Ion Channel Subunit Expression Changes in Cardiac Purkinje Fibers

A Potential Role in Conduction Abnormalities Associated With Congestive Heart Failure

Ange Maguy,* Sabrina Le Bouter,* Philippe Comtois, Denis Chartier, Louis Villeneuve, Reza Wakili, Kunihiro Nishida, Stanley Nattel

Abstract—Purkinje fibers (PFs) play key roles in cardiac conduction and arrhythmogenesis. Congestive heart failure (CHF) causes well-characterized atrial and ventricular ion channel subunit expression changes, but effects on PF ion channel subunits are unknown. This study assessed changes in PF ion channel subunit expression (real-time PCR, immunoblot, immunohistochemistry), action potential properties, and conduction in dogs with ventricular tachypacing–induced CHF. CHF downregulated mRNA expression of subunits involved in action potential propagation (Nav1.5, by 56%; connexin [Cx]40, 66%; Cx43, 56%) and repolarization (Kv4.3, 43%, Kv3.4, 46%). No significant changes occurred in KChIP2, KvLQT1, ERG, or Kir3.1/3.4 mRNA. At the protein level, downregulation was seen for Nav1.5 (by 38%), Kv4.3 (42%), Kv3.4 (57%), Kir2.1 (26%), Cx40 (53%), and Cx43 (30%). Cx43 dephosphorylation was indicated by decreased larger molecular mass bands (pan-Cx43 antibody) and a 57% decrease in Ser368-phosphorylated Cx43 (phospho-specific antibody). Immunohistochemistry revealed reduced Cx40, Cx43, and phospho-Cx43 expression at intercalated disks. Action potential changes were consistent with observed decreases in ion channel subunits: CHF decreased phase 1 slope (by 56%), overshoot (by 32%), and phase 0 dV/dt_max (by 35%). Impulse propagation was slowed in PF false tendons: conduction velocity decreased significantly from 2.2±0.1 m/s (control) to 1.5±0.1 m/s (CHF). His-Purkinje conduction also slowed in vivo, with HV interval increasing from 35.5±1.2 (control) to 49.3±3.4 ms (CHF). These results indicate important effects of CHF on PF ion channel subunit expression. Alterations in subunits governing conduction properties may be particularly important, because CHF-induced impairments in Purkinje tissue conduction, which this study is the first to describe, could contribute significantly to dysynchronous ventricular activation, a major determinant of prognosis in CHF-patients. (Circ Res. 2009;104:1113-1122.)

Key Words: heart failure ■ remodeling ■ ventricular dyssynchrony ■ connexins ■ specialized conducting system

Cardiac Purkinje fibers (PFs) play an important role in ensuring rapid and appropriately timed conduction of electric impulses to the ventricles and are an important arrhythmogenic source for a variety of cardiac arrhythmias. Congestive heart failure (CHF) changes ion channel distribution and function, with important electrophysiological consequences, and sudden arrhythmic death contributes importantly to CHF-related mortality. We previously demonstrated discrete CHF-induced remodeling of repolarizing ion current function in canine cardiac PFs. In particular, decreases in transient-outward (I_{to}) and inward-rectifier (I_{K1}) K^+ currents reduce repolarization reserve and enhance the action potential (AP)-prolonging effects of class III antiarrhythmic agents, potentially accounting for the increased Torsades de Pointes risk conferred by CHF. Virtually no work has been done on CHF-induced remodeling of PF ion channel subunit expression at the molecular level. In the present study, we began by examining CHF-induced changes in PF K^+ channel subunit mRNA and protein expression, to understand previously noted repolarization current alterations. We then moved on to study effects on molecular determinants of PF impulse propagation: Na^+ channel and connexin (Cx) subunits. Substantial changes in these subunits led us to assess associated changes in PF conduction in vitro and in vivo.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

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Animal Model
CHF was induced by 2-week right ventricular tachypacing (VTP) at 240 bpm, as previously described. Under anesthesia (morphine, 2 mg/kg SC; α-chloralose 120 mg/kg IV load, 29.25 mg/kg per hour infusion), hearts were excised and immersed in oxygenated Tyrode solution. Free-running false tendons were removed for study. Animal care procedures were approved by the local animal research ethics committee.

RNA Isolation and Quantitative PCR
RNA was extracted with TRIzol (Invitrogen) and treated with DNAseI to minimize genomic DNA contamination. Quantitative PCR probes and primers are listed in Online Tables I and II. The 18S rRNA housekeeping gene was used as a reference. PCR efficiency was in the range of 95% to 100% for all assays. PCR cycle parameters were: 2 minutes at 50°C; 10 minutes at 95°C initial incubation, followed by 15 seconds at 95°C; and 1 minute at 60°C for 40 cycles. For each construct, analyses were performed separately for n=7 control, n=8 CHF hearts, each in duplicate. Data are expressed as 2−ΔΔCt [17].

