Familial Hypertrophic Cardiomyopathy-linked Alterations in Ca\(^{2+}\) Binding of Human Cardiac Myosin Regulatory Light Chain Affect Cardiac Muscle Contraction*

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The ventricular isoform of human cardiac regulatory light chain (HCRLC) has been shown to be one of the sarcomeric proteins associated with familial hypertrophic cardiomyopathy (FHC), an autosomal dominant disease characterized by left ventricular and/or septal hypertrophy, myofibrillar disarray, and sudden cardiac death. Our recent studies have demonstrated that the properties of isolated HCRLC could be significantly altered by the FHC mutations and that their detrimental effects depend upon the specific position of the missense mutation. This report reveals that the Ca\(^{2+}\) sensitivity of myofibrillar ATPase activity and steady-state force development are also likely to change with the location of the specific FHC HCRLC mutation. The largest effect was seen for the two FHC mutations, N47K and R58Q, located directly in or near the single Ca\(^{2+}\)-Mg\(^{2+}\) binding site of HCRLC, which demonstrated no Ca\(^{2+}\) binding compared with wild-type and other FHC mutants (A13T, F18L, E22K, P95A). These two mutants when reconstituted in porcine cardiac muscle preparations increased Ca\(^{2+}\) sensitivity of myofibrillar ATPase activity and force development. These results suggest the importance of the intact Ca\(^{2+}\) binding site of HCRLC in the regulation of cardiac muscle contraction and imply its possible role in the regulatory light chain-linked pathogenesis of FHC.

The regulatory light chain (RLC)\(^{3}\) of myosin is a major regulatory subunit of smooth-muscle and non-muscle myosins and a modulator of the troponin (Tn) -controlled regulation of the striated muscle contraction. The crystal structures of chicken skeletal S1 (1) and the regulatory domain of scallop myosin, consisting of one RLC, one essential light chain (ELC), and a part of the myosin heavy chain (2), have revealed that the RLC is localized at the head-rod junction of the myosin heavy chain and, together with the ELC, stabilizes the \(\alpha\)-helical neck of the myosin head. The N terminus of RLC is noncovalently bound to the myosin heavy chain between Asn-825 and Leu-842, whereas its C terminus wraps around the region located between Gln-808 and Val-826 of the myosin heavy chain (1). The N-terminal domain of the RLC contains a divalent cation-binding site, located in the first helix-loop-helix motif, which binds both Ca\(^{2+}\) and Mg\(^{2+}\) (Fig. 1A). The N-terminal region of RLC also contains the myosin light chain kinase-specific phosphorylation site (Ser-15), which is located in the proximity of the cation-binding site (3).

Recent studies have revealed that the ventricular RLC is one of the sarcomeric proteins associated with familial hypertrophic cardiomyopathy (FHC) (4–6). FHC is an autosomal dominant disease characterized by left ventricular hypertrophy, myofibrillar disarray, and sudden cardiac death. It is caused by missense mutations in various genes that encode for \(\beta\)-myosin heavy chain (7), myosin-binding protein C (8), ventricular RLC and ELC (4–6, 9), troponin T (10), troponin I (11), troponin C (TnC) (12), \(\alpha\)-troponyosin (13), actin (14), and titin (15). Depending on the affected gene and the site of the mutation, FHC has a variable presentation with regard to its degree, severity, and extent of myocardial disarray. The clinical manifestations of FHC range from benign to severe heart failure and to sudden cardiac death. The best characterized clinical cases include patients with \(\beta\)-myosin heavy chain mutations, who show a high level of cardiac hypertrophy, and those with troponin T mutations, who have less hypertrophy but a higher incidence of sudden cardiac death in young adults.

To date 10 RLC FHC mutations have been identified, eight of which are single point mutations and two are intronic splice site mutations (Fig. 1B). The first three mutations, identified in an American population, A13T, E22K, and P95A, were shown to have a rare cardiac phenotype that involved massive hypertrophy of the cardiac papillary muscles and adjacent ventricular tissue causing a mid-cavity obstruction (4). Two other RLC mutations, F18L and R58Q, identified in a French population (5), were associated with a classic form of hypertrophic cardiomyopathy that causes increased left ventricular wall thickness and abnormal electrocardiogram findings with no mid-cavity obliteration. A new report by Richard et al. (16) identified two additional mutations of French origin, D166L and a splice site mutation, intervening sequences 5-2 A \(\rightarrow\) G of intron 5. Three subsequent RLC FHC mutations were found in the Danish cohort, N47K, K104E, and another splice site mutation, intervening sequences 6-1 G \(\rightarrow\) C of intron 6 (6) (Fig. 1).

