Focal Energy Deprivation Underlies Arrhythmia Susceptibility in Mice With Calcium-Sensitized Myofilaments

Sabine Huke, Raghav Venkataraman, Michela Faggioni, Sirish Bennuri, Hyun S. Hwang, Franz Baudenbacher, Björn C. Knollmann

Rationale: The Ca\(^{2+}\) sensitivity of the myofilaments is increased in hypertrophic cardiomyopathy and other heart diseases and may contribute to a higher risk for sudden cardiac death. Ca\(^{2+}\) sensitization increases susceptibility to reentrant ventricular tachycardia in animal models, but the underlying mechanism is unknown.

Objective: To investigate how myofilament Ca\(^{2+}\) sensitization creates reentrant arrhythmia susceptibility.

Methods and Results: Using hypertrophic cardiomyopathy mouse models (troponinT-I79N) and a Ca\(^{2+}\) sensitizing drug (EMD57033), here we identify focal energy deprivation as a direct consequence of myofilament Ca\(^{2+}\) sensitization. To detect ATP depletion and thus energy deprivation, we measured accumulation of dephosphorylated Connexin 43 (Cx43) isoform P0 and AMP kinase activation by Western blotting and immunostaining. No differences were detected between groups at baseline, but regional accumulation of Connexin 43 isoform P0 occurred within minutes in all Ca\(^{2+}\)-sensitized hearts, in vivo after isoproterenol challenge and in isolated hearts after rapid pacing. Lucifer yellow dye spread demonstrated reduced gap junctional coupling in areas with Connexin 43 isoform P0 accumulation. Optical mapping revealed that selectively the transverse conduction velocity was slowed and anisotropy increased. Myofilament Ca\(^{2+}\) desensitization with blebbistatin prevented focal energy deprivation, transverse conduction velocity slowing, and the reentrant ventricular arrhythmias.

Conclusions: Myofilament Ca\(^{2+}\) sensitization rapidly leads to focal energy deprivation and reduced intercellular coupling during conditions that raise arrhythmia susceptibility. This is a novel proarrhythmic mechanism that can increase arrhythmia susceptibility in structurally normal hearts within minutes and may, therefore, contribute to sudden cardiac death in diseases with increased myofilament Ca\(^{2+}\) sensitivity. (Circ Res. 2013;112:1334-1344.)

Key Words: conduction velocity dispersion ■ Connexin 43 ■ familial hypertrophic cardiomyopathy ■ myofilament Ca\(^{2+}\) sensitivity ■ sudden cardiac death ■ ventricular arrhythmias

Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac disease with a prevalence of about 1 in 500. In most cases, the underlying molecular defect is an autosomal-dominant mutation in a sarcomeric protein (disease of the sarcomere). Mutation carriers have an increased risk for ventricular arrhythmia and sudden cardiac death (SCD), but how sarcomeric mutations predispose to ventricular arrhythmias and SCD remains unclear.

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With few exceptions, proteins with HCM mutations integrate together with wild-type protein into the sarcomere thereby altering the force-producing process. Through an as yet unidentified pathway, this initiates progressive structural changes that typically involve hypertrophy, focal and interstitial fibrosis, and myofibrillar disarray, but with very large individual variability. The structural remodeling likely creates an anatomic substrate and contributes to the increase in arrhythmia susceptibility in HCM. However, SCD risk assessment on the basis of structural features alone is inaccurate, and current algorithms to classify individual patients are unreliable. Hence, additional factors likely contribute to arrhythmia risk, and there is need to investigate the molecular events that cause SCD.

Considerable effort has been invested into identifying a common sarcomere property conferred by HCM mutations, which would explain the similar downstream effects of the many different HCM mutations. A frequent finding among HCM mutations is increased myofilament Ca\(^{2+}\) sensitivity, which is also observed in acquired conditions associated with arrhythmias. Myofilament Ca\(^{2+}\) sensitization has several immediate downstream effects and impacts Ca\(^{2+}\) homeostasis (by affecting cytosolic Ca\(^{2+}\) buffering), mechanics (positive inotropic, negative lusitropic), and energy demand (proportional increase in myosin ATPase...
activity), as reviewed. We have previously demonstrated that myofilament Ca\(^{2+}\) sensitization is proarrhythmic, in part using transgenic mouse models that express human troponin T (TnT) HCM mutations. The degree of Ca\(^{2+}\) sensitization correlated with the risk for ventricular reentrant-type arrhythmias (eg, the highest arrhythmia risk was found in mice with the most sensitized myofilaments: TnT-I79N). \(\text{Ca}^{2+}\)-sensitized hearts exhibited an increased dispersion of conduction velocity (CV; ie, regional CV slowing), which is a highly proarrhythmic state, but was not the result of structural remodeling in the TnT mutant hearts. Accordingly, treating control mice with the Ca\(^{2+}\) sensitizer drug EMD57033 (EMD) increased CV dispersion and induced arrhythmias, whereas a compound with Ca\(^{2+}\) desensitizing properties, blebbistatin (BLEB), prevented CV dispersion and arrhythmia induction during rapid pacing. Thus, increasing myofilament Ca\(^{2+}\) sensitivity raises arrhythmia risk, and lowering Ca\(^{2+}\) sensitivity to control level prevents the occurrence of arrhythmias. How myofilament Ca\(^{2+}\) sensitization causes increased CV dispersion and arrhythmias is not understood, and the objective of this study was to investigate the underlying pathway.

Here, we used the TnT-I79N mouse model, EMD, and BLEB to determine the link between myofilament Ca\(^{2+}\) sensitivity and arrhythmias. We identify regional energy deprivation as a direct consequence of myofilament Ca\(^{2+}\) sensitization. Subsequent focal gap junctional uncoupling likely contributes to regional CV slowing forming the substrate that can support reentry. This mechanism generates arrhythmia susceptibility in structurally normal hearts within a short period of time and can cause lethal arrhythmias.

