Published in final edited form as:

J Theor Biol. 2011 September 7; 284(1): 71–81. doi:10.1016/j.jtbi.2011.06.014.

Cross-bridge Kinetics in Myofibrils Containing Familial Hypertrophic Cardiomyopathy R58Q Mutation in the Regulatory Light Chain of Myosin

P. Mettikolla¹, N. Calander^{1,3}, R. Luchowski¹, I. Gryczynski², Z. Gryczynski¹, J. Zhao⁴, D. Szczesna-Cordary^{4,*}, and J. Borejdo^{1,*,∞}

¹ Dept of Molecular Biology & Immunology and Center for Commercialization of Fluorescence Technologies, University of North Texas, Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107

² Dept of Cell Biology & Genetics and Center for Commercialization of Fluorescence Technologies, University of North Texas, Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, TX 76107

³ Dept of Physics, Macquarie University, Balacalava Rd, NSW 2109, Australia

⁴ Dept of Molecular and Cellular Pharmacology, University of Miami Miller School of Medicine, 1600 NW 10th Ave., Miami, FL 33136

Abstract

Familial hypertrophic cardiomyopathy (FHC) is a heritable form of cardiac hypertrophy caused by single-point mutations in genes encoding sarcomeric proteins including ventricular myosin regulatory light chain (RLC). FHC often leads to malignant outcomes and sudden cardiac death. The FHC mutations are believed to alter the kinetics of the interaction between actin and myosin resulting in inefficient energy utilization and compromised function of the heart. We studied the effect of the FHC-linked R58Q-RLC mutation on the kinetics of transgenic (Tg)-R58Q cardiac myofibrils. Kinetics was determined from the rate of change of orientation of actin monomers during muscle contraction. Actin monomers change orientation because myosin cross-bridges deliver to it periodic force impulses. An individual impulse (but not time average of impulses) carries the information about the kinetics of actomyosin interaction. To observe individual impulses it was necessary to scale down the experiments to the level of a few molecules. A small population (~4 molecules) was selected by using (deliberately) inefficient fluorescence labeling and observing fluorescent molecules by a confocal microscope. We show that the kinetic rates are significantly smaller in the contracting cardiac myofibrils from Tg-R58Q mice than in control Tg-wild type (WT). We also demonstrate a lower force per cross-section of muscle fiber in Tg-R58Q *versus* Tg-WT mice. We conclude that the R58Q mutation-induced decrease in cross-bridge kinetics underlines the mechanism by which Tg-R58Q fibers develop low force and thus compromise the ability of the mutated heart to efficiently pump blood.

© 2011 Elsevier Ltd. All rights reserved.

*corresponding author: Julian.Borejdo@unthsc.edu.

∞dedicated to the memory of Prof. Manuel F. Morales

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Mutations in genes that encode for the major contractile proteins of the heart underlie the genesis of Familial Hypertrophic Cardiomyopathy (FHC), an autosomal dominant disease manifested by ventricular hypertrophy, myofibrillar disarray and sudden cardiac death (SCD) [1]. The most prevalent mutations occur in the beta-myosin heavy chain, myosin binding protein C, tropomyosin, troponin and actin (reviewed in [2–4]). The mutations observed in the genes encoding the ventricular regulatory (RLC) and essential (ELC) light chains of myosin are less common but often lead to malignant outcomes [5–13]. The lack of therapeutic strategies in FHC highlights the importance of finding the mechanisms responsible for the pathogenic phenotypes caused by the FHC mutations.

