**Ca**$^{2+}$ activation of myofilaments from transgenic mouse hearts expressing R92Q mutant cardiac troponin T

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**IN EXPERIMENTS** reported here, we have determined mechanical properties of preparations from transgenic (TG) mouse hearts expressing R92Q mutant cardiac troponin T. Am J Physiol Heart Circ Physiol 280: H705–H713, 2001. —The functional consequences of the R92Q mutation in cardiac troponin T (cTnT), linked to familial hypertrophic cardiomyopathy (FHC), are not well understood. We have studied steady- and pre-steady-state mechanical activity of detergent-skinned fiber bundles from a transgenic (TG) mouse model in which 67% of the total cTnT in the heart was replaced by the R92Q mutant cTnT. TG fibers were more sensitive to Ca$^{2+}$ than nontransgenic (NTG) fibers [negative logarithm of half maximally activating molar Ca$^{2+}$ (pCa$_{50}$) = 5.84 ± 0.01 and 6.12 ± 0.01 for NTG and TG fibers, respectively]. The shift in pCa$_{50}$ caused by increasing the sarcomere length from 1.9 to 2.3 μm was significantly higher for TG than for NTG fibers (ΔpCa$_{50}$ = 0.13 ± 0.01 and 0.29 ± 0.02 for NTG and TG fibers, respectively). The relationships between rate of ATP consumption and steady-state isometric tension were linear, and the slopes were the same in NTG and TG fibers. Rate of tension redevelopment was more sensitive to Ca$^{2+}$ than for NTG fibers (pCa$_{50}$ = 5.71 ± 0.02 and 6.07 ± 0.02 for NTG and TG fibers, respectively). We concluded that overall cross-bridge cycling kinetics were not altered by the R92Q mutation but that altered troponin-tropomyosin interactions could be responsible for the increase in myofilament Ca$^{2+}$ sensitivity in TG myofilaments.

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extracted fiber bundles from TG mice that express the mutant mouse cTnT (R92Q cTnT), which replaced 67% of the native protein in the cardiac myofilaments. Our results provide the first evidence for the molecular basis of the effects of the R92Q mutation in cTnT on cardiac myofilament activation. The data fit with a model in which an altered Tn-Tm interaction, due to the mutation in cTnT, is responsible for the decrease in the threshold for Ca\(^{2+}\)-dependent activation of strong cross-bridge interaction with actin.

**METHODS**

**TG mice.** TG mice were generated as described by Tardiff et al. (39, 40). A full-length adult mouse cTnT was used to construct the transgene. To estimate the level of expression in the heart, cTnT was tagged at the NH\(_2\) terminus with an 11-amino acid human \(\varepsilon\)-myc epitope. Cardiac-specific expression of the mutant cTnT was under the control of a 2,996-bp rat \(\alpha\)-MHC promoter (39).

**Simultaneous measurement of force and ATPase activity.** Left ventricular papillary muscle fiber bundles from freshly dissected mouse hearts were used for skinned fiber experiments. Fiber bundles were extracted overnight in a high-relaxing (HR) solution containing 20 mM MOPS (pH 7.0), 10 mM EGTA, 1 mM free Mg\(^{2+}\), and 10 mM creatine phosphate, 10 IU/ml creatine kinase (bovine heart; Sigma Chemical), 0.5 mM dithiothreitol, and 1% Triton X-100. Detergent-skinned muscle fiber bundles were attached to a displacement generator (model AE 801, SensoNor) on one end and to a force transducer element (model AE 601, SensoNor) on the other end with the use of aluminum T clips. The natural frequency of the force transducer was \(\sim 2\) kHz. After two cycles of full activation and relaxation, the resting sarcomere length was readjusted to 2.3 \(\mu\)m using a He-Ne laser diffraction system (12). With the use of this approach, we find that the resting sarcomere length remains stable throughout the experiment (11). Fiber width and diameter were measured at three points along the fiber, and the cross-sectional area was estimated on the basis of an elliptical model. Tension was expressed as force per cross-sectional area. A computer program (13, 16) was used to calculate the composition of activating and relaxing solutions. To determine the relationship of the negative logarithm of molar free Ca\(^{2+}\) concentration (pCa) to tension, skinned fiber bundles were sequentially bathed in solutions with pCa values ranging from 8.0 to 4.3, and the corresponding force was measured on a chart recorder. Fibers were discarded if the maximally activated tension decreased by \(>20\%\) of the initial tension.

