Molecular mechanisms underlying $K^+$ current downregulation in canine tachycardia-induced heart failure

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Heart failure (HF) is characterized by marked prolongation of action potential duration and reduction in cellular repolarization reserve. These changes are caused in large part by HF-induced $K^+$ current downregulation. Molecular mechanisms underlying these changes remain unclear. We determined whether downregulation of $K^+$ currents in a canine model of tachycardia-induced HF is caused by altered expression of underlying $K^+$ channel $\alpha$- and $\beta$-subunits encoding these currents. $K^+$ channel subunit expression was quantified in normal and failing dogs at the mRNA and protein levels in epicardial (Epi), midmyocardial (Mid), and endocardial (Endo) layers of left ventricle. Analysis of mRNA and protein levels of key $K^+$ channel $\alpha$- and $\beta$-subunits encoding these currents, $K^+$ channel subunit expression was quantified in normal and failing dogs at the mRNA and protein levels in epicardial (Epi), midmyocardial (Mid), and endocardial (Endo) layers of left ventricle. Analysis of mRNA and protein levels of candidate genes encoding the transient outward $K^+$ current ($I_{to}$) revealed marked reductions in canine $cKv4.3$ expression in HF in Epi (44% mRNA, 39% protein), Mid (52% mRNA, 34% protein), and Endo (49% mRNA, 73% protein) layers and a paradoxical enhancement (41% Epi, 97% Mid, 113% Endo) in $cKv1.4$ protein levels, without significant changes in $Kv$ channel-interacting protein $cKChIP2$ expression. Expression of $cKir2.1$, the gene underlying inward rectifier $K^+$ current ($I_{kr}$), was unaffected by HF at mRNA and protein levels despite significant reduction in $I_{kr}$, whereas canine $e1$e2-\$\tilde{a}$-go-go-related gene ($cERG$), which encodes the rapidly activating component of the delayed rectifier current ($I_{K1}$), exhibited increased protein expression. HF was not accompanied by significant changes in $cKvLQT1$ or $cMinK$ mRNA and protein levels. These data indicate that 1) downregulation of $I_{to}$ in HF is associated with decreased $cKv4.3$ and not $cKv1.4$ or $cKChIP2$, and 2) alterations in both the rapidly activating and slowly activating components of $I_{K1}$ as well as $I_{K1}$ in nonischemic dilated cardiomyopathy are not caused by changes in either transcript or immunoreactive protein levels of relevant channel subunits, which suggests posttranslational modification of these currents by HF.

Potassium current; inward rectifier; transient outward; myocardial infarction

Over 4 million Americans suffer from congestive heart failure (HF), and $\geq$400,000 die annually (32). Up to 50% of deaths in patients with HF are sudden and unexpected, and the majority of those are due to ventricular arrhythmias (8, 32). Recent investigations have highlighted the importance of repolarization abnormalities secondary to action potential duration (APD) prolongation in the genesis of arrhythmias in HF (2, 18, 24). Although APD prolongation is associated with downregulation of key $K^+$ currents, the molecular basis for such downregulation remains unclear (7, 17, 19, 20, 33). Recently, altered expression of $K^+$ channels was reported in a rat model of myocardial infarction-induced hypertrophy (16, 29). Because of major phenotypic differences in the time course of cellular repolarization and the underlying repolarizing $K^+$ currents between rodents and humans, we sought to investigate the molecular basis for $K^+$ current downregulation in the canine pacing-tachycardia HF model, which emulates many key electrophysiological, structural, and hemodynamic features of human nonischemic dilated cardiomyopathy (2, 18).

In both dogs and humans, a host of $K^+$ currents including the transient outward ($I_{to}$), the inward rectifier ($I_{K1}$), and both the rapidly and slowly activating components of the delayed rectifier ($I_{Kd}$ and $I_{Ks}$, respectively) control the duration and shape the profile of cardiac action potentials. Differences in expression of these currents are thought to underlie regional (14), transmural (20), and disease-induced changes in APD (18).

In the present study, the molecular basis for APD prolongation and $K^+$ current downregulation in a relevant model of HF was investigated by directly measuring changes in mRNA and protein levels of key $K^+$ channel $\alpha$- and $\beta$-subunits that encode $I_{to}$ ($cKv4.3$, $cKv1.4$, and $cKChIP2$), $I_{Kd}$ ($cERG$), $I_{Ks}$ ($cKvLQT1$ and $cMinK$), and $I_{K1}$ ($cKir2.1$) in epicardial (Epi), midmyocardial (Mid), and endocardial (Endo) layers of normal and failing hearts.