Protein Extraction and Immunoblots
Protein-enriched samples were obtained by pooling all usable false tendons from 2 dogs, to provide sufficient protein to load in a well. Results were obtained from 8 dogs per group (n=4 determinations each for control and CHF) unless otherwise indicated. To enrich membrane proteins, snap-frozen free-running false tendons were homogenized in extraction buffer. A preliminary 1000g centrifugation (10 minutes, 4°C) was performed to pellet debris. The supernatant was further ultracentrifuged at 100 000 g for 1 hour to pellet membrane-protein fractions. Pellets were resuspended with extraction buffer supplemented with 1% Triton-X100. Protein concentration was determined with Bradford assay. Protein samples (100 μg) were separated with SDS-PAGE. The separated proteins were transferred by electrophoresis to poly(vinylidene fluoride) transfer membranes. Membranes were blocked 2 hours with TTBS solution (Tris-HCl 50-mmol/L, NaCl 500-mmol/L, pH 7.5, 0.1% Tween) containing 5% nonfat dried milk and incubated overnight with primary antibodies (Online Table III) in TTBS with 5% nonfat dried milk. Corresponding secondary antibodies conjugated to horseradish peroxidase were used for detection. Staining was quantified by chemiluminescence. All expression data are provided relative to GAPDH staining for the same samples on the same gels.

AP Recordings and Conduction Velocity Measurements
Preparations containing free-running false tendons were pinned to the floor of a 30-mL Lucite tissue chamber (Figure I in the online data supplement) and superfused with Krebs–Henseleit solution (in mmol/L: NaCl 120, KCl 4, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, CaCl2 1.25, dextrose 1g/L; 95% O2/5% CO2, pH 7.4) at 35°C. Two floating glass microelectrodes with tip resistances of 15 to 20 MΩ were used to impale cells in the same longitudinal orientation. One microelectrode (E1) was placed at one fiber end near a bipolar platinum stimulation electrode (SE) positioned on ventricular muscle and a second (E2) was placed at various points distal to E1 (Online Figure I). Square wave 2-ms pulses (1.5×late diastolic threshold current) were delivered with an electronic stimulator. Phase 0 upstroke was analyzed by electronic differentiation. Activation time was defined as the time of dV/dtmax. Conduction velocity was determined by dividing the distance between E1 and E2 by the difference between their respective activation times and averaging conduction velocity estimates at all E2 positions.

Immunohistochemistry
After AP recording and conduction velocity measurement, false tendons were fast-frozen. Cryosections (12-μm thickness) were fixed with a 1× PBS solution containing 4% paraformaldehyde (pH 7.3), blocked, and permeabilized with 1× PBS solution containing 2% normal donkey serum and 0.5%-Triton X-100. Primary antibod-
was not changed in CHF, unlike mRNA. Like KChIP2 mRNA, KChIP2 protein expression was unchanged.

We then examined genes encoding proteins underlying outward currents affecting phase 3 repolarization. Transcript levels corresponding to \( I_{Ks} \) subunits underlying the major delayed-rectifier and inward-rectifier currents (\( KCNQ1 \) encoding the \( I_{Ks} \) subunit underlying \( I_{Ks} \), \( KCNH2 \) encoding the ERG \( I_{Kr} \), \( KCNJ2 \) encoding the \( I_{Kr} \) subunit of \( I_{Kr} \), \( KCNJ3/KCNJ5 \) encoding Kir3.1/3.4 \( I_{KaCh} \) subunits) were unchanged in CHF (Figure 2A). The \( I_{Ks} \) subunit (minK)-encoding gene \( KCNE1 \) was downregulated at the mRNA level. The putative \( I_{Kr} \) subunit \( MiRP1 \) was weakly expressed and not altered by CHF. No changes in protein expression were observed for \( KvLQT1 \), minK, or \( MiRP1 \) (Figure 2B and 2C). Although \( KCNJ2 \) mRNA was unchanged, Kir2.1 protein was reduced significantly, by 26%.

We noted 4 bands on ERG immunoblots with an antibody directed at the ERG C-terminus. The 160- and 140-kDa isoforms (corresponding to mature and immature forms of ERG 1a respectively)\(^{14}\) were upregulated in CHF, whereas 95- and 83-kDa bands (corresponding to mature and immature ERG 1b\(^{14}\)) were unchanged.