For simultaneous measurement of force and ATPase activity, a system described by de Tombe and Stienen (11) was used. In these experiments, detergent-skinned fiber bundles were mounted, and the sarcomere length was adjusted to 2.3 \(\mu\)m as described above. Force was recorded on a chart recorder and sampled via an analog-to-digital converter to a computer. To measure ATPase activity, a near-ultraviolet (UV) light was projected through the quartz window of the bath (30 \(\mu\)l volume) and detected at 340 nm. The bath was milled in an aluminum block and placed on top of a base that allowed water circulation for temperature control (20°C). For efficient mixing, the solution in the bath was continuously stirred by means of motor-driven vibration of a membrane positioned at the base of the bath. Experimental conditions were as described by de Tombe and Stienen (11) and Wolska et al. (47). ATPase activity of the skinned fiber bundles was measured as follows: ATP regeneration from ADP was coupled to the breakdown of phosphoenolpyruvate to pyruvate and ATP catalyzed by pyruvate kinase, which was linked to the synthesis of lactate catalyzed by lactate dehydrogenase. The breakdown of NADH, which is proportional to the amount of ATP consumed, was measured on-line by UV absorbance at 340 nm. The ratio of light intensity at 340 nm (sensitive to NADH concentration) to light intensity at 410 nm (reference signal) was obtained by means of an analog divider. After each recording, the UV absorbance signal of NADH was calibrated by multiple rapid injections of 0.25 nmol of ADP (0.025 \(\mu\)l of 10 mM ADP) into the bathing solution with a motor-controlled calibration pipette.

**Force measurements at short and long sarcomere length.** Left ventricular papillary muscle fiber bundles from TG and non-TG (NTG) hearts were used for skinned fiber experiments. After dissection, fiber bundles \(-150\)–\(200\) \(\mu\)m wide and 3–4 mm long were placed in HR solution. A computer program (16) was used to calculate the amount of Ca\(^{2+}\) required to generate a range of pCa values. A cocktail of protease inhibitors was included in all the buffers (4). Skinning was performed in HR solution containing 1% Triton X-100 for 30 min at room temperature. pCa-force relations at short and long sarcomere lengths were measured after the sarcomere length was adjusted to 1.9 and 2.3 \(\mu\)m, respectively. All measurements were made at room temperature.

**Measurement of rate of tension redevelopment.** A large slack/release approach, originally described by Brenner and Eisenberg (9), was used to disengage force-generating cross bridges from the thin filaments, which were isometrically activated. The resting sarcomere length was set at 2.3 \(\mu\)m. Fast activation of the fiber was achieved by transferring the skinned fibers from the preactivation solution containing a low concentration of EGTA (pCa 9.0) to an activating solution containing various amounts of free Ca\(^{2+}\). Once the steady state was reached, a slack equivalent to 10% of the muscle length was rapidly induced at one end of the muscle with the use of a motor-moving coil that decreased the force to zero within 1 ms. This was followed immediately by an unloaded shortening for 20 ms. The remaining bound cross bridges were mechanically detached by rapidly (1 ms) restretching the muscle fiber to its original length, after which tension redeveloped. The rate of tension redevelopment increased with increasing Ca\(^{2+}\) concentrations. In all cases, tension redevelopment could be fitted with a first-order exponential equation. There was \(-10\%\) residual tension in the fibers in the slack/release-and-restretch protocol. Buffer conditions were the same as those used for simultaneous measurement of force and ATPase rate.

**Stiffness measurements.** Fiber stiffness was measured by applying length perturbations (1% muscle length). The resulting force response at 500 Hz was measured by means of a dual-phase lock-in amplifier (Stanford Instruments). Fiber stiffness was calculated as the ratio of change in force to change in corresponding muscle length.