**Methods**

A detailed description of all procedures and information about mouse models and drugs can be found in the online Data Supplement.

**Results**

**Arrhythmias in TnT-I79N Mice In Vivo Are Associated With Altered Cardiac Gap Junctions**

To investigate the mechanism responsible for the ventricular arrhythmias in the TnT-I79N HCM mouse model, we used a previously established catecholamine challenge protocol (isoproterenol [Iso], 1.5 mg/kg). TnT-I79N mice frequently exhibited ventricular ectopy (56%) and ventricular tachycardia (VT, 25%), whereas only 1 mouse expressing wild-type TnT (TnT-WT) had 1 single premature ventricular contraction (Figure 1B; \(P<0.006\) by Fisher exact test). The heart rate increase was the same in both groups (from 464±11 to 620±6 in TnT-WT compared with 457±12 to 624±16 bpm in TnT-I79N). ECG analysis revealed a progressive prolongation of QRS duration in TnT-I79N mice after Iso injection (Figure 1C). A longer QRS duration reflects a delay in whole heart activation, which may be a consequence of the slower regional conduction previously reported.\(^{17}\)

Gap junction channels are important for conduction properties, they lower membrane resistance by directly connecting the cytoplasm of neighboring cells and thus facilitate electric propagation. Thus, we investigated the major gap junction–forming protein expressed in ventricle, Connexin 43 (Cx43), in hearts collected after Iso injection and found a lower expression (72±6%; Figure 1E; for baseline expression comparison see Figure 3C and 3D). Cx43 has several phosphorylation sites, and at least 3 different phosphorylation states of Cx43 are separated by SDS-PAGE (marked as P2, P1, and P0 indicated) after Iso treatment.

![Figure 1. Isoproterenol (Iso) injection in vivo induces ventricular arrhythmias, slowed ventricular depolarization, and is associated with reduced expression of the gap junction protein connexin 43 (Cx43) in troponin T (TnT)-I79N mice.](https://example.com/figure1.jpg)

**Figure 1.** Isoproterenol (Iso) injection in vivo induces ventricular arrhythmias, slowed ventricular depolarization, and is associated with reduced expression of the gap junction protein connexin 43 (Cx43) in troponin T (TnT)-I79N mice. A, Representative examples for a premature ventricular contraction (PVC) and ventricular tachycardia (VT). Summary data are presented in B, n=16 each. C, QRS duration was prolonged within a few minutes after Iso injection in TnT-I79N, but not TnT-wild-type (WT) mice. D, Typical Western blot pattern for anti-Cx43 (isoforms P2, P1, and P0 indicated) after Iso treatment. E, Total expression of Cx43 and (F), P0/P2 isoform ratio analyzed by Western blot. n, numbers are indicated in columns and are the same for E and F. *\(P<0.05\) vs WT.
as dephosphorylated Cx43. The ratio between the Cx43 isoforms P0/P2 was increased after Iso treatment (Figure 1F), indicating that in addition to the overall Cx43 decrease, altered phosphorylation may be associated with changes in gap junctional function.26

Rapid Pacing Induces VT, Prolongs QRS Duration, and Increases Anisotropy Ratio in TnT-I79N Hearts

To further dissect the mechanism responsible for the QRS prolongation and ventricular arrhythmias, we used a rapid pacing protocol in isolated hearts that led to VT in >50% of TnT-I79N, but not in wild-type TnT (TnT-WT) and was prevented by blebbistatin (BLEB, 3 μmol/L; n=3–18). Online Figure IC shows an example for VT induced during rapid pacing in an isolated TnT-I79N heart. Similar to our findings in vivo after Iso, rapid pacing of isolated hearts caused progressive QRS prolongation in TnT-I79N hearts but not in TnT-WT (Figure 2A). QRS prolongation was prevented by the Ca2+ desensitizer BLEB, which at 3 μmol/L reduces myofilament Ca2+ sensitivity of TnT-I79N hearts to that of nontransgenic control hearts.17 We next performed optical mapping and measured the CV along the fiber (longitudinal [CVL], fast) and across the fiber (transverse [CVT], slow) as illustrated in Figure 2B.17 CVT in TnT-I79N was similar to control (Figure 2C), consistent with previous observations that the average CV is not altered.17 However, CVT was reduced by >20% in TnT-I79N mice compared with TnT-WT (Figure 2C), which resulted in an increased anisotropy ratio (CVL/CVT). CVT slowing and increased anisotropy were prevented by pretreatment with BLEB (Figure 2D).

Because the anisotropy ratio and, in particular, CVT are strongly influenced by the number, activity, and spatial arrangement of gap junctions,27,28 we next investigated gap junction properties in detail at the biochemical level.

Rapid Changes in Cx43 Abundance, Solubility, and Isoform Distribution in TnT-I79N Hearts

Gap junctions are formed by connexins; the only connexin isoform expressed in adult ventricular myocardium is Cx43.29 The junctional (=insoluble) Cx43 is found at the cell surface forming gap junction channels at the intercalated disc, whereas the nonjunctional (=soluble) Cx43 is intracellular. Cardiac tissue was fractionated into 3 fractions (the insoluble fraction was divided into a low spin and high spin fraction) to measure the relative distribution between...
junctonal (light and dark gray) and nonjunctional Cx43 (white) as illustrated in Figure 3A. The pellet is discarded after each processing step and the supernatant analyzed via Western blot. α-Tubulin is completely soluble and illustrates equal loading of all fractions. The distribution of junctional:nonjunctional Cx43 before pacing was ≈50:50 (Figure 3B) and not significantly different between TnT-WT and TnT-I79N mice. After pacing (see Online Figure IA for protocol), the control groups (nontransgenic and WT) tended to have increased junctional Cx43, whereas TnT-I79N tended to have decreased junctional Cx43 (Figure 3B), although neither result was significantly different from baseline. However, there was a difference between nontransgenic control and TnT-I79N after pacing because they changed into opposite directions. This effect was not observed in the presence of BLEB. Increased solubility of Cx43 has been described in end-stage human ischemic cardiomyopathy and was detected here also after global ischemia (Figure 3B).