A C \(\rightarrow\) A transversion in exon 3 of the RLC gene, resulting in the N47K substitution (Fig. 1B), was identified in the 60-year-old proband of a Danish family (6). The patient had severe septal and ventricular hypertrophy, abnormal electrocardiogram findings, and a high, relatively fixed mid-ventricular flow gradient, as well as diastolic filling abnormalities. The mid-ventricular flow gradient was caused not only by the pro-
Functional Study of Cardiac Myosin Regulatory Light Chain

MATERIALS AND METHODS

Mutation, Expression, and Purification of Wild-type HCRLC and the FHC Mutants—The cDNA for wild-type (WT) human cardiac RLC (HCRLC) was cloned by reverse transcription PCR using primers based on the published cDNA sequence (GenBank™ accession number AP020788) and standard methods (17). The FHC RLC mutants, A13T, F18L, E22K, N47K, R58Q, and P95A, were generated using overlapping sequential PCR (17). Wild-type and mutant cDNAs were constructed with an NcoI site at the N-terminal ATG and a BamHI site following the stop codon, to facilitate ligation into the NcoI-BamHI cloning site of the pET-3d (Novagen) plasmid vector and transformation into BL21 expression host cells, and proteins were expressed in large (16 liters) cultures. Expressed proteins were purified as described previously (18).

Flow Dialysis—Flow dialysis was performed in a solution of 100 mM KCl, 20 mM imidazole buffer, pH 7.0 (22 °C). All proteins were equilibrated in this buffer prior to the measurements. The flow dialysis experiments were performed according to Colowick and Womack (19) with slight modifications described in detail previously (18). Data were calculated using Scatchard analysis (20) as described (18).

CD Measurements—Far-UV circular dichroism (CD) spectra were obtained using a 1-mm-path quartz cell in a Jasco J-720 spectropolarimeter. Spectra were recorded at 195–250 nm with a bandwidth of 1 nm at a speed of 50 nm/min and a resolution of 0.2 nm. Analysis and processing of data were done using the Jasco system software (Windows standard analysis, version 1.20). Ten scans were averaged, base lines subtracted, and no numerical smoothing applied. Values of mean residue ellipticity (θ_max, in degree cm^2/dmol) for the spectra were calculated (utilizing the same Jasco system software) using the following equation (21–23).

\[
\theta_{\text{max}} = \theta_0 / (10 \times \text{Cr}^{-1})
\]

where \(\theta_0\) is the measured ellipticity in millidegrees, \(\text{Cr}\) is the mean residue molar concentration, and \(l\) is the path length in cm. The optical activity of the buffer was subtracted from relevant protein spectra. The \(\alpha\)-helical content for each protein was calculated using the standard equation for \(\theta\) at 222 nm (24).

\[
\theta_{\alpha,222} = -30.30 \times f_\alpha + 2.340
\]

where \(f_\alpha\) is the fraction of \(\alpha\)-helical content (\(f_\alpha \times 100\), expressed in %). The measurements were performed at 5 and 22 °C in 30 mM NaCl, 0.3 mM EGTA, 0.7 mM MgCl_2, and 3 mM Tris-HCl buffer at pH 7.4. Spectra were presented as mean residue ellipticity as shown previously (18). Depletion of Endogenous RLC from Porcine Cardiac Myofibrils (CMF) Followed by Reconstitution with CTrC and FHC HCRLC Mutants—CMF were prepared from the left ventricular walls of porcine hearts of healthy young adult animals according to Solorio et al. (25). They were stored at a concentration of about 20 mg/ml in SB buffer containing 30 mM imidazole, 60 mM KCl, 2 mM MgCl_2, pH 7, and 50% glycerol at −20 °C. Before experiments were performed, CMF were removed from 50% glycerol stock, diluted with an equal volume of SB solution containing 1 mM dithiothreitol (SB1), and pelleted at 800 × g. They were re-suspended, tested for protein concentration (determined by a Coomassie protein assay), and diluted to 2.5 mg/ml in the same buffer. The RLC extraction was initiated with a wash of CMF with a solution containing 5 mM CDTA, 50 mM KCl, 1% Triton X-100, 40 mM Tris-HCl, pH 8.4, 1 µM pepstatin A, 0.6 mM NaN_3, and 0.2 mM phenylmethylsulfonyl fluoride for 5 min at room temperature. Then the CMF were pelleted, resuspended to 2.5 mg/ml, and incubated in the same solution for another 30 min at room temperature. They were washed three times with SB1 buffer, assayed for protein concentration, and diluted to 2.5 mg/ml. The RLC-depleted

The amino acid sequence analysis of cardiac RLC from different organisms reveals a high sequence homology among RLC from different species and demonstrates that the FHC-mutated residues of HCRLC are highly conserved in all of the presented RLC sequences (Fig. 2A). The structure of the N terminus of RLC is similar to other EF-hand Ca^{2+}-binding proteins such as calmodulin (CaM) or TnC (Fig. 2B), whereas the C terminus is considerably less similar (1). Interestingly, sequence comparison of HCRLC and other EF-hand Ca^{2+}-binding proteins reveals that amino acids of both Ca^{2+}-binding site mutations, N47K and R58Q, in which the Ca^{2+}-binding ability was lost due to the FHC mutation, increased Ca^{2+}-sensitivity of myofibrillar ATPase activity and steady-state force. Our results suggest that the FHC-associated perturbations of the HCRLC Ca^{2+}-binding site that lead to its inactivation and produce alterations in the Ca^{2+}-dependent ATPase/force could be a key mechanism of the RLC-linked pathogenesis of FHC. We also propose the importance of the intact Ca^{2+}-binding site of HCRLC in the regulation of cardiac muscle contraction in the normal and diseased state of the heart.

