Thin Filament Disinhibition by Restrictive Cardiomyopathy Mutant R193H Troponin I Induces Ca\(^{2+}\)-Independent Mechanical Tone and Acute Myocyte Remodeling

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Abstract—Inherited restrictive cardiomyopathy (RCM) is a debilitating disease characterized by a stiff heart with impaired ventricular relaxation. Mutations in cardiac troponin I (cTnI) were identified as causal for RCM. Acute genetic engineering of adult cardiac myocytes was used to identify primary structure/function effects of mutant cTnI. Studies focused on R193H cTnI owing to the poor prognosis of this allele. Compared with wild-type cTnI, R193H mutant cTnI more effectively incorporated into the sarcomere, where it exerted dose-dependent effects on basal and dynamic contractile function. Under loaded conditions, permeabilized myocyte Ca\(^{2+}\) sensitivity of tension was increased, whereas the passive tension–extension relationship was not altered by R193H cTnI. Normal rod-shaped myocyte morphology acutely transitioned to a “short-squat” phenotype in concert with progressive stoichiometric incorporation of R193H in the absence of altered diastolic Ca\(^{2+}\). The specific myosin inhibitor blebbistatin fully blocked this transition. Heightened Ca\(^{2+}\) buffering by the R193H myofilaments, and not alterations in Ca\(^{2+}\) handling by the sarcoplasmic reticulum, slowed the decay rate of the Ca\(^{2+}\) transient. Incomplete mechanical relaxation conferred by R193H was exacerbated at increasing pacing frequencies independent of elevated diastolic Ca\(^{2+}\). R193H cTnI–dependent mechanical tone caused acute remodeling to a quasicontacted state not elicited by other Ca\(^{2+}\)-sensitizing proteins and is a direct correlate of the stiff heart characteristic of RCM in vivo. These results point toward targets downstream of Ca\(^{2+}\) handling, notably thin filament regulation and actin–myosin interaction, in designing therapeutic strategies to redress the primary cell morphological and mechanical underpinnings of RCM. (Circ Res. 2007;100:1494-1502.)

Key Words: cardiomyopathy ■ troponin ■ contraction

Inherited cardiomyopathies represent a clinically diverse group of progressive heart muscle diseases that can be caused by mutations in specific sarcomeric genes.1 Cardio- myopathies are classified into several distinct clinical subtypes based on a range of morphological and functional criteria.1 The most malignant and least studied of the subtypes is restrictive cardiomyopathy (RCM).2 The distinguishing clinical features of RCM patients include markedly impaired ventricular filling, pronounced diastolic dysfunction from an extremely stiff heart,1,2 and the potential for rapid progression to overt heart failure.2,3

Recently, mutations were identified in the gene encoding cardiac troponin I (cTnI), TNNI3, in human patients with autosomal dominant RCM.3 RCM-linked mutant cTnIs result in amino acid substitutions in the most highly conserved regions of cTnI.4 cTnI is known to function as a molecular switch within the sarcomere by regulating the Ca\(^{2+}\) dependence of cardiac muscle contraction.4 During diastole, myocyte intracellular Ca\(^{2+}\) is low and cTnI binds tightly to actin inhibiting strong actin–myosin interactions. Elevation of intracellular Ca\(^{2+}\) initiates systole by weakening cTnI–actin interactions, promoting a strong TnI–TnC interaction, permissive of cross-bridge cycling.4

Recent biochemical reconstitution studies provide evidence that RCM mutant cTnIs hypersensitize the myofilaments to Ca\(^{2+}\) relative to hypertrophic cardiomyopathy (HCM) mutant TnIs.5–7 These data in reconstituted preparations are important, but the primary effects of RCM mutants on membrane-intact cardiac myocytes studied under physiologic conditions remain unknown. This is important as recent evidence suggests a vital interplay between myocyte Ca\(^{2+}\) handling and the sarcomere in diseased myocardial tissue.8 Accordingly, we used an acute genetic engineering strategy to study myocyte structure and function. This study focused on the RCM-linked mutant cTnI R193H owing to its severe phenotype in humans.3 Simultaneous dual gene transfer of wild-type (WT) and cTnI mutant R193H showed a selective advantage of RCM cTnI over WT cTnI for stoichiometric incorporation into the sarcomere. This outcome has implications for gene dose–response relationships given the autosomal dominant genetic underpinnings of inherited RCM. Functional studies demonstrated that R193H cTnI blunted the response to positive inotropes and resulted in a myofilament-based remodeling of the Ca\(^{2+}\) transient in the absence of...
changes in $Ca^{2+}$ cycling protein expression and sarcoplasmic reticulum (SR) $Ca^{2+}$ load. A novel $Ca^{2+}$-independent precontracted state was observed in intact mutant myocytes coinciding with the progressive incorporation of R193H cTnI into the cardiac sarcomere. This R193H mediated cellular remodeling cannot be explained by $Ca^{2+}$ sensitivity alterations alone as other $Ca^{2+}$-sensitizing TnI molecules, including slow skeletal TnI and HCM R146G TnI, do not cause a “quasi-contracted” basal state.4,9,10 These findings suggest that events downstream of $Ca^{2+}$ handling are causal for the RCM R193H defects and points to the thin filament as a unique target for possible therapeutic intervention.

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

Mutagenesis and Generation of Recombinant Adenovirus
Stratagene Quick Change site directed mutagenesis kits were used to engineer the RCM-linked R193H mutation into the full-length rat cTnI cDNA. The Admax vector system was used to generate recombinant adenoviral vectors, yielding a titer of $10^{10}$ to $10^{12}$ pfu/mL.

Isolated Cardiac Myocyte Preparation and Adenoviral Gene Transfer
Hearts were removed from heparinized and anesthetized adult rats, and ventricular cardiac myocytes were isolated by collagenase-hyaluronidase digestion.10

Unloaded Dynamic Sarcomere Contractility, Calcium Transients, and Isometric Tension
Methods are detailed in the online data supplement.

Myocyte Morphology
Isolated adult rat cardiac myocytes from control (WT) and flag- and R193H-transduced experimental groups were cultured in DMEM with or without 10 μmol/L blebbistatin (Sigma), with or without 10 mmol/L 2,3-butanedione monoxime (BDM) (Sigma), or with or without acidic DMEM (pH 6.5, without NaHCO$_3$ and HEPES modified) for 96 hours and microscopically examined using ImagePro Express software (Media Cybernetics, Silver Spring, Md).