**METHODS**

Experimental model of HF. A total of 34 (18 normal and 16 HF) dogs were used as a source of cardiac tissue for measuring the expression of various $K^+$ channel $\alpha$- and $\beta$-subunits and 16 (8 normal and 8 HF) dogs were used for myocyte isolation and electrophysiological recordings. In 6 normal and 5 HF dogs, both $K^+$ channel subunit expression and cellular electrophysiological measurements were performed. All procedures involving the animals were approved by Institutional Animal Care and Use Committee of the Johns Hopkins University.

Details of the canine pacing-tachycardia model of HF have been described in detail previously (18, 23). Briefly, adult male mongrel dogs (20–30 kg body wt) underwent invasive hemodynamic measurements and pacemaker implantation as described previously. Dogs were sedated with thiamylal sodium, intubated, and anesthetized with 1–2% halothane. $O_2$ saturation and expired $CO_2$ values were maintained within physiological ranges throughout the procedure by continuous-volume ventilation. A left lateral thoracotomy was performed, and two pacing leads (Medtronic; St. Paul, MN) were positioned on...
the right ventricular apex. Animals were allowed to fully recover from surgery for 1–2 days, after which rapid (250 beats/min) right ventricular pacing was initiated for 3–4 wk. This resulted in HF in all dogs as evidenced by elevated left ventricular (LV) end-diastolic pressure (LVEDP, 30–40 mmHg), depressed dP/dt max (<2000 mmHg/s), delayed time constant of relaxation (>45 ms), and evident clinical symptoms of HF including lethargy, loss of appetite, dyspnea, and ascites. Hearts were harvested as previously described (18). Briefly, the chest was opened by a left lateral thoracotomy, the great vessels were banded, and the coronary arteries were perfused retrogradely with cold cardioplegic solution that contained (in mmol/l) 110 NaCl, 16 KCl, 16 MgCl2, 10 NaHCO3, and 1 CaCl2, pH 7.45 with NaOH to induce cardiac arrest. Hearts were quickly excised and submerged in cold (<4°C) cardioplegic solution. The time from initial organ harvest to tissue and cell isolation was uniformly <10 min. To obtain normal control tissue and cell samples, hearts were harvested from adult mongrel dogs in the same manner but without prior pacing.

Western blot measurements. LV tissue slices were carefully dissected from the Epi (<1 mm), Mid (central third of the wall), and Endo (<1 mm) layers of a region midway between the left anterior descending (LAD) coronary artery and the first diagonal branch and were rapidly frozen in liquid nitrogen. Proteins were prepared from whole tissue lysates and isolated myocytes as previously described (3, 12) or from membrane fractions prepared by spinning the supernatant of whole tissue lysates for an additional 30 min at 100,000 g. All samples were ran in duplicate or triplicate on 7.5% (Kv4.3, Kv1.4, ERG, KvLQT1, and Kir2.1), 12.5% (KChIP2), or 18% (MinK) Tris•HCl precast gels (Bio-Rad; Hercules, CA) in 25 mM Tris•HCl, 192 mM glycine, and 0.1% (wt) SDS running buffer. A standard control sample was analyzed on all gels to allow for comparisons across gels. GAPDH, which in preliminary experiments was repeatedly shown to exhibit no transmural or HF-induced changes in protein expression, was used to normalize for gel loading. Primary antibody incubations were performed overnight at 4°C using either commercially available or custom-developed antibodies including the following: antibodies to Kv4.3 (AB5194; human, 1:300 dilution), Kir2.1 (AB5374; human, 1:200 dilution), and MinK (human, 1:1000 dilution) were purchased from Chemicon (Temecula, CA), and antibodies to Kv1.4 (APC007; rat, 1:300 dilution) were from Alomone. Anti-ERG antibodies from both Chemicon (ABS908; 1:400 dilution) and Alomone (APC062) were used. A polyclonal antibody raised to the epitope SYDQLTDSVDDE that spans the splice-excision site in KChIP2 was used (12). The anti-KvLQT1 antibody (human) was generated by immunization of rabbits with a conjugated peptide with the sequence DPPEERLHDSVGDYDSSVK (Research Genetics; 1:5,000 dilution).

