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EFFECTS OF R92 MUTATIONS IN MOUSE CARDIAC TROPONIN T ARE INFLUENCED BY CHANGES IN MYOSIN HEAVY CHAIN ISOFORM

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INTRODUCTION

Mutations in cardiac troponin T (cTnT) account for approximately 5–15% of familial hypertrophic cardiomyopathy (FHC) cases in humans [1–4]. Despite extensive *in vitro* and TG mouse studies, there is a lack of understanding as to how different mutations in cTnT lead to divergent cardiac phenotypes. Although transgenic (TG) mouse models of FHC-related mutations in cTnT have provided useful information, species-dependent variations in key contractile regulatory proteins between humans and mice raise important questions. In this context, it is important to note that the impact of mutations is studied against a background of α -myosin heavy chain (MHC) isoform in TG mice, whereas in the adult human heart, β -MHC is the predominant isoform. Because these MHC isoforms have greatly varied enzymatic properties, they are expected to impart different contractile dynamics in cardiac muscle [5, 6]. An important question then is, “how do mutations in cTnT affect contractile dynamics and function against a background of β -MHC?”

Some critical observations further emphasize the importance of MHC isoform in studying the effect of cTnT mutations: 1) β -MHC significantly slows contractile dynamics [6–8]; 2) troponin (Tn)-based effects on contractile function and dynamics are modulated by an

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DISCLOSURES

None declared

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interplay with β -MHC [5, 9]; and 3) MHC isoform in human hearts containing FHC mutations undergoes a near-complete shift to β -MHC during end-stage cardiac failure [10, 11]. Therefore, studying the impact of cTnT mutations on cardiac function against a background of β -MHC may yield new information relevant to human heart failure.

In this study, we focused on two important cTnT mutations at Arg 92 (R92): R92L and R92Q. Mutations at R92 lead to different cardiac phenotypes and are characterized by varying degrees of ventricular hypertrophy or incidences of sudden cardiac death (SCD). For example, R92L is associated with a hypertrophic remodeling of the heart, but has low incidences of SCD [12]. Conversely, R92Q is associated little hypertrophy, but has high incidences of SCD [12]. The mechanisms by which R92 mutations impart such differential cardiac phenotypes have been studied extensively against α -MHC using TG mice approaches [13–17]. A common trend observed in these studies was that R92 mutations resulted in an increase in myofilament Ca^{2+} sensitivity, without affecting the maximal tension production of cardiac muscle. Whether these R92 mutant effects are influenced by β -MHC remains unclear.

The goal of our study was to understand how R92 mutations affected contractile dynamics at the myofilament level and, importantly, to understand how the effects of R92 mutations differed against β -MHC. We used TG mouse models, where mice expressing R92L or R92Q against α -MHC were crossed with TG mice expressing β -MHC [12], to mimic the conditions found in the human heart. A major finding from our study was that the R92L mutation blunted the SL-dependence of tension development and Ca^{2+} sensitivity against β -MHC, an effect previously not observed in the presence of α -MHC. Another significant finding suggested that R92Q sped rates of crossbridge (XB) recruitment and XB detachment against β -MHC, but not against α -MHC. Furthermore, R92Q affected the way strained XBs modulated allosteric/cooperative mechanisms in the thin filament. Our study provides significant new findings regarding the impact of R92 mutations on the length-dependent activation of cardiac myofilaments and XB cycling dynamics under conditions that better mimic the MHC isoform composition in the human heart.

METHODS

Animal treatment protocols and transgenic mice

The treatment of animals used in this experiment followed the established guidelines of the Washington State University Institutional Animal Care and Use Committee. Transgenic (TG) mice were generated and well characterized, as previously described [12]. TG mice expressing R92Q or R92L mutations against native α -MHC (R92L/ α -MHC or R92Q/ α -MHC) were crossed with TG mice expressing predominantly β -MHC, resulting in R92L/ β -MHC or R92Q/ β -MHC coexpression. Breeding heterozygous mice resulted in a mixed litter of nontransgenic (WT) and TG α -MHC and TG β -MHC mice, which were genotyped using PCR. Expression profiles were as follows: R92L, 60% of total cTnT; R92Q, 67% of total cTnT; α -MHC ~100% of total MHC; β -MHC 80% of total MHC, as estimated from ventricular homogenates [12]. The expression levels of cTnT R92 mutations were similar under either α - or β -MHC backgrounds.

Preparation of detergent-skinned cardiac muscle fiber bundles (fibers)

Mice were deeply anesthetized by inhalation of isoflurane; hearts were quickly excised and placed into an ice-cold relaxing solution that contained the following: 50 mM N, N-bis (2-hydroxyethyl)-2-amino-ethane-sulfonic acid (BES), pH 7.0, 30.83 mM K Propionate, 10 mM Na Azide, 20 mM ethylene glycol tetraacetic acid (EGTA), 6.29 mM MgCl_2 , 6.09 mM Na_2ATP , 1.0 mM DTT, and 20 mM 2, 3-butanedione monoxime (BDM). A fresh cocktail of

protease inhibitors (4 μ M benzamidine-HCl, 5 μ M bestatin, 2 μ M E-64, 10 μ M leupeptin, 1 μ M pepstatin and 200 μ M phenylmethylsulfonyl fluoride (PMSF)) were added to all buffered solutions. Papillary muscle bundles were carefully removed from the left ventricles of mouse hearts. Small muscle fiber bundles (~150–200 μ m in width and 2.0 mm in length) were dissected and skinned overnight using 1% triton X-100 in relaxing solution.

