Mutations in Human Cardiac Troponin I That Are Associated with Restrictive Cardiomyopathy Affect Basal ATPase Activity and the Calcium Sensitivity of Force Development*

Received for publication, January 10, 2005, and in revised form, June 10, 2005
Published, JBC Papers in Press, June 15, 2005, DOI 10.1074/jbc.M500287200

Aldrin V. Gomes‡, Jingsheng Liang, and James D. Potter§

From the Department of Molecular and Cellular Pharmacology, the University of Miami Miller School of Medicine, Miami, Florida 33136

Human cardiac Troponin I (cTnI) is the first sarcomeric protein for which mutations have been associated with restrictive cardiomyopathy. To determine whether five mutations in cTnI (L144Q, R145W, A171T, K178E, and R192H) associated with restrictive cardiomyopathy were distinguishable from hypertrophic cardiomyopathy-causing mutations in cTnI, actomyosin ATPase activity and skinned fiber studies were carried out. All five mutations investigated showed an increase in the Ca2+ sensitivity of force development compared with wild-type cTnI. The two mutations with the worst clinical phenotype (K178E and R192H) both showed large increases in Ca2+ sensitivity (ΔpCa50 = 0.47 and 0.36, respectively). Although at least one of these mutations is not in the known inhibitory regions of cTnI, all of the mutations investigated caused a decrease in the ability of cTnI to inhibit actomyosin ATPase activity. Mixtures of wild-type and mutant cTnI showed that cTnI mutants could be classified into three different groups: dominant (L144Q, A171T and R192H), equivalent (K178E), or weaker (R145W) than wild-type cTnI in actomyosin ATPase assays in the absence of Ca2+. Although most of the mutants were able to activate actomyosin ATPase similarly to wild-type cTnI, L144Q had significantly lower maximal ATPase activities than any of the other mutants or wild-type cTnI. Three mutants (L144Q, R145W, and K178E) were unable to fully relax contraction in the absence of Ca2+. The inability of the five cTnI mutations investigated to fully inhibit ATPase activity/force development and the generally larger increases in Ca2+ sensitivity than observed for most hypertrophic cardiomyopathy mutations would likely lead to severe diastolic dysfunction and may be the major physiological factors responsible for causing the restrictive cardiomyopathy phenotype in some of the genetically affected individuals.

* This work was supported by National Institutes of Health Grants HL42325 and HL67415 (to J. D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.
‡ Present address: Rm 1609, MRL Bldg., Cardiovascular Research Laboratories, Dept. of Physiology, University of California, Los Angeles, CA 90095.
§ To whom correspondence should be addressed: Professor and Chairman, Dept. of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, 1600 N.W. 10th Ave., Miami, FL 33136. Tel.: 305-243-5874; Fax: 305-324-6024; E-mail: jdpotter@miami.edu.

The abbreviations used are: cTnI, cardiac troponin I; RCM, restrictive cardiomyopathy (RCM) (1). cTnI is one of three subunits (the other subunits being troponin T (TnT) and troponin C (TnC)) that make up the regulatory troponin (Tn) complex. The regulatory proteins Tn and tropomyosin (Tm) form a complex on the actin filament that inhibits actomyosin ATPase and force development under resting intracellular Ca2+ concentrations (2, 3). Although the activation and inhibition of muscle contraction are primarily achieved by TnC and TnI, respectively, TnT anchors the Tn complex to the thin filament and also contributes to the Ca2+-dependent regulation of muscle contraction. Therefore, any functional and structural defects in any of these Tn subunits may cause alterations in the Ca2+ regulation of muscle contraction. More than 20 mutations in the TnI gene that are linked to hypertrophic cardiomyopathy (HCM) are now known and investigations on these mutations show different physiological effects depending on the mutation (4). No study on any RCM causing mutations in TnI or any other protein associated with RCM has been previously reported.