The R58Q mutation in the ventricular RLC was found in patients with various ethnic backgrounds and has been associated with a malignant disease phenotype including multiple cases of SCD [6, 8–10]. Using transgenic (Tg) mice expressing the R58Q-RLC mutation in the heart, we have previously shown that, compared with the wild type (WT) mice, the R58Q mutant fibers were characterized by increased Ca^{2+} sensitivity of force, lower level of maximal force, and impaired muscle relaxation [14]. This potentially abnormal diastolic function determined in Tg-R58Q mouse papillary muscles was later confirmed by the echo-Doppler assessment showing prolonged deceleration times in Tg-R58Q mice compared with Tg-WT [15]. We have also shown an R58Q-dependent increase in the actin activated myosin ATPase activity and increased actin filament sliding velocity in the *in vitro* motility assays [16]. The latter report confirmed, at the molecular level, the R58Q-dependent decrease in force generation [14, 16]. Based on our previous findings we hypothesized that the R58Q mutation of RLC may cause alterations in the kinetics of the acto-myosin interaction. Consequently, the focus of the current investigation was to determine the effect of R58Q on cross-bridge kinetics, using our recently described Single Molecule Detection (SMD) approach [17].

In this work we labeled actin. It was advantageous to observe actin rather than myosin molecules, because labeling actin with fluorescent phalloidin does not alter the enzymatic properties of the actomyosin system [18, 19] and because labeling is stoichiometric allowing for the strict control of the degree of labeling. In addition, actin was labeled in a non-covalent manner, and therefore rigidly, which is extremely important when orientation of a dipole moment of the probe is measured. When a myosin cross-bridge delivers a force impulse to actin, it induces actin to change orientation. There is evidence that it is a whole segment of actin changes orientation, not just actin monomer with which a cross-bridge interacts directly [19–21]. This segment can encompass few actin monomers [22]. The segment is indicated by colored circles in Fig. 1. The bottom line is that actin assumes certain orientation when a cross-bridge is strongly bound to a segment, different orientation when it is weakly attached to it, and still different orientation when it is not attached at all. This is illustrated in Fig. 8.

The kinetic information obtained by measuring isometric force is a temporal average of trillions of individual impulses that myosin delivers to actin in each second [23]. Averaging causes the loss of this kinetic information. SMD approach is used to avoid averaging by measuring the impulses delivered by only a few cross-bridges. To accomplish this we employ fluorescence to measure rotational motion: myofibrillar actin is very lightly labeled with a fluorophore, and fluorescence is observed from a small volume ($\sim 1 \mu\text{m}^3$) by a confocal microscope. This volume is defined by ellipsoid of revolution (green in Fig. 1) and is centered on a fluorescent area. This area contains both actin (fluorescent) and myosin cross-bridges (invisible). Since sarcomere length is short (Fig. 2), the I-bands are short and myosin and actin overlap along nearly the whole length of a sarcomere. The bottom line is

that we are collecting data from the O-band, the area that contains both actin and myosin heads. This arrangement assures that only a few labeled molecules are observed. When a myosin cross-bridge delivers a force impulse to actin, the adjacent segment of the thin filament changes its orientation. These orientational changes can be followed by the polarization of fluorescence (PF), which is a sensitive indicator of the state of muscle contraction [24–31]. PF is a normalized ratio of the difference between parallel and perpendicular polarized intensities: $PF = (I_{||} - I_{\perp}) / (I_{||} + I_{\perp})$, where $I_{||}$ and $I_{\perp}$ are intensities of fluorescence obtained when exciting light is polarized parallel and perpendicular with respect to the myofibrillar axis. The signals analyzed in the current work are the orthogonal intensities $I_{||}$ or $I_{\perp}$, rather than PF because PF, being a ratio of two noisy intensities, was also very noisy. But over a short period of time the $I_{||}$ and $I_{\perp}$ behave similarly to PF (see Discussion and [32]).