Preparation of cardiac muscle protein samples

After removing the heart, ventricles were rapidly frozen and stored at -80°C until use for protein preparations. Hearts were submerged in liquid nitrogen and pulverized using a mortar and pestle. The tissue was then resuspended in 10 μ L of a protein extraction buffer per 1 mg of pulverized tissue. The protein extraction buffer contained the following: 2.5% SDS, 10% glycerol, 50 mM tris base (pH 6.8 at 4 C), 1 mM DTT, 1mM PMSF, 4 mM benzamidine HCl, and contained a fresh cocktail of phosphatase inhibitors (PhosSTOP, Roche Applied Science) and protease inhibitors (E-64, leupeptin, and bestatin). Resuspended tissue was further homogenized on ice using a “Tissue Tearor” (Model 985370–395 Biospec Products, Inc.), sonicated in a water bath at 4°C , and then centrifuged at 10K rpm.

Analysis of sarcomeric protein levels and phosphorylation status in cardiac muscle preparations

Loading amounts were standardized between each sample by estimating total protein concentration and further optimized based on relative actin concentrations. Equal amounts of protein were loaded on a 12.5% SDS gel, separated, and stained with Pro-Q Diamond staining solution (Invitrogen P33300, stain; P33310, destain) to determine the levels of phosphorylated protein in ventricular samples. Imaging was done using UV transillumination and a BioRad Chemi Doc XRS camera. Following Pro-Q Diamond staining, the gels were then stained with coomassie blue stain to determine total protein content and check for any changes in the expression of protein isoforms. Relative phosphorylation levels were quantified by densitometric analysis using ImageJ software (NIH, acquired at <http://rsbweb.nih.gov/ij/>) for Pro-Q or coomassie blue-stained gel images, respectively.

Measurement of isometric tension in detergent-skinned cardiac muscle fibers

Muscle fibers were mounted between a motor arm and a force transducer using aluminum T-clips [5, 18]. Muscle fiber sarcomere length (SL) was determined using He-Ne laser diffraction measurements and adjusted to the desired SL of 1.9 or 2.3 μ m, following 2–3 repetitions of maximal activation and relaxation. Isometric tension production was measured during steady-state activation using a SensorOne AE-801 force transducer. SL was readjusted again if necessary. Next, the muscle fiber was activated by immersion into a 15 μ L measuring chamber (temperature controlled to 20°C) that contained activating solutions. Motor-driven vibration of a membrane at the bottom of the measuring chamber kept the activating solution continuously stirred during muscle fiber activations. Maximal activating solution (pCa 4.3) contained the following: 10 mM EGTA, 10.11 mM CaCl_2 , 6.61 mM MgCl_2 , 5.95 mM Na_2ATP , 30.83 mM potassium propionate, 10 mM phosphoenol pyruvate, 0.5 mg mL^{-1} pyruvate kinase, 0.05 mg mL^{-1} lactate dehydrogenase, 20 μ M diadenosine pentaphosphate (A_2P_5), and a cocktail of protease inhibitors. Relaxing solution (pCa 9.0) contained the same makeup as the pCa 4.3 solution except the following: 0.02416 mM CaCl_2 , 6.87 mM MgCl_2 , 5.83 mM Na_2ATP , 51.14 mM potassium propionate. Steady-state tension was measured over a range of pCa between 4.3 and 9.0 to construct the pCa-tension relationships in each group of fibers. The Hill equation was fitted to the pCa-tension relationships according to the following:

$$T = T_{MAX} \frac{pCa^{n_H}}{pCa^{n_H} + pCa_{50}^{n_H}} \quad \text{Eq. 1}$$

where T_{MAX} is maximal tension (measured at pCa 4.3), n_H is the Hill coefficient, and pCa_{50} is the pCa value required to reach 50% of T_{MAX} .

Rate constant of tension redevelopment (k_{tr})

The rate constant of tension redevelopment (k_{tr}), following rapid slackening and re-stretching of an activated muscle fiber, was as described previously [19]. During steady-state activation, the muscle fiber was rapidly (within 1 ms) slackened by 10% of muscle length (ML) and held for 25 ms using a high-speed length control device (Aurora Scientific Inc.). After this brief shortening period, the fiber was rapidly stretched past its original length by 10% for 1 ms to break any residually-bound XBs. The fiber was then rapidly returned to its original ML in a step-wise fashion (within 1 ms), from which force began to redevelop. k_{tr} was estimated by fitting the monoexponential function to force data:

$$F(t) = (F_{obs} - F_0)(1 - e^{-k_{tr}t}) + F_0 \quad \text{Eq. 2}$$

where $F(t)$ is the force at time t , F_{obs} is the steady state redeveloped force, and F_0 is the residual force from which tension begins to redevelop.

Muscle fiber mechano-dynamics

Once the muscle fiber attained steady-state activation, step-like length changes were applied to the fibers as described previously [20]. In brief, ML was rapidly stretched (by 0.5%, 1.0%, 1.5%, and 2.0% of ML) and held at the increased length for 5 seconds. The motor arm was then commanded to rapidly return (representing release by 0.5%, 1.0%, 1.5%, and 2.0% of ML) to the initial ML where it was held for another 5 seconds. Representative force responses to large-amplitude stretch and release are shown in Fig. 1. A previously-developed nonlinear recruitment distortion (NRD) model [20] was fitted to the entire family of force responses to characterize the XB cycling dynamics in the muscle fiber. The force responses, $F(t)$, to step-like length changes were represented as a product of the net stiffness of the fiber, $\eta(t)$, times the distortion of the fiber, $x(t)$:

$$F(t) = \eta(t)x(t) \quad \text{Eq. 3}$$

$\eta(t)$ represents the stiffness of the fiber, and is proportional to the number of XBs bound at time t . The change in ML, $\Delta L(t)$, due to the step perturbation, results in a change in recruitment of the number of bound XBs, and $\eta(t)$ changes with time as shown in Eq. 4. b is a rate constant that determines the speed at which the recruitment variable $\eta(t)$ changes and γ is a parameter that represents how XB distortion, $x(t)$, influences XB recruitment. $x(t)$ represents the mean distortion of bound XBs, and changes with respect to the velocity of ML change, $\frac{dL(t)}{dt}$, as shown in Eq. 5. c is a rate constant that determines the speed at which XB distortion dissipates, as XBs that were distorted due to $\frac{dL(t)}{dt}$ detach and repopulate into a non-distorted state. Therefore, c is considered a measure of the rate of XB detachment and b is considered a measure of the rate of XB recruitment. γ is described as a parameter that characterizes how XB strain influences the dynamics of XB recruitment. Therefore, γ may be considered a measure of thin filament-based cooperative/allosteric mechanisms that modulate XB-mediated effects on other XBs.