RCM patients demonstrate a condition in which the walls of the ventricles are abnormally rigid and lack the elasticity to expand as the ventricles fill with blood. RCM can be idiopathic or secondary to a number of rare cardiac and systemic disorders such as endomyocardial fibrosis, infiltrative disorders (amyloidosis, sarcoidosis), and rare metabolic disorders (Gau- cher’s disease and Fabry’s disease) (5, 6). Patients with the idiopathic form of RCM may have a family history of cardiomyopathy, and recent evidence suggests that the disease may be caused by the same genetic abnormalities that result in the more common HCM (1, 7). Patients with RCM generally show impaired diastolic function but normal or near normal systolic function until later stages of the disease, eventually leading to heart failure. Several cases of idiopathic restrictive cardiomyopathy in infants and young children (9 and 12 years) were characterized by severe and slowly progressive congestive heart failure (8, 9). RCM has also been shown to be sometimes associated with skeletal muscle and orthopedic abnormalities (10). The intermediate filament protein Desmin has been linked to familial cases of RCM (11, 12). Clinical features of these families included syncope, right atrial enlargement, left bundle branch block, and heart failure. In the familial case of RCM although the age of onset, severity, and rate of progression were variable most (if not all) cases ultimately result in heart failure.

Five cTnI mutations associated with RCM (L144Q, R145W, A171T, K178E, and R192H) were investigated using several in vitro assays to determine the physiological effects of these mutations on the function of cTnI. The cTnI mutations K178E and K178R inactivations are possibly one of the mechanisms that may be responsible for the development of RCM.
and R192H were not present in either of their parents and were classified as de novo mutations (1). The K178E mutation was present in a patient that was diagnosed with idiopathic RCM at the age of 6. This patient was awaiting cardiac transplantation at the time of the report. The patient with the R192H mutation died of heart failure at the age of 19 while awaiting cardiac transplantation (1). This patient showed severe biventricular enlargement with normal systolic function. Two patients containing the R145W mutation were diagnosed with RCM in their late 50s. These patients showed both left and right atrial enlargement together with symptoms of heart failure. Another mutation, A171T was identified in a person in his late 50s who was diagnosed following a stroke (1). The L144Q RCM mutation resulted in symptoms of heart failure at the age of 17 years in the person carrying this mutation. This person died of heart failure at the age of 31 while awaiting cardiac transplantation. Other members of her family were known to have died suddenly. Biopsies from several TnI RCM patients showed nonspecific interstitial fibrosis and in some cases HCM like features.

Reconstituted fibers containing any of the five cTnI mutations associated with RCM showed increased Ca\(^{2+}\) sensitivity of force development (\(\Delta pC_{50} = 0.14–0.47\)). These results suggest that these mutations in cTnI are able to modulate Ca\(^{2+}\) sensitivity similar to what is observed in HCM mutations. However, the increase in Ca\(^{2+}\) sensitivity of force development was generally greater for these mutations than for HCM mutations. The ability of Tn complexes to inhibit ATPase activity was lower for all five cTnI mutants investigated relative to wild-type TnI. Our results suggest that mutations in cTnI associated with RCM result in significant increases in the Ca\(^{2+}\) sensitivity of force development and also affect the basal and maximal force and the basal and maximal actomyosin ATPase activity. Because some patients classified as RCM have family members diagnosed as HCM (1), it is likely that the cTnI mutation is not the only factor that influences the RCM phenotype.

**MATERIALS AND METHODS**

**Expression and Purification of Wild-type and Mutant cTnI**

Human cTnI and mutants were made using a sequential overlapping PCR method as described previously (13). Standard methods previously used in this laboratory were utilized for expression and purification of the different cTnI mutants (14). Briefly, bacterially expressed and extracted cTnI was purified on a Fast Flow S-Sepharose column. cTnI was eluted from this column with a NaCl gradient (0–0.5 M). The semipurified cTnI was then further purified on a cTnC affinity column. cTnI was eluted from this column with a double gradient of urea and EDTA (0–6 M and 0–5 M, respectively). All steps were performed at 4°C unless otherwise indicated. The purity of the isolated cTnI (>95%) was determined by SDS-PAGE.

**Formation of the Troponin Complex**

Formation of the human cardiac Tn complexes containing human recombinant cTnT, cTnC, and cTnI was carried out utilizing a modified method of the one recently described by Szczesna et al. (15). Proteins were first dialyzed against a solution containing 3 M urea and 1 M KCl and then in a solution containing 0 M urea and 1 M KCl. Complexes were formed in the presence of 0 M urea and 1 M KCl and then dialyzed against successively lower concentrations of KCl as described previously (15). Proper stoichiometry was verified by SDS-PAGE. Gel filtration of these formed Tn complexes showed that this reconstitution method resulted in a single complex.