We then turned our attention to ion channel subunits involved in cardiac conduction. The \( SCN5A \) gene encoding the predominant \( Na^+ \) channel \( \alpha \) subunit Nav1.5 is strongly expressed in cardiac Purkinje cells, along with the \( \beta \) subunit \( SCN1B \) (Nav1.1) and the more weakly expressed \( \alpha \) subunit gene \( SCN1A \) (Nav1.1).\(^{15}\) Nav1.5 and Nav1.1 transcripts were expressed at similar levels, \( \approx \)4 orders of magnitude greater than Nav1.1 (Figure 3A). Nav1.1 and Nav1.1 were unaffected by CHF, but Nav1.5 mRNA was strongly downregulated, by \( \approx \)56%. We also examined expression of genes encoding the hemichannels mediating low-resistance cell-to-cell cardio-

myocyte communication, \( GJA5 \) (Cx40), \( GJA1 \) (Cx43), and \( GJA7 \) (Cx45). Cx43 mRNA was very strongly expressed, at an order of magnitude greater than Cx40, Nav1.5, and Nav1.1. Cx45 expression was much weaker than Cx40. Cx40 and -43 transcripts were strongly and significantly downregulated in CHF, by \( \approx \)66% and 56%, respectively. Cx45 mRNA was upregulated.

Western blots (Figure 3B) confirmed 48% downregulation of Nav1.5 protein expression (Figure 3C). Cx40 band intensity was reduced in CHF by 53%. Cx45 bands were too weak for accurate detection. Cx43 migrated as 2 discrete bands, previously identified as nonphosphorylated (smaller-molecular-mass) and phosphorylated (larger-molecular-mass) bands, respectively.\(^{16}\) Estimating total Cx43 protein expression based on the sum of both indicated a 30% reduction. Cx43 dephosphorylation contributes to CHF-induced conduction impairment in ventricular muscle.\(^{17}\) The larger-molecular-mass band intensity decreased as a fraction of the smaller-molecular-mass band in CHF, suggesting reduced phosphorylation. Probing with an antibody specific to Ser368-phosphorylated Cx43 revealed a dominant band at the expected molecular mass (Figure 3D, left), which decreased in CHF by \( \approx \)57% (Figure 3D, right).

### Cx Distribution at Intercalated Disks

Changes in Cx expression and phosphorylation may be reflected in changed distribution within intercalated disks, the principal structures governing cardiomyocyte coupling.\(^{18}\) Figure 4A shows representative single en face intercalated disks. Figure 4B shows corresponding lateral views. Cx immunostaining exhibited a typical localization pattern, with small central gap junctions in plicate regions surrounded by larger gap junction plaques located in interplicate regions at
the disk periphery. In PFs from CHF dogs, peripheral gap junction immunostaining was preserved for Cx40 and total Cx43. However, immunostaining of Ser368-phosphorylated Cx43 was reduced at the periphery of the intercalated disks. Quantitative analyses of CHF-induced changes in Cx distribution are illustrated in Figure 5A (left), which shows representative 3D surfaces of Cx staining at central intercalated disk regions for 1 sample each in control and CHF groups. Quantitative analysis (Figure 5A, right) showed significant 25% reductions in Cx40 density in central intercalated disk regions in CHF dogs, with no significant change in the periphery (Figure 5B). Similarly, Cx43 immunostaining was reduced by 27% in central regions but not significantly altered in the periphery. In contrast, central phosphor-
ylated Cx43 staining was unaffected by CHF, but a 27% decrease was noted in peripheral zones. Average intercalated disk surface areas and numbers of Cx plaques per intercalated disk were not significantly altered in CHF (Online Figure II).

AP Properties and Conduction

AP properties and conduction velocity were determined at 1 Hz. Resting potentials were not significantly altered by CHF (Table), but AP amplitude, overshoot, and phase 0 upstroke velocity were decreased, consistent with reduced Nav1.5 expression. The slope of phase 1 was also significantly decreased, compatible with reduced \( I_{Na} \) subunit expression. Overall, AP duration was not significantly altered, consistent with previous findings.8

Typical AP recordings used for conduction velocity assessment in a control dog are illustrated in Figure 6A, with the upstrokes shown on an expanded time scale in Figure 6B. Recordings from a CHF dog with a similar E1–E2 distance to
the control dog illustrate the longer conduction times that were typically noted in CHF false tendons. Figure 6C provides mean conduction velocity values, with a statistically significant, ∼30% reduction noted in CHF.

In Vivo Purkinje System Conduction Changes

To determine whether changes in ventricular conduction properties are altered in vivo in relation to PF $I_{Na}$ and Cx remodeling, we measured QRS duration and HV intervals in control and CHF dogs. Typical recordings used for QRS and HV interval measurement are shown in Figure 7A. Because of the noninvasive nature of ECG recordings, we were able to measure QRS duration at baseline and after tachypacing in CHF dogs. QRS durations were very similar in control dogs and at baseline in CHF dogs (Figure 7B). However, with tachypacing, the QRS increased significantly, by a mean of 13.5 ms (30%), in CHF dogs. Similarly, the HV interval (which reflects PF-mediated His-Purkinje conduction) was substantially greater in CHF than in control dogs (Figure 7C), by 13.8 ms (39%). No modification of the AH interval was observed (Figure 7D).