$$\frac{d\eta(t)}{dt} = -b\eta(t) - \gamma \left(\frac{x(t) - x_0}{x_0} \right)^2 \eta(t) + b\Delta L(t) \quad \text{Eq. 4}$$

$$\frac{dx(t)}{dt} = -cx(t) + \frac{1}{v_0} \frac{dL(t)}{dt} \quad \text{Eq. 5}$$

Data Analysis

Contractile function parameters and mechano-dynamic parameters were compared using three-way analysis of variance (ANOVA). One factor in this analysis was cTnT R92 variant (WT, R92L, or R92Q), the second was MHC isoform (normal α -MHC or TG β -MHC), and the third was sarcomere length (SL 1.9 or 2.3 μm). Following analysis by three-way ANOVA, planned multiple pairwise comparisons were made using Fisher's LSD method. Comparisons were made to determine the effects of the R92 mutations, MHC isoform expression, or SL on contractile function parameters. Hill's equation (Eq. 1) was fitted to data from normalized pCa-tension measurements to obtain pCa₅₀ and the Hill coefficient (n_H). The NRD model was fitted to force response data from step-length perturbations, as described above. Each contractile parameter is reported as the mean of fitted parameters $\pm$ SEM. Each measurement was determined from at least 7 fibers, from 2–4 hearts, for each experimental group. Asterisks in figures indicate significance by post-hoc (Fisher's LSD) comparisons.

RESULTS

Effects of R92 mutations and β -MHC on isoform expression and phosphorylation of sarcomeric proteins

Results from SDS-PAGE and subsequent coomassie blue staining and Pro-Q diamond staining are shown in Figs. 2A and 2B, respectively. Fig. 2A indicates that the isoform expression profile of sarcomeric proteins (aside from α -MHC and β -MHC) were not different between hearts from each of the mouse groups. The migration pattern of bands corresponding to sarcomeric proteins (cardiac myosin binding protein C (cMyBP-C), cTnT, tropomyosin, cardiac TnI, myosin light chain 1 (MLC-1), MLC-2, or cardiac TnC) were not different in samples from WT or any of the TG mouse hearts. No additional bands were found to indicate expression of different isoforms of other sarcomeric proteins in cTnT or β -MHC TG hearts (Fig. 2A, Lanes 2–6). Fig. 2B shows Pro-Q diamond staining of the same gel, and indicates that the phosphorylation levels of sarcomeric proteins were not different between α -MHC and β -MHC tissue preparations. Densitometric analyses of Pro-Q diamond stained gel images demonstrated that phosphorylation levels of myofilament proteins were not different between groups of hearts. As shown in Table 1, the band intensities (such as those shown in representative image Fig. 2B) were consistent in each of the cardiac preparations except for MLC-2. Basal phosphorylation of MLC-2 was slightly depressed in R92L and R92Q hearts when compared to WT hearts. More importantly, however, there was no difference in the phosphorylation levels of either MLC-2 or any of the other sarcomeric proteins between α -MHC and β -MHC hearts (within the same cTnT group, e.g. compare lanes 1 vs. 2, 3 vs. 4, and 5 vs. 6). Since protein isoform expression and phosphorylation levels were not significantly affected by TG expression of β -MHC, our results could be interpreted in terms of the MHC isoform influence on the outcome of cTnT mutation-mediated impact on contractile function.

Effects of R92 mutation on Ca^{2+} -activated maximal tension in α - vs. β -MHC fibers

Ca^{2+} -activated maximal tension was estimated in each fiber group at SL 1.9 and 2.3 μm (values are listed in Table 2). A three-way ANOVA revealed a significant interaction effect on maximal tension ($P < 0.05$), suggesting that MHC isoform influenced the cTnT effect on the SL-dependence of tension development. To probe the cause of this three-way interaction effect, we compared the SL-dependence of maximal tension, represented as the percentage increase in tension observed at SL 2.3 μm vs. SL 1.9 μm , in all groups of fibers (Fig. 3). In fibers containing WT cTnT, the SL-dependent percent increase in maximal tension was 51 or 53 under α -MHC or β -MHC, respectively (Fig. 3A). In fibers containing R92L cTnT, the SL-dependent percent increase in maximal tension was 53 or 17 under α -MHC or β -MHC, respectively (Fig. 3B). In fibers containing R92Q cTnT, the SL-dependent percent increase in maximal tension was 37 or 29 under α -MHC or β -MHC, respectively (Fig. 3C).

Therefore, the significant three-way interaction was likely due to the blunting effect of R92L on the SL-dependent increase in tension under a β -MHC background (indicated by an arrow in Fig. 3B).

