood from the heart, collagenase (Worthington type 2, 0.65 mg ml\(^{-1}\)) and protease-free bovine serum albumin (0.65mg ml\(^{-1}\)) were added to the perfusate. The left main coronary artery was cannulated for myocyte isolation as previously described(28). In hearts from the post-MI animals, a clear margin of the infarct was visible as scar tissue. After 30-45 minutes of perfusion, the digested mid-myocardial section of the left ventricle up to 6 cms from the infarct center (scar tissue) was separated from the epicardial and endocardial sections; digested tissue was shaken in a water bath at 37°C for an additional 5-10 minutes. This typically yielded 50-70% rod shaped myocytes with staircase ends and sharp margins. The myocytes were stored at room temperature in a standard incubation buffer solution containing (in mM) NaCl 118, KCl 4.8, MgCl\(_2\) 1.2, KH\(_2\)PO\(_4\) 1.2, glutamine 0.68, glucose 10, pyruvate 5, CaCl\(_2\) 1, along with 1 µmol l\(^{-1}\) insulin, and 1% BSA until use. All myocyte experiments were conducted within ten hours of isolation. Myocyte aliquots and left ventricular tissues were snap frozen in liquid nitrogen and stored at -80°C for protein analyses.
Electrophysiological Protocols:

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

APs were recorded with perforated whole cell patch techniques, as described above. APs were measured as the average of the last 10 (steady-state) APs, obtained during a train of twenty-five APs at each stimulation rate. To analyze beat-to beat variability in the AP recordings, the standard deviation (SD) of the AP duration at 90% repolarization in each myocyte was calculated for beats 15 to 25. The amplitude of
phase 2 was measured as the maximal potential following phase 1 of the action potential. We observed arrhythmia in some cells; myocytes exhibiting cellular arrhythmias were excluded from APD measurements. In a second series of current clamp experiments, cellular arrhythmias were quantified by recording action potentials in the presence and absence of isoproterenol.

Transient outward potassium current \( (I_{to}) \) was elicited from a holding potential of -60 mV by a series of 100 ms test potentials from -20 to +50 mV, and measured as peak current minus steady-state current. \( I_{K1} \) was elicited by voltage steps from -140 to +40 mV from a holding potential of -40 mV. The current was measured at the end of each 100 ms test pulse. \( I_{K1} \) inward conductance \( (\text{mS/cm}^2) \) was determined by calculating the slope of the linear portion of the current density-voltage relationship from -140 mV to -100 mV(11). Peak outward \( I_{K1} \) density was measured as the current at -60 mV \( (I_{-60}) \).

Rapid \( (I_{Kr}) \) and slow \( (I_{Ks}) \) components of the delayed rectifier current were elicited using 10 mV incremental voltage steps from -40 mV to +50 mV from a holding potential of -50 mV. \( I_{Kr} \) was measured as the d-sotalol-sensitive \( (100 \, \mu\text{M}) \) current, while d-sotalol-insensitive tail currents were used to measure \( I_{Ks} \) (18; 27). During \( I_{Kr} \) and \( I_{Ks} \) recordings the bath solution also contained 4-aminopyridine \( (100 \, \mu\text{M}) \) to prevent any potential contamination by “\( I_{Kur} \)-like” plateau current (44).

Sustained outward potassium current was elicited from a holding potential of -40 mV using 10 mV voltage steps from -20 mV to +50 mV. A combination of a -40 mV holding potential and an 80 ms prepulse to +30 mV was used to inactivate \( I_{to} \). A sustained 4-AP-sensitive plateau current was measured as the steady-state difference current, recorded after a minimum of four minutes of superfusion with 4-AP(44).
D-sotalol (30 and 100 µM) was used to inhibit I\(_{Kr}\) during action potential recordings in VF- myocytes and control myocytes. These concentrations selectively inhibit I\(_{Kr}\) in canine ventricular myocytes\((43; 50)\).