Discussion

In this study, we investigated changes in transcript and protein expression of $K^+$ channel, $Na^+$ channel, and Cx subunits induced by CHF in canine cardiac PFs. We observed decreases in the expression of $I_{Na}$-related α subunits, little change in inward and delayed-rectifier $K^+$ channel subunits, and concerted downregulation of $Na^+$ channel α subunit and Cx40/43 proteins. In addition, Cx43 phosphorylation was reduced, and Cx expression in intercalated disks was altered in quantity and spatial distribution. These changes were associated with in vitro and in vivo evidence of slowed Purkinje system conduction.

PF Electrical Remodeling in CHF

Relatively little work has been performed to address CHF-induced changes in PF electrophysiology. We previously reported an ∼32% decrease in $I_{Na}$ density and ∼22% decrease in peak outward $I_{Kr}$ density as a result of tachypacing-induced heart failure in dogs, without any change in delayed-
Figure 5. Quantification of central and peripheral intercalated disk Cx expression. A, left, Representative Cx staining (normalized intensity levels) at the central region of the intercalated disk. A, right, Mean±SEM central densities. B, left, Representative peripheral regions of the same intercalated disks as in A. B, right, Mean±SEM results for peripheral regions. Control: n=13 disks/4 dogs; CHF: n=11 disks/5 dogs. *P<0.05.
rectifier currents. The precise molecular basis of cardiac PF $I_{\text{Kr}}$, has not been definitively established, but there is evidence supporting a role for Kv4.3 and/or Kv3.4 subunits, both of which were downregulated in the present study. Kir2.1 protein expression decreased by ~26%, sufficient to account for the previously described $I_{\text{K1}}$ downregulation, but mRNA levels were unaffected, suggesting posttranscriptional changes. The lack of change in KvLQT1 and minK protein is consistent with unaltered $I_{\text{Ks}}$, but the picture is more complex for $I_{\text{Kr}}$. ERG transcript expression was unaltered, as measured by primers directed to a C-terminal sequence shared by both ERG isoforms. Both mature and immature forms of ERG 1a protein were increased in CHF PFs, whereas ERG 1b protein expression was unchanged. Recent evidence points to hetero-

greenary protein were increased in CHF PFs, whereas ERG 1b protein expression having been reported. The discrepancies may relate to varying experimental conditions, species, duration, and type of disease, etc.

Cx43 is consistently downregulated in failing ventricular muscle, with corresponding changes in mRNA and protein generally reported. Cx43 is believed to be an important contributor to ventricular conduction slowing.

Novelty and Potential Significance

This study is the first, to our knowledge, to address CHF-induced ion channel subunit remodeling in cardiac PFs. Our results regarding $I_{\text{Na}}$ and $I_{\text{Ks}}$ subunits are largely consistent
with previously reported changes in ventricular muscle and corresponding ion current density alterations in PFs.\textsuperscript{6,7} We are not aware of previous studies that report differential disease-induced changes in the cardiac expression of ERG 1a versus ERG 1b subunits. In conjunction with our previous observation of unchanged \( I_{Kr} \) in PFs from CHF dogs, our finding of increased ERG 1a protein with unchanged ERG 1b would suggest that ERG 1b expression is a particularly important determinant of \( I_{Kr} \) function. These results are consistent with recent observations pointing to an essential role of ERG 1b in \( I_{Kr} \).\textsuperscript{19}

Our observations point to particularly important changes in the ion channel subunits determining the primary conduction function of cardiac PFs. Decreases in \( I_{Na} \) \( \alpha \) subunits and Cx hemichannel subunits correspond to impairments of the active and passive determinants of PF conduction respectively. These alterations were associated with substantial and statistically significant decreases in directly measured PF conduction velocity in vitro, as well as corresponding His-Purkinje system conduction slowing (as indicated by increased HV interval) in vivo. This novel finding may have important bearing on a clinically significant abnormality frequently observed in CHF patients, dyssynergic ventricular excitation/contraction. Conduction through the His-Purkinje system is an important determinant of the sequence of ventricular activation and contraction.\textsuperscript{1} Cardiac conduction abnormalities are a strong independent predictor of mortality in CHF patients.\textsuperscript{27} Evidence of disturbed His-Purkinje system function like bundle-branch block is common in CHF, predicting increased disease progression and mortality.\textsuperscript{28} Dyssynchronous cardiac contraction has important deleterious effects on cardiac function mediated by adverse remodeling related to deleterious changes in cardiac gene expression.\textsuperscript{29} Resynchronization therapy improves morbidity and mortality in CHF patients with ventricular activation abnormalities.\textsuperscript{30} Antiarrhythmic effects of resynchronization therapy\textsuperscript{31} suggest an important role of dyssynchrony in ventricular arrhythmogenesis, either directly or via ventricular dysfunction-related arrhythmogenic remodeling. Our results point to remodeling of PF ion channel subunits (particularly \( I_{Na} \) \( \alpha \) subunits and connexins) as a potentially important contributor to ventricular activation abnormalities in CHF.