Effects of R92 mutation on myofilament Ca^{2+} sensitivity and cooperativity of tension development in α - vs. β -MHC fibers

The pCa-tension relationships, measured at SL of 1.9 μm and 2.3 μm , were constructed by plotting normalized tension values against a range of pCa (Fig. 4). Myofilament Ca^{2+} sensitivity (pCa_{50} , the pCa value required to generate half-maximal tension) was estimated by fitting the Hill equation to the pCa-tension relationships (Fig. 5A, 5B, and Table 2). Under an α -MHC background, a SL-dependent increase in pCa_{50} was observed for all the groups of fibers, resulting in a leftward shift in the pCa-tension relationships at SL of 2.3 μm (Fig. 4A, 4C, and 4E, respectively). Under a β -MHC background, a SL-dependent leftward shift in the pCa-tension relationship was observed only for WT (Fig. 4B) and R92Q (Fig. 4F) fibers. A significant observation was that the SL-dependent increase in pCa_{50} was ablated in R92L fibers under a β -MHC background (indicated by arrows in Fig. 4D). Three-way ANOVA revealed a significant interaction effect ($P < 0.01$) on pCa_{50} , which demonstrated that a shift in MHC influenced how cTnT modulated the SL-dependence of Ca^{2+} sensitivity. In agreement with previous studies, we found that pCa_{50} was significantly higher in fibers containing R92 mutations when compared to the WT fibers; this trend was maintained under either MHC backgrounds and at either SL (Fig. 5A and 5B, Table 2).

Cooperativity of tension development (n_H) was also estimated by fitting the Hill's equation to pCa-tension relationships (values are listed in Table 2). Three-way ANOVA showed no significant interaction effect, but it revealed significant main effects of cTnT R92 mutants, MHC isoform, and SL on n_H . Similar to the effects of R92L/ β -MHC on the SL-dependence of tension and pCa_{50} , under a β -MHC background, the SL-dependence of n_H was ablated in R92L/ β -MHC fibers.

Effects of R92 mutation on the nonlinear XB strain-dependent effect on XB recruitment dynamics in α - vs. β -MHC fibers

The NRD model contains a parameter, γ , that is essential to reproduce the nonlinearity observed in the family of force responses to various amplitude quick stretches and releases (see Fig. 1 for large amplitude responses). Physiologically, γ represents an effect whereby XB strain negatively impacts the dynamics of XB recruitment via its effect on allosteric/cooperative mechanisms in the thin filament. Three-way ANOVA revealed no significant interaction effects on γ ; accordingly, we report subsequent post-hoc analysis results only. Against α -MHC, R92Q had an overall effect of decreasing γ (Fig. 6A), but this effect was only significant at SL 2.3 μm . Against β -MHC, R92Q either increased γ at SL 1.9 μm or decreased γ at SL 2.3 μm (Fig. 6B). γ 's interpretation of XB strain affecting XB

recruitment differently by the R92Q mutation suggests that the allosteric mechanisms are weakened by R92Q at SL 2.3 μm , regardless of MHC isoform. However, these allosteric mechanisms are enhanced at SL 1.9 μm when β -MHC is present. R92L mutations did not affect such allosteric/cooperative mechanisms, as indicated by no changes in γ values at either SL and under either MHC backgrounds.

Effects of R92 mutation on XB recruitment and distortion dynamics in α - vs. β -MHC fibers

R92 mutation-mediated effects on the dynamics of ventricular function have been shown to depend on the MHC isoform [12]. Therefore, we sought to determine how these R92 mutations influence contractile dynamics at the myofilament level against either α - or β -MHC. We used a previously-described NRD model to characterize contractile dynamics based on force responses to step-like length perturbations [20]. Based on our model analyses, R92L and R92Q mutations had an apparent effect of speeding the rate constant of XB recruitment, b , against β -MHC (Fig. 7B), an effect that was not seen against α -MHC (Fig. 7A). This gave rise to a significant cTnT-MHC interaction effect ($P < 0.05$) on b , indicating that R92 mutations influenced XB recruitment dynamics in a MHC-dependent manner. Post-hoc analyses revealed that b was not altered by R92L or R92Q in α -MHC fibers, but that b was sped by R92L (~28% or 23% increase at SL 1.9 or 2.3 μm , respectively) or R92Q (~50% or 25% increase at SL 1.9 or 2.3 μm , respectively) mutations in β -MHC fibers. The rate constant of tension redevelopment (k_{tr}) showed a similar trend, where k_{tr} was not affected by R92 mutations against α -MHC (Fig. 7C), but R92L and R92Q mutations sped k_{tr} against β -MHC (Fig. 7D). Three-way ANOVA revealed a significant cTnT-MHC interaction effect on k_{tr} ($P < 0.01$), further substantiating the notion that XB recruitment dynamics are affected by R92 mutations in cTnT in a way that depends on MHC isoform.

Similarly, the XB distortion rate constant, c , was differently affected by R92Q mutations depending on the type of MHC isoform present (Fig. 8). This gave rise to a significant cTnT-MHC interaction effect on c ($P < 0.01$). Post-hoc analyses revealed that c was not affected by R92 mutations in α -MHC fibers (Fig. 8A); however, c was faster in R92Q fibers (~43% or 21% increase at SL 1.9 or 2.3 μm , respectively) against β -MHC (Fig. 8B), when compared to WT/ β -MHC. Since c is largely governed by the rate of XB detachment, these findings suggest that R92Q speeds XB detachment in the presence of β -MHC, but not in the presence of α -MHC.

DISCUSSION

Given the importance of interplay between MHC and troponin (Tn) actions [5], we hypothesized that the effects of cTnT mutations on contractile function would depend on the type of MHC isoform. To test our hypothesis, we used two different experimental models to probe the functional effects of R92L or R92Q mutations in mouse cTnT: 1) R92 mutant TG mice with predominantly α -MHC cardiac expression; and 2) R92 mutant TG mice with predominantly β -MHC cardiac expression. Because isoform expression and phosphorylation levels of sarcomeric proteins were similar in hearts containing α - or β -MHC, we were confident in interpreting how the cTnT mutation-mediated effect on contractile function is influenced by MHC isoform. Novel findings from our study demonstrate the following: 1) R92L blunts length-mediated myofilament activation only in the presence of β -MHC; 2) R92Q mutations have an effect on allosteric mechanisms that underlie nonlinear contractile behavior; and 3) R92 mutations speeds XB cycling dynamics only in the presence of β -MHC, an observation that provides mechanistic insights for the previously-reported rescuing effect of β -MHC on systolic dysfunction in R92Q TG mouse hearts [12].