Next, the total expression levels of Cx43 were analyzed in the low spin supernatant, while loading equal amounts of total protein as shown in Figure 3C. Cx43 protein levels were the same before pacing (baseline) in both groups, but pacing caused a reduction in Cx43 in the TnT-I79N (94.8±12.2 before pacing and 69.2±5.1 after pacing; Figure 3D), whereas Cx43 levels were not affected by pacing in TnT-WT (100±4.8 before pacing and 97.8±15.4 after pacing). BLEB prevented the pacing-induced decrease in Cx43 in the TnT-I79N (Figure 3D).

We also analyzed the Cx43 isoform distribution as in Figure 1. No difference was observed before pacing, but pacing induced distinct changes in the Cx43 isoform distribution in TnT-I79N: a significant decrease in P2 isoform in TnT-I79N compared with baseline (61.5±5.1 versus 93.7±12.9, respectively), but no change in P1 and a moderate increase in P0 with pacing (136.8±9.3 versus 106.5±10.5; n, numbers as in columns). Together, this caused an increase in the P0/P2 ratio in TnT-I79N after pacing that was prevented by BLEB (Figure 3E). Pacing does not induce changes in TnT-WT, and earlier experiments with a slightly different pacing protocol showed no effect of BLEB on Cx43 expression and isoform distribution in TnT-WT (data not shown).

Taken together, at baseline no differences in Cx43 solubility, amount, and isoform distribution exist between groups. After rapid pacing, the solubility of Cx43 increases in TnT-I79N and isoform P2 is specifically lost, which is the phospho-isofom considered to be part of the functional gap junctional plaque. These results indicate a decrease in Cx43 at the intercalated disc induced by rapid pacing similar to what we observed after Iso treatment in our in vivo model (Figure 1D–1F).

**Focal Accumulation of Cx43-P0 in TnT-I79N During Rapid Pacing**

To determine the effect of rapid pacing on gap junction localization, frozen heart sections were obtained before and after pacing and immunostained with anti-Cx43 antibodies, specific for either total Cx43 or the lower phosphorylated and fast migrating isoform of Cx43 (Cx43-P0; Figure 4A and 4B). Cx43-P0 isoform staining was barely detectable, but typically homogeneous before pacing (Figure 4A) or after pacing in TnT-WT or TnT-I79N+BLEB (Figure 4B, top). In TnT-I79N, however, areas of intense staining for P0 isoform were observed after pacing (2 examples; Figure 4B, bottom). The distribution of Cx43-P0 was nonhomogeneous, with small regions of varying shape, size, and intensity (patches) where Cx43-P0 accumulated (characterized by high P0/Cx43 ratio; Figure 4D, red/green). The patches were often found in endocardial regions, whereas accumulation in epicardial and midmyocardial tissue seemed less frequent. Areas with Cx43-P0 accumulation had for the most part clear borders with abrupt transition to areas with low level of Cx43-P0 staining.

The measurement of Cx43 expression levels by immunostaining (Figure 4C, green) was consistent with the results predicted from the Western blot experiments depicted in Figure 3. No significant difference between TnT-I79N and control hearts was observed before pacing (integrated Cx43 density 3094±640 versus 3692±586 in TnT-WT versus TnT-I79N, respectively), but after pacing the amount of total Cx43 was lower in TnT-I79N than in TnT-WT hearts (3800±426 in TnT-WT versus 2143±399 in TnT-I79N; Figure 4B and 4C). This difference in the total Cx43 is the result of an opposite trend in TnT-I79N and TnT-WT; the TnT-WT tended to increase Cx43 at the intercalated disc (>20%↑), whereas the TnT-I79N reduced it during pacing.
and, as in TnT-I79N, VT first occurred after a time delay (see Online Figure IB). Analogous to the results in TnT-I79N hearts (Figure 4B), EMD caused focal accumulation of Cx43-P0 (patches; Figure 5D, middle and right; see also Figure 5F). In contrast, the decrease in Cx43 density at the intercalated disc was not observed in EMD-treated hearts, indicating that this reduction is not a prerequisite for the development of arrhythmias (Figure 5E). These results indicate that myofilament Ca\(^{2+}\) sensitization, via patchy Cx43-P0 accumulation, is likely the key molecular property that is responsible for the increased arrhythmia susceptibility.

We further characterized changes in CV induced by EMD treatment (Online Figure IV). Optical mapping revealed that the transverse CV decreased within minutes after perfusion with EMD (Online Figure IVA). This led to increased anisotropy which was prevented by the presence of BLEB (3 μmol/L; Online Figure IVB). After longer perfusion with EMD we detected conduction abnormalities consistent with the formation of conduction blocks (example shown in Online Figure IIC). Similar to the TnT-I79N hearts, pacing according to the protocol in Online Figure IA led to moderate QRS prolongation over time, whereas no prolongation was observed with vehicle only (Online Figure IID).