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

**Solutions and Chemicals:**

All chemicals for buffer and stock solution preparation were purchased from Fisher Scientific (USA), Sigma Aldrich (St.Louis, MO, USA) and Invitrogen Inc (Carlsbad, CA, USA). Stock solutions of nifedipine, amphotericin B and 4-aminopyridine were prepared daily. Isoproterenol solutions were prepared daily from commercially available injectable solutions (Sanofi Winthrop Pharmaceuticals, New York NY). D-sotalol was obtained from Merck Research Laboratories, West Point PA. All nifedipine, isoproterenol, and amphotericin B solutions were protected from exposure to light.

**K\(^+\) channel protein subunit measurements**

hERG was detected using left ventricular tissue homogenates. Tissue samples were pulverized using an ice-cold mortar and pestle. Integral membrane proteins were isolated as described previously\((26)\). Isolated membrane proteins (3 mg) were cleared with protein G sepharose beads (Amersham Biosciences) for 1-2 hours at 4 °C. Cleared lysates were then incubated with 15 µl of mouse anti-hERG antibody (Axxora, San Diego CA) on a rotating platform for 12-16 hours at 4 °C. 30 µl of protein G beads were added to the samples and incubated for an additional 2 hours at 4 °C. Beads were collected by centrifuging at 1,000 x g and washed 3 times in 150 mM NaCl, 25 mM
Tris-HCl, pH 7.4, and 5 mM NaEDTA, 1% (v/v) Triton X-100. Proteins were eluted by incubating beads at 65 °C for 10 minutes in 30 µl 4x LSB (25 mM Tris-HCl, pH 6.8, 2% (v/v) sodium dodecylsulfate, 10% glycerol, 200 mM DTT). Eluted proteins were separated by 7.5% SDS-PAGE using the Criterion system (Bio-Rad Laboratories). Proteins were transferred onto Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford MA) with the use of Criterion transfer system (Bio-Rad). hERG protein was visualized by Western blotting with rabbit anti-hERG antibody as described previously.(39)

All other K+ channel subunits were assessed by immunoblot analysis. Myocytes were lysed with Ripa buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Cell lysate proteins (10 µg) were subjected to 4% to 20% SDS-PAGE, blotted onto nitrocellulose membranes (Bio-Rad Labs, Hercules, CA, USA). Anti-Kv1.5, -Kv4.3, -KChQ1, -KChIP2, and -Kir2.1 antibodies were from Santa Cruz. Anti-GAPDH antibody was from Abcam (Cambridge, MA). Each sample had total protein assayed to provide equal loading on the gels, and GAPDH was measured as an internal control. Blots were developed with Super Signal West Pico (PIERCE) and quantified using ImageJ (NIH, USA) and Origin 7 (OriginLab, Northampton, MA USA) software.

Statistical Analysis:

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

There was no evidence of impaired global LV structure or function after infarction, as has been previously reported in this model\(^{(6;\ 7;\ 22)}\). LV fractional shortening was unchanged after MI (41±1.6% vs. 40.6±0.9%, baseline and 8 weeks post-MI, respectively, p=NS). LV diameters at end systole (2.43 ± 0.05 vs. 2.40 ± 0.03cm, baseline and 8 weeks post-MI, respectively, p=NS) and end diastole (4.14 ± 0.14 vs. 4.13 ± 0.05 cm, baseline and 8 weeks post-MI, respectively, p=NS) were unchanged. However, myocyte capacitance was significantly increased post-MI in both VF+ and VF- groups (p<0.05) compared to controls (Controls: 157 ± 19; VF +: 202 ± 14; VF -: 191 ± 8 pF), but did not differ as a function of susceptibility to VF.

VF+ myocytes exhibited a significant increase in action potential durations at 50% (APD\(_{50}\)) and 90% (APD\(_{90}\)) repolarization at both 0.5 and 1 Hz (p<0.05), while the VF- myocytes did not differ from controls (Figure 1A-1C). The plateau potential did not differ significantly between the VF+, VF-, and control myocytes (27.3 ± 0.8 35.6 ± 2.8, 31 ± 1.6 mV at 0.5 Hz in control, VF+ and VF- groups, respectively, p=0.08). The resting membrane potential was also similar in the three groups (-82 ± 2.6 mV in control, -79.6 ± 0.9 mV in VF+ and -79.8 ± 0.4 mV in VF- group respectively).