Results
Targeted Stoichiometric Replacement and Localization of RCM cTnI R193H
All vector-constructed cTnI (mutant and WT) were engineered with or without C-terminal flag epitope tag to directly assess expression relative to native cTnI. The flag epitope had no detectable effect on myocyte structure or contractile performance as shown previously.10–12 Myofilament stoichiometry after TnI gene transfer was unchanged across time in all groups as determined by cTnI/α-Tm ratio.10 The rate of replacement was significantly greater for R193H than cTnI flag (Figure 1A), such that R193H mutant cTnI almost fully replaced the native cTnI, whereas cTnI flag had reached $\approx 45\%$ replacement at 4 days after gene transfer. By day 6, cTnI flag can achieve complete replacement without affecting $Ca^{2+}$-activated tension.10 There was no significant difference in the relative expression of the mutant cTnIs between permeabilized and nonpermeabilized myocytes (Figure 1A, column P; t test, $P>0.05$), evidence that the mutant cTnIs were incorporating into the sarcomeric lattice.

The increased magnitude of replacement by vector-derived R193H relative to cTnI flag suggested that the R193H mutant has an advantage over WT cTnI when incorporating into the sarcomere (Figure 1A). To directly test this hypothesis, a dual gene transfer approach was used, whereby myocytes were simultaneously transduced with 2 recombinant vectors and then assessed for incorporation efficiency (Figure 1B and supplemental Figure I). As cTnI WT titer was increased, cTnI flag (held at a constant titer, 20 multiplicities of infection [MOI]) and increasing viral titers (represented by the wedge, 0 to 500 multiplicities of infection) of nontagged WT cTnI. Myocytes were collected at 4 days after gene transfer. Western blot analysis was with an anti-cTnI antibody. C, Representative confocal projection images of isolated adult cardiac myocytes using dual labeling with anti-α-actinin antibody conjugated to Alexa 488 and anti-flag antibody conjugated to Texas Red. Images are shown for nontransduced myocytes (WT) and those treated with adenovirus containing flag epitope-tagged WT cTnI (cTnI Flag) and R193H mutant cTnI. The striated pattern labeling seen in the inset of the merged image represents appropriate incorporation of the mutant cTnI. Bar=20 μm.

Figure 1. Targeted stoichiometric replacement and localization of RCM-linked mutant cTnI. A, Western blot analysis with an anti-cTnI and anti-flag antibody showing expression of epitope-tagged RCM mutant cTnI in transduced adult rat cardiac myocytes. Lanes marked “day 4” are from intact cells at 96 hours after gene transfer. Lanes marked “P” represent permeabilized myocytes at day 4. There is a progressive increase in the percentage expression of the higher-molecular-weight flag-tagged cTnI, with a concomitant decrease in expression of the native cTnI over time. An anti–α-tropomyosin (α-Tm) antibody was used as a loading control. There was no significant difference in myofilament stoichiometry across time, as determined by cTnI/α-Tm ratios. B, Competition assays (supplemental Figure I). Myocytes were cotransduced with flag epitope–tagged R193H or cTnI flag (held at a constant titer, 20 multiplicities of infection [MOI]) and increasing viral titers (represented by the wedge, 0 to 500 multiplicities of infection) of nontagged WT cTnI. Myocytes were collected at 4 days after gene transfer. Western blot analysis was with an anti-cTnI antibody. C, Representative confocal projection images of isolated adult cardiac myocytes using dual labeling with anti–α-actinin antibody conjugated to Alexa 488 and anti-flag antibody conjugated to Texas Red. Images are shown for nontransduced myocytes (WT) and those treated with adenovirus containing flag epitope–tagged WT cTnI (cTnI Flag) and R193H mutant cTnI. The striated pattern labeling seen in the inset of the merged image represents appropriate incorporation of the mutant cTnI. Bar=20 μm.
left). These findings are evidence for the preferential incorporation of the cTnI R193H over cTnI WT into the intact sarcomere.

Confocal imaging of myocytes dual labeled with α-actinin and flag antibodies provided evidence of the correct sarcomeric localization and striated incorporation of cTnI R193H (Figure 1C). More than 95% of the myocytes incorporated mutant cTns, and only R193H mutant myocytes had a distinct alteration in cellular morphology, whereby >87% of R193H myocytes had a short-squat phenotype after gene transfer (Figure 1C).

**R193H cTnI Increases Myofilament Ca2+-Activated Tension**

The steady-state isometric tension–Ca2+ relationship was determined at a preset sarcomere length (SL) (2.1 μm) in single permeabilized cardiac myocytes after gene transfer. There was no significant difference in maximal isometric force generation or inhibition at nominal [Ca2+] between R193H and control myocytes (supplemental Table II). R193H mutants demonstrated higher isometric tension than control myocytes at submaximal [Ca2+] as represented by a marked leftward shift in the tension–Ca2+ relationship (Figure 2A and 2B). Under acidic conditions, R193H mutants showed heightened myofilament Ca2+ sensitivity relative to controls (Figure 2C). The passive tension–SL extension relationships for R193H and control myocytes were not significantly different at nominal [Ca2+], providing functional evidence that titin-dependent myocyte elasticity is not altered by R193H mutant cTnI (Figure 2D).

**RCM cTnI R193H Causes a Ca2+-Independent Shortening of Resting SL and Slows Relaxation and Ca2+ Transient Decay**

Basal contractile function and Ca2+ transients were simultaneously measured in unloaded intact adult rat cardiac myocytes at 37°C (Figure 3). A summary of contractility, defined as the amplitude of SL shortening, and Ca2+ transient parameters from myocytes 4 days after gene transfer is presented in supplemental Table I. Efficiency of gene transfer was >95%, with 90±3% replacement of native cTnI with vector-derived R193H mutant cTnI (Figure 1A).