**Actin-Tm-activated Myosin-ATPase Assay**

Porcine cardiac myosin, rabbit skeletal F-actin, porcine cardiac Tm, and recombinant human cardiac TnC were prepared as described previously (14). The ATPase inhibitory assay was performed in a 100-μl reaction mixture of 100 mM KCl, 4 mM MgCl\(_2\), 1.0 mM EGTA, 2.5 mM ATP (Mg\(^{2+}\)-ATP), 0.1 mM dithiothreitol, 10 mM MOPS, pH 7. The ATPase activation assay was carried out in the same 100-μl reaction mixture with 1 mM EGTA replaced with 0.5 mM CaCl\(_2\). F-actin (3.5 μM), myosin (0.8 μM), and Tm (1 μM) were homogenized and added to the reaction tube after the addition of buffer and Tn to the assay tube. Different Tn complexes each containing either wild-type or mutant TnI, TnT, and cTnC were used. The ATPase reaction was initiated with the addition of ATP and stopped after 20 min with trichloroacetic acid (4% final concentration). After sedimenting the precipitate, the inorganic phosphate concentration in the supernatant was determined according to the method of Fiske and SubbaRow (16). The ATPase rates were measured at 30°C by single time points that were predetermined to be linear with time.

**Determination of the Ca\(^{2+}\) Dependence of Force Development**

**Fiber Preparation**—All skinned muscle experiments were performed with glycereinated porcine papillary muscle preparations. Hearts from recently slaughtered pigs were obtained from a nearby slaughterhouse. Strips of muscle, 3–5 mm in diameter and ~5 mm in length were dissected from the papillary muscle of the left ventricle and skinned for 24 h in a 1% Triton X-100 (by volume) containing pCa 8.0 relaxing solution (15). Fibers were then transferred to a similar replacement solution without Triton X-100. The skinned cardiac muscle (~140 μm in diameter) was mounted using stainless steel clips to a force transducer and immersed in a relaxation solution (pCa 8.0) containing 10−8 M Ca\(^{2+}\), 5 mM Mg\(^{2+}\), 7 mM EGTA, 20 mM imidazole, pH 7.0, 5 mM Mg\(^{2+}\)-ATP, 15 mM creatine phosphate, and 20 units/ml phosphocreatine kinase and between 78 and 92 mM potassium propionate to achieve a constant tension in all solutions and in all solutions incubation (pCa 4.0) was the same composition as the pCa 8.0 solution except that the Ca\(^{2+}\) concentration was 10−4 M and was used to measure the initial force. All fiber studies were carried out at room temperature (22°C). To determine the Ca\(^{2+}\) dependence of force development, the contraction of skinned cardiac muscle was tested in solutions containing intermediate concentrations of Ca\(^{2+}\). The various pCa solutions were prepared using the room temperature (22°C) binding constants of Fabiato and Fabiato (18).

Treatment of the skinned fibers with 0.8 mg/ml of cTnT for 2 h at room temperature (with fresh cTnT solution changed after 1 h of incubation) resulted in a loss of Ca\(^{2+}\) dependence of force, and the fibers became unregulated. The protein concentration of cTnT was adjusted to 0.8 mg/ml to obtain maximal displacement of Tn subunits and loss of Ca\(^{2+}\) regulated force. Upon incubation of these fibers with a preformed cTnT-cTnC or cTnI mutant-cTnC complex (30 μM), in relaxing solution (pCa 8.0), the fibers underwent a gradual relaxation as the Tn activity was reconstituted (15, 19). Once fully relaxed (after 1 h incubation with the TnT-cTnC complex), the force became entirely regulated by Ca\(^{2+}\).

**Western Blot Analysis of Experimental Fibers**—Western blotting was carried out to determine the amount of TnI reconstituted in the TnT-displaced fibers. The skinned fibers used for this analysis include control fibers (treated with 1% Triton X-100 for 30 min), fibers displaced with 0.8 mg/ml of TnT for 2 h, and fibers displaced with 0.8 mg/ml of TnT for 2 h and reconstituted with different TnT-cTnC complexes. The fibers were incubated for 5 min at 95°C in 0.4% SDS, 6 M urea, 500 mM Tris, pH 6.8, 14.2 mM β-mercaptoethanol (β-mercaptoethanol), 0.03% bromophenol blue (w/v) for SDS-PAGE on 12% polyacrylamide gels. Proteins on the SDS-PAGE gels were transferred to nitrocellulose membrane and probed with either monoclonal anti-cardiac TnI 6F9 (Research Diagnostics Inc.) (1:2000 dilution) or rabbit polyclonal regulatory light chain antibody (generated in our laboratory) at 1:250 dilution for 2 h at room temperature. Following this, the membranes were washed and incubated for 1 h with peroxidase-labeled secondary antibody and visualized with the chemiluminescence substrate from Amersham Biosciences. The regulatory light chain was used as a loading control to verify that an equal amount of skinned fiber was loaded in each well. The intensity of the bands on the x-ray film were quantified on the stained gel by densitometric scanning using NIH Scion Image Beta 4.02 (Scion Corp.).