R92L mutation blunts length-mediated activation of thin filaments only in the presence of β -MHC

Length-dependent activation (LDA) is an important phenomenon by which an increase in SL increases the myofilament responsiveness to Ca^{2+} [21, 22]. LDA is more pronounced in cardiac muscle than in fast or slow skeletal muscles [23]. Despite extensive *in vitro* and TG mouse studies, there is a lack of understanding of how R92 mutations affect LDA, especially with regard to their effect in the presence of β -MHC. This is an important issue because the human heart expresses predominantly β -MHC (~92%), a predominance that shifts to nearly 100% in the late pathological stages of FHC [10]. TG mice used in our study provided an ideal model to address the R92 mutation effects on LDA under either an α -MHC or β -MHC background.

In our study, α -MHC and β -MHC had differential impacts on R92L- or R92Q-mediated effects on maximal tension and myofilament Ca^{2+} sensitivity. The SL-dependence of maximal tension development was normal in R92L/ α -MHC fibers, but was significantly blunted in R92L/ β -MHC fibers (Fig. 3B). On the other hand, the MHC-dependent trend observed in R92Q fibers was not different between α -MHC and β -MHC fibers (Fig. 3C), suggesting that the effect of the R92Q mutation on the SL-dependence of maximal tension was not altered by MHC isoform. Thus, against β -MHC, R92L attenuated the SL-mediated increase in maximal tension, an important aspect underlying the Frank-Starling mechanism. A dramatic finding in our study was that the SL-mediated increase in Ca^{2+} sensitivity was completely ablated in R92L/ β -MHC fibers (Figs. 4D and 5B), but not in R92Q/ β -MHC fibers (Figs. 4F and 5B). Collectively, these observations highlight two important findings: 1) α - and β -MHC differentially impact cTnT mutation-mediated effect on contractile function; and 2) the blunting effect of β -MHC on the SL-mediated increase in myofilament Ca^{2+} sensitivity depends on the type of mutation in cTnT.

Our previous observations [5, 7], in agreement with experimental evidence presented here, are strongly suggestive of differential effects of α - and β -MHC on XB recruitment mechanisms and contractile function. A presumptive conclusion is that α - and β -MHC alter XB recruitment through modified cooperative mechanisms. For example, the slower cycling β -MHC may amplify thin filament cooperativity through increased XB-dwell time in the strongly-bound state. Because cooperativity is fundamental to the length-mediated effects on XB recruitment, kinetic properties of α - and β -MHC could provide a basis for the varying effects of α - and β -MHC on cooperativity. However, the thin filament structure – and, therefore, cooperative/allosteric mechanisms – is also strongly dependent on the nature of the cTnT [24]. Therefore, the interplay between XB and cTnT actions plays a collective role in modulating cooperativity, XB recruitment mechanisms, and thin-filament contractile activation. The differential contractile behaviors imposed by MHC and cTnT variants collectively led to a different outcome for R92L mutations on SL-dependent tension development against α - or β -MHC (Fig. 3).

R92L ablated the SL-mediated increase in Ca^{2+} sensitivity under a β -MHC background (Figs. 4D and 5B), but R92Q did not (Figs. 4F and 5B). Similarly, SL-mediated decrease in cooperativity of tension development was ablated by R92L, but not by R92Q, under a β -MHC background (Table 2). Therefore, the presumptive conclusion is that the SL-based mechanisms that modulate Ca^{2+} sensitivity and cooperativity at different SL are normal in R92Q/ β -MHC fibers, but such mechanisms are altered significantly in R92L/ β -MHC fibers, especially at SL of 1.9 μm . For example, pCa_{50} of R92L/ β -MHC fibers (at SL of 1.9 μm) was augmented to such an extent that an increase in SL did not result in any further increase in Ca^{2+} sensitivity. At the same time, n_H of R92L/ β -MHC fibers (at SL of 1.9 μm) was at such a level that an increase in SL did not result in any further change in cooperativity (Table 2). Thus, against β -MHC, but not α -MHC, R92L affects the structure of the thin

filament in such a way that the SL-based effects on the thin filaments fail to bring about a significant increase in myofilament Ca^{2+} sensitivity. Interestingly, such effects are only evident in the presence of slow cycling β -MHC isoform, suggesting that the β -MHC and R92L-induced effects on the thin filament are interacting to produce a distinct effect in the case of R92L/ β -MHC fibers.

These observations suggest that structural/functional consequences on thin filament activation are different for R92L and R92Q, and that their action is dependent on the MHC isoform present in the sarcomere. Although there are no direct studies comparing the effects of this pair of mutations on cTnT structure, *in silico* studies done on another pair of cTnT mutants (R92L and R92W) may provide a suggestive clue. For example, molecular dynamic studies [15] showed that R92L and R92W differently affected the helical structure of a cTnT peptide (cTnT_{70–170}). Furthermore, a recent *in silico* study [25] showed that R92L and R92W differently affected the flexibility of a hinge region (~residues 105–106) of cTnT. Despite such structural variability, both R92L and R92W cTnT mutants have been shown to incorporate stably into the sarcomere [15]. However, the functional behavior imposed by different R92 mutations is quite variable, as evidenced here and other studies [12]. Even though the cause for these functional differences is currently speculative, the answer may lie in the subtle yet divergent effects of R92 mutations on the structure of tropomyosin-Tn regulatory units (Tm-Tn; RU) of the thin filament.