RCM R193H gene transfer and sarcomeric incorporation significantly altered the normal rod-shaped cell morphology and decreased resting SLs in the absence of altered baseline [Ca2+] (P<0.001; Figure 3B and supplemental Table I). The R193H myocytes had baseline SLs that were on average 110 nm per SL shorter than control myocytes (P<0.001; Figure 3A and supplemental Table I). The significant Ca2+-independent shortening of SLs in R193H mutant myocytes could be attributed to the heightened magnitude of replacement by RCM R193H mutant relative to cTnI flag at this time point; therefore, the relationship between gene dosage and resting SL was determined by linear regression. With progressive cTnI flag incorporation, there was no change in resting SL (Figure 3A; y = −4.1e−6 + 1.801). By contrast SLs were significantly shorter even at 20% R193H replacement and demonstrated a significant dose-dependent response (Figure 3A; R2 = 0.88, y = −0.0011x + 1.7815).

Representative sarcomere shortening traces and the corresponding Ca2+ transients of nontransduced (control), cTnI flag, and RCM-linked R193H mutant myocytes are shown in Figure 3B. RCM R193H cTnI caused a marked slowing of relaxation with a concomitant slowing of the Ca2+ transient decay, whereas cTnI flag had no effect. There was no significant difference in peak shortening/fluorescence amplitudes between groups (supplemental Table I). The length of time from peak shortening to 50%, 75%, and 90% relaxation in R193H mutant myocytes was more than tripled relative to...
Figure 3. Intact single myocyte SL shortening and Ca\(^{2+}\) transients. A (left and middle), Summary of baseline SLs and Ca\(^{2+}\) for all of the experimental groups. A (right), Linear regression analysis of resting SL as a function of percentage native cTnI replacement. At 0%, replacement values for cTnI flag and R193H was set at 1.8 \(\mu\)m because this is the average resting SL of nontransduced control myocytes across time. Values are expressed as means±SEM. *Different from control and flag (\(P<0.05\); n=45 to 55). B, Representative raw sarcomere shortening and the corresponding Ca\(^{2+}\) transients from WT and cTnI flag- and R193H-transduced myocytes. Traces were normalized to peak shortening or the peak height of the Ca\(^{2+}\) transient. C (left and middle), Summary of 90% sarcomere relaxation time and the corresponding time to 90% of the Ca\(^{2+}\) transient decay. Relaxation and decay times were determined by calculating the difference from the time of peak shortening/fluorescence to 90% relaxation/decay. C (right), Linear regression analysis of 90% relaxation time as a function of percentage (Continued)
controls; likewise, the corresponding decay time of the R193H mutant myocyte \(Ca^{2+}\) transient was more than doubled (Figure 3C and supplemental Table I). Progressive incorporation of R193H cTnI into the sarcomere produced dose-dependent slowing of the time to 90% relaxation even at 20% replacement in comparison to control and cTnI flag myocytes (Figure 3C; \(R^2=0.79, y=0.003x+0.179\)). The cTnI flag myocytes had no change in relaxation time with increasing incorporation of cTnI flag (Figure 3C; \(R^2=0.69, y=4e^{-0.05}+0.128\)). The shortened resting SLs of the R193H myocytes are not predicted to affect relaxation times, as the dynamics of tension decay are independent of preloaded SL.\(^{14,15}\)

Pair-wise comparisons of myocyte contractile function was measured with 10 mmol/L isoproterenol (Figure 3D) or with or without 5.0 mmol/L extracellular \(Ca^{2+}\) (supplemental Figure IA and IIB). Relative to controls, the R193H myocytes did not significantly increase contractility in the presence of either isoproterenol or 5.0 mmol/L extracellular \(Ca^{2+}\) (Figure 3D and supplemental Figure IIA) but did have a significant lusitropic response to isoproterenol, which almost completely corrected their slow relaxation kinetics (Figure 3D). Steady-state \(Ca^{2+}\)-activated tension experiments revealed that the mutant myofilaments responded to the protein kinase A (PKA) catalytic subunit with the same magnitude of desensitization of the myofilaments to \(Ca^{2+}\) as seen with WT myocytes (Figure 3D). These data suggest that R193H cTnI does not abrogate the intramolecular handling protein expression between groups, suggesting that the R193H cTnI may alter \(Ca^{2+}\) cycling. To test this hypothesis, transduced myocytes were rapidly exposed to caffeine (10 mmol/L) to induce \(Ca^{2+}\) release from the SR. There was no significant difference (\(P=0.1\)) in \(Ca^{2+}\) transient amplitude between control (0.19±0.02) and R193H (0.24±0.02) myocytes, as summarized in Figure 3E and 3F, suggesting that the R193H cTnI does not directly affect functional SR \(Ca^{2+}\) load. Expression of critical \(Ca^{2+}\) handling proteins including Na\(^+\)/\(Ca^{2+}\) exchanger, SR \(Ca^{2+}\)-ATPase pump, phospholamban, and the serine 16 phosphorylated form of phospholamban were assessed by Western blot (Figure 3G). There were no significant differences in \(Ca^{2+}\) handling protein expression between groups, suggesting that \(Ca^{2+}\) reuptake and extrusion processes are functioning appropriately in R193H mutant myocytes.

**Increased Electrical Pacing Exacerbates \(Ca^{2+}\)-Independent Diastolic Tone in RCM Mutant Myocytes**

Frequency-response experiments highlighted a significant frequency-dependent diastolic dysfunction in R193H mutant myocytes (Figure 4A and 4B), as demonstrated by R193H SLs remaining significantly shorter during relaxation throughout a train of pulses relative to controls. This effect, which is summarized in Figure 4C, becomes more pronounced at higher frequencies, indicating that unlike controls the mutant myofilaments only partially relax with increasing demands. To quantify this effect the average resting SLs and fluorescence ratios were measured at each frequency >0.2 Hz, and these values were subtracted from the diastolic SL/fluorescence ratio measured at 0.2 Hz, yielding the following values (Figure 4C): \(\Delta \Delta (0.5 \text{ Hz}), \Delta B (1.0 \text{ Hz}), \Delta C (2.0 \text{ Hz})\). Control mechanical transients remained tightly coupled to the \(Ca^{2+}\) transient, but the R193H mutant myocytes developed progressively shorter diastolic SLs that were 18±3 nm shorter at 2.0 Hz compared with baseline, despite the \(Ca^{2+}\) transient returning to baseline values (Figure 4C). Five seconds after the pacing protocol ("off" bar in Figure 4A and 4B), resting SLs in R193H mutant myocytes were 10% (17±5 nm) shorter than their prefrequency response values in the absence of elevated diastolic \(Ca^{2+}\) (Figure 4C, \(\Delta D\)). In control myocytes, however, there was no significant difference in diastolic SLs and \(Ca^{2+}\) ratios before and after frequency response. Thus, pacing exacerbated the already increased basal mechanical tone in R193H myocytes independent of the \(Ca^{2+}\) transient, providing further evidence that RCM R193H mutation causes myofilament disinheritance at rest.