**Data Analysis**—Values are presented as mean ± S.D. The statistical significance of the differences between the mean values was analyzed by the Student’s t test.

**RESULTS**

**Actomyosin ATPase Studies on Reconstituted Troponin Complexes Containing Different Troponin I RCM Mutants**

The effects of the five cTnI RCM mutations on the inhibition of ATPase activity were examined using actomyosin ATPase assays (Fig. 1). Tn containing wild-type cTnI was able to inhibit ATPase activity in a concentration-dependent manner similar to what has been previously reported (19). Most of the ATPase inhibition occurred at ~1 μM Tn but maximal inhibition oc-
curved at 2 μM (Fig. 1A). Tn containing cTnI RCM mutants all showed maximal inhibition at 2 μM Tn (data not shown). Interestingly, all five of the mutants showed a significant decrease in their ability to inhibit ATPase activity in the absence of Ca²⁺ (Fig. 1B). Two mutations, L144Q and R145W, showed the greatest inability to inhibit ATPase activity. These mutations both occur within the known inhibitory region of cTnI. The very low inhibitory activity of three RCM mutants, L144Q, R145W, and K178E, suggest that these mutations would have major physiological effects on the function of cardiac muscle of patients containing these mutations. To further examine these mutations, different ratios of wild-type cTnI:mutant cTnI were investigated. Fig. 2 shows the RCM mutants show varying physiological properties with respect to ATPase activity when mixed with wild-type cTnI. All the ATPase assays shown in Fig. 2 were carried out using 1 μM Tn (total concentration) to make sure neither mutant nor wild-type cTnI would be in excess. The amount of Tn used in this assay was 1 μM. Previous reports suggest that one Tn molecule occurs for each Tm molecule. Mixtures of wild type and mutant cTnI showed that cTnI mutants could be classified into three different groups: dominant (L144Q, A171T, and R192H), equivalent (K178E), or weaker (R145W) than wild-type cTnI in actomyosin ATPase assays in the absence of Ca²⁺. These results show the complexity of these RCM mutations. This is the first report to demonstrate that RCM mutations in cTnI show different levels of dominance over wild-type cTnI.

The effects of the five cTnI RCM mutations on the activation of ATPase activity was also examined in reconstituted actomyosin ATPase assays (Fig. 3). The myosin ATPase activity in the absence of Tn was considered to be 100% activity (0% inhibition). The ATPase activity result is presented as a bar chart so that the different cTnIs can be directly compared. In all experiments the amount of Tn required for maximal ATPase activation was 1 μM, which is consistent with a ratio of Tn:Tm of 1:1. Three of the cTnI RCM mutations (R145W, A171T, and R192H) showed no change in maximal ATPase activity when compared with wild-type cTnI (Fig. 3). The L144Q mutation showed significantly lower ATPase activity than any of the other RCM mutants or wild-type cTnI. The K178E mutation showed a larger maximal ATPase activity than any of the other RCM mutants or wild-type cTnI (Fig. 3).
Wild-type (WT) showed a significant decrease in maximal ATPase activity when compared with unregulated force of 90.2% (S.D.) of the endogenous cTnI after TnT displacement is expressed as mean ± S.D. The Tn containing the L144Q mutation showed a significant increase in maximal ATPase activity when compared with wild-type (WT) Tn. * p < 0.001.