There was increased beat-to beat variability in the APD\(_{90}\) from the VF+ myocytes, quantified as the standard deviation (SD) of APD\(_{90}\) (Figure 1D).\(^{(3)}\) The VF+ myocytes exhibited a significantly increased variability in APD\(_{90}\) at both 0.5 and 1 Hz compared to both control and VF- myocytes (p<0.05).

\(I_o\) was reduced to a similar extent in both the VF+ and VF- groups (Figure 2) compared to control values (p<0.05). No differences in the kinetics of inactivation or
recovery from inactivation of $I_{Mo}$ were found between groups (data not shown). Inward $I_{K1}$ slope conductance was significantly reduced in the VF+ group compared to either the control or the VF- groups (Figure 3). The peak outward component of $I_{K1}$ did not differ between the three groups.

$I_{Kr}$ was significantly reduced in myocytes from the VF+ group to levels that were almost undetectable ($p<0.05$, Figure 4); $I_{Kr}$ density was unchanged in VF- compared to controls. The slow component of the delayed rectifier current ($I_{Ks}$) did not differ between the three groups (Figure 5A). We recently identified a 4-AP sensitive “$I_{kur-like}$” plateau current in a majority (~70%) of left ventricular mid-myocardial myocytes(44) The VF+ myocytes had a reduced density of “$I_{kur-like}$” plateau current compared to controls and the VF- groups ($p<0.05$, Figure 5B). The VF- myocytes did not differ from control values in 4-AP-sensitive plateau current density.

In experiments to quantify cellular arrhythmias, we observed early afterdepolarizations (EADs) in 8/12 VF+ myocytes (Figure 6A). We observed no evidence of EADs in eleven control and eight VF- myocytes. When treated with isoproterenol, there was no significant change in the number of cells showing EADs (9/12 in presence of isoproterenol vs. 8/12 at baseline in the VF+ group). No delayed afterdepolarizations were observed in any experiments. In ambulatory ECGs, we observed frequent ventricular premature depolarizations and episodes of non-sustained ventricular tachycardia in dogs from the VF+ group (Figure 6B), specifically those with myocytes exhibiting EADs.
To verify that loss of $I_{Kr}$ is a critical contributor to AP variability and afterdepolarizations, a series of separate experiments were conducted in control and VF- myocytes. $I_{Kr}$ was inhibited by superfusion of d-sotalol, a selective $I_{Kr}$ blocker (Figure 7). In control myocytes, both concentrations of d-sotalol resulted in APD prolongation (data not shown). In control myocytes 30 µM sotalol did not induce any EADs (n=6) at 0.5,1 or 2 Hz; 100 µM d-sotalol resulted in only a single isolated EAD in one of six myocytes at 0.5 Hz. In the VF- myocytes, superfusion with 30 or 100 µM d-sotalol consistently resulted in early afterdepolarizations at all frequencies (6 of 8 myocytes at both concentrations tested, compared to 0 of 8 at baseline, Figure 7).

Consistent with the reduction in $I_{K1}$ in the VF+ myocytes, we observed a significant reduction in Kir2.1 expression (figure 8). KChIP2 was significantly reduced to a similar extent in both VF+ and VF- myocytes compared to controls. hERG1a was expressed in control, VF+ and VF- left ventricular tissues (data not shown); both mature and immature forms of the subunit were found in all groups. There were no significant between group differences in the expression of KCNQ1, Kv1.5 or Kv4.3.
DISCUSSION:

Sudden cardiac death is a leading cause of cardiovascular mortality, and pre-existing coronary artery disease is a major risk factor for SCD. In the present study, a distinct form of electrophysiologic remodeling was observed in myocytes isolated from the hearts of animals with reproducible ischemia-induced sustained ventricular tachyarrhythmias. (6; 46). Specifically, myocytes from dogs with healed myocardial infarctions and lethal arrhythmias also exhibited early afterdepolarizations, action potential prolongation, and increased beat-to-beat variability in repolarization. These electrophysiological changes resulted from specific K⁺ current abnormalities. Two discrete mechanisms appear to contribute to the observed abnormalities in K⁺ currents: 1) altered function due to altered expression of ion channel subunits (e.g., KChIP2 and I₁K) and 2) post-translational modification of function (e.g. hERG1a and IKr).