![Diagram](http://example.com/diagram.png)

**FIG. 1.** Graphic diagrams of HCRLC. A, three-dimensional representation of HCRLC. The structure was derived from Protein Data Bank code 1WDC (2). FHC mutations and the Ca^{2+}-binding site are labeled. Two N-terminal mutations, A13T and F18L, are localized in the region of RLC that was not resolved in any of the available RLC structures (1, 2). B, exon organization of HCRLC and the sites of all FHC mutations identified to date. The first FHC HCRLC mutations identified in an American population, A13T, E22K and P95A, were shown to be associated with a particular subtype of cardiac hypertrophy defined by a mid-left ventricular obstruction (4). Two other RLC mutations, F18L and R58Q, identified in a French population, were associated with a typical form of hypertrophic cardiomyopathy that causes increased left ventricular wall thickness and abnormal electrocardiograms. The presented data provide a functional seven FHC genes that were screened in parallel were unexpected. The N47K mutation was not identified in the 150 healthy controls among the 100 hypertrophic cardiomyopathy cases. The pronounced septal hypertrophy but also by a significant increase in the size of the papillary muscle apparatus. Interestingly, a marked progression in the septal hypertrophy, from 31 to 45 mm, was seen over a 2-year span from age 60 to 62 years. The N47K mutation was not identified in the 150 healthy controls or in the other 197 probands. No other mutations in the additional seven FHC genes that were screened in parallel were identified in this patient (6).

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were placed in oxygenated physiological salt solution of 140 mM NaCl, Subbarow (26). Released inorganic phosphate was measured according to Fiske and 150 mM adjusted with potassium propionate) containing 1% Triton 2.5 mM MgATP and terminated after 5 min with 5% trichloroacetic acid. The reaction was initiated with 30 mM imidazole, pH 7, 50 mM KCl, 2 mM MgCl2, and different concentrations of Ca2+ from 0.01 mM to 4.5. After a 5-min incubation at 30 °C, the reaction was initiated with 0.6 mM NaN3, 0.2 mM 20 mM imidazole, pH 7.0, 15 mM creatinine phosphate; ionic strength ionic CTnC and HCRLC-WT or A13T, F18L, E22K, N47K, R58Q, and 20 mM Mg2+ ATP and terminated after 5 min with 5% trichloroacetic acid. Released inorganic phosphate was measured according to Fiske and Subbarow (26).

**Skinned Cardiac Muscle Fibers**—Freshly isolated porcine hearts were skinned in a 50% glycerol, 50% solution of HCRLC-WT or A13T, F18L, E22K, N47K, R58Q, and 935 mM NaH2PO4, 5.5 mM M CTnC. The solution of CTnC was included in the Ca2+ buffer except [Ca2+]4M) and relaxed in the Ca2+ solution containing 40 mM MgATP2−. 20 mM imidazole, pH 7.9, 15 mM creatinine phosphate; ionic strength — 150 mM adjusted with potassium propionate) containing 1% Triton X-100 for about 2 months. The SDS-PAGE of the control, CDTA-depleted, and CTnC- and HCRLC-reconstituted proteins: human cardiac RLC (P10916), pig (P08733). H9262). Two Ca2+ binding site FHC HCRLC mutations, N47K and R58Q, and the equivalent amino acids in other EF-hand proteins are labeled in white in a black box. Other FHC mutations are in bold letters and underlined in the sequence of HCRLC. The Ca2+ binding sites of all proteins are listed in gray (light gray for the Ca2+ inactive site of cardiac TnC).

Reconstitution of the CDTA-depleted Fibers with CTnC and FHC HCRLC Mutants—Reconstitution of the RLC-depleted fibers with porcine CTnC and HCRLC-WT or A13T, F18L, E22K, N47K, R58Q, and P95A mutants was performed in pCa 8 solution containing 40 μM HCRLC and 15 μM CTnC. The solution of CTnC was included in the reconstitution protein mixture during the first 30 min of fiber incubation followed by a 30-min incubation with fresh HCRLC solution at room temperature. The addition of CTnC was to assure that the fibers were not deficient in CTnC, because its partial extraction could affect the Ca2+ sensitivity of force development. Reconstituted fibers were then washed in pCa 8 solution and subjected to force measurements. The SDS-PAGE of the control, CDTA-depleted, and CTnC- and HCRLC-WT- or FHC mutant-reconstituted fibers is presented in Fig. 6.