**Data analysis.** Values are means \(\pm\) SE. Data from the normalized pCa-force and pCa-MgATPase activity measurements were fitted to the Hill equation by using a nonlinear least-square regression procedure to obtain the pCa values required for half-maximal activation (pCa\(_{0.5}\)) and the Hill coefficient. Statistical differences were analyzed by an unpaired t-test with the criteria for significance set at \(P < 0.05\). The rate constant of monoeXponential tension redevelopment (k\(_{tr}\)) was determined by fitting the rise of tension to the following equation: \(F = F_{\text{obs}}(1 - e^{-k_{tr}t}) + F_{\text{res}}\), where \(F\) is force at time \(t\), \(F_{\text{obs}}\) is force observed at steady state, and \(F_{\text{res}}\) is the residual force.
RESULTS

TG mice. TG mouse hearts expressing the R92Q mutant cTnT (~67% of the total myocardial cTnT) were generated as described by Tardiff et al. (39, 40). A control, TG mouse hearts expressing wild-type (WT) cTnT at ~50% of total myocardial cTnT were used (39, 40). Proteins had c-myc epitope tags at their NH2 termini. These TG mice demonstrated a reduced level of endogenous cTnT, which was downregulated in response to an increase in the expression of TG protein in the myocardial cell (39, 40). The net effect was to maintain the level of total cTnT in the TG heart similar to that of NTG hearts. In previous studies, TG mice expressing the WT cTnT (~50% of the total) showed no changes in myofibrillar organization or cardiac function (39, 40). Thus neither the transgene expression nor the presence of the myc tag at the NH2 terminus had any effect on the normal function of the heart. The hearts of TG mice that express ~67% of R92Q cTnT demonstrated significant hypercontractility, diastolic dysfunction, and impaired relaxation (40), hallmarks of FHC in humans. We wished to explore the biochemical basis for their functional phenotypes.

Our first set of experiments using TG mice was performed to address two important issues: 1) Does the fusion of the 11-amino acid c-myc epitope to mouse cTnT alter its activity in the detergent-skinned muscle preparations? 2) Does transgene expression itself have any deleterious effect on the mechanical properties? Figure 1A demonstrates that the Ca2+ regulation of cardiac myofilaments was not altered by the presence of c-myc epitope or by the presence of transgene product WT cTnT (WTG) in cardiac myofilaments. The myofilament response to Ca2+ (pCa50) and the Hill coefficient values were not significantly affected by the presence of transgene product WT cTnT in the myofilament. Furthermore, as shown in Fig. 1A, inset, Ca2+-activated maximal tension was not significantly altered in the WTTG fibers compared with the NTG fibers.

Effect of R92Q mutation in cTnT on Ca2+-regulated myofilament activation in detergent-skinned fiber bundles. Figure 1B illustrates the pCa-tension relations of the R92Q TG and the NTG fibers measured at sarcomere length of 2.3 μm. At all submaximally activating Ca2+ concentrations, there was a significant increase in the tension development in the R92Q TG fiber bundles compared with the NTG fiber bundles. Thus R92Q TG fiber bundles demonstrated a nearly twofold increase in Ca2+ sensitivity. There was no significant change in the cooperativity of the R92Q TG fiber bundles. As shown in Fig. 1B, inset, there was no significant difference in the Ca2+-activated maximal tension between the fibers from the R92Q TG and the NTG mouse hearts.

Whether an increase in tension at submaximal Ca2+ concentrations was proportional to changes in ATPase activity was tested by measuring the relationship between ATPase activity and Ca2+-activated isometric force generation in detergent-skinned muscle fiber bundles from the R92Q TG and the NTG mouse hearts. The results of these experiments are illustrated in Fig. 2. The data shown in Fig. 2 demonstrate that a Ca2+-activated increase in ATPase activity was proportional to an increase in Ca2+-activated tension. The pCa50 values representing the midpoints of pCa-ATPase relations were similar to those of pCa-tension relations (Fig. 1B). In agreement with pCa-tension relations, tension-ATPase relations of R92Q TG fiber bundles...
demonstrated a nearly twofold increase in Ca\(^{2+}\) sensitivity. As illustrated in Fig. 2, inset, there was no significant difference in the Ca\(^{2+}\)-activated maximal ATPase activity between the fibers from the TG and NTG mouse hearts.