### Potential Limitations

The canine VTP model of CHF provides a highly reproducible and robust experimental model exhibiting key hemodynamic, electrophysiological, and molecular changes associated with human CHF. However, CHF results from many different etiologies and extrapolation of our results to other forms of CHF should be cautious. Rapid activation of ventricular cardiomyocytes modifies \( I_{Na} \) expression in vitro.\textsuperscript{32} It is therefore possible that some of the changes we observed were attributable to rapid ventricular rates and not CHF per se; however, the ventricular ion channel function changes noted in animals with VTP-induced CHF do correspond closely to results obtained from explanted terminally failing human hearts.\textsuperscript{6} We investigated modifications in transcript and protein expression of \( K^+ \) channel, \( Na^+ \) channel, and Cx subunits induced by CHF in canine cardiac PFs, based on a desire to understand previously observed functional \( K^+ \) channel changes and to extend our understanding to ion channels governing cardiac conduction. A complete analysis of the molecular basis of CHF-induced PF electric remodeling would require studies of \( Ca^{2+} \) current, \( Ca^{2+} \) handling, HCN, \( Na^+/Ca^{2+} \) exchange, and \( Na^+,K^+ \)-ATPase gene/protein expression and function,\textsuperscript{33} work that is beyond the scope of the present article but should be performed in follow-up studies.

We observed decreases in PF Cx40 and -43 expression and increases in Cx45. It was traditionally assumed that all cardiac connexins contribute to conduction and decreases in any Cx isoform would impair cell coupling.\textsuperscript{19} Recent work has raised questions about this notion. Cx45 overexpression impairs cell coupling and increases ventricular tachyarrhythmia susceptibility in transgenic mice.\textsuperscript{34} Similarly, pattern-cultured neonatal atrial cardiomyocytes from Cx40-knockout mice show increased conduction velocity.\textsuperscript{35} Thus, functional effects of changed Cx expression may be difficult to predict directly,
particularly when alterations in multiple isoforms are observed. On the other hand, Cx40 knockout clearly slows His-Purkinje system conduction in the mouse, and decreases in Cx43 expression slow ventricular conduction. There is evidence that colocalization of Cx40 and Cx43 in PFs may be functionally important. The mRNA concentrations of Cx43 that we measured in PFs were more than 10-fold greater than those of Cx40 and more than 100-fold greater than Cx45. This observation, combined with decreased Cx43 phosphorylation, an important determinant of Cx43 localization and function, suggests that Cx43 alterations are likely to have played a particularly important role in the PF conduction slowing that we observed.

Species differences in Cx isoform distribution may be important. Therefore our results should be extrapolated with caution and follow-up studies in other species, including humans, would be of interest. Our studies were performed at a particular time point in a particular experimental model of CHF. Remodeling of the determinants of ventricular conduction are time-dependent but are well developed at 2 weeks of VTP and are qualitatively consistent at different time-points.

We have discussed K+ channel and Cx remodeling as separate entities; however, there is recent thought-provoking evidence that changes in cell coupling may importantly influence remodeling of K+ channels and repolarization properties. A wide range of ion channel subunits localize to intercalated disks, including Na+ channel α subunits. There may therefore be coordinate regulation of intercalated disk channel subunits involved in impulse propagation (eg, connexins and Na+ channels) that underlies the changes we observed. This would be a potentially interesting subject for future evaluation.

Conclusions

We report for the first time CHF-induced changes in the expression of K+, Na+, and Cx channel subunits in cardiac PFs. K+ channel subunit expression changes are consistent with and provide mechanistic insights into previously observed alterations in PF K+ channel function. Na+ and Cx subunit expression changes, including downregulation of Nav1.5, Cx40, and Cx43 mRNA and protein expression, decreased Cx43 phosphorylation at Ser368 and altered Cx distribution at intercalated disks, were associated with significant slowing in His-Purkinje conduction. These results may be important for understanding the alterations in ventricular activation and synchrony that contribute to deleterious outcomes in CHF patients.

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Disclosures

None.