**Steady-state Force Development**—A bundle of 3–5 single fibers isolated from a batch of glycine treated fibers was attached by tweezer clips to a force transducer, placed in a 1-ml cuvette, and bathed in pCa 8 solution containing 1% Triton X-100. The fibers were then tested for steady-state force development in pCa 4 solution (composition is the same as pCa 6 buffer except [Ca2+]4M = 10−4 M) and relaxed in the pCa 8 solution utilized for CMF extraction, containing 5 mM CTnC, 40 mM Tris, 50 mM KCl, 1 μg/ml pepstatin A, 0.6 mM NaN3, 0.2 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100, pH 8.4. The fibers were incubated in this solution for 5 min at room temperature and then transferred to the fresh solution of the same composition for another 30 min. The extent of RLC extraction was tested by SDS-PAGE (Fig. 6A). Depletion of the endogenous RLC may result in partial extraction of the endogenous TnC, and therefore, both of these proteins were added back into the CDTA-treated fibers (Fig. 6).

**CDTA Extraction of Cardiac Muscle Fibers**—Endogenous RLC depletion from porcine cardiac muscle fiber preparations was achieved in the same manner as described previously (44). The CDTA-treated fibers were washed twice with SB1 buffer, assayed for protein concentration, and diluted to 2.5 mg/ml in SB1 buffer. The SDS-PAGE of the control, CDTA-depleted, and CTnC- and HCRLC-reconstituted fibers is presented in Fig. 6.

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solution. Steady-state force development was monitored for control, CDTA-depleted, and then CTnC- and WT-, A13T, F18L, E22K, N47K, R58Q, and P95A-reconstituted fibers.

**Ca**^{2+} Dependence of Force Development—After the initial steady-state force was determined, the fibers were relaxed in the pCa 8 buffer and then exposed to solutions of increasing Ca^{2+} concentrations (from pCa 8 to 4). The maximal force was measured in each pCa solution followed by a short relaxation of the fibers in pCa 8 solution. Data were analyzed using the following equations:

\[ \text{% change in force} = 100 \times \frac{\text{pCa}^{2+} - \text{pCa}_{50}^{2+}}{\text{pCa}_{50}^{2+}} \]  

where \( \text{pCa}_{50}^{2+} \) is the free Ca^{2+} concentration that produces 50% force change.

**SDS Gel Electrophoresis**—Control, CDTA-depleted and CTnC/WT-, A13T, F18L, E22K, N47K, R58Q, and P95A-reconstituted porcine cardiac RLC and fibers were run on 15% SDS-PAGE according to Laemmli (27) (Figs. 4 and 6). The respective HCRLC protein bands were quantified utilizing densitometry with the Scion Image for Windows (version beta 4.0.2). The percent of RLC depletion and/or reconstitution was calculated from the net intensity of the RLC bands compared with the control untreated fibers (100%). Differences in gel loading were assessed further at 5°C.

**Statistical Analysis**—The significant difference between the pCa_{50} values of the Ca^{2+} sensitivity of myofibrillar ATPase activity and force development among WT and respective FHC HCRLC mutants was determined utilizing an unpaired Student’s t test (Sigma Plot 8.0), with significance defined as \( p < 0.05 \).

## RESULTS

**Effect of N47K Mutation in HCRLC on Ca^{2+} Binding and the CD Spectrum**—We have recently published the Ca^{2+} binding properties and the CD spectra of human cardiac RLC and five recombinant FHC HCRLC mutants (A13T, F18L, E22K, R58Q, and P95A) (18). Three of the FHC mutations, A13T, F18L, and P95A, decreased the K_{Ca}^0 -fold, whereas two other mutations, E22K and R58Q, changed the Ca^{2+} binding properties in a more drastic way. Compared with WT-HCRLC (K_{Ca} = 6.67 ± 0.21 \times 10^8 \text{ M}^{-1}), the E22K mutation decreased the K_{Ca} value by -17-fold, whereas the R58Q mutation eliminated Ca^{2+} binding to HCRLC (18) (Table I). Flow dialysis experiments using 45Ca^{2+} performed for the new N47K mutant showed no Ca^{2+} binding to the single Ca^{2+}-Mg^{2+} site of HCRLC (Table I). Therefore, the Asn → Lys substitution in the second from last coordinating position of the Ca^{2+} binding loop of HCRLC abolished its Ca^{2+} binding.

Far-UV CD spectroscopy was used to analyze whether the N47K mutation altered the secondary structure of HCRLC. As shown in Fig. 3, the mutation did not introduce any significant changes to the CD spectrum of HCRLC, and the calculated α-helical content (at wavelength 222 nm) of N47K was 18.7 versus 18.5%, determined for WT-HCRLC (n = 10). These values of α-helical content were calculated from the mean residue ellipticity at 222 nm (at 22°C), \([\theta]_{MRE}^0 \sim -8020 \text{ or } -7930\) for N47K or HCRLC-WT, respectively. As demonstrated by others (23, 28), they were similar to the values presented for rabbit skeletal RLC, although the percent of α-helical content calculated by these authors was shown to be higher (23, 28). The effect of the N47K mutation on the CD spectrum of HCRLC was tested further at 5°C. This was to assess the difference between these proteins at a low-temperature-induced, possibly more folded, structure. Surprisingly, the CD spectra of these proteins did not significantly change, and their calculated α-helical content was about 19%. Interestingly, there was no change in the CD spectra of WT and N47K when monitored at 5°C and then at 22°C followed by the measurement at 5°C. This suggests that the secondary structure of HCRLC is quite stable in this range of temperatures.