Intensities were obtained by measuring the orthogonal components of polarized fluorescence of rhodamine dipoles attached to actin filaments. Sparse labeling was achieved by tagging with 0.1 nM dye mixed with 10 μ M blocking peptide. We estimate that under these conditions about one in 100,000 actin monomers were labeled. By observing sparsely labeled myofibrils with a confocal microscope we were able to limit the number of observed actin molecules to ~4. To minimize the noise and estimate kinetics we used the Fluorescence Correlation Spectroscopy (FCS) [33–35]. [18, 19]

We have carried out the SMD measurements in an *ex vivo* heart, thus accounting for the effects of molecular crowding characterizing a dense muscle environment. The apparent concentrations of actin and myosin in muscle are ~0.6 and ~0.2 mM, respectively [36]. Consequently, the access to proteins might be limited to only small solvent molecules leading to over hydration *in vivo* [37, 38]. Such over hydration could be overlooked in the *in vitro* measurements. Furthermore, myosin activity might depend on various cofactors and on the local geometry of filament arrangements [39]. It is thus important that the measurements be carried out under conditions that closely reproduce a crowded *in vivo* environment.

We demonstrate that the rate of orientational changes of a rhodamine dipole is significantly slower in isometrically contracting cardiac myofibrils from Tg-R58Q mice compared to control, Tg-WT preparations. At the same time, we observe a lower force per cross-sectional area of muscle in isometrically contracting Tg-R58Q fibers compared with Tg-WT. We conclude that the R58Q-dependent decrease in the myosin cross-bridge kinetics and the resulting lower level of force observed in R58Q mutated hearts are most likely responsible for the overall compromised cardiac function and inability of the mutated hearts to efficiently pump blood.

MATERIALS AND METHODS

Chemicals and solutions

Fluorophores, rhodamine-phalloidin (RP) and Alexa-488-phalloidin (AP), were obtained from Molecular Probes (Eugene, OR). All the fluctuation experiments were done using RP. AP was only used for imaging. All other chemicals were from Sigma (St Louis, MO). The EDTA-rigor solution contained 50 mM KCl, 2 mM EDTA, 1 mM DTT, 10 mM Tris-HCl buffer pH 7.5. The Ca^{2+} -rigor solution contained 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 10 mM Tris-HCl buffer pH 7.5. The Mg^{2+} -rigor solution contained 50 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl buffer pH 7.5. Contracting solution was prepared by adding 5 mM ATP to Ca^{2+} -rigor solution. The composition of the pCa 8 solution (for skinned muscle fiber preparation and force measurements) was: 10^{-8} M $[Ca^{2+}]$, 1 mM $[Mg^{2+}]$, 7 mM EGTA, 5 mM $[MgATP^{2+}]$, 20 mM imidazole, pH 7.0, 15 mM creatine phosphate, ionic strength = 150 mM adjusted with KPr.

Transgenic muscle

All animal studies were conducted in accordance with institutional guidelines. After euthanasia, the hearts from 2.5, 5 and 13-month old male Tg-R58Q and respective age- and gender matched control Tg-WT mice were quickly removed and rinsed briefly with ice-cold 0.9% NaCl. Muscle strips from left and right ventricles and papillary muscles were dissected at 4°C in a cold room in ice-cold pCa 8 solution containing 30 mM BDM and 15% glycerol [17]. After dissection, muscle strips were transferred to pCa 8 solution mixed with 50% glycerol and incubated for 1 hr on ice. Then the muscle strips were transferred to fresh pCa 8 solution mixed with 50% glycerol and containing 1% Triton X-100 for 24 hr at 4°C. Muscle strips were finally transferred to a fresh batch of pCa 8 solution mixed 1:1 with glycerol and kept at -20°C until used for force measurements and for the preparation of myofibrils.

Preparation of myofibrils

Myofibrils from Tg-WT and Tg-R58Q mouse ventricles and papillary muscles were first washed with an ice-cold EDTA-rigor solution for 1.5 hr followed by an extensive wash with Mg^{2+} -rigor solution, followed by washing with Ca^{2+} -rigor solution. The fiber bundle was then homogenized using a Heidolph Silent Crusher S homogenizer for 20 s (with a break to cool after 10 s) in Mg^{2+} -rigor solution.