**Force Development and the Ca\(^{2+}\) Dependence of Force Development**

To exchange the mutant cTns into porcine skinned fibers, endogenous Tn in the skinned fibers was first displaced with recombinant human cardiac TnT by treating the fibers with an excess amount of TnT (0.8 mg/ml) under slightly acidic conditions (pH 6.2) (15, 19). Under these conditions, the endogenous Tn complex is displaced after the TnT treatment. A measure of the extent of displacement of the endogenous cTnI after TnT displacement is the amount of force development in the presence of a very low concentration of Ca\(^{2+}\) (pCa 8.0) (called the Ca\(^{2+}\)-unregulated force). In the absence of cTnI, fibers are unable to relax as the inhibitory activity of cTnI is lacking (15). We make use of this measurement of Ca\(^{2+}\)-unregulated force to ensure that all of the skinned fibers are displaced to the same extent. All of the results presented in this work had a Ca\(^{2+}\)-unregulated force of 90.2 ± 7% (S.D.). It has been previously shown that high levels of Ca\(^{2+}\)-unregulated force of >90% corresponds to high levels of displacement (~95%) of the endogenous cTnI (19, 20). It is impossible to get 100% displacement, because the region of the skinned fibers that are held by the clip and force transducers are not accessible to displacement by exogenous recombinant TnT, resulting in some cTnI in the skinned fibers. The fibers that were displaced with TnT were then reconstituted with cTnI TnC or cTnI RCM mutant/TnC. After displacement and reconstitution, the maximal force obtained for each fiber was measured. This force is measured relative to the initial force of the skinned fibers before TnT displacement. Consistent with previous reports, the maximum force of fibers displaced and reconstituted with wild-type Tn was ~79% (15, 19, 20). Fibers reconstituted with cTn I L144Q/TnC, cTnI R145W/TnC, cTnI A171T/TnC, or cTnI K178E/TnC all showed lower maximal force than fibers reconstituted with wild-type cTnI/TnC (Fig. 4A). Because Tn containing the RCM mutants R145W or K178E showed decreases in maximal force while maximal ATPase activity for Tn containing either of these two mutations was not decreased suggests that no direct correlation between the ATPase activity and force development occurs.

The ability of TnT displaced and cTnI TnC reconstituted skinned fibers to inhibit force development at a very low concentration (pCa 8.0) was also measured (Fig. 4B). Three of the RCM mutants investigated (L144Q, R145W, and K178E) showed considerable inabilities to inhibit force development at basal Ca\(^{2+}\) levels. The RCM mutants that showed the greatest inability to inhibit ATPase activity, L144Q and R145W, also showed the worst ability to inhibit force development at basal Ca\(^{2+}\) levels (Fig. 4B).

Porcine skinned fibers showed a decrease in Ca\(^{2+}\) sensitivity (ΔpCa\(_{50}\) = 0.16) after being displaced with TnT and reconstituted with cTnI TnC (Fig. 5A). This is consistent with previous reports using porcine skinned fibers (19–21). Investigation of the cTnI-RCM mutations showed that all five mutations investigated had greater Ca\(^{2+}\) sensitivity of force development than wild-type cTnI (Table 1). The two RCM mutations that are associated with sudden cardiac death (K178E and R192H) both showed large increases in Ca\(^{2+}\) sensitivity (ΔpCa\(_{50}\) = 0.47 and 0.36, respectively) when compared with wild-type cTnI (Table 1). The cTnI A171T mutation that caused the smallest increase in Ca\(^{2+}\) sensitivity of force development of all the RCM mutations still showed a significant increase in Ca\(^{2+}\) sensitivity (ΔpCa\(_{50}\) = 0.14). Most cTnI HCM mutations show increases in Ca\(^{2+}\) sensitivity of force development that are <0.14 pCa units.
The greater increase in $\text{Ca}^{2+}$ sensitivity may be a reason for the poorer prognosis of cTnI RCM patients when compared with cTnI HCM patients. The possibility that the skinned fibers may not be completely replete with the cTnI TnC complex was investigated by Western blotting (Fig. 5A, inset). Using the skinned fibers before TnT displacement as the control fibers (100% cTnI), the amount of wild-type or mutant Tn incorporated into skinned fibers was 98.7 ± 3.6% for wild-type, 101.5 ± 4.8% for L144Q, 92.5 ± 6.9% for R145W, 100.8 ± 4.1% for A171T, 102 ± 6% for K178E, and 99.5 ± 6.2% for R192H. The location of these RCM mutations may also be important for the major physiological effects that were observed. The recently resolved tertiary structure of cardiac Tn revealed a significant alteration when compared with wild-type or mutant cTnIs (Table I).