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Animal Model

CHF was induced by 2-week right-ventricular tachypacing (VTP) at 240 bpm, as previously described. Dogs were then anesthetized (morphine, 2 mg/kg SC; \(\alpha\)-chloralose 120 mg/kg IV load, 29.25 mg/kg per hour infusion) and a median sternotomy performed. The heart was excised and immersed in oxygenated Tyrode solution (mmol/L: NaCl 136, KCl 5.4, MgCl\(_2\) 1, CaCl\(_2\) 1, NaH\(_2\)PO\(_4\) 0.33, HEPES 5, dextrose 10; pH 7.4, NaOH). Free-running PF false tendons were then removed for study. Animal care procedures were approved by the local animal research ethics committee and were in agreement with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

RNA Isolation and Quantitative Polymerase Chain Reaction (QPCR)

RNA was extracted with Trizol® (Invitrogen) and then treated with DNaseI (Sigma-Aldrich) to minimize genomic DNA contamination. RNA concentration was determined spectrophotometrically. Integrity was assessed by electrophoresis and DNA contamination excluded by reverse transcription (RT)-negative PCR. High-capacity cDNA archive Kit for RT-PCR (Applied Biosystems) was used to synthesize first-strand cDNA from 2 \(\mu\)g total RNA. Probes and primers used for QPCR are listed in Online Tables I and II. The 18-S rRNA housekeeping gene was used as a reference. Standard curves were performed to determine PCR-efficiency, which was in the range of 95-100% for all assays. FAM fluorogenic Taqman probes with Taqman Universal Master Mix or primer pairs with Sybr Green Universal Master Mix (Applied Biosystems) were used for real-time PCR on the MxPro3000P Detection System (Stratagene). PCR cycle parameters were: 2 min at 50°C, 10 min at 95°C initial incubation
followed by 15 sec at 95°C and 1 min at 60°C for 40 cycles. mRNA was quantified with comparative threshold-cycle relative quantification and △△Ct methods. For each construct, analyses were performed separately for n=7 control, n=8 CHF hearts, each in duplicate. Data are expressed as 2-△Ct*10^7.

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Protein-enriched samples were obtained by pooling all usable false tendons from 2 dogs, to provide sufficient protein to load in a well. Results were obtained from 8 dogs per group (n=4 determinations each for control and CHF) unless otherwise indicated. To enrich membrane proteins, snap-frozen free-running false tendons were homogenized (PT3100, Polytron) in an extraction buffer containing (mmol/L): Tris 25, EGTA 5, EDTA 5, Na3VO4 10, AEBSF 0.5 iodoacetamide 1, microcystin 1, β-glycerolphosphate 50, β2-mercaptoethanol 1; aprotinin 10 μg/ml, leupeptin 10 μg/ml, pepstatin 1 μg/ml. A preliminary 1000×g centrifugation (10 minutes, 4°C; RC5C plus, Sorvall) was then performed to pellet debris. The supernatant was further ultracentrifuged at 100 000×g for 1 hour (Optima™ Max Ultracentrifuge, Beckman-Coulter) to pellet the membrane-protein fraction. The pellet was resuspended with extraction buffer supplemented with 1% Triton-X100. Protein-concentration was determined for each sample with Bradford assay (Biorad). Protein-samples (100-μg) were separated with sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). High molecular-weight proteins were separated on 7.5%-polyacrylamide gels (Kv1.4, Kv4.3, Kv3.4, KvLQT1, ERG, Nav1.5, Kir2.1) and low molecular-weight proteins were separated on 10%-gels (Cx43, Cx40, KChIP2, MiRP1, minK). The separated proteins were transferred by electrophoresis to polyvinylidene fluoride (PVDF) transfer membranes (Immobilon™, Millipore). Membranes
were blocked 2 hours with TTBS solution (Tris-HCl 50-mmol/L, NaCl 500-mmol/L, pH 7.5, 0.1%-Tween) containing 5% non-fat dried milk and incubated overnight with primary antibodies (see Online Table III) in TTBS 5% non-fat dried milk. Corresponding secondary antibodies conjugated to horseradish-peroxidase (HRP) were used for detection. Staining was revealed with chemiluminescence (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer) and quantified with Quantity-One software (Biorad). All expression data are provided relative to GAPDH staining for the same samples on the same gels.