Our data suggest that early afterdepolarizations may trigger reentrant ventricular tachyarrhythmias in this model of sudden death. Early afterdepolarizations occur when repolarization is prolonged; repolarization depends on the balance between multiple currents (K⁺ currents, late Na⁺ current, NCX and I₉a) and alterations in this balance can result in prolongation of the action potential. Reductions in a single repolarizing K⁺ current, IKr, may be sufficient to result in AP prolongation in the canine ventricle(50). Recently, it was shown that block of IKs in isolation is insufficient to prolong the APD unless IKr is also inhibited in normal canine ventricular myocytes(50). This requirement for the block of multiple currents has been attributed to the presence of a “repolarization reserve”, where multiple repolarizing K⁺ currents can compensate for the absence of a single repolarizing current(36; 37). In the present study, early afterdepolarizations were...
observed in the VF+ myocytes on the background of reduced $I_{to}$, $I_{kr}$ and a 4-AP sensitive “$I_{kur}$-like” current. As such, it was not clear which defect or defects in repolarization were required for the initiation of the afterdepolarizations in this model of SCD. However, the observation that the block of $I_{kr}$ in the VF- myocytes prolonged APD and provoked afterdepolarizations (i.e., converted these cells to the VF+ phenotype) strongly suggests that inhibition of $I_{kr}$ is required for arrhythmogenesis. Furthermore, since d-sotalol (30 µM) did not induce EADs in the control myocytes, it appears that APD prolongation due to $I_{kr}$ inhibition alone is not sufficient to induce EADs. Multiple repolarizing currents must therefore be impaired before “repolarization reserve” is sufficiently compromised to allow for the induction of cellular arrhythmias. As a consequence, repolarization reserve in normal canine ventricular myocytes prevents induction of EADs at physiologically relevant stimulation rates (0.5 and 1 Hz) following inhibition of $I_{kr}$ in isolation. As previously noted, EADs were induced with $I_{kr}$ blockade in the VF- myocytes (where $I_{to}$ is reduced, Figure 7). Thus, the combination of the inhibition of $I_{to}$ and $I_{kr}$ is sufficient to induce EADs. When considered together, these data suggest that in the VF+ myocytes, the pathophysiologic inhibition of multiple repolarizing $K^+$ currents results in APD prolongation, APD variability, and induction of EADs, thereby providing a substrate for the initiation of ventricular tachyarrhythmias.

The myocytes in the present study were derived from the left ventricular anterior mid-myocardium, and $I_{kr}$ blockers have been shown to produce greater action potential prolongation in the mid-myocardium compared to the epicardium or the endocardium. While AP prolongation is more prominent at slower stimulation rates, an elegant study by Hua and Gilmour demonstrated the contribution of $I_{kr}$ to rate-dependent action
potential dynamics in canine endocardial myocytes, with “baseline” $I_{Kr}$ (current activated preceding the upstroke of the action potential during continuous AP clamp stimulation) increasing significantly at faster stimulation rates (23). In contrast, they observed that “peak” $I_{Kr}$ was increased at slower stimulation rates (23). Furthermore, decreasing $I_{Kr}$, both in isolated myocytes and in-silico, increased the amplitude of APD alternans. In a separate study, these authors demonstrated the converse, that hERG overexpression in canine ventricular myocytes successfully abolished APD alternans (24). Additional studies have linked the loss of $I_{Kr}$, and APD alternans, to ventricular fibrillation (12; 16; 17). Although we did not observe true APD alternans in the VF+ myocytes, we did observe a significant increase in variability of APD$_{90}$. Notably, increased variability of repolarization has also been associated with the development of SCD in a different canine model of heart disease, a model that induced electrophysiologic remodeling by chronic AV block (47). The action potential variability we observed, due at least in part to the absence of $I_{Kr}$, triggered EADs in 8/12 VF+ myocytes tested. Interestingly, the absence of $I_{Kr}$ in left ventricular midmyocardial myocytes is consistent with a known lack of efficacy of $I_{Kr}$ blockers in preventing VF in this model (6; 49).