R92Q alters cooperative and/or allosteric mechanisms that underlie nonlinear contractile behavior

An important phenomena observed in cardiac muscle is the so-called “stretch activation response,” whereby a rapid increase in length leads to a delayed rise in force in activated muscle (i.e. Phase 3 in Fig. 1A). Stretch activation is being recognized as a prominent behavior in cardiac muscle. For example, stretch activation has been reported as being affected by HCM-related mutations in MLC1 [26]. Such effects on stretch activation have been linked to the frequency-dependence of power output, and thus, alterations in the stretch activation response may have major implications for optimal cardiac output in a diseased heart.

The NRD model used in this study characterizes “stretch activation” with physiologically descriptive parameters. For example, the delayed rise in force is considered an effect of a slow, but eventual recruitment of additional XBs and is characterized by the recruitment rate constant, b (results discussed later). The presence of an obvious delay to the second rise in force in the stretch response (Nadir in Fig. 1A), but not in the release response, is characterized by the parameter γ . The NRD model parameter γ is a nonlinear interaction term that represents the effect that strained XBs have on the recruitment of other XBs [20]. Because the effect of one XB on another XB is sensed through allosteric mechanisms in the thin filament, γ reflects cooperative and/or allosteric mechanisms operating within the thin filament. At SL 2.3 μm , R92Q attenuated γ in both α - and β -MHC backgrounds. At SL 1.9 μm , γ was unaffected against α -MHC, but γ increased against β -MHC background (Fig. 5). On the other hand, γ remained unaltered in R92L fibers, be it under α - or β -MHC background. Therefore, the cooperative/allosteric interactions are modulated in such a way that the XB strain-dependent effect on recruitment of other XBs was affected by R92Q, but not R92L. Although the molecular basis of this altered cooperative/allosteric mechanism is unclear, the source of this change is likely to be due to mutation-induced effects on the RU, whose activation is tightly coupled to both Ca^{2+} - and XB-induced changes in the thin filament (for review, see [27]). Because XBs themselves affect the balance between RU ‘on’ and ‘off’ states through cooperative activation, RU-related cooperativity in these mutants could have an impact on XB recruitment and XB detachment dynamics. Again, these

observations show that R92 mutations have divergent effects on the thin filament and that their effects are modified by the MHC isoform.

R92 speeds XB recruitment and detachment dynamics in the presence of β -MHC, but not α -MHC

Rice et al. (2010) reported that diminished rates of ventricular pressure development in R92Q/ α -MHC TG mouse hearts were rescued in R92Q/ β -MHC TG mouse hearts. Alternatively, β -MHC expression did not rescue depressed rates of ventricular relaxation in hearts possessing R92L or R92Q. The differences observed in R92Q hearts were explained in terms of alterations in the Ca^{2+} handling mechanisms of the cardiomyocyte. For example, slower cardiac pump dynamics were attributed to slower Ca^{2+} release/reuptake transients in the R92Q/ α -MHC cardiomyocytes, an effect that was rescued in R92Q/ β -MHC cardiomyocytes [12]. Our model analysis suggests that the speeding effect of R92Q on XB recruitment dynamics may have also contributed to rescuing effect observed by Rice et al. (2010) under β -MHC. For example, the rate constant of XB recruitment, b , of R92Q/ β -MHC fibers was ~25–50% faster than WT/ β -MHC fibers (Fig. 6B), whereas b was unaffected in R92Q/ α -MHC fibers (Fig. 6A). k_{tr} showed a similar trend (~22–26% increase in R92Q/ β -MHC fibers), supporting the notion that R92Q sped XB recruitment against β -MHC (Fig. 6D), but not against α -MHC (Fig. 6C). The effects of R92Q on b may suggest that diminished rate of ventricular pressure development observed in R92Q/ α -MHC hearts [12] may be rescued in R92Q/ β -MHC hearts [12] due to faster XB recruitment dynamics imposed by R92Q against β -MHC. In addition, faster XB recruitment rates in R92L/ β -MHC and R92Q/ β -MHC indicate a shift in the equilibrium between non-cycling and cycling XBs. This effect may prolong contractile activation in the heart and result in the slower rates of ventricular relaxation in R92L/ β -MHC and R92Q/ β -MHC hearts reported by Rice et al. (2010).

A similar speeding trend was also observed in the rate constant of XB distortion, c (Fig. 7). The R92Q effects on c imply that the reduced rate of ventricular relaxation observed in R92Q/ α -MHC hearts might be rescued in R92Q/ β -MHC hearts due to faster XB detachment dynamics imposed by R92Q against β -MHC. However, ventricular relaxation was not rescued by β -MHC [12], which suggested that an effect of R92Q on XB detachment kinetics was not strong enough to overcome slower relaxation rates in R92Q/ β -MHC hearts. Instead, the enhanced XB recruitment dynamics by R92 mutations against β -MHC appears to have the dominant effect in delaying ventricular relaxation. These data further support the notion that the outcome of a cTnT mutation depends on the interplay between the MHC isoform and cTnT.

Conclusions

Our findings provide new evidence to demonstrate that R92 mutation effects on cardiac contractile function and dynamics are influenced by the MHC isoform. The R92L mutation blunted length-mediated activation of thin filaments in the presence of the slower cycling β -MHC, an effect not observed in R92L fibers containing the faster cycling α -MHC. Therefore, hypertrophic remodeling seen in R92L human hearts may arise as a compensatory mechanism to overcome the attenuation of length-mediated effect on tension and ablation of length-mediated effect on Ca^{2+} sensitivity. The R92Q mutation sped XB recruitment and XB detachment dynamics in the presence of β -MHC, but these effects were not observed in the presence of α -MHC isoform. Our findings have important implications not only for the divergent effects of R92 mutations on cardiac phenotype, but also for the distinct effects of MHC isoforms in determining the outcome of mutations in cTnT. A further implication from our study is that results from TG mouse studies on HCM should be

cautiously interpreted with respect to effects being extrapolated to β -MHC expressing human hearts.