| cTn   | $pCa_{50}$ | Hill coefficient | Maximum force | No. of experiments |
|-------|------------|------------------|---------------|-------------------|
| Wild-type cTn | 5.49 ± 0.04 | 1.64 ± 0.08 | 79.2 ± 3.3 | 4 |
| cTnL144Q | 5.80 ± 0.05a | 1.12 ± 0.12a | 42.7 ± 7.4 | 3 |
| cTnR145W | 5.88 ± 0.04a | 1.23 ± 0.16a | 34.9 ± 4.9 | 3 |
| cTnA171T | 5.63 ± 0.01a | 2.01 ± 0.21a | 67.5 ± 2.7 | 3 |
| cTnK178E | 5.96 ± 0.05a | 1.42 ± 0.22 | 66.2 ± 2.0 | 3 |
| cTnR192H | 5.85 ± 0.04a | 1.37 ± 0.14a | 85.0 ± 8.5 | 6 |

a Indicates that the $pCa_{50}$ values for the wild-type cTnI is significantly different from cTnI RCM mutants ($p < 0.001$).

b Indicates that the Hill coefficient ($n_H$) values for the wild-type cTnI is significantly different from cTnI RCM mutants ($p < 0.05$).
of cTnI which is extended away from the other Tn subunits in the for this structure is 1J1E.

Six mutations in cTnI were recently identified in RCM patients (1). RCM has the worst prognosis and least therapeutic options of all of the major cardiomyopathies, and studies on children with RCM found that 28% of them die of sudden cardiac death, usually within 1 year of diagnosis (22). Recent evidence suggests that the disease may be caused by the same genetic abnormalities that result in the more common HCM (1, 7). Post-mortem microscopy of cardiac tissue from two cTnI RCM patients showed typical HCM histology (1). The lack of significant clinical/genetic data on patients containing these RCM-associated mutations is a major problem that compromises the designation of these mutants as RCM. Also, many family members of the index RCM patient (D190G) showed HCM, not RCM. Patients with other cTnI mutations (some of which are presented in this report) also show some HCM associated features (1). The RCM-associated cTnI mutations can cause features of HCM, RCM, or both, depending on the genetic background of the subject and possibly on the specific mutation. To determine whether the mutations classified as RCM by Mogensen et al. (1) are distinguishable from HCM-causing mutations in cardiac TnI, in vitro studies on these mutants were carried out.

The inability of all the Tn complexes containing RCM mutations to properly inhibit actomyosin ATPase activity was unexpected. This is different from cTnI HCM mutations in the C-terminal region of cTnI (residues 145–203), of which, some mutations have defective inhibition, whereas others do not (4). Tn complexes containing either cTnI L144Q or cTnI R145W were both less effective at inhibiting ATPase activity than the other complexes. The main inhibitory region of cTnI is localized to a small region (residues 128–145). The mutations L144Q and R145W both occur within this inhibitory region. The R145W mutation occurs on a residue at which two other mutations have been previously shown to be associated with HCM (R145G and R145Q). The TnI mutation R145G is the most investigated HCM mutation in vitro. This mutation significantly decreased the inhibition of ATPase activity in independent studies from different laboratories (14, 23–25). The other mutation at residue 145 of cTnI, R145Q, also caused decreased inhibition of ATPase activity and decreased activation of ATPase activity when compared with wild-type cTnI. The decreases in inhibitory ATPase activity of RCM mutants are generally larger than the decreases observed for those HCM mutations that show decreased inhibitory ATPase activity.