Previous studies with this model by other investigators have found prolongation of the QT interval in susceptible dogs (2; 46). Interestingly, electroanatomic mapping of the left ventricular endocardium revealed marked intra-ventricular heterogeneity in VF+ animals, with discrete regions demonstrating longer repolarization times (46). This regional dispersion of repolarization suggests that a substrate for reentry may result from dispersion of refractoriness (i.e., the possibility of unidirectional block of premature beats). The afterdepolarizations and triggered beats we observed may contribute to the
initiation of reentry, given the underlying substrate. The relevance of our findings is underscored by a clinical study which reported that in patients with a previous MI, consistent prolongation of the QT interval was associated with a significantly increased risk of sudden death (42).

**Comparisons to other experimental canine MI studies:**

Multiple studies have documented the cellular electrophysiological remodeling process occurring within hours to days following MI in dogs. Most of the original studies in this area have focused on the epicardial border zone and the Purkinje fibers as sites of re-entry initiation (4; 34). In contrast to these previous studies, we studied dogs with healed myocardial infarctions and a known predisposition to ischemia-induced sudden death, and our action potential measurements were restricted to the mid-myocardial layer where we found increases in APD50 and APD90. It is possible that additional regional differences in repolarization within the LV, either transmural or regional, could contribute to arrhythmogenesis.

Independent of arrhythmia risk, we found a decrement in $I_{to}$ density in both post-MI groups. This reduction is consistent with a previous report of reduced $I_{to}$ on day 5 post-infarction in the epicardial border zone (EBZ) (31). In a separate report from these investigators, $I_{to}$ was also reduced at 14 days post-infarction in EBZ myocytes, but restored to control values two months post-infarction (13). In contrast to this report, we found a significant reduction in $I_{to}$ at 8-10 weeks post-MI in both the VF+ and VF- groups. However, our data suggest that $I_{to}$ plays only a very minor role in modulating APD, as has been previously suggested (20). This interpretation is supported by the observation that $I_{to}$ density was reduced in the VF- myocytes, yet these cells exhibited
normal APDs. In fact; comparable reductions in $I_{to}$ were noted for both VF+ and VF- myocytes, while only the VF+ myocytes had APD prolongation.

There was no difference in the expression in Kv4.3 in the VF+ and VF- myocytes. The reduction in $I_{to}$ may be explained by the known modulatory effects of KChIP2 on Kv4-encoded $I_{to}$(29; 33; 38); and a similar pattern of Kv4.3 and KChIP2 subunit expression with reduced $I_{to}$ has recently been described in a canine model of ischemic cardiomyopathy(30). Thus, the reduction in $I_{to}$ we observed may be attributed to the reduction in KChIP2 expression.

Delayed rectifier currents have also been studied previously(14; 25) in canine post-infarction models. Jiang et al reported a reduction in both $I_{Kr}$ and $I_{KS}$ in myocytes isolated from the EBZ of 5 day post-MI canine hearts, which was associated with reduced mRNA for the subunits encoding $I_{Kr}$ and $I_{KS}$.(25) While we observed a lack of $I_{Kr}$ in the VF+ myocytes, there was not an associated absence of hERG1a in these tissues. This observation may be consistent with post-translational modulation of hERG channel function. In fact, oxidative modulation of hERG channels has been recently reported to alter $I_{Kr}$(45).