Labeling of myofibrils

1 mg/ml myofibrils were mixed in a Vortex with 0.1 nM rhodamine-phalloidin (RP) + 10 μ M unlabeled-phalloidin (UP) in Ca^{2+} -rigor solution. A large excess of unlabeled phalloidin was necessary to assure that actin monomers were uniformly labeled. If it were not present, myofibrils closest to the pipette tip (used to add the dye) would have been more heavily labeled than the distant ones. After application to a coated coverslip, myofibrils were washed with 5 volumes of the Ca^{2+} -rigor solution [40].

Coverslips

In contrast to skeletal myofibrils, which adhere to a glass surface very stably, the attachment of cardiac myofibrils to glass is weak. To assure a stable adhesion, we found it necessary to coat the coverslips with polylysine. Glass slides were cleaned with 100% ethanol, and spin coated with Poly-L-lysine solution (Sigma 0.1%) at 3,000 rpm for 120s using a Spincoater P6700 (Specialty Coating Systems, Indianapolis, Indiana).

Preventing shortening of contracting muscle

In the presence of Ca^{2+} and ATP the myofibrils can shorten (sometimes even in the absence of Ca^{2+} due to the damage to troponin or tropomyosin), making it impossible to record the FCS spectra during contraction. To measure fluorescence during contraction, it was therefore necessary to prevent myofibrils from shortening. This was done by cross-linking with a water-soluble cross-linker EDC [41]. Myofibrils (1 mg/ml) were incubated with 20 mM EDC for 20 min at room temperature. The reaction was stopped by adding 20 mM DTT. The lack of shortening was checked under the differential contrast conditions [42]. Cross-linking had no effect on contraction [43].

ATPase measurement

About 0.2 mg/mL of a myofibrillar suspension was incubated with 0.1 mM ATP for 30, 60, 90 and 120 s. After these time intervals, the reaction was stopped with 1 mM HCl. The samples were filtered through a cotton ball in a 200 μ L pipette tip. The Malachite Green (MG) reagent from the SensoLyte Phosphatase assay kit (AnaSpec, San Jose, CA) was added and incubated for 5 minutes. The 1.5 μ L of phosphate contained in the kit was

dissolved in deionized water along with 1mM HCl and MG reagent used as a standard. 1 mL of a 10 μ M standard contained 10^{-9} moles of phosphorus. The concentration of phosphate was measured at 650 nm. The Ca^{2+} - rigor along with HCl and MG reagent was used as a blank. The amount of $[\text{P}_i]$ was calculated as $\text{mol}/1 \text{ mol/min} = \text{Abs (sample)} * [\text{standard mol}]/\text{Abs (standard)}/[\text{myosin mol}]/\text{minutes}$. The amount of myosin in 150 μ L of 0.2 mg/mL myofibrils was $0.03 * 10^{-9}$ mole. Specific ATPase activities of x-linked Tg-WT and Tg-R58Q myofibrils were 0.56 s^{-1} and 0.64 s^{-1} , respectively (see Supplement, Fig 1S). Cross-linking had no effect on the ATPase of Tg-WT muscle and only marginally increased the ATPase of R58Q muscle.

Anisotropy measurements

FCS was measured by ISS-Alba-FCS (ISS Co, Urbana, IL) confocal systems coupled to an Olympus IX 71 microscope. Before each experiment, the instrument was calibrated and optimized with 50 nM solution of rhodamine G. The excitation was provided by a 532 nm CW laser. The linearly polarized laser beam was delivered by a single mode fiber, but to ensure that the exciting light was polarized, a sheet analyzer was inserted before the entrance to the microscope. The laser polarization was vertical on the microscope stage. Confocal pinhole was 50 μ m. Orthogonally, linearly polarized analyzers were placed before Avalanche PhotoDiodes (APD's). The myofibrils were always placed with the axis pointing vertically on the microscope stage. Channel 1 and 2 measured the polarized intensities oriented perpendicular and parallel to the myofibrillar axis, respectively.