These experiments also revealed that R193H mutant myocytes have an abnormal contractile response to increasing stimulation frequency. Classically, rodent myocytes respond to increased pacing with decreased shortening and \(Ca^{2+}\) transient amplitudes (negative staircase), as seen in control myocytes (Figure 4D). The sarcomere shortening and \(Ca^{2+}\) transient amplitudes of the R193H mutant remained unchanged with escalating frequency (Figure 4D). In all experimental groups, relaxation and \(Ca^{2+}\) transient decay increased as a function of increasing stimulation frequency (data not shown). Despite the global increase in sarcomere relaxation rates, R193H mutant myocytes were still 50% slower than control myocytes at each frequency. The \(Ca^{2+}\) transient decay rate, however, was only significantly slower than controls at the lower frequencies (0.2 and 0.5 Hz), providing further evidence of a frequency-dependent uncoupling between mechanical and \(Ca^{2+}\)-handling events. The frequency-dependent hastening of R193H mutant \(Ca^{2+}\) transients also suggests that SR \(Ca^{2+}\) handling is not directly impaired.

**RCM cTnI Causes a \(Ca^{2+}\)-Independent Cellular Mechanical Tone**

Whole cell morphology of R193H myocytes was 18% shorter and 32% wider (\(P<0.05\)) than WT myocytes, which main-
tained a classic rod-shaped morphology in primary culture (P<0.05, Figure 5A and 5B). To gain insight into the mechanism of this acute cellular remodeling, myocytes were treated with BDM (10 mmol/L), a compound that has pleiotropic inhibitory effects on myocyte E-C coupling. Chronic application of BDM in cell culture blocked the morphological transition of R193H mutant myocytes (Figure 5C), suggesting that acute remodeling is mediated either through Ca^{2+} handling or downstream at the level of actin-myosin interaction. Application of the specific myosin II inhibitor, blebbistatin, also sustained the R193H mutant myocyte with normal cellular morphology with a relative 30% increase in length and 50% decrease in width (Figure 5C), an effect not seen with the Ca^{2+} channel blocker, diltiazem (data shown in supplemental Figure IV). These data suggest that the R193H mutant myocyte remodeling that we refer to as basal mechanical tone is caused by insufficient actin–myosin inhibition at physiologic diastolic [Ca^{2+}].

The loss of the inhibitory function of R193H mutant cTnI that results in an elevated mechanical tone may be a function of the loss of positive charge in the highly basic C-terminal domain of cTnI that is associated with the arginine-to-

Figure 4. Frequency-dependent escalation in mechanical tone of R193H myocytes. A, Representative stimulation–frequency traces of sarcomere shortening normalized to peak height for R193H mutant (left) and control (right) myocytes. B, Zoomed-in traces of A, delineated by frequency. Δ values (ΔA to ΔD) represent the change in SLs from the dashed line to prestimulation resting SLs at each frequency. C, Summary of the average change in dynamic diastolic SL and the corresponding Ca^{2+} level (ΔA to ΔD, depicted in B) at each frequency. D, The change in sarcomere shortening amplitude as a function of frequency. Values are expressed as means±SEM; *different from control (P<0.05, n=25 to 30).
histidine substitution at codon 193. Because histidine becomes protonated at a pH of 7.0, mimicking the positive charge of arginine, we tested the hypothesis that an acidic media (pH 6.5) would block R193H mutant myocyte from developing diastolic tone. To test this hypothesis, transduced myocytes were chronically cultured in an acidic media (pH 6.5) without bicarbonate. Figure 5C demonstrates that acidic media blocked R193H cTnI-mediated alterations in myocyte length and width (DMEM, pH 7.4) and were similar to control myocyte dimensions (supplemental Figure III). Acidosis (pH 6.2) in steady-state Ca²⁺-activated tension assays showed that the R193H myocytes had a blunted pH-dependent desensitization of the myofilaments to Ca²⁺, unlike WT myocytes, which showed more significant desensitization of the myofilaments to Ca²⁺ (Figure 2C).

Discussion

We report the new finding that the RCM mutant cTnI R193H produces a direct cellular defect of a Ca²⁺-independent heightened mechanical tone at baseline (Figure 6). Myofilament Ca²⁺ sensitization cannot account for these findings as other potent TnI Ca²⁺ sensitizers, shown previously to confer equal or greater increases in Ca²⁺ sensitivity, do not produce these effects when tested under identical experimental conditions. The increased basal mechanical tone occurred without associated alterations in diastolic [Ca²⁺], thus uncoupling cellular mechanical function from Ca²⁺ signaling.

Blebbistatin, the specific inhibitor of myosin II function, fully blocked this effect, providing evidence that active myosin–actin interaction is required and is secondary to effects initiated by RCM cTnI sarcomeric incorporation.

The primary and novel defect of increased basal mechanical tone manifests as progressively shortened SLs commensurate with the replacement-dependent increase in incorporation of RCM TnI R193H into the sarcomere. Recent echocardiography results on a R193H transgenic mouse model reported significantly reduced left ventricular end-diastolic volumes. This organ-level phenotype under physiologic loaded conditions is interesting and may relate to our finding of reduced SLs after R193H gene transfer in vitro. Based on these findings, we hypothesize that R193H myocytes operate at reduced SLs under load in vivo. Passive tension–extension measurements at nominal [Ca²⁺] were not affected by cTnI R193H, providing mechanical evidence that passive elements (titin) are not responsible for the heightened basal mechanical tone. We interpret this effect as a partial disinhibition of the thin filament causing a quasicontracted state of the intact myocyte at physiologic diastolic [Ca²⁺].