In the aforementioned canine model (EBZ 5 days post-MI), Dun et al. described a more complex form of remodeling in myocytes from the infarcted EBZ(13). They reported an upregulation in the TEA-sensitive component of delayed rectifier current (possibly Kv2.1). Notably, similar to our finding in the mid-myocardial region of a 4-AP-sensitive plateau current, Dun et al reported a C9356-sensitive (C9356 is a selective blocker of Kv1.5 channels) current in myocytes from both the EBZ and normal zone of canine hearts. We recently reported the presence of a similar current which activates at
plateau voltages and modulates canine midmyocardial APD(44). Due to its similarities with canine atrial $I_{Kur}$ (both in properties and inhibition with micromolar 4-aminopyridine), we suggest that this is a 4-aminopyridine sensitive “$I_{Kur}$-like” plateau current. “$I_{Kur}$-like” current was selectively reduced only in myocytes from the VF+ group, which has the potential to alter the plateau potential as well as the APD.

**Limitations**

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

The myocardium undergoes a process of dynamic remodeling following a myocardial infarction. We only studied animals that were 8-10 weeks post-infarction. This time point was selected as one where ventricular remodeling is complete(9; 15; 48). Time-dependent pathologic remodeling of myocyte electrophysiology was beyond the scope of the present study.

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

In the present study, an increased risk of lethal arrhythmias in the late phase after infarction are associated with prolonged action potentials, increased beat-to-beat variability in repolarization, and cellular arrhythmias. The repolarization abnormalities that predispose to lethal arrhythmias were associated with a profound reduction in I_{Kr}. In the normal canine ventricle, multiple repolarizing potassium currents provide repolarization reserve. After a myocardial infarction, susceptibility to lethal arrhythmias arises from abnormalities in multiple repolarizing currents; at the myocyte level these abnormalities are manifested as repolarization variability, afterdepolarizations and triggered activity. Further studies are warranted to examine other potential contributors to arrhythmogenesis (e.g. abnormalities in myocyte calcium handling or autonomic modulation of electrophysiology) in this model.
Acknowledgements

This study was supported in part by NIH grants HL-68609, HL086700 and HL081780.

The studies were performed as a partial fulfillment for the requirements for the Doctor of Philosophy degree (Arun Sridhar).
Reference List


channel-interacting protein 2 (KChIP2) gene leads to a complete loss of I(to) and confers susceptibility to ventricular tachycardia. *Cell* 107: 801-813, 2001.


35. **Pu J, Robinson RB and Boyden PA.** Abnormalities in Ca(i)handling in myocytes that survive in the infarcted heart are not just due to alterations in repolarization. *J Mol Cell Cardiol* 32: 1509-1523, 2000.


47. **Thomsen MB, Truin M, van Opstal JM, Beekman JD, Volders PG, Stengl M and Vos MA.** Sudden cardiac death in dogs with remodeled hearts is associated with


FIGURE LEGENDS

Figure 1: VF+ myocytes exhibit prolonged APD$_{50}$ and APD$_{90}$ and increased variability in APD$_{90}$. Panels A and Panel B show representative action potential tracings from control (black), VF + (red) and VF- (Blue) recorded at 0.5 and 1 Hz respectively. The line indicates zero mV potential. Panel C depicts the summary APD$_{50}$ and APD$_{90}$ values at the two stimulation rates in the three groups. Panel D shows the averaged standard deviation in APD$_{90}$ measured from each myocyte in the three groups plotted as the function of stimulation frequency (* p<0.05 vs. control, § p<0.05 vs VF-).

Figure 2: Transient outward K$^+$ current (I$_{to}$) is reduced in both VF+ and VF- myocytes. Panel A depicts traces recorded from control, VF+ and VF- myocytes using the voltage protocol shown in the inset. Panel B depicts the average I-V relationship for I$_{to}$ in the three groups. The numbers within parentheses in panel B indicate the number of animals, while n is the number of myocytes. Panel C shows the slope conductance of I$_{to}$ measured from the linear part of the I$_{to}$ I-V curve (panel B) from +10mV to +50 mV (* p<0.05 vs. control).