Secondary horseradish peroxidase-conjugated antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Membranes and bands developed using ECL chemiluminescence (Amer sham Pharmacia Biotech; Piscataway, NJ) according to the manufacturer’s instructions. Band densities were quantified with a laser scanner and the ImageQuant software package (Molecular Dynamics). Differences in protein loading were compensated for by probing all gel strips with a constant amount of GAPDH, which in preliminary experiments was repeatable across gels. GAPDH, which in preliminary experiments was repeatedly shown to exhibit no transmural or HF-induced changes in protein expression, was used to normalize for gel loading. Primary antibody incubations were performed overnight at 4°C using either commercially available or custom-developed antibodies including the following: antibodies to Kv4.3 (AB5194; human, 1:300 dilution), Kir2.1 (AB5374; human, 1:200 dilution), and MinK (human, 1:1000 dilution) were purchased from Chemicon (Temecula, CA), and antibodies to Kv1.4 (APC007; rat, 1:300 dilution) were from Alomone. Anti-ERG antibodies from both Chemicon (ABS908; 1:400 dilution) and Alomone (APC062) were used. A polyclonal antibody raised to the epitope SYDQLTDSVDDE that spans the splice-excision site in KChIP2 was used (12). The anti-KvLQT1 antibody (human) was generated by immunization of rabbits with a conjugated peptide with the sequence DPPEERLHDSVGDYDSSVK (Research Genetics; 1:5,000 dilution).

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Ribonuclease protection assay measurements. Templates for preparing canine cRNA probes were generated by subcloning small fragments of the cDNAs encoding ion channel and control genes into BlueScript-SK, BlueScript II-SK (Stratagene), pGEM7, or pSP73 (Promega). cDNA fragments were isolated by amplification of regions of the full-length cDNA clones using polymerase chain reaction (PCR) and creating appropriate restriction sites for subsequent subcloning. All constructs were confirmed by DNA sequencing. Probe sequences were selected such that each probe contained a unique, long, uninterrupted region of identity with only the transcript being tested. There was no evidence for cross-hybridization between the probes and nonspecific transcripts. Protected fragments of the anticipated size confirmed the specific interaction of the cRNA probe and its target transcript. Total RNA was prepared either with TRIzol reagent (GIBCO-BRL) according to the manufacturer’s instructions or by centrifugation through a CsCl cushion. The integrity of all RNA samples was confirmed by analysis on a denaturing agarose gel and was quantified by absorbance measurements at a wavelength of 260 nm.

RNase protection assays (RPAs) were performed as previously described (17). Yeast tRNA (10 μg) was used as a negative control to determine the presence of probe self-protection. At least duplicate determinations were performed on each ventricular sample, and intrasample variability was mandated to be <15%. For each sample point, 10 μg of total RNA was used in the assay.

A probe hybridizing to the 3′-linked probe of the canine cardiac isoform of the Na+ channel (cH1) was used as a myocyte-specific normalizing control. The level of the protected fragment of the cH1 probe was normalized to that of a segment of the 28S ribosomal RNA to correct for differences in RNA loading. In general, the specific activity of the control probes was approximately fivefold lower than that for the target channel probes.

Steady-state mRNA levels were quantified by exposure of the gels on a storage phosphor screen and subsequent scanning with a PhosphorImager (Molecular Dynamics). Quantification of the transcript levels was performed using ImageQuant software. The level of target gene expression is given as the relative density of the protected fragment, normalized to the density of the control protected fragment to control for RNA loading and the fraction of the ventricular sample consisting of cardiac myocytes (cH1 probe).

Real-time PCR measurements. Real-time kinetic PCR (RT-PCR) was performed using the ABI Prism 7900 sequence-detection system (Applied Biosystems). One-step PCR was conducted using the Qiagen RNeasy Midi Kit, and total RNA was isolated from cardiac tissue samples. A trio of oligonucleotides with appropriate melting temperatures and structural features were then selected and synthesized. At the 5′ end, the probe was covalently linked to the fluorescent reporter dye 6-carboxyfluorescein (FAM), and the 3′ end was linked to the quenching dye 6-carboxy-N,N,N′,N′-tetramethylrhodamine (TAMRA). During PCR, the 5′ nuclease activity of the AmpliTaq Gold DNA polymerase cleaves the reporter dye from the quenching dye and thereby releases a fluorescent signal that is proportional to the quantity of the target template. Optimization of both the primer (50–900 nM) and probe (50–250 nM) to yield the lowest threshold cycle (Ct) and maximum change in normalized reporter signal (ΔRn) was determined. Each amplicon Ct was normalized against the 18S RNA Ct.