**Effect of FHC HCRLC Mutations on ATPase Activity in Reconstituted CMF**—Extraction of endogenous RLC from porcine CMF was achieved with a 5-min wash followed by a 30-min incubation in the CDTA-containing solution (see Materials and Methods). Even though the CDTA solution contained 50 mM KCl to prevent extraction of TnC, the RLC- and possibly CTnC-depleted CMF were reconstituted with HCRLC and porcine CTnC. Fig. 4 demonstrates an example of the SDS-PAGE of the control (not treated), RLC-depleted, and CTnC/HCRLC-reconstituted myofibrils utilized in the ATPase activity assays presented in Fig. 5. The CDTA extraction method resulted in a 78–88% depletion of the endogenous RLC from CMF (Fig. 4, A, lanes 2 and 9, and B, lanes 5 and 7; see Fig. 4 legend for exact percent of RLC depletion). These RLC-depleted CMF could be easily reconstituted with exogenous HCRLC-WT and all FHC HCRLC mutants (Fig. 4). The SDS-PAGE of reconstituted CMF demonstrated in Fig. 4 and the gels that are not shown revealed that all mutants were able to bind to the RLC-depleted CMF and restore their function (Fig. 5). As depicted in the legend of Fig. 4, the percent of CMF reconstitution with various HCRLC proteins exceeded 100%, the value assumed for the RLC in the control, non-treated CMF. This could be because of nonspecific binding of these FHC mutants to the RLC-depleted CMF or incomplete washout of unbound proteins. Measurements of the ATPase activity of the control, RLC-depleted and CTnC- and WT-, A13T-, F18L-, E22K-, R58Q-, and P95A-reconstituted myofibrils are presented in Fig. 5. Myofibrils lacking myosin RLC demonstrated dramatic impairment of the Ca^{2+} regulation of ATPase activity (at pCa 8 and 4.5) and also conferred lowered Ca^{2+} sensitivity to ATPase compared with

### Table I

| HCRLC protein | Isolated HCRLC<sup>a</sup> (M) | Myofibrils<sup>b</sup> (M) | Fibers<sup>c</sup> (M) | Force recovery compared with intact fibers |
|---------------|-----------------|-----------------|----------------|---------------------------------|
| WT           | 1.50 ± 0.02     | 0.200 ± 0.009   | 2.88 ± 0.19    | 84.1 ± 4.5 (8)                  |
| A13T         | 4.85 ± 0.31     | 0.191 ± 0.015   | 3.02 ± 0.06    | 76.7 ± 3.8 (4)                  |
| F18L         | 4.20 ± 0.26     | 0.224 ± 0.062   | 2.63 ± 0.10    | 74.3 ± 2.1 (5)                  |
| E22K         | 25.64 ± 3.48    | 0.178 ± 0.001   | 2.63 ± 0.10    | 66.4 ± 2.8 (6)                  |
| N47K         | No binding      | 0.141 ± 0.009   | 2.51 ± 0.10    | 74.0 ± 3.2 (8)                  |
| R58Q         | No binding      | 0.170 ± 0.004   | 2.19 ± 0.10    | 77.7 ± 2.0 (4)                  |
| P95A         | 4.74 ± 1.05     | 0.200 ± 0.011   | 2.75 ± 0.25    | 70.7 ± 4.7 (4)                  |

<sup>a</sup> Apparent Ca^{2+} dissociation constants (1/K_{app}) of isolated HCRLC-WT and FHC mutants (from Ref. 18). Flow dialysis was performed in a solution of 100 mM KCl, 20 mM imidazole buffer, pH 7.0 (22°C).

<sup>b</sup> K_{app} of reconstituted cardiac myofibrils expressing the free Ca^{2+} concentration, which produces 50% of myofibrillar ATPase activity (pCa_{50} values ±S.E.) (n is specified in Fig. 5C).

<sup>c</sup> K_{app} of reconstituted skinned fibers expressing the free Ca^{2+} concentration, which produces 50% of steady state force (pCa_{50} values ±S.E.) (n is specified in Fig. 7).
control CMF (Fig. 5A). Partial extraction of TnC could contribute in part to this effect. Reconstitution of the RLC-depleted CMF with CTnC and HCRLC (WT and FHC mutants) recovered the Ca^{2+} regulation of ATPase activity at low and high Ca^{2+} concentrations (Fig. 5, B and C). However, the maximal level of ATPase activation was slightly different for various FHC mutants, and the highest level was obtained for R58Q-reconstituted CMF, whereas the lowest level was for P95A-reconstituted CMF (Fig. 5B). The Ca^{2+} sensitiv-
ity of myofibrillar ATPase activity was significantly increased for N47K mutant and only slightly increased for E22K and R58Q mutants compared with WT-reconstituted CMF. As shown in Fig. 5C, a difference of ΔpC麾 = 0.15 was observed between WT and N47K mutant (p = 0.003). A slight decrease of the Ca\(^{2+}\) sensitivity of ATPase activity was observed for F18L mutant, whereas no change was monitored for A13T and P95A mutants (Fig. 5C). Interestingly, three of the mutants that increased the Ca\(^{2+}\) sensitivity of ATPase (Fig. 5C) shared the same property of altered Ca\(^{2+}\) binding to the single Ca\(^{2+}\)-Mg\(^{2+}\) binding site of HCRLC (Table I). As shown previously for E22K and R58Q mutants (18) and in this paper for N47K, these mutations either decreased affinity for Ca\(^{2+}\) (E22K) or totally inactivated the HCRLC Ca\(^{2+}\)-binding site (N47K and R58Q).