All five cTnI mutants investigated caused significant increases in the Ca$^{2+}$ sensitivity of force development in skinned fibers. The R145W mutation caused a significantly larger increase in Ca$^{2+}$ sensitivity of force development (0.39 pCa units) than the R145G HCM mutation. The cTnI R145G mutation increased the Ca$^{2+}$ sensitivity of force development by 0.13 pCa units when compared to wild-type cTnI (14). The R145Q HCM mutation was previously investigated by another research group, and this mutation was found to cause an increase in Ca$^{2+}$ sensitivity of force development by 0.10 pCa units (25). The R145G and R145Q HCM mutations had no effect on the affinity of Tn for TnC (using fluorescently labeled TnC or surface plasmon resonance-based assays) (14, 23, 25). The cTnI HCM mutations R145G and R145Q were previously found to reduce the intrinsic inhibitory activity of cTnI without changing the apparent affinity for actin (25). Because the increase in the Ca$^{2+}$ sensitivity of force development must be due to some change in the affinity of TnC for Ca$^{2+}$, the TnI R145G and R145Q (and possibly the R145W) mutations must be exerting their effects on other thin filament proteins (such as TnT or actin), which then affect TnC. These results suggest that the magnitude of the alterations in Ca$^{2+}$ sensitivity may influence the development of RCM over HCM. However, the genetic background of the patient is also an important factor that needs further investigation. The maximal ATPase level in Tn containing the TnI L144Q mutation was significantly decreased when compared with wild-type Tn, consistent with the lower level of force seen in the reconstituted fibers. However, the lower level of force seen with Tn containing the TnI R145W mutation was not accompanied by a decrease in the maximal ATPase level suggesting that this R145W mutation would cause impaired energetics (lower force for the same ATPase activity as wild-type Tn) similar to what is seen in animal models of HCM (26, 27). The R145G mutation caused a 25% decrease in the inhibitory ATPase activity of cTnI (14), whereas the R145W mutation caused an 80% decrease in the inhibitory activity of cTnI. The residues A171 and K178 are part of the second actin-Tm binding region (residues 173–181), which has been shown to potentiate the inhibitory action of the inhibitory region (28). cTnI contains two known actin binding sites at residues 128–148 and 173–181 (4, 8). This may explain the lower inhibition of basal ATPase activity and force for fibers reconstituted with cTnI containing either of these two mutations (A171T and K178E).

It has been long known that under a variety of conditions when the thin filament is not shut off in the absence of Ca$^{2+}$, its apparent Ca$^{2+}$ affinity increases, and this may be a factor in determining the extent of the increased Ca$^{2+}$ sensitivity seen with some of these mutations (35). The only mutant without a large effect on Ca$^{2+}$ sensitivity is A171T, which is also the only mutant investigated to have a nearly normal ability to switch off the thin filament in the absence of Ca$^{2+}$. Based upon the in vitro results it is possible that this A171T mutation may result in a different phenotype (more similar to HCM) than the other mutations investigated. More clinical/genetic data are required to determine whether this mutation clinical phenotype is more similar to HCM or RCM.

The impaired myocardial relaxation seen in RCM patients may be due mainly or in part to the inability of the Tn-contain-
Mutations in cTnI associated with RCM show similar in vitro physiological characteristics as cTnI HCM mutations. However, some significant differences between these mutations and HCM mutations include 1) generally greater increases in Ca\(^{2+}\) sensitivity of force development for these mutations when compared with HCM mutations, 2) generally higher levels of basal sensitivity and 2) the poor relaxation properties of the fibers containing these mutations that would lead to severe diastolic dysfunction. In general the results suggest that these RCM mutations cause functional effects that parallel findings seen for many HCM-causing mutations, but tend to be more severe. This greater functional severity may be pivotal in causing the RCM phenotype in some of the genetically affected individuals. Further investigations are required to determine what causes this functional phenotype (RCM) to be manifested in some patients and not in others possessing the same mutation.