Myocyte isolation. Canine Epi, Mid, and Endo myocytes were isolated from the anterior LV wall by perfusion of a diagonal branch of the LAD with a nominally Ca2+-free solution that contained collagenase and protease as described previously (3). Myocytes were stored at room temperature (22–23°C) in Tyrode solution that consisted of (in mmol/l) 130 NaCl, 4.5 KCl, 5 MgCl2, 23 HEPES, 21 glucose, 20 taurine, and 5 Na+–pyruvate, adjusted to pH 7.4 with NaOH. The concentration of CaCl2 was gradually raised from 100 μM to 2 mM. Only rod-shaped cells that exhibited clear cross-
striations and no spontaneous contractions were selected for electrophysiological study.

**Electrophysiological measurements.** Current recordings were obtained at 24°C using the patch-clamp technique in whole cell configuration as previously described (3). Glass pipettes were prepared to have a fine tip resistance of 2–3 MΩ when filled with internal solution that contained (in mmol/l) 120 K⁺-glutamate, 10 KCl, 10 HEPES, 5 EGTA, and 5 Mg²⁺-ATP; pH was adjusted to 7.2 with KOH, yielding a final K⁺ concentration of 140 mmol/l. Cells were perfused with Tyrode solution that contained (in mmol/l) 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose and adjusted to pH 7.4 with NaOH. For I_K, measurements, CdCl₂ (0.3 mmol/l) was added to the bath solution to eliminate voltage-activated L-type Ca²⁺ and Ca²⁺-dependent currents. Cell capacitance was estimated by integrating the area under an uncompensated depolarizing step of 10 mV from a holding potential of −80 mV. Whole cell currents were elicited using a family of depolarizing voltage steps (−40 to +80 mV) from a holding potential of −50 mV. Currents were low-pass filtered at 2 kHz and digitized at 10 kHz for offline analysis. I_K was defined as the difference between the peak transient current and steady-state current at the end of a 500-ms voltage-clamp pulse. I_K, was elicited using a family of test potentials from −120 to 0 mV (10-mV increments) from a holding potential of −40 mV. I_K density was measured at the end of each 300-ms test pulse.

For action potential measurements, myocytes were superfused at 37°C with Tyrode solution that contained (in mmol/l) 138 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 0.33 Na₂HPO₄, 10 glucose, and 10 HEPES, pH 7.4 with NaOH. The pipette solution contained (in mmol/l) 130 K⁺-glutamate, 9 KCl, 10 NaCl, 0.5 MgCl₂, 5 Mg²⁺-ATP, and 10 HEPES, pH 7.2 with KOH. The pipette-to-bath liquid junction potential was approximately −17 mV and was corrected. Cells were stimulated at 1 Hz for multiple (≥50) beats to ensure steady state. APD values at 90% repolarization (APD₉₀) of Epi, Mid, and Endo myocytes from normal and failing hearts were calculated offline after steady state was ensured.

**Immunohistochemical and cytochemical staining.** Epi, Mid, and Endo tissue slices were mounted on glass slides, and myocytes were plated on coverslips. Incubations at the indicated dilutions were performed overnight at 4°C with rabbit polyclonal Kv1.4 (1:500 dilution) and ERG (1:400 dilution) antibodies (Chemicon). Incubation with the secondary anti-rabbit antibody conjugated with the fluorescent reporter Alexa Fluor 488 (Molecular Probes; Eugene, OR) was performed at room temperature for 1 h. Tissue and myocyte samples were imaged using an inverted epifluorescence microscope (Nikon Diaphot 300) attached to a PCM-2000 laser confocal scanning microscope system (Nikon; Melville, NY). Fluorescence was excited at 488 nm using an argon laser (Spectra Physics; Mountainview, CA), and the emitted light was subsequently band-pass filtered (at 505–535 nm), digitized, and recorded. The sections were examined at low magnification to determine the overall tissue architecture and at high magnification to determine the subcellular distribution of antibody staining.