Fluorescence data analysis

Fluorescence was collected every 10 μ s for 20 s (~2 million data points). The correlation functions were computed and fitted using the ISS Vista version 4.0.29 software. For plotting, the signal was smoothed by binning ~1000 data points.

Steady-state Force Development

The transgenic mutant mice used in these experiments were ~5 month old males that were age and gender matched with Tg-WT controls. A bundle of fibers approximately 100–150 μ m in diameter was isolated from a batch of glycerinated mouse papillary fibers and attached by tweezer clips to a force transducer. The fibers were relaxed in a 1 ml of pCa 8 solution and then tested for maximal steady state force in pCa 4 solution (composition identical to pCa 8 solution except the $[\text{Ca}^{2+}] = 10^{-4} \text{ M}$). Steady state force development was monitored for the Tg-R58Q fibers and compared to control, Tg-WT fibers.

Ca^{2+} Dependence of Force Development

After the initial steady state force was determined, the fibers were relaxed in pCa 8 solution and then exposed to solutions of increasing Ca^{2+} concentrations (from pCa 8 to pCa 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 equation: % Change in Force = $100 \times [\text{Ca}^{2+}]^{n_H}/([\text{Ca}^{2+}]^{n_H} + [\text{Ca}^{2+}_{50}]^{n_H})$ where “[Ca^{2+}_{50}]” is the free Ca^{2+} concentration which produces 50% force and “ n_H ” is the Hill coefficient.

Statistical Analysis

Data are expressed as the average of n experiments $\pm$ SE (standard error of the mean). Comparisons between two groups (Tg-R58Q and Tg-WT) were performed using an unpaired Student's t-test (Sigma Plot 11; Systat Software, Inc., San Jose, CA, USA). The significance was defined as $P < 0.05$.

RESULTS

Imaging

A typical lifetime image of a rigor Tg-WT myofibril from the mouse heart labeled with a fluorescent phalloidin is shown in Fig. 2A (the decay of fluorescence intensity and the histogram of lifetimes are shown in Supplement Fig. 2S). Note that the entire sarcomere is labeled. This is because sarcomere length is short, the I-bands are nearly absent and the O-band is nearly equal to the A-band. As mentioned earlier, the data is collected from the O-band. In contrast, in skeletal muscle the fluorescent phalloidin labels only the overlap zone of the sarcomere [44]. This is because in skeletal muscle there is nebulin, which allows phalloidin to bind only to the pointed ends of actin filaments [45]. Since there is no nebulin in cardiac muscle, the entire I-bands are stained. The H-bands appear dark and this is because they contain no actin. The parallel ($I_{||}$) and perpendicular ($I_{\perp}$) polarization images shown in Fig. 2B and C, respectively, show that fluorescence is highly anisotropic, as is expected from the aligned array of polar actin filaments [46].

Immobilization of the probe

For the quantitative measurement of orientation, it is important to know whether the probe is immobilized by the protein so that the transition dipole of the fluorophore reflects the orientation of the protein. Fig 2D shows that this is the case for the AP-labeled thin filaments. We chose to fit the decay of anisotropy, defined as $(I_{||}-I_{\perp})/(I_{||}+2I_{\perp})$, by a simple mobile-immobile model. The best fit was obtained with the exponential function $r(t)=R_{\infty}+a \cdot \exp(-t/\theta)$, where $R_{\infty} = 0.29$ is the value of anisotropy at infinite time, $a = 0.07$, is the amplitude of the anisotropy change, and $\theta = 1.3$ ns is the rotational correlation time. The short correlation time is due to the rotation rate of AP, which is consistent with the rotation rates of a small molecule, MW=1,250 Da. The fact that the anisotropy did not decay to zero from the maximum value of $R_0 = 0.35$, but decayed to a large asymptotic value of $R_{\infty} = 0.29$, shows that this constant anisotropy of fluorescence may be attributed to immobile actin filaments. The mobile fraction thus constitutes $(0.35-0.29)/0.35 = 17\%$ of fluorophores. Thus ~83% of fluorescent phalloidin is immobilized by F-actin. This is consistent with the fact that the probes attached to proteins through the interactions that stretch over the large surface areas, e.g. hydrophobic or Van der Waals interactions, are attached more rigidly than the probes attached by the covalent links. We conclude that fluorescent phalloidin is a good dye for monitoring the anisotropy of actin protomers in cardiac myofibrils.