**Myocardial Regions With Cx43-P0 Accumulation Are Energetically Compromised**

What causes the rapid focal Cx43 dephosphorylation detected as Cx43-P0 accumulation? The only other condition that has been described to induce very rapid changes in Cx43 is acute ischemia.\(^{31}\) Ischemia has several downstream effects, but an elegant study by Turner et al\(^{32}\) demonstrated that the trigger for the Cx43 dephosphorylation during ischemia is the drop in cellular ATP levels. Thus, the Cx43 dephosphorylation observed here is possibly a marker for intracellular ATP depletion. To further confirm that low ATP levels are also the underlying mechanism for Cx43 dephosphorylation in this study, we measured AMPK-dependent kinase (AMPK) activation. We assessed cellular ATP levels by testing AMPK activation: When [ATP] drops and subsequently [AMP] rises, AMP binds to AMPK-\(\gamma\) subunit and promotes phosphorylation of the \(\alpha\)-subunit in the activation loop at threonine 172 (phosphorylated AMPK [pAMPK]).\(^{33–36}\)

Before pacing, Western blotting of whole heart homogenates detected no difference in \(\alpha\)AMPK-T172 (pAMPK) phosphorylation and was similarly low in TnT-WT and TnT-I79N (Figure 6A and 6B). Rapid pacing increased pAMPK to 225±47% in TnT-I79N, and BLEB prevented the increase in pAMPK in TnT-I79N with pacing. TnT-I79N hearts exposed to 21 minutes of global no-flow ischemia served as a positive control (Figure 6B and 6C). Any differences in pAMPK signal were not caused by changes in \(\alpha\)AMPK expression owing to pacing or ischemia (data not shown).

After rapid pacing challenge, the tissue distribution of pAMPK was heterogeneous in TnT-I79N hearts, and the highest intensity was found in the regions where Cx43-P0 accumulated (Figure 6C, green). No regions with intense
pAMPK staining were observed in TnT-WT, nontransgenic, or hearts treated with BLEB. No-flow ischemia also activated AMPK, but the distribution was largely homogeneous (Figure 6C, bottom right). These data indicate that the myocardium in the areas of Cx43-P0 accumulation is energetically compromised.

**Assessment of Gap Junctional Coupling Using Lucifer Yellow Dye Spread**

Next we assessed whether the accumulation of Cx43-P0 is associated with altered gap junctional function using a modified scrape loading technique. Monitoring the passage of fluorescent dyes through gap junctions from one cell to another is a simple and rapid technique to assess intercellular communication. Lucifer yellow (LY) spreads from the site of injury through gap junctions to the neighboring cells, the faster it diffuses the more are cells interconnected. The LY spread distance through intact cardiomyocytes in the longitudinal direction was 364±34 μm and in the transverse direction 135±14 μm in TnT-WT after pacing (Figure 7A–7C and 7K). For TnT-I79N, sites were classified as Cx43-P0 accumulation negative or positive if Cx43-P0 accumulation was detected along at least 2 sides (Figure 7A–7K). Sites that could not be clearly classified were not included in the analysis. Consistent with the lower amount of Cx43 found in TnT-I79N compared with TnT-WT after pacing (reduced by ≈43%), LY dye spread was reduced in TnT-I79N as well (Cx43-P0 accumulation negative): in the longitudinal direction was 182±18 μm and in the transverse direction 86±10 μm (both P≤0.05 versus TnT-WT). This is a 50% reduction in the longitudinal direction and ≈36% reduction in the transverse direction compared with TnT-WT. Intercellular coupling was even further reduced in Cx43-P0 accumulation positive areas: the LY spread was 157±35 in the longitudinal direction (>12% ↓; not significant versus Cx43-P0 negative) and, strikingly, only 34±4 μm in the transverse direction (>60% ↓; P≤0.05 versus TnT-I79N Cx43-P0 negative). The gap junctional coupling in Cx43-P0 positive areas, when compared with TnT-WT, is reduced >56% in the longitudinal direction and >74% in the transverse direction. Thus, Cx43-P0 identifies areas where significant intercellular uncoupling has occurred.

**Discussion**

This study investigates the underlying mechanisms for the increased occurrence of reentrant arrhythmias in myofilament Ca2+-sensitized hearts. Here, we show that myofilament Ca2+ sensitization predisposes to the generation of focal energy deprivation during stress. Myocytes in the affected regions display signs of metabolic stress, as evidenced by AMP kinase activation and Cx43-P0 accumulation. To test the dependence of patchy Cx43-P0 accumulation on myofilament Ca2+ sensitization, we used different models and protocols: Ca 2+-sensitized mutant hearts show this phenomenon in isolated perfused hearts as well as in vivo, it can be induced by Ca2+ sensitizing drugs in controls and is prevented by Ca 2+ desensitization. Thus, the formation of patchy regions with ischemia-like changes during high heart rates seems to be a direct downstream effect of increased myofilament Ca2+ sensitivity. We further demonstrate that gap junctional coupling is reduced in areas with Cx43-P0 accumulation to a sufficient degree to affect CV (see below). The heterogeneous Cx43-P0 accumulation may lead to heterogeneous CV slowing (increased dispersion of CV),
which constitutes a highly proarrhythmic state predisposing to conduction block and reentry. These new results are in agreement with our previous report that increased CV dispersion is induced by rapid pacing in Ca^{2+}-sensitized hearts. The induction of focal energy deprivation that rapidly decreases gap junctional coupling in Ca^{2+}-sensitized hearts is a novel proarrhythmic mechanism that can generate arrhythmia susceptibility in structurally normal hearts within minutes.

Critical Role of Focal Cx43 Dephosphorylation for Arrhythmias?

We find a strong association between focal Cx43-P0 accumulation and arrhythmias. The focal Cx43-P0 accumulation is found under all conditions with raised arrhythmia susceptibility, but we did not detect Cx43-P0 accumulation in any conditions without increased arrhythmia susceptibility. In TnT-I79N mice, we find in addition to focal Cx43-P0 accumulation also a decline in Cx43 levels and both may contribute to arrhythmia generation. However, in EMD-treated hearts we only observed focal Cx43-P0 accumulation, not an overall Cx43 decrease (Figure 5E), but the same type and time of onset for the arrhythmias (Online Figure IB), indicating the Cx43-P0 accumulation is potentially critical.