Since the change in the rate of ATP hydrolysis reflects a change in cross-bridge cycling kinetics, we determined the relationship between the steady-state isometric tension and the rate of ATP hydrolysis in fiber bundles from NTG and R92Q TG mouse hearts. In Fig. 3, data from seven sets of experiments demonstrated that the relationships between the rate of ATP consumption and the steady-state isometric tension remained linear. The slopes of tension-ATPase relations were similar for NTG and R92Q TG fiber bundles, which suggested that the rate of cross-bridge detachment was not altered by the presence of R92Q cTnT. The rate of ATP consumption at pCa 8.0 and 4.3 was 23 \pm 4 and 332 \pm 29 pmol \cdot \mu l^{-1} \cdot s^{-1}, respectively, for the NTG fibers and 17 \pm 28 and 324 \pm 28 pmol \cdot \mu l^{-1} \cdot s^{-1}, respectively, for the R92Q TG fibers.

To test whether the Ca\(^{2+}\)-dependent increase in the tension at submaximal Ca\(^{2+}\) concentration (leftward shift in the pCa-tension relation) seen in the R92Q TG fibers was mediated through alteration in cross-bridge turnover kinetics, we measured \(k_{tr}\) as a function of free Ca\(^{2+}\) concentration in detergent-skinned cardiac fiber bundles (Fig. 4). Previously, it was shown that Ca\(^{2+}\) has a direct effect on the \(k_{tr}\) after a brief isotonic unloaded shortening and restretch of cardiac muscle fiber at optimal sarcomere length (3, 43, 46). In NTG and R92Q TG fiber bundles, there was a nearly threefold increase in \(k_{tr}\) with Ca\(^{2+}\) activation (see Fig. 6). Such an increase in \(k_{tr}\) with Ca\(^{2+}\) activation, had been demonstrated from studies using detergent-skinned rat right ventricular trabeculae (46), skinned rat ventricular myocytes (2, 43), and intact right ventricular trabeculae (3).

We observed that the Ca\(^{2+}\)-\(k_{tr}\) relations for the NTG and the R92Q TG mouse cardiac fiber bundles were sigmoidal. This suggested that Ca\(^{2+}\) activation of strong cross-bridge binding to the thin filament was cooperative. As shown in Fig. 5, the pCa\(_{50}\) value (Ca\(^{2+}\) concentration required for half-maximal activation of \(k_{tr}\)) of the R92Q TG fiber was shifted toward the left by 0.36 pCa unit. Thus the R92Q mutation in cTnT resulted in a 2.2-fold increase in the Ca\(^{2+}\) sensitivity of the \(k_{tr}\) response to Ca\(^{2+}\) activation, which is in excellent agreement with pCa-tension and pCa-ATPase measurements. Such a sigmoidal relationship between \(k_{tr}\) and Ca\(^{2+}\) activation has also been observed in detergent-skinned rat right ventricular trabeculae (46) and detergent-skinned rat ventricular myocytes (43). Interestingly, similar to the Ca\(^{2+}\)-dependent increase in tension and ATPase (pCa 6.33–5.84), \(k_{tr}\) in the TG fibers was elevated only at intermediate Ca\(^{2+}\) concentrations (pCa 6.20–5.80). Therefore, these observations indicated that the increase in \(k_{tr}\) at submaximally activating Ca\(^{2+}\) concentrations was due to an increase in the number of force-generating cross bridges. To determine whether increases in \(k_{tr}\) in R92Q TG myofilaments were due solely to an increase in force-generating cross bridges, steady-state tension was plotted against \(k_{tr}\). Figure 6 illustrates that the \(k_{tr}\)-tension relation was not significantly altered by the R92Q mutation in cTnT.