**Statistical analysis.** Channel subunit mRNA and protein levels in Epi, Mid, and Endo layers of normal hearts were reported as means ± SD and were compared with their respective layers in failing hearts. APDs and ionic current densities are reported as means ± SE. Comparisons were performed using unpaired Student’s t-test. Data were considered significantly different if *P* < 0.05. NS denotes statistical nonsignificance (P ≥ 0.05) between compared samples.

**RESULTS**

**Altered repolarization in HF.** The canine pacing-tachycardia model of nonischemic dilated cardiomyopathy is characterized by key hemodynamic features that are reminiscent of human HF. For example, in randomly selected normal (n = 5) and failing (n = 5) dogs, the LVEDP was markedly (P < 0.0001) elevated in HF (31.4 ± 6.0 mmHg; n = 5) compared with normal (6.6 ± 4.3 mmHg; n = 5) dogs, whereas dP/dt₉₀ values in the same animals were significantly (P < 0.0003) reduced from 2,840 ± 411 in normal dogs to 1,236 ± 417 mmHg/s in HF dogs.

In addition to changes in mechanical function, the pacing-tachycardia HF model is characterized by major electrophysiological changes including prominently abnormal repolarization. Shown in Fig. 1A are representative action potential traces recorded from Epi, Mid, and Endo cells isolated from normal and failing hearts. Figure 1B shows the average (and SE) values of APD in all myocytes studied. HF resulted in a significant (P < 0.021) prolongation of APD in Mid cells, whereas the change in Endo and Epi cells did not reach statistical significance. Although in normal hearts the average difference in APD of cells from the three transmural layers was only 33 ms, this difference was markedly (141 ms) enhanced in HF (Fig. 1B), which indicates that the increased transmural APD heterogeneity reported previously in canine wedge preparations from failing hearts (2) may be related at least in part to changes in intrinsic action potential properties of myocytes.

**Molecular basis for I_K downregulation: expression of cKv4.3, cKv1.4, and cKChIP2.** We previously showed (18) that altered repolarization of Mid cells in this model of HF was accompanied by major changes in key K⁺ currents (namely, I_K and I_Ca,L). In the present study, we investigated potential underlying molecular mechanisms. First, we tested the hypothesis that functional downregulation of I_K in HF is due to altered expression of the α- and β- (cKChIP2) subunits that underlie this current. In addition, because Kv1.4 underlies I_K in several species (6, 34), its expression was also measured. Quantitative analysis of mRNA and protein expression levels of cKv4.3 revealed a marked reduction in expression in HF at both mRNA and protein levels in Epi (44% mRNA, 39% protein), Mid (52% mRNA, 34% protein), and Endo (49% mRNA, 73% protein) layers (Fig. 2). Interestingly, cKv4.3 did not exhibit a clear transmural gradient in mRNA (Fig. 2A) or protein (Fig. 2D) levels, which is consistent with a previous report (28). In contrast to reduced cKv4.3 expression, which correlated with significant reduction in I_K density (Fig. 2E), there was an increase (by 41% Epi, 97% Mid, 113% Endo) in cKv1.4 protein levels (Fig. 3) that argues against major involvement of this current in the formation of native canine I_K, which is consistently downregulated in HF. Moreover, the time constant of I_K inactivation was not altered in HF (Fig. 2F), which further argues against a relative isoform switch from Kv4.3 to Kv1.4 (34). Although the function of cKv1.4 remains unknown, our results indicate that it is abundantly expressed in canine myocardium at both mRNA (not shown) and immunoreactive protein (Fig. 3) levels. Moreover, as shown in a representative Mid LV myocyte, cKv1.4 is highly colocalized with α-actinin (Fig. 3C; yellow area), which suggests abundant T-tubular distribution of the protein across cardiac myocytes. Such distribution was not altered by HF (not shown).