**AP Recordings and Conduction Velocity Measurements**

Preparations containing free-running false tendons and attached ventricular-muscle were pinned carefully (to avoid stretching the fiber) to the floor of a 30-ml Lucite tissue chamber (Online Figure I) and superfused with Krebs-Henseleit solution (mmol/L: NaCl 120, KCl 4, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, CaCl2 1.25, dextrose 1g/L; 95% O2/5% CO2, pH 7.4) at 35°C. All preparations were stimulated at 1 Hz for 1 hour to allow equilibration. Two floating glass microelectrodes with tip resistances of 15 to 20 MΩ when filled with 3-mol/L KCl coupled to a high-impedance amplifier (Axoclamp 2B; Axon Instruments) were used to impale cells in the same longitudinal orientation. One microelectrode (E1) was placed at one fiber-end near a bipolar platinum stimulation electrode (SE) positioned on ventricular muscle and a second microelectrode (E2) was placed at various points distal to E1 (Online Figure I). The E1-E2 distance was determined with a 64× binocular magnifier. Square-wave 2-ms pulses (1.5× late-diastolic threshold current) were delivered with an S-95 Trilevel stimulator (Medical Systems Corporation). Phase-0 upstroke was analyzed with a DDT differentiator (World Precision Instruments). Amplified waveforms were digitized (DigiData 1200; Axon), acquired and
analyzed with Axoscope 8.1 and Clampfit 9.1 software (Axon Instruments). Activation time was
defined as the time of \( \frac{dV}{dt_{\text{max}}} \). Conduction velocity was determined by dividing the distance
between the two electrodes (E1 and E2) by the difference between their respective activation
times, and averaging the conduction velocity estimates at all E2 positions in each preparation.

**Immunohistochemistry**

After AP-recording and conduction velocity measurement, false tendons were fast-frozen in
Optimal Cutting-Temperature Solution (Sakura). Cryosections (12-μm thickness) were fixed
with a 1×Phosphate-Buffer Saline (PBS) solution containing 4% paraformaldehyde (PFA, pH 7.3), blocked and permeabilized with 1×PBS solution containing 2%-normal donkey serum
(NDS) and 0.5%-Triton X-100. Primary antibodies (rabbit anti-Cx40, mouse anti-Cx43,
Chemicon; mouse anti-ser 368-phosphorylated Cx43, Cell Signaling) were diluted 1/200 in
1×PBS solution containing 2%-NDS and 0.1%-Triton X-100 for overnight incubation with
cryosections (for details see Online Table III). Alexa Fluor-conjugated donkey anti-rabbit
(488-nm, Invitrogen) or donkey anti-mouse (555-nm, Invitrogen) were used as secondary
antibodies (1/600 dilution). Alexa Fluor-conjugated phalloidin (647-nm) was used as an
actin-filament marker (1/600 dilution). Slides were mounted in DABCO/Glycerol (25%/75%)
and examined with a Zeiss Axiovert 100-M microscope coupled to a Zeiss LSM-510
laser-scanning confocal system. Identical settings were used to image samples from control and
CHF dogs. Images were deconvolved with the Maximum Likelihood Estimation algorithm
(Huygens software, Scientific Volume Imaging). Three-dimensional (3D) rendering was
performed with Volocity software (PerkinElmer).
Connexin-protein density was quantified from deconvolved confocal images. Each intercalated disk was separated into peripheral and central regions and respective connexin-density calculated as described below. All procedures were performed with MatLab v6.5 (Mathworks). The number of samples was 13 disks/4 dogs for the control group and 11 disks/5 dogs in the CHF group.

**Periphery:** Transverse two-dimensional images obtained from each individual gap junction were analyzed. Binary images of the gap junction were constructed by thresholding at 0.15 of the normalized amplitude. Normalized 2-dimensional (2D) histograms were obtained by binning (4×4 pixels) each binary image. Cumulative probability-density matrices were then computed from the 2D histograms. The sum of regions having a cumulative density amplitude between 0.05 and 0.95 was considered the gap junction area. The center of the region was approximated by averaging a set of points representing the outer contour. The tissue contour was segmented by dividing the 360 degrees around the center into 250 segments. The inner boundary of the peripheral region was defined as segments with positions 1.24 microns from the outer contour. Connexin-protein expression density of the periphery was calculated from the region encompassed between outer and inner boundaries of the normalized initial projection images.

**Central region:** The 3D-volume was divided into sub-volumes, each containing a gap junction, and the intensity was normalized. A binary 3D-matrix was constructed by thresholding with “on” voxels having a level greater than 0.15. Signal-filtering was performed by cluster analysis of the binary matrix to exclude signals from other gap junctions, with a threshold distance of 3 μm. The filtered binary matrix was then skeletonized to decrease the total positions in the surface-fitting step. The gap junction surface was approximated by a set of non-constrained Delaunay triangles (Qhull algorithm) from the “on” positions of the skeletonized
binary matrix. The intensity level of each triangle was set as the mean of vertex intensities. The central region was determined as the inner area 1.8 microns away from the surface boundary. Connexin-protein density was calculated as the sum of triangle intensities multiplied by their surface areas divided by total surface area.

**ECG Recording, AH and HV-Interval Measurements**

Standard ECG leads were recorded to determine QRS-duration. ECGs were recorded once in control dogs, and twice (at baseline and after 2 weeks of VTP) in CHF dogs. QRS-durations were based on averages of 5 consecutive complexes in sinus rhythm. AH and HV-intervals were recorded with intracardiac quadripolar catheters (Biosense-Webster). Bipolar signals were amplified, filtered (40 to 400 Hz) and recorded with a digital recording system (Bloom Associates).