REFERENCES

1. Mogensen, J., Kubo, T., Duque, M., Uribe, W., Shaw, A., Murphy, R., Gimeno, J. R., Elliott, P., and McKenna, W. J. (2003) J. Clin. Investig. 111, 209–216
2. Tobacman, L. S. (1996) Am. J. Cardiol. 78, 110F–115F
3. Gomes, A. V., and Poter, J. D. (2002) Ann. N. Y. Acad. Sci. 963, 214–224
4. Gomes, A. V., and Poter, J. D. (2004) Mol. Cell. Biochem. 263, 99–114
5. Arzt, G., and Wynne, J. (2000) Curr. Treat Options Cardiovasc. Med. 2, 331–348
6. Ammass, N. M., Seward, J. B., Bailey, K. R., Edwards, W. D., and Tajik, A. J. (2000) Circulation 101, 2490–2496
7. Amlani, A., Calzolari, V., Thiene, G., Bozza, G. M., Volante, M., Daliento, L., Basso, C., Calabrese, F., Razzolini, R., Livi, U., and Chionn, R. (1997/1998) Am. J. Cardiol. 80, 1046–1050
8. Palka, P., Lange, A., and Ward, C. (2003) Cardiol. Young 13, 469–471
9. Pazzoli, C., Ortolani, P., Binetti, G., Pichiecchio, F. M., and Magnani, B. (1990) Int. J. Cardiol. 29, 121–126
10. Schwartz, M. L., and Colan, S. D. (2003) Am. J. Cardiol. 92, 636–639
11. Zachara, E., Bertini, E., Loi, E., Boldrini, R., Prati, P. L., and Bosman, C. (2005) J. Biol. Chem. 280, 4348–4352
12. Zhang, J., Kumar, A., Stalker, H. J., Virdi, G., Ferrans, V. J., Horiba, K., Fricker, J. F., and Wallace, M. R. (2001) Clin. Genet. 59, 248–256
13. Ausubel, F. M., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) in Current Protocols in Molecular Biology (Janssen, K., ed) pp. 8.0.1–8.5.9, John Wiley & Sons, New York
14. Lang, R., Gomes, A. V., Zhao, J., Housmans, P. R., Miller, T., and Poter, J. D. (2002) J. Biol. Chem. 277, 11670–11678
15. Szczesna, D., Zhang, R., Zhao, J., Jones, M., Guzman, G., and Poter, J. D. (2000) J. Biol. Chem. 275, 624–630
16. Fiske, C. H., and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
17. Guth, K., and Poter, J. D. (1987) J. Biol. Chem. 262, 13627–13635
18. Fabiato, A., and Fabiato, F. (1978) J. Physiol. (Lond.) 276, 233–255
19. Gomes, A. V., Guzman, G., Zhao, J., and Poter, J. D. (2002) J. Biol. Chem. 277, 35341–35349
20. Venkatasaran, G., Harada, K., Gomes, A. V., Kovacik, G., and Poter, J. D. (2003) J. Biol. Chem. 278, 41670–41676
21. Venkatasaran, G., Kovacik, G., Davis, J. P., Tikhonova, S. B., Engel, P., Solaro, R. J., and Poter, J. D. (2004) J. Biol. Chem. 279, 49579–49587
22. Rivesen, S. M., Kearney, D. L., Smith, E. O., Towbin, J. A., and Denfield, S. (2002) Circulation 106, 876–882
23. Elliott, K., Watkins, H., and Redwood, C. S. (2000) J. Biol. Chem. 275, 22069–22074
24. Takahashi-Yanagita, F., Morimoto, S., and Ohitsuki, I. (2000) J. Biochem. (Tokyo) 127, 355–357
25. Takahashi-Yanagita, F., Morimoto, S., Harada, K., Nakamaki, R., Shiraiishi, F., Ohta, M., Lu, Q. W., Sasaguri, T., and Ohitsuki, I. (2001) J. Mol. Cell. Cardiol. 33, 2093–2107
26. Jayagopalan, M. M., Tarffid, J. C., Finz, I., and Ingwall, J. S. (2003) J. Clin. Investig. 112, 768–775
27. Spindler, M., Saule, K. W., Christe, M. E., Sweeney, H. L., Seidman, C. E., Seidman, J. G., and Ingwall, J. S. (1998) J. Clin. Investig. 101, 1775–1783
28. Tripet, B., Van Eyk, E. J., and Hedges, R. S. (1997) J. Mol. Biol. 271, 728–750
29. James, J., Zhang, Y., Oisinska, H., Sanbe, A., Klevisky, R., Hewett, T. E., and Robbins, J. (2000) Circ. Res. 87, 805–811
30. Parsons, B., Szczesna, D., Zhao, J., Van Shuloten, G., Kerrick, W. G., Potek, R. J. A., and Poter, J. D. (1997) J. Muscle Res. Cell. Motil. 18, 599–609
31. Miller, T., Szczesna, D., Housmans, P. R., Zhao, J., and Freitas, F., Gomes, A. V., Creek, J., McCue, J., Wang, Y. X., Yu, K., Kerrick, W. G., and Potter, J. D. (2003) J. Biol. Chem. 278, 3743–3748
32. Schwaertl, S., Lehrer, S. S., and Geeske, M. A. (1995) Biochemistry 34, 15890–15894
33. Hill, L. E., Mehegan, J. P., Butters, C. A., and Tobacman, L. S. (1992) J. Biol. Chem. 267, 16106–16113
34. Kashwaha, S. S., Fallon, J. T., and Fuster, V. (1997) Nat. Engl. J. Med. 336, 267–276
35. Bremel, R. D., and Weber, A. (1972) Nat. New Biol., 248–256