Neither cKChIP2 mRNA (Fig. 4A) nor protein (Fig. 4C) expression was altered in any layer by HF. In contrast with a recent report (27), despite a marked (more than eightfold) transmural gradient of cKChIP2 mRNA expression, cKChIP2 protein expression in whole tissue lysates from Epi, Mid, and Endo layers did not significantly vary in either control or failing hearts.
Molecular basis for $I_K$ downregulation: expression of cERG, cKvLQT1, and cMinK. Li et al. (20) have previously reported changes in $I_K$ (namely, $I_{Ks}$) density in Epi, Mid, and Endo cells in a similar model of HF. The molecular basis for such changes was investigated in the present report by determining the expression of underlying $\alpha$- (cERG for $I_{Kr}$ and cKvLQT1 for $I_{Ks}$) and $\beta$- (cMinK for $I_{Ks}$) subunits in normal and failing ventricular myocardium. Shown in Fig. 5 is cERG expression in Epi, Mid, and Endo layers. As is evident, mRNA levels were not significantly changed (Fig. 5, A and B) despite a trend toward reduced expression by RT-PCR. In contrast, however, HF was associated with a significant increase in cERG protein levels (Fig. 5, C and D) in LV Mid (by 51%; $P = 0.027$), Epi (39%; $P = 0.044$), and Endo (46%; $P = 0.035$). Furthermore, to eliminate the potential confounding influence of cERG expression in nonmyocyte elements in heart, we specifically studied the expression of cERG in lysates prepared from isolated LV myocytes. Figure 5E shows a representative Western blot performed on lysates prepared from Mid LV myocytes that indicates comparable upregulation of cERG in HF as observed in whole tissue lysates.

MinK-related protein (MiRP)-1 in some tissues and species may form an important auxiliary subunit that interacts with human ERG channels to form native $I_{Kr}$ (1). In dog ventricles, however, staining with an anti-MiRP-1 antibody (not shown) did not produce a specific immunoreactive band, which is consistent with the findings of a recent study (26) that showed predominant expression of MiRP-1 in canine Purkinje fibers but not ventricular myocytes.

Mid cells are characterized by reduced $I_{Ks}$ density, which contributes to a longer APD compared with cells from Epi and Endo layers (5, 31). We quantified protein levels underlying the pore-forming $\alpha$- (cKvLQT1) and accessory $\beta$- (cMinK) subunits of $I_{Ks}$ in normal and failing left ventricle. Figure 6A illustrates that mRNA levels measured by RPA in the Mid left ventricle are not significantly different between normal and failing ventricular myocardium. Shown in Fig. 6, B and C, is a representative Western blot and summary data of cKvLQT1 expression in normal and HF samples. Despite known differences in $I_{Ks}$ density across layers (5, 31) as well as downregulation of the current by HF (20), there were no changes in cKvLQT1 protein expression either transmurally or with disease. The expression of cMinK, an ancillary subunit that modulates cKvLQT1 function and is a component of cardiac $I_{Ks}$ (26), was investigated. Figure 7A shows a representative Western blot that confirms abundant expression of cMinK in Epi, Mid, and Endo layers of normal and failing canine ventricular myocardium. Quantitative analysis of cMinK expression (Fig. 7B) revealed no significant changes in any layer in HF or in the gradient of expression across layers.
Molecular basis for $I_{K1}$ downregulation: expression of cKir2.1. To understand the molecular basis for $I_{K1}$ downregulation in HF, we measured transmural levels of the $I_{K1}$ pore-forming α-subunit (cKir2.1) in normal and failing hearts. Kir2.1 is the major Kir2-family subunit expressed in canine ventricle (data not shown). cKir2.1 mRNA levels exhibited a trend toward reduced expression in failing ventricular myocardium that did not reach statistical significance (Fig. 8A).

Similarly, cKir2.1 protein levels were not altered in HF in any of the LV myocardial layers (Fig. 8, B and C). These data indicate that significant functional downregulation of $I_{K1}$ in Epi, Mid, and Endo layers in HF (Fig. 8D) are not due to changes in the expression of the pore-forming channel subunit.

DISCUSSION

Sudden cardiac death due to ventricular arrhythmia is a major cause of mortality in patients with congestive HF (32). Recently, key electrophysiological changes occurring in response to myocardial failure (i.e., HF-induced electrical remodeling) have been identified (2, 20, 22, 30). Such alterations result in the prolongation of APD and enhancement of APD heterogeneity (2, 18). Although APD prolongation predisposes to the development of early afterdepolarizations that can act as arrhythmogenic triggers in HF (23), APD heterogeneity constitutes a suitable electrophysiological substrate for reentrant excitation that leads to malignant ventricular arrhythmias and sudden death (2, 4).