To determine whether the average force produced by the cross bridge is altered, we determined the relationship between fiber stiffness and Ca\(^{2+}\)-activated fiber tension. As illustrated in Fig. 7, the isometric tension-stiffness relations were linear for the NTG and R92Q TG fibers, and the slopes were not altered by the R92Q mutation in cTnT. In both cases, tension and stiffness increased proportionally, and the slopes from the linear fits were 0.95 \pm 0.04 for the NTG fibers and 0.90 \pm 0.06 for the R92Q TG fibers. There were no significant differences between the NTG and R92Q TG fibers.
The effect of the R92Q mutation on the length-dependent activation of cardiac myofilaments was tested by measuring the pCa-tension relation at sarcomere lengths of 1.9 and 2.3 μm. Data illustrating the effect of sarcomere length on the pCa-tension relation are shown in Fig. 8. The pCa50 values at 1.9 and 2.3 μm for the NTG fibers were 5.70 ± 0.01 and 5.83 ± 0.02, respectively (Fig. 8A). The pCa50 values at 1.9 and 2.3 μm for the R92Q TG fibers were 5.93 ± 0.01 and 6.22 ± 0.02, respectively (Fig. 8B). The shift in pCa50 caused by increasing the sarcomere length from 1.9 to 2.3 μm was significantly higher for the R92Q TG than for the NTG fibers. ΔpCa50 was 0.13 ± 0.01 for the NTG fibers and 0.29 ± 0.02 for the R92Q TG fibers. Thus the increase in Ca2+ sensitivity induced by the R92Q mutation was amplified at the longer sarcomere length.

DISCUSSION

Experiments presented here provide new evidence for the mechanism underlying the effects of the R92Q mutation on Ca2+-regulated activation of cardiac myofilament activation. To our knowledge, this is the first study that addresses several unresolved questions: 1) What is the effect of this mutation on myofilament response to Ca2+, and how is it linked to altered actin-myosin interactions? 2) What is the consequence of altered actin-myosin interaction on economy of force maintenance? 3) Are there any changes in cross-bridge cycling kinetics? These important questions have been addressed using a TG mouse model in which 67% of the total cTnT in the heart was replaced by the R92Q mutant cTnT. This ratio of mutant to WT cTnT approximates the situation in patients heterologous for the mutation.

Effect of R92Q mutation in cTnT on Ca2+-regulated activation of cardiac thin filaments. Our study provides evidence for the molecular basis for hypercontractility observed in the intact myocytes and hearts form the TG mouse model expressing the R92Q mutant cTnT (40). Increased myofilament response to Ca2+ was demonstrated by the observation that, at relatively low...
Fig. 8. Effect of the R92Q mutation in cTnT on length-dependent activation in detergent-skinned fiber bundles. Composition of the activation buffer (pCa 4.5) was 20 mM MOPS (pH 7.0), 10 mM EGTA, 9.96 mM CaCl₂, 1 mM free Mg²⁺, 5 mM MgATP₂⁻, 12 mM creatine phosphate, 10 IU/ml creatine kinase, and 0.5 mM dithiothreitol; ionic strength was adjusted to 180 mM with KCl. A: pCa-force relation of NTG fiber bundles at 1.9 μM (●, pCa₅₀ = 5.70 ± 0.01, n = 3.8 ± 0.2) and 2.3 μM (●, pCa₅₀ = 5.83 ± 0.01, n = 3.1 ± 0.1). B: pCa-force relation of R92Q TG fiber bundles at 1.9 μM (●, pCa₅₀ = 5.93 ± 0.01, n = 2.3 ± 0.2) and 2.3 μM (●, pCa₅₀ = 6.22 ± 0.02, n = 2.2 ± 0.2). Values are means ± SE of 6 determinations from 3 different hearts for each case.

Ca²⁺ concentrations, detergent-skinned fiber bundles from the TG mouse hearts expressing the R92Q mutant cTnT produced significantly higher tension than those of control NTG fibers. An important observation in the initial description of mice expressing the R92Q mutation was that those myocytes demonstrated a significant increase in lipid deposition and smaller mitochondria, although the sarcomeric structure was intact. An intriguing possibility is that, with an increase in basal sarcomeric activation, chronic mismatch between ATP synthesis and ATP consumption by the overall cross-bridge activity may result in observed changes in lipid content and mitochondrial morphology, reminiscent of ischemic cardiac myocytes (18, 26).