Another primary effect of cTnI R193H relates to its heightened incorporation efficiency into the adult cardiac sarcomere. Simultaneous dual gene transfer of cTnI R193H and cTnI WT to cardiac myocytes showed that R193H could not be readily “competed off” by increasing cTnI WT viral titer in contrast to nonmutated cTnI (Figure 1B). This is likely due to the increased basal mechanical tone of R193H myocytes.
interpreted as an advantage of R193H over cTnI WT to stoichiometrically replace endogenous cTnI and incorporate into the sarcomere. Because RCM is inherited in an autosomal-dominant manner, affected individuals have 1 normal cTnI allele and 1 R193H allele. Our results suggest, in contrast to the estimated 50:50 ratio of WT to diseased protein content reported in hearts for other inherited cardiomyopathy alleles,8,17 R193H individuals may be predicted to have greater than 50% R193H protein content in the sarcomere. This may prove physiologically important as genetic titration of R193H in this study demonstrated significant dose-dependent effects on myocyte structure/function (Figure 3A and 3C). In addition, novel gene suppression strategies for treating inherited cardiomyopathies18 may be more challenging for cTnI R193H or similar functioning alleles given its preferential incorporation into the sarcomere.

Chronic exposure of the R193H mutant myocytes with the myosin II–specific inhibitor blebbistatin fully blocked cellular remodeling and is evidence that the R193H weakens the inhibitory function of cTnI, resulting in a low level of actin–myosin interaction under normal diastolic [Ca\textsuperscript{2+}]. This partial loss of inhibitory function of cTnI attributable to R193H may be caused by the charge change that results from an arginine-to-histidine substitution in the highly basic C-terminal region of TnI. Exon 8, which encodes cTnI amino acids 185 to 211, is highly conserved across all TnI isoforms and species, ranging from human to fish (>72% similarity),4 indicating that there is high selection pressure in this region of TnI. Of the more than 10 known RCM and HCM mutations located within the cTnI C-terminal region, 40% result in a loss of positive charge. The elucidation of the crystal structure of TnI in conjunction with recent NMR studies show that the C-terminal region in the Ca\textsuperscript{2+} bound state is highly dynamic and lacking secondary structure.19,20 In the “fly casting” model,19,21 the cTnI C terminus is unstructured in the presence of Ca\textsuperscript{2+} and tethered through upstream TnI interactions with TnC.19 After Ca\textsuperscript{2+} removal, the cTnI switch domain interacts with actin and the C-terminal domain “reels in” the rest of the troponin complex to its new position on the thin filament.19,22 The fly casting model necessitates electrostatic interactions to facilitate rapid protein–protein interface transitions. We speculate, for cTnI R193H in particular, electrostatic tethering is critical and also pH-dependent. At physiologic pH, the histidine mutation is postulated to adversely affect cTnI–actin electrostatic interactions through loss of positive charge in this region of cTnI. We further hypothesize that at acidic pH, at which the imidazole group of histidine becomes positively charged, the cTnI–actin interaction would stabilize cTnI–actin interactions similar to control. In support of this idea, when R193H transduced cardiac myocytes were maintained in acidic media (pH 6.5) throughout the entire culture period, the increased basal mechanical tone was blocked. The caveat is that acidosis is a global inhibitor of contractile function affecting both Ca\textsuperscript{2+} handling and actin–myosin interactions; however, this experiment highlights the importance of actin–myosin interactions as a precursor to increased mechanical tone.

Our results of a primary effect of an increased cellular mechanical tone contrasts with an apparent secondary outcome of myocyte remodeling that accompanies HCM mutant troponin in the context of the working myocardium in vivo.23,24 Isolated myocytes from the α-myosin heavy chain R403Q mice exhibited shortened morphology, albeit in the absence of changes in resting SL,23 suggesting that different mechanisms, such as compensatory changes rather than the direct effect of heightening resting cross-bridge activation, govern the altered morphology. In addition, our results are distinct from previously reported shortened myocytes isolated from adult transgenic mice harboring HCM mutant cTnT, which could be readily reversed by acute application of BDM.24 In our study, acute application of BDM had no effect on the short-squat myocyte morphology, but after membrane permeabilization, the R193H myocytes could be readily mechanically extended to normal rod-shaped morphology and SLs via micropipette attachment to each end of the cell.25 Under these conditions, cell widths and resting passive tension were not different between RCM R193H and WT myocytes. Gomes et al did find that R193H mutants had a...
decreased ability to inhibit ATPase activity, but they did not see this result in isometric tension assays.⁵ Together, these results underscore the role of the intact myocyte and myofilament response to diastolic [Ca²⁺] as being required to induce cellular morphological alterations seen for R193H mutant cTnI. As such, our findings in intact myocytes would not be detected in protocols in which TnI is exchanged in membrane-permeabilized preparations at nominal [Ca²⁺].⁶–⁷

The slowing in SL relaxation and Ca²⁺ transient decay kinetics may be explained by cTnI-mediated thin filament dis inhibition, as there was no change in functional SR Ca²⁺ load or Ca²⁺-handling protein expression (Figure 3E and 3F) in R193H myocytes. Collectively, these data suggest a role of the mutant myofilaments in altering the Ca²⁺ transient and lend support to our proposed mechanism that the heightened Ca²⁺-buffering capacity of R193H myofilaments altered Ca²⁺-cycling dynamics to directly remodel the Ca²⁺ transient as previously suggested in mouse models of HCM.⁸

This study shows that the RCM-linked mutation R193H in TNNI3 directly induces a basal mechanical tone that cannot be explained simply by a gain in myofilament Ca²⁺ sensitivity. This basal quasicontracted state arises without detected alterations in resting [Ca²⁺].⁹ We infer that the primary cellular defect of cTnI R193H results from TnI-based dis inhibition in actin–myosin interaction at normal diastolic [Ca²⁺].¹⁰ This Ca²⁺-independent mechanical tone is blocked by direct chronic inhibition of actin–myosin interaction but not by diltiazem. Therapeutic strategies for inherited and acquired cardiomyopathies have primarily focused on myocyte Ca²⁺ handling.¹¹ The present results suggest that targets throughout Ca²⁺ handling, specifically thin filament regulation and actin–myosin interaction, may represent new targets to redress the primary cell morphological and mechanical underpinnings of RCM cTnI.

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Disclosures

None.

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Supplement

Expanded Materials and Methods

Experimental Model.

Female Sprague Dawley rats (~200g, Harlan, Indianapolis, IN) were used as the experimental model for this study. The care and use of the laboratory animals for this study was in agreement with the guidelines set forth by the Internal Review Board of the University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine.

Mutagenesis and generation of recombinant adenovirus.