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ABBREVIATIONS

cTnT	cardiac troponin T
FHC	familial hypertrophic cardiomyopathy
MHC	myosin heavy chain
ML	muscle length
NRD	Nonlinear Recruitment-Distortion
RU	regulatory unit
SL(s)	sarcomere length(s)
SCD	sudden cardiac death
TG	transgenic
Tn	troponin
Tm	tropomyosin
XB(s)	crossbridge(s)

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HIGHLIGHTS

- We studied hypertrophic cardiomyopathy using fibers from transgenic mouse hearts
- Cardiac troponin T (cTnT) mutations are studied against β -myosin heavy chain (MHC)
- The length-dependence of contractile activation was blunted by R92L against β -MHC
- Contractile dynamics were differentially affected by R92Q or R92L against β -MHC
- These findings aid in understanding human heart failure, where β -MHC predominates

Figure 1. Representative force responses to large amplitude step-length perturbations in a maximally-activated fiber

Force traces (**A**) were normalized to the steady-state isometric force, F_{Iso} , attained prior to the series of length perturbations. Muscle fiber length (**B**) was normalized to the basal muscle length, ML, determined under isometric conditions. Force response traces to largest-amplitude stretch or release only are shown in **A** to highlight important phases of the response: Phase 2, a fast phase of force decline from the incident force response, F_1 . The rate of force decline in Phase 2 is governed by the rate of crossbridge (XB) detachment and determines c ; Nadir, which is present in the large-amplitude stretch response but not in the release response. The presence of the Nadir in the stretch, but not in the release, demonstrates nonlinear behavior in $F(t)$ and determines γ ; and Phase 3, a slower but eventual rise in force in response to positive length change ($+\Delta L$). The rate of force development in Phase 3 is governed by the rate of XB recruitment and determines b .

Figure 2. Representative SDS gel images to show effects of R92 mutations or β -MHC TG expression on isoform changes and phosphorylation status of sarcomeric proteins
 Ventricular protein preparations were separated using SDS-PAGE. Panel **A** shows coomassie blue staining of a 12.5% gel, and illustrates that the overall isoform expression of sarcomeric proteins were not different. Panel **B** shows Pro-Q diamond staining of the same gel as panel **A**, and indicates that the phosphorylation levels of sarcomeric proteins are not different between α -MHC and β -MHC tissue preparations. In lanes containing R92L or R92Q mutant cTnT, an additional band just above cTnT corresponds to the mutant cTnT, which migrates slower due to the incorporated N-terminal c-Myc tag.

Figure 3. The effects of R92 mutations and MHC isoforms on the SL-dependent increase in Ca^{2+} -activated maximal tension
 Ca^{2+} -activated maximal tension (against α - vs. β -MHC) was measured at SL of 1.9 μm and 2.3 μm . To probe the main contributing factor(s) for the significant interaction effect ($P < 0.05$), the SL-dependent increase in maximal tension was calculated as the percentage increase in tension, followed by planned multiple pairwise comparisons (see methods). Panels **A**, **B**, and **C** show the percent increase in tension as a function of SL in WT, R92L, and R92Q (against α - vs. β -MHC), respectively. The SL-dependent rise in maximal tension was attenuated by 36% in R92L/ β -MHC fibers (attenuation indicated by an arrow in **B**), whereas attenuation was marginal (only 8%) in R92Q/ β -MHC fibers (**C**). Number of determinations was at least 8 for each group. Values are reported as mean $\pm$ SEM. *, $p < 0.05$; ***, $p < 0.001$ when comparing maximal tension values estimated at SL 1.9 μm vs. SL 2.3 μm within each group of fibers.

Figure 4. pCa-tension relationships in WT, R92L, and R92Q (against α - vs. β -MHC) fibers
 Normalized pCa-tension relationships were fitted using the Hill equation (see methods), and fitted curves are shown as connecting lines. (A), WT/ α -MHC; (B), WT/ β -MHC; (C), R92L/ α -MHC; (D), R92L/ β -MHC; (E), R92Q/ α -MHC; and (F), R92Q/ β -MHC. Arrows in panel D indicate that the SL-dependent shift in Ca^{2+} sensitivity is ablated in R92L/ β -MHC fibers. Number of determinations was at least 7 for each group. Data are represented as means $\pm$ SEM.

Figure 5. Effects of R92 mutations and MHC isoforms on SL-mediated increase in pCa₅₀

Hill's equation was fitted to normalized pCa-tension data (see methods) to determine pCa₅₀ values from measurements made at SL of 1.9 μm or 2.3 μm. The effects of R92 mutations on pCa₅₀ at SL 1.9 μm or 2.3 μm are shown in fibers from α-MHC (A) or β-MHC (B) mouse hearts. Three-way ANOVA revealed a significant interaction effect ($P < 0.01$) on pCa₅₀, suggesting that the cTnT mutant effect on SL-dependence of pCa₅₀ was influenced by MHC isoform. The main contributing factor for the significant interaction effect was evident as the ablation of the SL-mediated increase in Ca²⁺ sensitivity of R92L/β-MHC fibers (see arrows in panel B). Number of determinations was at least 7 for each group. Values are reported as mean ± SEM. ***, $p < 0.001$; NS, not significantly different, as determined by post-hoc comparisons.

Figure 6. Effects of R92 mutations, MHC isoforms, and SL on γ

The NRD model-predicted nonlinear interaction parameter, γ , characterizes the effect that XB strain has on XB recruitment dynamics [20]. γ was determined by fitting the NRD model to step response data (see methods). γ is shown from fibers containing α -MHC (**A**) or β -MHC (**B**). Three-way ANOVA revealed no significant interaction effect on γ . Subsequent planned multiple pairwise comparisons showed that γ decreased in fibers containing R92Q at long SL against both α -MHC and β -MHC backgrounds, but increased in R92Q/ β -MHC fibers at short SL. Number of determinations was at least 7 for each group. Values are reported as mean + SEM. *, $p < 0.05$.