How is intercellular coupling affected by Cx43-P0 accumulation? We know that the accumulation of Cx43-P0 is accompanied by junction closure (eg, in ischemia). Studies by Lampe et al indicate that unphosphorylated S365 is likely the critical feature for Cx43-P0–specific antibody binding. Phosphorylation at S365 is lost during hypoxia and this dephosphorylation is at the same time a prerequisite for PKC-dependent phosphorylation at S368, which reduces gap junctional conductivity. Here, we also directly demonstrate that in areas with Cx43-P0 accumulation the gap junctional coupling is reduced as measured by LY dye spread. It seems that Cx43-P0 is an excellent marker for cellular uncoupling, whether the exact molecular change that generates it is responsible for it or not.

Can the patchy gap junctional uncoupling affect CV? The number of gap junction channels is typically much larger than needed for excitation propagation. This is consistent with our finding that the CV (this study) or average CV is unaltered. Studies using mice with 50% decrease in gap junctions do not show a decrease in CV.
either no effect on CV,41,42 or a reduction of ≈25%.43,44 A loss of 90% of gap junctions leads to an ≈50% decrease in CV.45 The reduction in dye spread in this study in the areas with Cx43-P0 accumulation was 56% (longitudinal) and 74% (transverse), which is a range where effects on CV can be expected. Gap junctional uncoupling preferentially affects conduction in the transverse direction.46,47 It is quite possible that the patchy gap junctional uncoupling may importantly contribute to the decrease in CV during pacing demonstrated in this study.

A role of focal gap junction changes for reentrant arrhythmias? In general, regional gap junctional uncoupling can produce or unmask preexisting heterogeneities in cellular refractoriness and CV that can contribute to slow conduction and reentry. Similar changes in gap junctions occur in hypoxia or ischemia, and their role for arrhythmogenesis has been reviewed elsewhere.19,48,49 The arrhythmia susceptibility may arise from the increased anisotropy, predisposing to unidirectional conduction block and reentry, although the exact mechanism is debated.48 Another possible mechanism is that the abrupt exciton spread from poorly coupled into well coupled tissue may facilitate conduction block via a source-sink mismatch and assist in the generation of reentry.50

ATP Depletion as Cause for Cx43 Dephosphorylation

The rapid regional changes in Cx43 phosphorylation in myofilament Ca2+-sensitized hearts and the simultaneous AMPK activation are a striking result of this study. It is well established that ATP depletion is a mechanism that can cause rapid Cx43 dephosphorylation: Metabolic inhibition is frequently used experimentally to induce Cx43 dephosphorylation and it reduces gap junctional communication in cortical astrocytes.51,52 Beardslee et al.53 provided the first evidence for Cx43-P0 accumulation in the intact perfused heart during global ischemia. The most direct evidence that ATP depletion is the upstream requirement for Cx43 dephosphorylation was later provided by Turner et al.54 They dissected the individual contributions of hypoxia, extracellular factors (acidosis, build-up of metabolites), glucose, and ATP depletion to dephosphorylation of Cx43. They found that Cx43 dephosphorylation closely mirrored fluctuations in cellular ATP levels, requiring >50% of basal ATP levels to maintain Cx43 phosphorylation state. How ATP levels mediate the effect on Cx43 phosphorylation has not been solved. Turner et al.,54 however, also provide evidence that pharmacological activation of AMPK does not lead to Cx43 dephosphorylation. This demonstrates that these 2 events are not linked and that they are independent indicators of ATP depletion. AMPK phosphorylation primarily relies on AMP binding to cause a conformational change that then promotes increased phosphorylation of T172, but other pathways have been demonstrated. Recent evidence suggests that AMPK activity can be regulated receptor-dependent and dissociated from the energy charge of the cell (eg, by hormones like adiponectin or estrogen).53,54 It is unclear how such mechanisms would play a role in our study. Thus, the focal accumulation of Cx43-P0 and corresponding elevated levels of pAMPK

found in this study both indicate that intracellular [ATP] levels are decreased.

Why Is Cx43 Dephosphorylation Focal?

The focal nature of Cx43 dephosphorylation and AMPK activation indicates that in some areas demand exceeded supply, but we have not yet elucidated the origins of this mismatch. On one hand, the deficit may lie on the supply side and may be because of insufficient perfusion volume, whereas, on the other hand, the deficit may result from increased demand because of pathologically high energetic requirements of Ca2+-sensitized cardiac muscle. The positive inotropic effect conferred by myofilament Ca2+ sensitization will increase myosin ATPase activity and thereby proportionally increase energy demand.14,55 Increased heart rates can be expected to further raise energy demand. Moreover, increased tension cost (higher energetic cost of force production) has been described for HCM sarcomeric mutations, including TnT mutations.56 It is, however, known that the racemic mixture EMD53998 (50% EMD57033) has no effect on tension cost and actually lowers tension cost in the presence of inorganic phosphate.57 How myofilament Ca2+ sensitivity may impact supply is less clear, but the negative lusitropic effect and increased extramural compression may have negative effects on perfusion. In the heart, in contrast to all other organs, coronary flow is slower during phases of contraction than during diastole, and thus prolonging the duration of contraction would naturally impinge on coronary flow. Coronary perfusion also greatly relies on pressure waves and the largest is a suction wave generated by relaxation of the left ventricle, a critical driver of diastolic coronary blood flow.58 How these effects alone or in combination lead to energy deprivation in some regions and not others requires further studies, but the exact regional balance between raised demand and compromised supply may result in the observed phenotype. It is an intriguing feature of HCM that although the genetic mutation is expressed throughout the ventricle, fibrosis is often patchy and the hypertrophy asymmetrical or even focal.59,60