The wild type (WT) rat cTnI sequence was subcloned into a pGEM3Z vector with and without a C-terminal epitope Flag tag (DYKDDDDK, Sigma) as previously described\(^1-3\). Rat cTnI served as the background as it is 92% homologous to human cTnI and >99% identical in the mutated region of the protein. Stratgene Quick Change site directed mutagenesis kits were used to engineer the RCM linked R193H mutation into the full-length rat cTnI cDNA using the following primer pairs: sense 5’-ccgagggtggagactggcacaa gaatatcgatgcac-3’ and anti-sense 5’-gtgc atcgatatcttgtgccagttccacctcggc-3’. The complete cTnI mutants were confirmed by sequencing with the following primers: T7, SP6 and 2 custom primers (upstream 5’-gccgggctgtgctctgtg-3’ and downstream 5’-cctctcttgcctccattc-3’). The R193H cTnI mutant was subcloned into the Microbix pDC315 AdMax shuttle vector to form AdcTnIR193H with and without C-terminal Flag epitope. The Admax vector system was employed to generate recombinant adenoviral vectors. Viral particle lysates were
prepared for infection of a cell factory. Viral plaque assays were used to determine the
titer in plaque forming units/ml (10^{10} – 10^{12} pfu/ml).

**Isolated cardiac myocyte preparation for primary culture and adenoviral gene transfer.**

Hearts were removed from heparinized (1500 U/kg) and anesthetized adult rats
(Nembutal; 150 U/kg) and ventricular cardiac myocytes were isolated by collagenase-
hyaluronidase digestion previously described\(^4\). Post digestion the myocytes were
resuspended in DMEM (Dulbecco’s Minimum Eagle Media) plus 5% fetal bovine serum
(FBS). Myocytes were counted and approximately 20,000 cells were plated on laminin-
coated coverslips for two hours prior to replacing the media with serum-free DMEM.
Myocytes were incubated with adenovirus\(^+\) mutant cTnI for one hour. The average yield
from a single adult rat heart was approximately 1.5-2 X 10\(^6\) rod shaped myocytes with
70 ± 5% viability. For adenoviral gene transfer, isolated myocytes were transduced at
an experimentally predetermined optimal multiplicity of infection in terms of
stoichiometric cTnI replacement for each vector.

**Analysis of protein expression by SDS-gel electrophoresis and Western blot.**

In order to assess expression and incorporation of mutant cTnIs into the sarcomere,
cultured ventricular myocytes were permeabilized in relaxing solution containing 0.1%
Triton X-100 and washed three times in relaxing solution prior to collection. Myocytes
were scraped into Laemmli sample buffer and prepared for gel electrophoresis by
boiling and sonication. Proteins were separated on a 12% SDS-page gel and prepared
for Western blot analysis as previously described\(^5\). The following primary antibodies and dilutions were used for immunodetection: monoclonal antibody (MAB) directed against cTnI (MAB 1691, 1:1000, Chemicon), MAB directed against the Flag epitope (M2, 1:1000, Sigma) and a polyclonal antibody directed against cTnI (A1627, 1:1000, Chemicon). Horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody was used for chemiluminescence detection (1:2000, Amersham). Blots were probed for other myofilament components (e.g. tropomyosin; Figure 1A lower panel) and showed no changes in myofilament content or isoform expression, similar to previous reports of targeted sarcomeric gene transfer\(^3;7\).

**Indirect expression and incorporation by immunofluorescence and confocal microscopy.**

Dual labeling immunofluorescence was performed as previously described\(^4;8\). Briefly, cultured cardiac myocytes were fixed in 3% paraformaldehyde/PBS and blocked in 20% normal goat serum (NGS). Myocytes were incubated in the first primary antibody directed against the Flag epitope (M2, 1:500, Sigma) and detected with a Texas Red-conjugated secondary antibody directed against mouse IgG (1:100, Molecular Probes). Antibody binding sites were quenched using mouse FAB fragments and blocking in 20% NGS. Myocytes were incubated in the second primary antibody directed against \(\alpha\)-actinin (EA53, 1:500, Sigma) and detected with Alexa 488-conjugated secondary antibody directed against mouse IgG. All antibodies were diluted in 2% NGS + 0.5% Triton X-100/PBS. Immunofluorescence was visualized on a MIAC Olympus FV500 confocal microscope (University of Michigan Diabetes Core).
Unloaded dynamic sarcomere contractility and calcium transients.

Sarcomere length measurements were made using the Ionoptix system (Ionoptix Co., Milton, MA). A coverslip containing cultured myocytes was mounted to a microscope stage in a custom chamber where its bathing solution was kept at 37°C. Myocytes were bathed in HEPES buffered medium 199 (M199, 1.8 mM [Ca^{2+}]) and electrically stimulated at 7 volts at a frequency of 0.2 Hz. Sarcomere length and Ca^{2+} transients were measured in real time and averaged over 10-12 contraction cycles. Ca^{2+} transients were measured simultaneously using a fluorescent Ca^{2+} indicator, Fura-2 AM (Molecular Probes, 5 µM) loaded and measured as previously described^{9,10}. The kinetics of Ca^{2+} transients were analyzed in conjunction with myocyte mechanical measurements. In some experiments pairwise comparisons of myocytes + pharamacologic agents or 5.0mM [Ca^{2+}] M199 were made. Some experiments utilized pharmacologic agents in the following concentrations: 10nM isoproterenol (Sigma) or 1µM Diltiazem (Sigma). In addition, stimulation frequency experiments were performed utilizing the previously described mechanical and Ca^{2+} imaging methods under steady-state conditions^{10}. Stimulation frequency was varied from 0.2, 0.5,1.0 to 2.0 Hz. At each frequency a minimum of 12 contractions were used for the myocyte to achieve a steady state and 8 subsequent contractions were used for analysis^{10}.

Measurement of steady-state calcium activated isometric force.