Table I summarizes the apparent Ca\(^{2+}\) dissociation constants of the HCRLC-reconstituted myofibrils calculated from the pC麾 values of the ATPase-pCa relationship of WT- and FHC mutant-reconstituted myofibrils. As demonstrated, the half-activation of the ATPase activity occurred at about 0.2 μM Ca\(^{2+}\) concentrations.

**Effect of FHC HCRLC Mutations on Steady-state Force in Reconstituted Porcine Papillary Muscle Fibers**—HCRLC-WT and all FHC mutants (A13T, F18L, E22K, N47K, R58Q, and P95A mutants) were also tested for steady-state force development and the regulation of Ca\(^{2+}\) sensitivity of force. Skinned porcine papillary muscles were utilized in this study. Depletion of the endogenous RLC in these fibers was achieved in a manner similar to the procedure employed in porcine cardiac myofibrils (see “Materials and Methods”). As in CMF, the reconstitution of the CDTA-depleted fibers was performed with HCRLC and porcine CTnC due to the possibility of partial extraction of endogenous TnC during treatment with CDTA. The percent of RLC depletion (and reconstitution) was calculated from the net intensity of the RLC bands of the respective fibers compared with the control untreated fibers (100%) (Fig. 6). Differences in gel loading were corrected by a comparison of the RLC bands with the ELC bands, which are not affected by the RLC extraction/reconstitution procedure. As shown in Fig. 6, the CDTA extraction procedure resulted in 76–83% RLC depletion (Fig. 6A, lanes 2 and 3). Similar to CMF, the RLC-depleted fibers could be reconstituted easily with WT and/or FHC mutants during a 1-h incubation of the CDTA-depleted fibers. The solution of porcine CTnC was added to the first reconstitution solution of WT or FHC HCRLC mutants followed by incubation of the fibers with fresh HCRLC solution (see “Materials and Methods”). Analogous to the reconstituted myofibrils, the fibers also had a slight excess of reconstituted HCRLC compared with the RLC content in the control, non-treated fibers (Fig. 6). Depletion of the RLC accompanied by a partial extraction of TnC resulted in a decrease in the maximal level of force development from 100% (intact, non-treated fibers) to 46.3 ± 1.5% (n = 40). Reconstitution of RLC-depleted fibers with CTnC and HCRLC-WT restored the maximal level of force to 84.1 ± 4.5% (n = 8) (Table I). The lowest level of recovered force was observed for the E22K mutant (66.4 ± 2.8%, n = 6) whereas N47K- and R58Q-reconstituted fibers demonstrated 74 ± 3.2% (n = 8) and 77.7 ± 2.0% (n = 4), respectively (Table I). The effect of other FHC mutations on the percent of force recovery is listed in Table I. Fig. 7 demonstrates the pC麾 values of the force-pCa relationship of these reconstituted porcine cardiac fibers. Similar to the results of ATPase-pCa relationship, the two Ca\(^{2+}\) binding site mutants, N47K and R58Q, showed an increase in the Ca\(^{2+}\) sensitivity of force development, with R58Q demonstrating significantly higher Ca\(^{2+}\) sensitivity of force than that of WT-reconstituted fibers (p = 0.04). The apparent Ca\(^{2+}\) dissociation constants of these reconstituted cardiac muscle fibers are presented in Table I. They were calculated from the pC麾 values of the force-pCa relationship of WT- and FHC mutant-reconstituted fibers. As shown, the half-activation of the force-pCa curve occurred at 2.2–3 μM [Ca\(^{2+}\)]\(_{i}\), the range of Ca\(^{2+}\) concentrations that matches the approximate Ca\(^{2+}\)-Mg\(^{2+}\) binding site of HCRLC in the isolated state and in the absence of Mg\(^{2+}\) (Table I).