Relevance for Human Disease

There are important implications of this novel mechanism that extend beyond HCM patients and concern all patients with increased myofilament Ca2+ sensitivity. For example, it is well documented that myofilament Ca2+ sensitivity increases in patients after myocardial infarction.61,62 More mechanistic insights may also benefit HCM patients by improving risk stratification for arrhythmias, which is currently challenging and unreliable. Arrhythmia risk greatly varies between individual HCM mutation carriers, even between individuals with the same mutation and from the same family. The identification of new risk factors and how different risk factors may act synergistically to produce arrhythmias is clearly needed. Along the same lines, pharmacological strategies to prevent sudden death in HCM patients have not been very successful.63,64 and comprehensive understanding about the underlying mechanisms may lead to the development of much needed new treatments.
This molecular mechanism will also inform our efforts to develop new positive inotropic drugs for the treatment of heart failure. One new compound that was recently tested in phase 2 clinical trials is omecamtiv mecarbil, a myosin activator that prolongs systolic ejection time and increases stroke volume.55 The mechanism is promising, increasing the number of cross-bridges and enhance contraction without effect on the contraction rate and myocardial oxygen consumption.56 However, at high serum concentrations, ischemic episodes were triggered in a few healthy patients, potentially because of the shortening of the diastolic interval. More studies are needed.57

Conclusions

In this study, we present evidence that myofilament Ca\textsuperscript{2+} sensitization can cause focal energy deprivation during stress. This pathway can be activated within minutes and may explain the occurrence of SCD in young HCM patients even before significant structural remodeling occurs. This strongly suggests that lowering myofilament Ca\textsuperscript{2+} sensitivity to control level may be a promising new strategy to prevent ventricular arrhythmias and SCD. The mouse model and tools used in this study present the unique opportunity to investigate in the future the underlying cause for the regional energy deficit (ie, the contribution of supply and demand and test possible treatments).

Interestingly, the 2 properties that have been both independently proposed to constitute the critical impairment conferred by HCM sarcomeric mutations are (1) increased myofilament Ca\textsuperscript{2+} sensitivity and (2) increased energy cost of force production.58-70 As we demonstrate in this study, the pathological effect of increased myofilament Ca\textsuperscript{2+} sensitivity is linked to focal energy deprivation and this may at least in part unify both theories.

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Disclosures

None.

References


**Novelty and Significance**

Increased sensitivity of the myofilaments to Ca\(^{2+}\) has been linked to increased arrhythmia susceptibility and may contribute to sudden cardiac death. The present study aimed to determine how myofilament Ca\(^{2+}\) sensitization generates a myocardial substrate that promotes reentry arrhythmias. We Ca\(^{2+}\) sensitized myofilaments in mice either by expressing a mutant cardiac troponin-T or by treatment with a Ca\(^{2+}\) sensitizing drug, EMD57033, and in reverse desensitized myofilaments using blebbistatin. When hearts were stressed, reentry arrhythmias were observed after a few minutes delay. By measuring Cx43 and AMP-dependent kinase phosphorylation sensitive to cellular ATP levels, we identified myocardial regions of energy deprivation that developed within minutes only in Ca\(^{2+}\)-sensitized hearts. In the same regions intercellular coupling via gap junctions was reduced to a degree that is likely to slow excitation spread. This can be predicted to lead to conduction velocity heterogeneity, consistent with previously observed regional conduction velocity slowing, a very proarrhythmic state. These results provide new mechanistic insights into the genesis of arrhythmias that could facilitate the development of targeted new treatments to prevent arrhythmias.
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SUPPLEMENTAL MATERIAL

Mouse models and Drugs

All animal procedures were approved by the Animal Care and Use Committee of Vanderbilt University and conformed to 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). The generation of all transgenic mouse lines expressing human troponin T (TnT-WT (line 3)) or mutant TnT (TnT-I79N (line 8)) has been described previously.¹ A total of 232 age-matched mice (9-14 weeks old) of both genders were used for experiments. All lines had been backcrossed for >10 generations into B6SJLF1/J strain and were bred in-house while continuing to use female B6SJLF1/J breeder mice obtained from Jackson laboratories (stock# 100012). Non-transgenic littermates (NTG) were used for experiments with EMD57033 (3 mmol/L stock solution in DMSO; a generous gift of MERCK KGaA, Darmstadt, Germany). Phosphodiesterase inhibition, often a problem with myofilament Ca²⁺ sensitizing compounds, is not observed at the concentration of 3 μmol/L used here.² (-)-Blebbistatin (BLEB) was purchased from Sigma, kept as 30 mmol/L stock solution in DMSO and protected from light. BLEB is an inhibitor of the actin-myosin interaction without any effects on action potential duration.³ Effects of the TnT-I79N mutation, EMD and BLEB on myofilament Ca²⁺ sensitivity were published previously.⁴

Isolated Heart Perfusion / Volume conducted ECG

Hearts were excised after mice were deeply anesthetized with 1.2% Avertin (30 ml/kg) or 3-5% Isoflurane in 100% O₂ and the aorta was cannulated for retrograde perfusion at a constant pressure of 70 mm Hg as described.⁴ The tyrode solution used for perfusion contained in mmol/L: NaCl 139, KCl 4, NaHCO₃ 14, NaH₂PO₄ 1.2, MgCl₂ 1, CaCl₂ 1.5, Glucose 10, S-propanolol 0.0002. Tyrode solution was filtered before use and oxygenated with carbogen (95% O₂/5% CO₂) to achieve pH7.4. Hearts were lowered into a warm bath and volume conducted
ECGs recorded using two silver/silver chloride wire electrodes and a ground lead. ECG was recorded using a custom built pre-amplifier and amplifiers connected to a PowerLab station (ADI instruments) and LabChart5 software. Unless described otherwise, a two-prong platinum pacing wire was inserted near the apex into the left ventricle and hearts paced according to the protocol shown in Fig. I. Pacing was applied via an isolated stimulator connected to a PowerLab (AD instruments) and controlled by LabChart5 software. Stimulus duration was 2.5 ms and current was set to 30% above threshold. When applicable, EMD or 0.1% DMSO was added fresh to the perfusion buffer via a syringe pump either to the bubble trap reservoir or heating coil directly above the heart.