Single rod-shaped cardiac myocytes were attached to micropipettes coated in silicone adhesive and permeabilized in 0.2% Triton X-100 for one minute. All measurements
were made at 15°C in relaxation (RS) or activating solutions (AS). Both RS and AS contained 1mM/L free Mg$^{2+}$, 4 mM/L MgATP, 14.5 mM/L creatine phosphate, 20 mM/L imidazole, and KCL to yield an ionic strength of 180 mM/L. Solution pH was adjusted to 7.00 (or 6.20 for acidic pH experiments) with KOH/HCl. The pCa (-log[Ca$^{2+}$]) of the RS was 9.0 and the pCa of maximal AS was 4.0. Sarcomere length was set to 2.1 um and the isometric tension-pCa relation was constructed by measuring the calcium activated isometric tension at basal (pCa=9.0), maximal (pCa=4.0), and various submaximal calcium levels as previously described$^{11-13}$. Every third contraction was taken at maximal activating calcium concentrations in order to normalize tension values. A non-linear least squares fitting algorithm was used to determine Hill coefficient (n) and pCa$_{50}$, the calcium concentration at which 50% of maximal tension was produced.

For phosphorylation studies with the catalytic subunit of PKA, a coverslip containing the single myocytes was washed with relaxing solution (RS), briefly exposed to RS + 0.1% Triton X-100 for 30-60 seconds, and then washed three times with standard RS. Phosphorylation was achieved by exposing myocytes to a RS with dithiothreitol (DTT, 6 mg/ml) + 1 U/µl PKA (catalytic subunit) for ~25 min (range 15-60 min) at 25°C. Single myocytes were then attached to the recording apparatus as described above, and the tension-pCa relation was determined$^{13}$.

**Myocyte Morphology.**

Isolated adult rat cardiac myocytes from control (WT) and Flag and R193H transduced experimental groups were cultured in DMEM + 10µM blebbistatin (Sigma), ±10mM BDM.
(2,3-Butanedione monoxime, Sigma), or + Acidic DMEM (pH 6.5, without NaHCO$_3$ and HEPES modified) for 96 hours. Coverslips from each group were microscopically examined, images were captured, and myocyte dimensions were analyzed with Image-Pro Express software (Media Cybernetics, Silver Spring, MD).

Supplement Description: The proposed model of the cellular physiologic consequences associated with RCM linked mutant cTnl (Main Text Figure 6).

We propose a working model to account for the three physiologically relevant consequences of increased mechanical tone induced by R193H RCM mutant cTnl (Figure 6). First, as mutant Tnl incorporates into the cardiac sarcomere, the baseline diastolic sarcomere length decreases significantly, which is represented by a rightward shift of the baseline sarcomere length (Figure 6). At the cellular level, this translates to mal-adaptation in the sarcomere length-tension relationship that, in turn, places limits on cardiac myocyte cellular contractile reserve. Second, the R193H myocyte’s abrogated inotropic response to β-adrenergic agonists and high extracellular [Ca$^{2+}$] (Figure 3D) is represented in the model as a decreased area within the contraction-sarcomere length loop (shown in red, Figure 6) relative to WT. A third detrimental effect is evident during stimulus frequency-response testing of the myocytes. Escalating stimulation frequency further exacerbates the RCM myocyte’s heightened mechanical tone, the magnitude of which is represented by a whole-sale shift to the right in the contraction-length relationship (Figure 6; gray zone). Increased mechanical tone is indicative of incomplete relaxation during pacing that, unlike wild type myocytes, is uncoupled to
changes in diastolic Ca\textsuperscript{2+} in R193H myocytes. Heightened mechanical tone in the absence of elevated diastolic Ca\textsuperscript{2+} was also observed in rabbit cardiac myocytes after R193H gene transfer (data not shown), suggesting that this phenomena is also relevant in larger mammalian species with Ca\textsuperscript{2+} handling mechanisms similar to humans\textsuperscript{14}. Collectively, these alterations would be expected to compromise diastolic performance at the organ level. It is of note that our previous work with HCM mutant TnI R146G did not demonstrate cellular remodeling or mechanical tone\textsuperscript{3}. As the primary clinical feature of the RCM heart is marked diastolic dysfunction and stiff myocardium, even more so than HCM hearts\textsuperscript{15-18}, the present results provide a direct cellular mechanism to account, at least in part, for the stiffer RCM myocardium.
**Supplement Figure Legends**

**Supplemental Figure I.** Competition assay schematic. Simultaneous cTnI (green) and cTnIR193HFlag (red) gene transfer is represented by large arrows. In the instance where cTnI completely out competes cTnIR193HFlag, the myofilaments are represented with green troponin I molecules in the sarcomere, whereas if R193H is dominant then the myofilaments will contain only R193H (red spheres). If they both effectively incorporate, this is conceptualized by yellow troponin I in the sarcomere. In practice, myofilament composition is best evaluated in competition assays by Western blot analysis to quantify the relative amounts of cTnI and R193H as shown in Figure 1B.

**Supplemental Figure II.** The primary functional effects of RCM-linked R193H mutant cardiac myocytes in the presence of high extracellular Ca\(^{2+}\). (A) Summary of sarcomere length shortening amplitude + 5.0 mM [Ca\(^{2+}\)] in control and R193H transduced myocytes. (B) Summary of the average time from peak height to 75% SL relaxation + 5.0 mM [Ca\(^{2+}\)] in control and R193H transduced myocytes. Values are expressed as mean ± SEM,* P<0.05 2-way ANOVA treatment effect, + P<0.05 2-way ANOVA mutant allele effect, n=25. Basal condition is measured at 1.8 mM [Ca\(^{2+}\)].

**Supplemental Figure III.** Assessment of morphologic remodeling of R193H transduced isolated adult rat cardiac myocytes. (A) Representative brightfield images (10X and 40X) of non-transduced (control) and R193H transduced myocytes cultured in either DMEM (control media), 10mM BDM, or SDMEM at pH 6.5 (Acidosis). SDMEM is DMEM a HEPES buffered media without sodium bicarbonate. Bar = 40 µm (10X) and 20 µm.
(40X). (B) Summary of the average myocyte area of control and R193H mutant myocytes four days post gene transfer cultured in DMEM (control media), 10µM blebbistatin, 10mM BDM, or acidic media (pH 6.5). There was no significant difference in myocyte area. Values are expressed as mean ± SEM, P=0.483, 2-Way ANOVA, n=200-250.