**Statistical Analysis**

Average data are expressed as mean±SEM. Non-paired Student t-tests were used to compare control and CHF groups. Two-tailed $P<0.05$ indicated statistical significance. When multiple recordings were obtained from individual dogs, test-of-significance statistics were based on average values in each dog, i.e. using each dog as an n of 1, to reflect the non-independence of repeated recordings in each animal.

**Estimation of the average intercalated disk area**

The intercalated disk area of purkinje cardiomyocytes was estimated starting from the outer contour of each gap junction (see the periphery subsection of the Materials and Methods section
for details about the contour evaluation). An ellipse was fitted on the contour using a direct least-square algorithm (Halíř R., Flusser J., Numerically stable direct least squares fitting of ellipses, Proceedings of the 6th International Conference in Central Europe on Computer Graphics and Visualization. WSCG '98, 125-132, 1998) implemented in Matlab (The Mathworks, inc). The surface (S) was calculated using the equation $S=\pi ab$ where $a$ and $b$ are respectively the long and short radius of the fitted ellipse.

**Evaluation of the total number of connexins plaques per intercalated disk**

A 5x5 $\mu m^2$ window was manually positioned inside the gap junction contour. The 2D image of the connexin intensity were transformed in the binary image with “on” pixels corresponding to sites with normalized intensity greater than 0.25. The number of plaques inside the square window was calculated in each binary image assuming independent clusters from the 8-connected neighbourhood. The density of plaques ($D_P$) was then calculated as the number of plaques in the square window divided by its surface ($25 \ \mu m^2$). The total number of plaques was then estimated by $D_P$ multiplied by $S$, the ellipse surface.
Reference

ONLINE TABLE I. Gene-specific primers and Taqman probe sequences used in real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
<th>Probe sequence</th>
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<td>Kv1.4</td>
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### Primary Antibodies

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<thead>
<tr>
<th>Protein</th>
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<th>Company</th>
<th>Catalog Number</th>
<th>Dilution</th>
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<tbody>
<tr>
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<td>Santa Cruz</td>
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<tr>
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<td>Dr James Trimmer</td>
<td></td>
<td>1/1000</td>
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<tr>
<td>MinK</td>
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<td>Dr Jacques Barhanin</td>
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### Secondary Antibodies

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<td>Donkey anti-Goat IgG</td>
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## ONLINE TABLE IV. Hemodynamic indices at terminal study

<table>
<thead>
<tr>
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<th>Control (n=15)</th>
<th>CHF (n=16)</th>
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</thead>
<tbody>
<tr>
<td>Systolic BP (mmHg)</td>
<td>136±7</td>
<td>108±5**</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>77±3</td>
<td>67±4</td>
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<tr>
<td>mean BP (mmHg)</td>
<td>91±5</td>
<td>76±5*</td>
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<tr>
<td>LVEDP (mmHg)</td>
<td>4.0±0.4</td>
<td>12.7±0.9***</td>
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<tr>
<td>mean RAP (mmHg)</td>
<td>3.4±0.6</td>
<td>6.6±0.6***</td>
</tr>
<tr>
<td>mean LAP (mmHg)</td>
<td>4.1±0.6</td>
<td>9.0±0.5***</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001 versus control. BP: femoral arterial blood pressure; LVEDP: LV end-diastolic pressure; LAP and RAP: LA and RA mean pressure.
Online Figure II

A control

B CHF

C

D}

E

F

ns

ns

ns

ns
Online Figure legends

Online Figure I. Example of a preparation for study. A: Tissue bath with preparation mounted. B: Higher-power view of preparation. SE=stimulating electrode; E1, E2 are recording microelectrodes; PF=Purkinje fiber false tendon.

Online Figure II. Average intercalated disk surface area and number of connexins plaques per intercalated disk. A: representative fit of the intercalated disk contour with an ellipse using a direct square algorithm from a maximum projection en-face view in control conditions (a and b are respectively the long and short axis of the fitted ellipse). The example shown was analyzed for Cx43-staining. (Cx43-staining intensity is graded according to the scale at the right, with intensity increasing as colors progress from blue through yellow towards red.) B: similar example in CHF conditions. (Cx43-staining intensity is graded in a similar fashion to that shown in panel A.) C: Mean±SEM intercalated disk surface, calculated based on Cx43-stained images. Very similar results were obtained based on Cx40 and Cx43-P images. D: Mean±SEM Cx43 plaque number per intercalated disk. E: Mean±SEM phospho-368-Cx43 plaque number per intercalated disk. F: Mean±SEM Cx40 plaque number per intercalated disk. Control: n=13 disks/4 dogs; CHF: n=11 disks/5 dogs.