Figure 3: Inward I$_{K1}$ is reduced in the VF+ myocytes and preserved in the VF- myocytes. Panel A shows representative raw current tracings recorded in response to a hyperpolarizing voltage step to -140 mV in the three groups. Panel B shows the summary I-V relationship for the myocytes from the three groups. The numbers within parentheses indicate the number of animals, while n indicates the number of myocytes.
Panel C. $I_{K1}$ slope conductance is reduced in the VF+ group compared to controls.

Panel D. Outward $I_{K1}$ is not different between the three groups. (* p<0.05 vs. control)

Figure 4: $I_{Kr}$ is significantly reduced in VF+ myocytes. Panel A shows representative current traces from the three groups, elicited by the protocol shown in the inset. Panel B shows the summary I-V relationships from myocytes in the three groups. $I_{Kr}$ is substantially reduced in myocytes from VF+ animals, while VF- myocytes are comparable to controls (* p<0.05 vs. control, § p<0.05 vs. VF-).

Figure 5: $I_{Ks}$ is unchanged, while the 4-AP sensitive plateau current is reduced in the VF+ myocytes. Panel A depicts $I_{Ks}$ traces obtained from myocytes from the three groups with the summary current density-voltage relationships shown at right (p=NS). Panel B depicts 4-AP sensitive plateau current, which is reduced in VF+ myocytes; the summary current density-voltage relationships shown at right (* p<0.05 vs. control; § p<0.05 vs. VF-). The proportion of cells expressing the 4-AP sensitive current is shown for each group, and did not differ among groups.

Figure 6. Evidence of arrhythmia in isolated myocytes and in vivo. Panel A shows evidence of cellular arrhythmia from a VF+ dog recorded at 0.5 Hz. Panel B shows a Holter recording from the same VF+ animal, two normal sinus beats were followed by initiation (premature beat, marked with downward arrow) of nonsustained ventricular tachycardia at rest.
Figure 7: Inhibition of $I_{Kr}$ with d-sotalol (30 µM) in VF- myocytes results in the development of early afterdepolarizations at 0.5 and 1 Hz. Afterdepolarizations are marked with an *. This was a consistent finding and occurred in six of eight VF-myocytes tested. In separate experiments in VF- myocytes, zero of eight myocytes displayed EADs at baseline, and zero of six control myocytes displayed EADs with 30 µM d-sotalol.

Figure 8: Expression of K$^+$ channel subunits. A. Representative Western blots of K$^+$ channel subunits in left ventricular myocytes from control, VF- and VF+ animals. GAPDH was measured as an internal control. B. KChIP2 is reduced in both VF+ and VF- groups relative to control (p<0.05). C. Kir2.1 is reduced in the VF+ group relative to control and the VF- group (p<0.05). There were no significant differences in the expression of Kv1.5 (D), KCNQ1 (E), or Kv4.3 (F) between groups. Data were averaged from four samples in each group.
**Figure 1**

(A) Electrocardiogram recordings at 0.5 Hz showing control and VF+ and VF- conditions with a 50 mV scale and a 300 ms time scale.

(B) Electrocardiogram recordings at 1 Hz showing control and VF+ and VF- conditions with a 50 mV scale and a 200 ms time scale.

(C) Comparison of APD50 and APD90 at 0.5 Hz and 1 Hz for control, VF+, and VF- conditions. The control group (n=13) is represented by black bars, VF+ (n=11) by red bars, and VF- (n=12) by blue bars. Significant differences are indicated by asterisks and dollar signs.

(D) SD (APD90) at 0.5 Hz and 1 Hz for control, VF+, and VF- conditions. The control group is represented by open squares, VF+ by red triangles, and VF- by blue triangles. Significant differences are indicated by asterisks and dollar signs.
A

KChIP2

Kir2.1

KCNQ1

Kv1.5

Kv4.3

GAPDH

B

KChIP2

Control

VF -

VF +

C

Kv2.1

Control

VF -

VF +

D

Kv1.5

Control

VF -

VF +

E

KCNQ1

Control

VF -

VF +

F

Kv4.3

Control

VF -

VF +