One of the mechanisms by which the R92Q mutation in cTnT could increase ATPase activity would be by increasing the intrinsic cross-bridge detachment rates. An increase in the rate of cross-bridge detachment could lead to an increase in the rate of ATP consumption for a given level of steady-state isometric tension development, which would result in a decline in the economy of tension maintenance. Pertinent to this observation, Sweeney et al. (37) showed that incorporation of human R92Q mutant cTnT into quail skeletal myotubes led to a 1.8-fold increase in the unloaded shortening velocity, which indicated an increase in cross-bridge detachment rate. According to our results, it is unlikely that the high-strain force-generating cross-bridge detachment rate constant (g) is altered. Ca²⁺-activated maximal ATPase activity and tension were not altered by the R92Q mutation in cTnT. Moreover, slopes describing the isometric force-ATPase relations were not significantly different for myofilaments from the NTG fibers and the R92Q mutation in cTnT. Our estimates of the maximum rate of ATP consumption were 332 ± 29 and 324 ± 28 pmol·μl⁻¹·s⁻¹ for the NTG and R92Q TG fiber bundles, respectively. These values are similar to those previously reported for rat fibers (11, 20) and mouse fibers (47). If it is assumed that the myosin head concentration is ~160 pmol/μl (5), our data indicate a maximum cycling rate of 2 s⁻¹ per myosin head for NTG and R92Q TG fibers. This value is close to that determined by Potma et al. (30), Kentish and Stienen (20), and de Tombe and Stienen (11). Thus our data indicate that the R92Q mutation in cTnT does not alter “g” in cardiac myofilaments under conditions of high strain. However, the differences in the results between our work and that of Sweeney et al. (37) may be related to the fact that the use of heterologous protein in the work of Sweeney et al. may have contributed to the discrepancy between their results and our observations.

It is unlikely that the increased contractile response at submaximal Ca²⁺ is due to improper binding of the R92Q cTnT to the thin filaments, which would lead to uncoupling of the myofilament regulation at low Ca²⁺ concentrations. That the binding of the R92Q mutant cTnT to the thin filament is not altered is supported by the observation that the total cTnT content in the particulate fractions of myofibrillar protein preparations is similar in the R92Q TG and NTG cases (39). Moreover, the sarcomeric structure was found to be normal, with full registration of Z bands and a well-defined organization of thick and thin filaments (40). Although the binding of the R92Q cTnT to the actin-Tn complex is tight enough (42) for normal sarcomeric assembly, this does not preclude changes in myofilament response to Ca²⁺.

In fact, the mechanism by which the R92Q mutation increases the myofilament response to Ca²⁺ in myofilaments containing the R92Q cTnT appears to be exerted through the mutant cTnT on the activation of thin filaments rather than through any changes in intrinsic cross-bridge cycling rate. In the case of R92Q TG fibers, there was an enhancement in isometric tension and ATPase activity at submaximal Ca²⁺ concentrations, which indicated a nearly twofold increase in Ca²⁺ sensitivity compared with that of the NTG myofilament. For a given level of Ca²⁺, the active tension may be modulated by changing the number of strong cross bridges interacting with the thin filament or by increasing the average force produced by a single cross bridge. However, our data fit a model in which the R92Q mutation in cTnT promotes cross-bridge interactions with actin during submaximal activation. Evidence in favor of this hypothesis is provided
by our observation that the Ca$^{2+}$-activated increase in ATPase activity and tension occurred only at submaximal Ca$^{2+}$ concentrations and that the Ca$^{2+}$-activated maximum ATPase activity or tension was the same in controls and in the R92Q TG myofilaments. Moreover, tension and stiffness increased proportionally in NTG and TG myofilaments, and the slopes describing the relationship between tension and stiffness were similar in both cases. Furthermore, as was the case with tension and ATPase activity, the $k_{tr}$ at maximally activating Ca$^{2+}$ concentrations (pCa 4.3) was not affected by the R92Q mutation. For example, $k_{tr}$ values obtained at maximally activating Ca$^{2+}$ concentration (pCa 4.3) were 12.82 ± 0.85 s$^{-1}$ for the NTG fibers and 12.45 ± 0.63 s$^{-1}$ for the R92Q TG fibers. Taken together, these observations indicate that the average force produced by the cross bridge and the total number of cross bridges during maximal activation are not altered by the R92Q mutation in cTnT. It is apparent, therefore, that intrinsic cross-bridge cycling rates are not altered.