**Surface ECG**

Mice were anesthetized with 1.2% Avertin (30 ml/kg, 2,2,2-tribromoethanol/ tertiary amyl alcohol) and placed in prone position on a warm surface essentially as previously published. 24-gauge electrodes were inserted subcutaneously into all four forelimbs and connected to ECG leads. ECG (lead I and II) was recorded using amplifiers connected to a PowerLab station (AD instruments) and LabChart5 software. After a stable baseline was obtained, isoproterenol (Isuprel, 1.5 mg/kg) was injected i.p. and the ECG recordings continued for 12 min. Afterwards the animal was briefly subjected to 3% Isoflurane in 100% oxygen via nose-cone until loss of foot reflex, the heart harvested and rapidly processed.

**ECG Analysis**

The entire protocol was evaluated for the presence of ventricular arrhythmias from digitally stored files by the same investigator blinded to the treatment and/or genotype. Heart rate and QRS interval duration were determined by averaging 3 consecutive beats during sinus rhythm (in isolated paced hearts during short pacing breaks when pacing frequency was switched or pacing briefly stopped). Since mice do not show a flat ST-segment like humans to determine the
QRS duration, the S-peak was used. Traces were then examined for the presence of premature ventricular contractions (PVC) and ventricular tachycardia (VT; 3+ consecutive beats). Manual analysis and semi-automatic analysis using a built-in algorithm of LabChart7 software were compared and results closely matched.

Optical mapping
Hearts were stained with di-4-ANEPPS (Invitrogen, 0.5 mg/ml in DMSO) slowly injected through a port into the bubble trap above the perfusion cannula and fluorescence was excited by a diode-pumped, solid-state laser (Verdi, Coherent, CA) at a wavelength of 532 nm delivered to the heart via optical fibers. The left ventricle was stimulated in the center of the field of view with a platinum pacing electrode (0.1-1 mA, 2 ms duration, 10 Hz). To prevent tissue damage at the contact site, stimulation via this electrode was limited to 3 min. Images were recorded with a high-speed CCD camera (14-bit, 80x80 pixels, 1000 fps, RedShirt Imaging) equipped with a 52 mm standard lens and the emitted light passed through a high pass filter. The imaging area was adjusted and typically about 12×12 mm. Custom developed “C”-based software was used to control data acquisition, stimulus timing and laser illumination as described. Fast (longitudinal) and slow (lateral) CVs were identified by plotting local CVs against orientation. Local CVs were calculated after applying a Gaussian 3x3 spatial filter and 3 ms moving average temporal filter. Isochronal maps were computed by a custom Matlab-based program and activation times plotted versus distance from the center for each angle, plotted and the two fast and slow CVs averaged. No assumptions about the fiber orientation were necessary. Motion artifacts did not play a significant role, because the activation is fast and precedes contraction.

Sample Fractionation/Homogenization
Heart samples for protein analysis had been flash frozen and stored at -80°C. Samples were fractionated following a modified protocol according to Smyth et al. Each heart was pulverized
in a metal mortar cooled in liquid N\textsubscript{2} (Bessman tissue pulverizer) and transferred into a 2 ml Dounce homogenizer flask. Each sample was homogenized with the same number of strokes in 15 vol of homogenization buffer containing in mmol/L: Tris 50, Triton-X 100 1\%, sodium fluoride 10, sodium-ortho-vanadate 5, sodium pyrophosphate 1, \(\beta\)-glycerophosphate 10, pH 7.5 with protease inhibitors (Sigma, P8340) added fresh before use. Afterwards homogenates were agitated for 2 hrs at 4°C. SDS was added to a small aliquot to a final concentration of 1\% and samples flash frozen (= total). The remaining sample was centrifuged at 500\(\times\)g for 10 min at 4°C and an aliquot of the supernatant supplemented with SDS and flash frozen (= low spin). The remaining supernatant was transferred into a new tube and centrifuged at 7000\(\times\)g for 20 min at 4°C. One last aliquot was supplemented with SDS and flash frozen (= soluble). Protein concentration was determined according to Lowry.

Western Blotting

SDS polyacrylamid gels according to Sambrook et al.\textsuperscript{10} were made fresh before electrophoresis essentially as described before.\textsuperscript{11-13} Samples were diluted in homogenization buffer to achieve the same protein concentration, mixed with one volume of Laemmlı sample buffer (BioRad 161-0737) containing 5\% beta-mercaptoethanol and 40 mmol/L dithiothreitol and heated to 55°C for 5 minutes. Proteins were transferred overnight to nitrocellulose (0.45 µm pore size, BioRad) and membranes incubated in 6\% fat free dry milk in Tris-buffered saline containing 0.2% Tween20. The following antibodies were applied in Odyssey Blocking Buffer (LICOR Biosciences, NE): anti-Connexin43 (SIGMA, C6219 polyclonal rabbit), anti-alpha-tubulin (Abcam ab7291 mouse monoclonal), anti-AMP-kinase alpha (Cell Signaling 2603 (23A3) rabbit monoclonal) and anti-phospho Thr 172 AMP-kinase (Cell Signaling 2535 (40H9) rabbit monoclonal). Secondary fluorescent antibodies recognizing rabbit (IRDye 800 conjugated anti-rabbit IgG) or mouse IgG (IRDye 700DX conjugated anti-mouse IgG, both Rockland) were
detected with a LICOR Odyssey near-infrared scanner and signals were analyzed using Odyssey 3.0 software.