In the present report, we demonstrate marked prolongation of APD in cells isolated from Mid but not Epi or Endo cells in HF (see Fig. 1). These changes were accompanied by significant downregulation of $I_{K1}$ and $I_{to}$ in all cell layers. Therefore, we sought to investigate the specific molecular mechanisms that underlie $K^+$ current downregulation in this well-established, reproducible, and clinically relevant animal model of HF.
HF (18, 20, 24) by directly measuring transmural changes in K⁺/H11001 channel subunit expression and subcellular distribution across failing ventricles.

To date, several key changes in K⁺/H11001 currents have been identified in this and other animal models of HF (15, 16). The most consistent ionic current change in failing hearts is a reduction in the density of Iᵣ (17, 18). We previously demonstrated that such reduction correlated with decreased steady-state levels of cKv4.3 mRNA in failing human hearts (17). In the present study, we further demonstrate a reduction in cKv4.3 mRNA levels in HF that is paralleled by similar reductions in the immunoreactive protein (see Fig. 2), which is consistent with the findings of a recent report (35).

Kv1.4 is another K⁺ channel subunit that is expressed in the mammalian heart and encodes an “Iᵣ-like” current, albeit with much slower kinetics. Our present findings indicate that cKv1.4 is abundantly and uniformly expressed in Epi, Mid, and Endo layers of the LV. Interestingly, cKv1.4 protein expression increases across all layers in HF. The functional reduction of Iᵣ density in HF and its correlation with changes in cKv4.3 protein and mRNA levels but not cKv1.4 levels further discounts cKv1.4 as the pore-forming canine Iᵣ channel, which is consistent with our previous data (3). Moreover, because we and others (20) have shown that Iᵣ density but not kinetics are altered in this model of HF, it is unlikely that an isoform switch from Kv4.3- to Kv1.4-encoded current occurs (34). Therefore, the precise functional role of Kv1.4 in heart and its upregulation in HF requires additional investigation.

Accessory subunits are known to significantly alter the functional expression of several K⁺ currents. For example, KChIP2 is a Ca²⁺-binding protein that modulates the density and kinetics of Kv4-encoded currents (12, 13). In HF, no significant changes in cKChIP2 mRNA or protein expression levels are observed; hence, Iᵣ downregulation is unlikely to be

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Accessory subunits are known to significantly alter the functional expression of several K⁺ currents. For example, KChIP2 is a Ca²⁺-binding protein that modulates the density and kinetics of Kv4-encoded currents (12, 13). In HF, no significant changes in cKChIP2 mRNA or protein expression levels are observed; hence, Iᵣ downregulation is unlikely to be
the primary consequence of altered cKChIP2 expression. Instead, our data are consistent with the notion that downregulation of $I_{\text{to}}$ in HF is due to an altered balance between transcription and degradation of the mRNA encoding the pore-forming $\alpha$-subunit cKv4.3. Interestingly, in contrast with the findings of a recent report (27), our data do not demonstrate a significant transmural protein gradient of cKChIP2 in dogs, which suggests an alternative mechanism for the well-described transmural $I_{\text{to}}$ gradient. It is known that several splice variants of cKChIP2 exist in hearts, and therefore differences in the affinity of the antibodies used in different studies could account for observed differences in cKChIP2 expression as was recently demonstrated by Zicha et al. (35). We validated the sensitivity of the antibody used in the present study by measuring the expression of KChIP2 protein in HEK-293 cells that were transfected with either KChIP2 alone or KChIP2 and a small interfering (si)RNA construct that mediated the genetic silencing of KChIP2 (11). As expected, the immunoreactive band was completely suppressed when KChIP2 expression was knocked down in vitro. Similarly, suppression of KChIP2 in cultured neonatal rat ventricular myocytes by the same siRNA construct was also documented, which further indicates specificity of our antibody to KChIP2. Therefore, the present data suggest that an alternative mechanism must be responsible for establishment of the transmural $I_{\text{to}}$ gradient; this mechanism will be further elucidated as we better understand the exact molecular identity of $I_{\text{to}}$ and its regulatory components. Interestingly, a recent study has demonstrated the involvement of the novel 110-kDa molecule dipeptidyl aminopeptidase-related protein (DPPX) in the formation of native neuronal $I_{\text{to}}$ (21).
Unlike KChIP2, DPPX accelerates the inactivation kinetics of Kv4.3- and Kv4.2-expressed currents, thereby rendering them more similar to native $I_{K\alpha}$ (21). To date, it remains unknown whether DPPX or other related molecules participate in the formation of native cardiac $I_{K\alpha}$.