**DISCUSSION**

This report is a continuation of our previous study in solution (18), which characterized mutations in the regulatory light chains of human cardiac myosin that have been linked to familial hypertrophic cardiomyopathy. The current paper investigates the functional effects of these mutations when reconstituted in skinned cardiac muscle preparations. These muscle systems are designed to provide important physiological information such as myofibrillar ATPase activity and steady-state force development. Ca\(^{2+}\) sensitivity of ATPase/force, and therefore are suitable to use in characterizing the ability of the muscle to perform work. Our study describes the phenotype of six FHC mutations in HCRLC that were shown to cause various forms of cardiac hypertrophy in humans. FHC is an autosomal dominant disease known to be a heritable form of cardiac hypertrophy caused by mutations in genes encoding sarcomeric proteins. Affecting 1/500 of the population, FHC is the most common identified cause of sudden death in young people (29). Patients harboring RLC mutations develop ventricular and/or septal hypertrophy and heart failure (4–6,16). We
have examined the wild type and six FHC HCRLC mutants (A13T, F18L, E22K, N47K, R58Q, and P95A) that have been reconstituted in skinned porcine cardiac myofibril and fiber preparations. The ATPase assays performed on 75–85% RLC-depleted and fully reconstituted myofibrils (Fig. 4) showed that the N47K mutant increased the Ca\textsuperscript{2+} sensitivity of myofibrillar ATPase by \( \Delta p_{Ca_{0.5}} = 0.15 \) compared with WT (\( p = 0.003 \)) (Fig. 5, B and C). It was quite interesting that the Asn \( \rightarrow \) Lys mutation in HCRLC, which in fact resulted in abolishing Ca\textsuperscript{2+} binding to HCRLC, increased the Ca\textsuperscript{2+} sensitivity of myofibrillar ATPase activity. However, the same trait was observed for two other FHC HCRLC mutations, R58Q and E22K, which, respectively, eliminated or dramatically decreased Ca\textsuperscript{2+} binding to isolated HCRLC (18) (Table I). Both of these mutants increased the Ca\textsuperscript{2+} sensitivity of ATPase activity although the differences were not great compared with HCRLC-WT (Fig. 5C). The results from skinned fiber studies in which the Ca\textsuperscript{2+} sensitivity of steady-state force development was measured supported myofibrillar ATPase findings. In this case the R58Q mutant, which had its Ca\textsuperscript{2+} binding site inactivated because of the Arg \( \rightarrow \) Gln amino acid substitution caused a significant increase in the Ca\textsuperscript{2+} sensitivity of force compared with WT-reconstituted fibers (\( p = 0.04 \)) (Fig. 7). The N47K mutation also increased the Ca\textsuperscript{2+} sensitivity of force, but the change was not significant when compared with WT-reconstituted fibers (Fig. 7). As pictured in Fig. 1A, both of these mutations, which resulted in functionally important alterations in the Ca\textsuperscript{2+} sensitivity of ATPase/force, are located in or near the Ca\textsuperscript{2+} binding site of HCRLC. Even though different amino acid replacements occurred in the HCRLC molecule because of the FHC-mutated HCRLC gene (Asn \( \rightarrow \) Lys and Arg \( \rightarrow \) Gln), both mutations resulted in an inactivation of this site for Ca\textsuperscript{2+}, and both mutants produced an increase in the Ca\textsuperscript{2+} sensitivity of ATPase/force when reconstituted in porcine cardiac muscle preparations. To our knowledge this is the first physiological study assessing the functional consequences of these FHC-linked N47K and R58Q mutations in HCRLC. Interestingly, the sequence comparison of HCRLC and other EF-hand Ca\textsuperscript{2+}-binding proteins such as CaM or TnC (Fig. 2B) reveals that both of the FHC-mutated amino acids, N47K and R58Q, in HCRLC appear as Lys and Gln in the equivalent positions of the CaM and TnC Ca\textsuperscript{2+}-binding sites regardless of whether they bind (CaM, skeletal TnC) or do not bind (cardiac TnC) Ca\textsuperscript{2+}. Therefore, these two amino acids, Asn-47 and Arg-58, exist specifically in the HCRLC, and any perturbations in these residues may affect the essential interactions of RLC with the heavy chain of myosin. Other amino acid differences in the sequence of the Ca\textsuperscript{2+}-coordinating loops of HCRLC versus CaM versus TnC (Fig. 2B) may have been crucial in determining the specificity and affinity of these sites for Ca\textsuperscript{2+}. The results of this study imply the importance of an enduring binding of Ca\textsuperscript{2+} to the RLC during cardiac muscle contraction and suggest an important role for RLC in this troponin- and Ca\textsuperscript{2+}-regulated muscle system. As shown in Table I, the Ca\textsuperscript{2+} affinity of isolated HCRLC-WT in the absence of Mg\textsuperscript{2+} was about 1.5 \( \mu \text{M} \), and all tested FHC mutations decreased the Ca\textsuperscript{2+} binding, with N47K and R58Q abolishing it. The affinity of this site for Ca\textsuperscript{2+} in the presence of 2 mM Mg\textsuperscript{2+} was shown to be \(-2\)-fold lower (30). It is not known whether the site inactivated for Ca\textsuperscript{2+} does or does not bind Mg\textsuperscript{2+}. Holroyde et al. (31) demonstrated that cardiac and/or skeletal myosin can bind Ca\textsuperscript{2+} with 100-fold higher affinity than isolated RLC, and it is not impossible that Ca\textsuperscript{2+} binding to RLC increases further when myosin is bound to the thick filaments in muscle. It was shown that in the presence of 0.3 mM Mg\textsuperscript{2+}, cardiac myosin bound Ca\textsuperscript{2+} with a \( K_{Ca} = 3 \) \( \mu \text{M} \). As shown in Table I, the half-activation of force in our reconstituted porcine cardiac fibers (at 1 mM Mg\textsuperscript{2+}) occurred at about 2.2–3 mM Ca\textsuperscript{2+}. Therefore, the contribution of RLC to Ca\textsuperscript{2+}-dependent activation in skinned cardiac muscle preparation cannot be ignored. It is thought that under physiological conditions when muscles are in the relaxed state, the RLC Ca\textsuperscript{2+} binding site is occupied by Mg\textsuperscript{2+} (31) and may become partially saturated with Ca\textsuperscript{2+}, depending on the length of the [Ca\textsuperscript{2+}] transient (32). Furthermore, because of the slow dissociation of the Mg\textsuperscript{2+} bound to this site during muscle activation (33), it was thought not to play a primary regulatory role in muscle contraction. Although this may be true for myosin in solution, it may not reflect the true binding properties of this site when myosin is bound in muscle. As shown in this study, the FHC-linked mutations in HCRLC that led to inactivation of its Ca\textsuperscript{2+} binding site were able to affect the [Ca\textsuperscript{2+}] of force development even though the change was not large. The observed increase in the Ca\textsuperscript{2+} sensitivity of force/ATPase could result from RLC-induced alterations in the \( k_{off} \) of Ca\textsuperscript{2+} from TnC or could be a direct effect of the structural changes in RLC. Even though the RLC Ca\textsuperscript{2+} binding site cannot compete with the regulatory site(s) of TnC in muscle activation, it may contribute to or modulate the function of TnC during prolonged contractions.