**Immunohistochemistry**

Heart tissue was embedded horizontally in OCT and flash frozen in liquid N₂ cooled 2-methylbutane. 10-16 µm thick frozen cross-sections (frontal, four chamber view) were fixed in 4% formaldehyde for 30 min and then washed in phosphate buffered solution (PBS) 3 times for 10 min. After pre-incubation in PBS containing 5% goat serum and 0.4 % Triton X-100, primary antibodies were incubated overnight at 4°C. In addition to the antibodies listed under “Western Blotting”, a mouse monoclonal anti-connexin 43 antibody specific for the P0 isoform was used (Invitrogen 13-8300). This Cx43-P0 antibody was directly labeled with AlexaFluor 488 using the APEX kit according to the manufacturer’s instructions (Invitrogen A10468) or purchased pre-labeled (Invitrogen, 13-8388). The secondary anti-rabbit antibody (Alexa Fluor 568 Goat Anti-Rabbit IgG, Invitrogen) was applied in PBS containing 0.4 % Triton X-100 for 1 hr at RT. Sections were mounted in ProLong Gold antifade reagent (Invitrogen) and cured for 24 hrs before coverslip edges were sealed with nail polish.

**Imaging and Image Analysis**

Two channel composite images of immunostained sections were acquired using a Zeiss LSM 510 inverted confocal scanning microscope (part of the VUMC Cell Imaging Shared Resource (supported by NIH grants CA68485, DK20593, DK58404, HD15052, DK59637 and EY08126)) by an investigator blinded to the treatment and genotype of the sample. Settings to acquire images were identical within sets of samples and only minor adjustments were made between sets. At least five images (0.45x0.45 mm) per section were acquired from all regions of the left ventricle (LV free wall, apex and septum) while avoiding areas containing large vessels and regions where the pacing wire was attached. Image sampling was stratified that if areas with
Cx43-P0 accumulation were found after visual inspection, these areas were included during image acquisition. This reduced the number of images necessary to document these regions ("patches") and prevented false negative results.

Quantitative analysis was performed using ImageJ 1.43 (NIH) using only raw images as illustrated in Fig. II. Background signal was negligible in buffer perfused hearts, but was subtracted from non-perfused hearts. Signal area and signal integrated density both normalized to the area covered by tissue were determined for Cx43 and Cx43-P0 for each image by "thresholding" and conversion into binary images.

**Lucifer Yellow Dye transfer (modified scrape loading)**

Gap junctional function was assessed essentially as described previously.\textsuperscript{14, 15} Isolated perfused hearts were subjected to a standardized pacing protocol (Fig. IA) and then washed for 3 min with Ca\textsuperscript{2+}-free Dulbecco's PBS. The left ventricle was punctured at multiple sites with a 27-gauge needle and a solution injected containing 0.5% Lucifer yellow DH (LY) and 0.3% Tetramethylrhodamine dextran (TMR, MW 10,000, both Invitrogen) in 120 mM LiCl. LY will diffuse through gap junctions, while TMR is not permeable. After 15 min, the left ventricle was fixed in 4% formaldehyde for 30 min, embedded in OCT and flash frozen in liquid N\textsubscript{2} cooled 2-methylbutane. 18 µm sections were immunostained using Cx43-P0 specific antibody and analyzed using a Zeiss LSM 510 inverted confocal scanning microscope. For each needle puncture, three images were obtained detecting LY, TMR and Cx43-P0. Dye spread distance was analyzed using ImageJ, measuring the distance between the border of the TMR signal to the border of the LY signal. Four measurements per puncture were obtained, two for the maximal transverse spread and two for the maximal longitudinal spread on each side, which were then averaged.
**Statistics**

GraphPad Prism 5.0 (GraphPad Software Inc.) or tools from the website [http://vassarstats.net/](http://vassarstats.net/) were used for statistical analysis. Analysis was performed using unpaired two-tailed t-test with Welch’s correction, one-way ANOVA followed by Tukey’s multiple comparisons test, repeated measures ANOVA (mixed model) or Fisher exact probability test as appropriate. All values are mean ± SEM. P values of less than 0.05 were considered significant.
REFERENCES


Fig. 1: Rapid pacing induces VT in Ca\(^{2+}\) sensitized isolated perfused hearts. (A) Experimental pacing protocol to generate samples for all biochemical assays. All isolated perfused hearts were processed (e.g. frozen) after 21 min of perfusion time. ECG was monitored during all experiments and traces analyzed for occurrence of VT. (B) The incidence of VT during pacing ("arrhythmia survival") of isolated hearts. VT was not observed in TnT-WT or NTG and there were also no arrhythmias in TnT-I79N hearts treated with BLEB, but in 63% of TnT-I79N hearts and 52% of EMD treated hearts. (C) Representative ECG trace of a TnT-I79N heart with VT. VT induction in this heart occurred during pacing at 18 Hz.
Fig. II: Quantitative analysis of confocal images. All images were recorded with a Zeiss 510 confocal scanning microscope and analyzed using ImageJ 1.43 (NIH). Each channel was converted into a binary image using the “threshold” function (fixed threshold for each experimental set). The integrated signal density per channel is then calculated from the number of pixels and mean intensity per pixel determined using the histogram function and normalized to the area occupied by cells.
Fig. III: Effect of pacing on Cx43 (“green”). Shown is Cx43 staining only. Representative images for WT and I79N after 3 min of perfusion without pacing (top) and after pacing (bottom) including I79N treated with BLEB. Pacing protocol as shown in Fig. S1A. Summary data are shown in Fig. 3.
Fig. IV: Slowed lateral CV, QRS prolongation and arrhythmias in EMD treated control hearts. Longitudinal (fast) and transverse (slow) conduction velocity (A) and anisotropy ratio (B) in control (vehicle, VEH, 0.1% DMSO) and hearts treated with EMD. N numbers are indicated in columns. (C) Abnormal isochronal map observed after a few minutes of perfusion with EMD. (D) QRS prolongation observed in NTG hearts after perfusion with EMD. QRS duration was measured from sinus beats during short pacing breaks. EMD (3 µM) or vehicle (VEH, DMSO 0.1% final) were added after 5 min of equilibration (as indicated by arrow).