The expression of other K$^+$ currents such as $I_{Kr}$ and $I_{Ks}$ is also heterogeneous in mammalian heart. Functional reduction of these currents due to mutations in underlying channel subunits such as those in human ERG ($I_{Kr}$) or KvLQT1 ($I_{Ks}$) predisposes to torsades de pointes, an arrhythmia associated with the long-QT syndrome (9). The canine ortholog cERG does not exhibit transmural differences in expression at either mRNA or protein levels in the basal state. In HF, there is a significant upregulation of cERG immunoreactive protein expression that occurs in both tissue lysates and membrane preparations from ventricular myocardium and lysates from isolated ventricular myocytes. The significance of the upregulation in cERG protein levels in the failing heart remains uncertain.

Myocytes isolated from the Mid layer are characterized by longer APDs due in large part to reduced $I_{Ks}$ density compared with Epi and Endo cells (5, 31). Our data demonstrate the absence of a transmural protein (see Fig. 6) or mRNA (not shown) gradient of cKvLQT1 across normal and failing left ventricle, which suggests that reduced $I_{Ks}$ content in Mid cells is likely to be posttranslationally mediated. It should be noted, however, that two isoforms of cKvLQT1 have been described previously (10, 25). Interestingly, the truncated version (isoform 2) is thought to act as a dominant-negative regulator that reduces the density of $I_{Ks}$. Because the antibody used in this study recognizes both isoforms of KvLQT1, we cannot definitively rule out a relative change in the ratio of isoforms 1 and 2 in HF from our data. Finally, cKvLQT1 associates with the transmembrane accessory protein cMinK to form native $I_{Ks}$. cMinK exhibits no transmural differences in protein expression in normal or failing hearts (see Fig. 7).

Reduction in $I_{K1}$ density is a well-established feature of the pacing-induced HF model and is thought to underlie, in part, the enhanced propensity of failing myocytes for development of arrhythmogenic afterdepolarizations and spontaneous depo-

**Fig. 7.** A: representative Western blot of cMinK expression in Epi, Mid, and Endo layers of normal and HF left ventricles. B: summary plot of cMinK expression in all normal and HF dogs.

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Reduction in $I_{K1}$ density is a well-established feature of the pacing-induced HF model and is thought to underlie, in part, the enhanced propensity of failing myocytes for development of arrhythmogenic afterdepolarizations and spontaneous depo-
larizations (18, 23). Therefore, reduced $I_{K1}$ density could underlie the triggering beats for reentrant excitation in addition to contributing to APD prolongation in HF. Although a number of Kir2 family members encode inward rectifiers, cKir2.1 is the dominant subunit in the canine ventricle. Despite reduction in $I_{K1}$ density (see Fig. 8D), there are no changes in cKir2.1 mRNA or protein levels in failing heart, which suggests a posttranslational mechanism of functional current downregulation.

In conclusion, there are several $K^+$ current changes that contribute to the remodeling action potential profile of the failing heart. The mechanism of current downregulation varies depending on the specific channel under consideration. In the case of $I_{Na}$, there is ample evidence for transcriptional downregulation of the $\alpha$-subunit, Kv4.3. In this model of nonischemic dilated cardiomyopathy, we find no evidence for transcriptionally mediated changes in current density for the other $K^+$ channels investigated, which is in contrast with a model of postmyocardial infarction-induced hypertrophy in rats (15). Additionally, we see no evidence for large-scale changes in channel distribution as would occur with aberrant trafficking to explain the alterations in $K^+$ currents. Hence it seems likely that altered cellular signaling in HF modulates channel function leading to $K^+$ current downregulation in this disease state.

Limitations. There are several limitations to our study that should be recognized. This model is one of HF in the context of dyssynchronous ventricular contraction produced by rapid right ventricular pacing. There may be regional differences and differential regulation of $K^+$ channel subunit expression within the left ventricle of this model that have not been quantified. In this study, all $K^+$ channel measurements were done in tissue sections isolated from approximately the same region within the left ventricle of normal and failing hearts.

Downregulation of steady-state levels of Kv4.3 mRNA have been demonstrated in the present study. However, no measure of RNA synthesis rates for Kv4.3 were made to determine whether such alterations are caused by changes in the rate of transcription or mRNA stability.

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