In the present study, sarcomere lengths were not controlled during the measurements of $k_{tr}$. However, we are confident that the observations made in this study are valid on the basis of work reported by Wolff et al. (46). In their study, using skinned rat cardiac trabeculae, they observed that the relation between $k_{tr}$ and free Ca$^{2+}$ concentration was not significantly different whether sarcomere lengths were controlled or not. The only significant effect of lack of sarcomere length control on the $k_{tr}$ measurement was that $k_{tr}$ values obtained from studies with no sarcomere length control were found to be an average of 12% lower than those obtained from studies in which sarcomere lengths were carefully controlled. Moreover, $k_{tr}$ values at low and high Ca$^{2+}$ concentrations determined in the study of Wolff et al. were more or less similar to the values reported in our study. For example, $k_{tr}$ values of 4.20 ± 0.38 s$^{-1}$ (at pCa 5.9) and 12.82 ± 0.85 s$^{-1}$ (at pCa 4.3) for the control fibers reported in our study are comparable to those obtained by Wolff et al. (3.57 ± 0.82 and 9.51 ± 1.29 s$^{-1}$ at pCa 5.9 and 4.5, respectively). Furthermore, pCa$^{50}$ of $k_{tr}$ in the control NTG fiber was 5.71 ± 0.02 s$^{-1}$, which is almost identical to the value reported by Wolff et al. (5.75 ± 0.18).

**Molecular basis for the R92Q-induced increase in Ca$^{2+}$ sensitivity of TG myofilaments.** The unique structural features of TnT may aid in explaining the mechanism by which the mutant Tn may alter myofilament response to Ca$^{2+}$. Results from biochemical studies of sTnT suggested that it is the NH$_2$-terminal region of sTnT (residues 70–159) that promotes cooperative interactions between the functional units (19, 33). The corresponding region in cTnT spans residues 102–189. Interestingly, 4 of the 13 known mutations in cTnT are located within residues 92–94 of human cTnT (3 at position 92 and 1 at position 94), which lies close to the domain in cTnT that may play an important role in extending the cooperative unit size of the thin filament. The R92Q mutation-induced leftward shift in the pCa-tension, pCa-ATPase, and pCa-$k_{tr}$ relations suggests that altered TnT-Tm interactions may be an important determinant of the increased Ca$^{2+}$ sensitivity. Well-coordinated Tm-TnT interactions are important for the highly cooperative activation of muscle contraction, which is tightly coupled to the movement of the head-to-tail linked Tm on the actin filament (23, 24). Although the molecular basis of this cooperative mechanism is not well understood, there is evidence that it is tightly coupled to Ca$^{2+}$- and cross-bridge-induced changes in the movement of the Tn-Tm complex on the actin filament (31). These may include cooperativity induced by interactions between the thin-filament regulatory units (17) or between cross bridges themselves (7, 8) or cross-bridge feedback effects on the position of Tn-Tm on the actin filament (21, 34).

An increase in myofilament response to Ca$^{2+}$ could result from an increase in Ca$^{2+}$ affinity for the Tn complex or a Tn-induced partial inhibition of the thin filaments, which would decrease the threshold for Ca$^{2+}$ activation. On the basis of our understanding of the structure of the Tn complex, it is unlikely that the R92Q mutation has a direct effect on the affinity of TnC for Ca$^{2+}$ (10). Clues as to how the myofilament response to Ca$^{2+}$ can be enhanced, which is independent of direct TnC involvement, are provided by reports on the action of a Ca$^{2+}$-sensitizing agent EMD-57033 (35) and partial removal of Tn (28) on thin-filament activation. In both studies, strong cross-bridge binding linked to partial activation of the thin filaments increased myofilament response to Ca$^{2+}$ by increasing the Ca$^{2+}$ sensitivity of the neighboring regulatory unit (28, 35). This increase in cooperativity, between a strongly bound cross bridge and the neighboring regulatory unit, increases myofilament response to Ca$^{2+}$ significantly (28, 35). If this observation holds true for the R92Q-induced changes in the Ca$^{2+}$ sensitivity of TG myofilaments, it would suggest that a partial dis inhibition of actin monomers, linked to altered Tn-Tm interactions, is responsible for the decrease in threshold for the Ca$^{2+}$-dependent activation of cross-bridge interaction with actin. This leads to an increase in the population of force-bearing cross bridges at submaximal Ca$^{2+}$ concentrations and, thus, greater force at lower Ca$^{2+}$ concentrations.