Figure 7. Effects of R92 mutations, MHC isoforms, and SL on b and k_{tr}

The NRD model-predicted rate constant of XB recruitment, b , was determined from fibers containing α -MHC (A) or β -MHC (B). The rate constant of tension redevelopment, k_{tr} , following rapid slack/restretch protocol [19], was also determined from fibers containing α -MHC (C) or β -MHC (D). Three-way ANOVA revealed significant cTnT-MHC interaction effects ($P < 0.05$) for both b and k_{tr} , suggesting that R92 mutations influenced XB recruitment dynamics in a MHC-dependent manner. Post-hoc multiple pairwise comparisons revealed that neither b nor k_{tr} were altered by R92 mutations in fibers containing α -MHC (A or C), but each were sped by either R92L or R92Q mutations against β -MHC (B or D), when compared to respective WT fibers. Number of determinations was at least 7 for each group. Values are reported as mean + SEM. *, $p < 0.05$; ***, $p < 0.001$.

Figure 8. Effects of R92 mutations, MHC isoforms, and SL on c

The NRD model-predicted XB distortion rate constant, c , was determined from fibers containing α -MHC (A) or β -MHC (B). Three-way ANOVA revealed no significant interaction effects on c . Subsequent planned multiple pairwise comparisons showed that c was unaltered by R92 mutations in fibers containing α -MHC (A), but was faster in R92Q/ β -MHC fibers (B), when compared to respective WT fibers. Number of determinations was at least 7 for each group. Values are reported as mean + SEM. *, $p < 0.05$; **, $p < 0.01$.

Quantitative analysis of phosphorylated protein levels in ventricular preparations
 12.5% SDS gels were stained by Pro-Q diamond (Invitrogen) to assess the phosphorylation levels of sarcomeric proteins.

Table 1

	WT/ β -MHC	R92L/ α -MHC	R92L/ β -MHC	R92Q/ α -MHC	R92Q/ β -MHC
cMyBP-C	104.31 $\pm$ 6.20	104.14 $\pm$ 2.41	103.12 $\pm$ 4.14	96.99 $\pm$ 2.62	98.37 $\pm$ 14.47
Desmin	106.40 $\pm$ 3.79	97.70 $\pm$ 4.02	104.48 $\pm$ 5.85	106.27 $\pm$ 5.56	93.93 $\pm$ 6.71
Tm	112.40 $\pm$ 7.54	105.56 $\pm$ 2.78	109.18 $\pm$ 9.55	103.05 $\pm$ 5.93	114.62 $\pm$ 16.59
cTnI	97.49 $\pm$ 3.47	101.41 $\pm$ 2.67	98.80 $\pm$ 3.45	88.45 $\pm$ 7.31	101.93 $\pm$ 7.81
MLC-1	101.64 $\pm$ 5.56	92.63 $\pm$ 4.00	104.54 $\pm$ 12.12	107.84 $\pm$ 14.39	115.64 $\pm$ 20.60
MLC-2	99.20 $\pm$ 7.11	39.18 $\pm$ 6.97 ^{***}	30.93 $\pm$ 2.94 ^{***}	60.62 $\pm$ 3.81 ^{**}	49.47 $\pm$ 6.44 ^{**}

Phosphorylation levels of cardiac myosin binding protein C (cMyBP-C), desmin, tropomyosin (Tm), cardiac troponin I (cTnI), and myosin light chains-1, and -2 (MLC-1 and MLC-2) were determined by densitometric analysis of the corresponding phosphorylated bands (representative gel shown in Fig. 2B) for each group of WT and TC hearts. Phosphorylation levels are represented as the intensity of each protein band as a percentage of the intensity of the same protein in WT heart preparations. Number of gels analyzed was at least 3 for each group.

Values are reported as mean $\pm$ SEM.

^{**} p < 0.001 when compared to the intensity of the same protein in WT heart preparations.

Ca²⁺-activated maximal tension, pCa₅₀, and n_H values

Ca²⁺-activated maximal tension (mN mm⁻²) was measured at pCa 4.3. Normalized pCa-tension relationships were fitted to the Hill's equation (see methods) to derive pCa₅₀ and n_H values, as described in Fig. 3. Number of determinations was at least 7 for each group.

α-MHC									
WT			R92L			R92Q			
	SL 1.9 μm	SL 2.3 μm	SL 1.9 μm	SL 2.3 μm	SL 2.3 μm	SL 1.9 μm	SL 2.3 μm	SL 2.3 μm	
Max Tens.	31.0±1.36	46.6±1.31	31.8±2.38	48.4±3.17	31.7±1.72	43.4±1.22			
pCa₅₀	5.61±0.018	5.71±0.016	5.78±0.027	5.91±0.017	5.81±0.017	5.88±0.019			
n_H	3.00±0.19	2.29±0.10	2.33±0.20	1.81±0.09	2.13±0.14	1.80±0.12			
β-MHC									
WT			R92L			R92Q			
	SL 1.9 μm	SL 2.3 μm	SL 1.9 μm	SL 2.3 μm	SL 2.3 μm	SL 1.9 μm	SL 2.3 μm	SL 2.3 μm	
Max Tens.	33.5±1.17	51.1±2.80	37.3±1.63	43.8±2.25	32.3±0.99	41.6±1.15			
pCa₅₀	5.66±0.012	5.73±0.011	5.88±0.010	5.86±0.015	5.86±0.014	5.95±0.010			
n_H	3.19±0.32	2.55±0.16	2.25±0.11	2.20±0.16	2.55±0.09	1.94±0.15			

Values are reported as mean ± SEM.