Some of the mutations examined in this paper have been studied in RLC-depleted and RLC mutant-reconstituted rabbit skeletal muscle fibers (34). In contrast to our porcine papillary muscle fibers and the human ventricular RLC, these authors (34) utilized rabbit psoas muscle fibers and the rat cardiac isoform of the RLC with the FHC mutations inserted in the amino acid positions that were homologous to human ventricular RLC (G13T, F18L, E22K, and P95A); no N47K or R58Q mutations were included in their studies. Despite the hetero-
neyticy of their system, the study provided important information on the E22K mutation, which, in agreement with our results, slightly increased the Ca$^{2+}$ sensitivity of force (34). In contrast, we did not observe dramatic changes in the maximal isometric tension, cooperativity, and Ca$^{2+}$ sensitivity of force generated by the F18L mutation. This discrepancy could be because of their skeletal versus our cardiac fibers, the variability of their control fibers, with $\rho_{Ca_{50}}$ values varying between 5.8 and 6.3, and the fact that only 50% of the endogenous RLC has been extracted from their skeletal fibers (34). Therefore, their observed effects on force development were partially produced by skeletal RLC and in part by cardiac RLC. As was shown by other laboratories, these two different RLC isoforms could generate different fiber kinetics, force development, Ca$^{2+}$ sensitivity, and the like. A study from the Robbins lab (35) analyzed the effect of complete or partial replacement of the cardiac RLC with the isoform that is normally expressed in fast skeletal muscle fibers in the atria and ventricles and showed that the replacement of the ventricular with the skeletal isoform reduced left ventricular contractility and relaxation, although the unloaded shortening velocity of isolated ventricular cardiomyocytes was not significantly different. Furthermore, Sanbe et al. (36) have shown that fiber kinetics are not affected when transgenically encoded protein is expressed in its endogenous compartment but that ectopic replacement invariably leads to changes in the cross-bridge kinetics of the fiber.

Other studies utilizing slow skeletal muscle fibers from an E22K-diseased patient also demonstrated increased Ca$^{2+}$ sensitivity of force development (37). However, in vitro motility studies utilizing E22K-mutated myosin isolated from cardiac biopsies of affected individuals showed normal actin filament translocation (4). Interestingly, this E22K mutation is the only FHC-linked mutation in HCRLC expressed in a transgenic mouse model (36). Transgenic mice expressing E22K showed no detectable hypertrophy in mature adult animals at the chamber or cellular levels. However, no functional study of these E22K-transgenic mice has been performed or reported (38).

In summary, this is the first study that characterizes FHC HCRLC mutant proteins when reconstituted in porcine cardiac muscle preparations. Furthermore, we provide new information for two intriguing HCRLC Ca$^{2+}$ binding site mutants, N47K and R58Q, that has not been reported by others using myosin and between myosin and other contractile proteins the patients harboring these FHC HCRLC mutations. Furthermore, the interactions between the RLC and the heavy chain of myosin and between myosin and other contractile proteins could also be affected by these FHC-linked HCRLC mutations. It is plausible, for instance, that the Ca$^{2+}$ affinity of TnC could indirectly be altered in this HCRLC-mutated myocardium. Cardiac hypertrophy and/or heart failure are perhaps the end result of these functional perturbations among the contractile proteins. Further in vivo work is needed to assess the physiological consequences of these FHC HCRLC mutations and to identify specific mechanisms involved in the pathogenesis of RLC-linked FHC.

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Familial Hypertrophic Cardiomyopathy-linked Alterations in Ca$^{2+}$ Binding of Human Cardiac Myosin Regulatory Light Chain Affect Cardiac Muscle Contraction
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