Positive-feedback effect of cross bridges on myofilament activation has been considered to be an important determinant of the length-dependent activation in cardiac myofilaments, which enables the heart to respond appropriately to prevailing hemodynamic demands. That the length-dependent increase in Ca$^{2+}$ sensitivity is amplified in the R92Q TG fibers suggests that the feedback effect of cross bridges on thin-filament activation is enhanced in the R92Q TG myofilaments. Our hypothesis is that the altered interactions between TnT and Tm, due to the R92Q mutation in cTnT, shift the equilibrium from the "off" to the "on" state toward the "on" state. The consequence of this shift in the equilibrium is a decrease in the threshold
for Ca\(^{2+}\) activation, thereby increasing the Ca\(^{2+}\) sensitivity of myofilament activation. Moreover, at longer sarcomere lengths, this mechanism is likely to enhance the Ca\(^{2+}\) sensitivity even further, because an increase in the population of strong cross bridges itself leads to an increase in myofilament response to Ca\(^{2+}\). This may result from the cooperative effect of cross bridges on the near-neighbor Tn-Tm movement (14, 25) or strengthening of the Tm-actin association induced by cross-bridge-induced changes in actin structure (21).

**Physiological implications for the effect of R92Q mutation on cardiac function.** Our findings at the myofilament level are corroborated by the impact of the R92Q mutation in cTnT at the whole heart level (40). Hearts from R92Q TG mice demonstrated hypercontractility and diastolic dysfunction, features that are commonly observed in FHC patients. An increase in Ca\(^{2+}\) sensitivity of tension development and \(k_\text{rel}\), and the length-dependent increase in the enhancement of Ca\(^{2+}\) sensitivity correspond to the significant increase (+18%) in the maximal rates of pressure development and a significant decrease (−30%) in the time required to reach peak intraventricular pressure as load was increased (40). In the R92Q TG mouse, significant diastolic dysfunction was present (40) during the late phase of relaxation, which was indicated by a 136% increase in the time constant of ventricular relaxation (\(\tau\)). In the absence of any abnormalities in the Ca\(^{2+}\)-handling system of the myocyte, increased Ca\(^{2+}\) sensitivity of myofilaments could play a significant role in slowing the rate of relaxation.

How the primary dysfunction at the myofilament level translates into complications associated with FHC has been the subject of many investigations. It is now becoming clear that no single mechanism can explain how different mutations in diverse sarcomeric proteins express themselves. However, what is strikingly common in many experimental observations is that the R92Q mutation in cTnT leads to hypercontractility (increase in Ca\(^{2+}\) sensitivity) of cardiac myofilaments. An attractive hypothesis that may serve to link hypercontractility and cardiac dysfunction is that severely affected hearts fail to meet the increased energy demand, especially when stress is imposed on the heart. Given that ~75% of cellular ATP is consumed to support the contractile activity (for review see Ref. 22), an imbalance in contractile activity itself may have serious consequences on myocardial function. For example, impaired myocardial relaxation has been associated with the accumulation of MgADP, which may result from altered actin-myosin interactions (41). Associated with such alterations is an increase in P, and a decrease in intracellular pH, which can further compromise actin-myosin interactions. This has significant implications for ischemia and ventricular arrhythmia associated with sudden cardiac death in FHC patients (1). Recent observations showing that Ca\(^{2+}\) sensitization induced by the R92Q mutation in human cTnT is amplified under acidic conditions (27, 44) may be highly relevant.

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