**Supplemental Figure IV.** The primary functional effects of diltiazem on myocyte function. (A) Summary of diltiazem’s (10µM) effects on the Ca^{2+} sensitivity of tension generation (pCa_{50}) in permeabлизed wild type (WT) myocytes. Values are expressed as mean ± SEM, P<0.05, n=7. (B) Summary of diltiazem’s effects on resting sarcomere lengths (SL) in intact cardiac myocytes at physiologic temperatures. There was no significant effect of diltiazem treatment on resting sarcomere length, P=0.77. Values are expressed as mean ± SEM, R193H effect = P<0.001 (*), 2-Way ANOVA, n=40.
**Supplement Table**

**Supplement Table I**

<table>
<thead>
<tr>
<th></th>
<th>Baseline SL (µm)</th>
<th>Amplitude (nm)</th>
<th>Dep V (%peak)</th>
<th>Ret V (%peak)</th>
<th>50% RTP (ms)</th>
<th>75% RTP (ms)</th>
<th>90% RTP (ms)</th>
<th>½ Width (ms)</th>
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<tbody>
<tr>
<td><strong>WT</strong></td>
<td>1.802 ± 0.004</td>
<td>166 ± 5.5</td>
<td>30 ± 0.73</td>
<td>19.5 ± 0.45</td>
<td>45 ± 2</td>
<td>69 ± 3</td>
<td>122 ± 5</td>
<td>72 ± 2</td>
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<tr>
<td><strong>Flag</strong></td>
<td>1.801 ± 0.006</td>
<td>163 ± 1.1</td>
<td>32 ± 1.77</td>
<td>18.5 ± 0.86</td>
<td>43 ± 2</td>
<td>71 ± 3</td>
<td>128 ± 5</td>
<td>74 ± 2</td>
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<tr>
<td><strong>R193H</strong></td>
<td>1.69 ± 0.006**</td>
<td>179 ± 6.4</td>
<td>27 ± 0.75</td>
<td>10.1 ± 0.45**</td>
<td>115 ± 7**</td>
<td>200 ± 1**</td>
<td>268 ± 2**</td>
<td>185 ± 8**</td>
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<tr>
<th></th>
<th>Baseline (360/380)</th>
<th>Amplitude (%BL)</th>
<th>Rise V (%peak)</th>
<th>Decay V (%peak)</th>
<th>50% DTP (ms)</th>
<th>75% DTP (ms)</th>
<th>90% DTP (ms)</th>
<th>½ Width (ms)</th>
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<tr>
<td><strong>WT</strong></td>
<td>0.79 ± 0.006</td>
<td>13 ± 0.9</td>
<td>96 ± 4</td>
<td>13.1 ± 1.3</td>
<td>107 ± 5</td>
<td>166 ± 8</td>
<td>257 ± 12</td>
<td>126 ± 3</td>
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<tr>
<td><strong>Flag</strong></td>
<td>0.82 ± 0.006</td>
<td>12 ± 0.3</td>
<td>93 ± 6</td>
<td>16.0 ± 2.7</td>
<td>106 ± 4</td>
<td>171 ± 8</td>
<td>263 ± 13</td>
<td>129 ± 4</td>
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<tr>
<td><strong>R193H</strong></td>
<td>0.81 ± 0.008</td>
<td>14.13 ± 0.9</td>
<td>100 ± 7</td>
<td>8.9 ± 1.0</td>
<td>190 ± 8**</td>
<td>310 ± 15**</td>
<td>512 ± 25**</td>
<td>230 ± 8**</td>
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</table>

Wild type (WT) is the control group and consists of combined data from non-transduced and AdcTnIwildtype as an unpaired t-test indicated these data were not significantly different (P>0.27), thus there is no effect on intact myocyte function due to adenovirus. Shortening and relaxation velocity is normalized to the peak shortening amplitude.

Relaxation time is calculated from peak shortening to 50, 75, or 90% of relaxation. ½ width is the time from 50% peak contraction to 50% relaxation. Values are represented as the mean ± SEM, * P<0.05 different from control and + P<0.05 different from flag, ANOVA, Post Hoc Newman-Keuls Test, n=45-55 myocytes. Key: BL= baseline, Dep V = sarcomere length shortening velocity, Ret V = sarcomere length relaxation velocity, RTP = relaxation time from peak, Rise V = rate of rise of the Ca²⁺ transient, Decay V = Ca²⁺ transient decay rate, and DTP = Ca²⁺ transient decay time from peak.
### Supplement Table II

<table>
<thead>
<tr>
<th></th>
<th>pCa_{50} (pH 7.0)</th>
<th>n_H (pH 7.0)</th>
<th>P_0 (kN/mm^2)</th>
<th>pCa_{50} (pH 6.2)</th>
<th>n_H (pH 6.2)</th>
<th>P/P_0</th>
<th>ΔpCa_{50}</th>
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<tbody>
<tr>
<td>Control</td>
<td>6.10± 0.02</td>
<td>2.80± 0.23</td>
<td>20.6± 2.1</td>
<td>4.85± 0.02</td>
<td>2.39± 0.22</td>
<td>0.57± 0.02</td>
<td>1.25± 0.02</td>
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<tr>
<td>R193H</td>
<td>6.40± 0.07**</td>
<td>2.61± 1.05</td>
<td>21.1± 1.3</td>
<td>5.26± 0.07*</td>
<td>2.50± 0.62</td>
<td>0.54± 0.01</td>
<td>1.21± 0.03</td>
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</table>

Summary of Ca^{2+} activated isometric tension parameters at pH 7.0 and 6.2 for control and R193H permeabilized myocytes. Results were compared at each pH by an unpaired t-test with P<0.01 (*) or P<0.001 (**) defining significant differences relative to control. Values are represented as mean±SEM, n=7-8 myocytes per group. Key: n_H = Hill coefficient, P_0 = maximum tension.
Supplement Figure I
Supplement Figure II

A

![Bar graph showing SL shortening amplitude (μm) with Basal and 5.0 mM Ca^{2+} conditions for Control and R193H](image)

B

![Bar graph showing 75% SL relaxation time (s) with Basal and 5.0 mM Ca^{2+} conditions for Control and R193H](image)
Supplement Figure III

A

Control

R193H

B

<table>
<thead>
<tr>
<th></th>
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<th>R193H</th>
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<td>DMEM</td>
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<td>Acidosis</td>
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Area (μm²)

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<th>BDM</th>
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</table>
Supplement Figure IV

A  Permeabilized WT Myocytes

B  Intact Myocytes

- Control
- R193H

Resting SL (μm)

Diltiazem  -  +

pCa50

Diltiazem  -  +
Reference List