Number of detected molecules

The red circle in Fig. 2A indicates the projection of the confocal aperture on the image plane. Its diameter (1.2 μm) is equal to the diameter of the confocal pinhole (50 μm) divided by 40x (magnification of the objective). The Detection Volume (DV) is equal approximately to the surface area of the projection (1.1 μm^2) multiplied by the height of a myofibril (1 μm). To calculate the number of fluorophores in the DV, we recall that the myofibril is labeled with 0.1 nM RP + 10 μM UP (unlabeled phalloidin), so only one in ~100,000 of actin monomers carries the fluorescent phalloidin. The length, width and height of a typical half-sarcomere (HS) are 1, 1, and 1 μm , respectively, and therefore the DV volume is $\sim 10^{-15}$ L. Since the concentration of actin in muscle is ~0.6 mM [36], this volume contains ~4 actin monomers. This estimation carries significant error arising from uneven labeling of muscle, uncertainty of the fibril's thickness, and a possible underestimation of the diameter of the focused laser beam. The error may be as large as 100%, but it should be emphasized that the exact number of fluorescent molecules in the DV does not matter as long as the number is small (i.e. fits within the mesoscopic range required for the FCS method [47]).

Myofibrils

The signal utilized in this study was the intensity of polarized fluorescence, $I_{||}$ or $I_{\perp}$. The intensity of polarized fluorescence was measured by fixing the laser beam positioned at the center of the O-band and collecting the perpendicular and parallel components of fluorescence in ch1 and ch2, respectively. Fig. 3 compares the traces of polarized fluorescence intensity obtained from rigor and contracting myofibrils. It demonstrates that during contraction, but not in rigor, it is possible to distinguish individual impulses by inspection. However, while the impulses were readily visible, it was difficult to extract from them the kinetic rates because of their variable shapes and duration. Also, the signal was noisy due to the fact that the impulses originated from ~4 molecules amongst ~200,000 present in the HS. Nevertheless, because the signal originated from a small number of molecules, the kinetic information could be extracted from stochastic fluctuations of the signal by computing the correlation function of fluctuations. The correlation function at a given delay time τ is a sum of the products of the signal multiplied by a signal shifted by a delay time τ . If the signal is periodic, the autocorrelation function will also be periodic with the same periodicity as the original signal. At the same time, the autocorrelation of noise is zero, because there is no correlation between the value of signal at a given time and its value at the later time. Thus, correlation greatly decreases the noise of the signal. Figs. 4B and 5B compare the correlation functions of the signals from Tg-WT myofibril in rigor and during contraction. The signal and the correlation functions obtained from contracting Tg-R58Q myofibril is shown in Fig. 6.

The theoretical expression for correlation function (see eq. 1 of Discussion) suggests that the best fit should consist of 3 exponents characterized by cross-bridge rate constants k_1 , k_2 , and k_3 . The experimental data was therefore fitted by three experimental rate constants α , β and γ . In a majority of cases γ was 0. The Discussion describes the model used to relate model constants k_1 , k_2 , and k_3 to experimental constants α and β . Table 1 and Fig. 3S compare the results from 35 WT and 46 Tg-R58Q myofibrils. There is a statistically significant difference between the WT and mutated myofibrils. The difference of 8115 between means for A was significant at the level better than 5% ($t = -3.146$, $P = 0.002$ with 78 degrees of freedom). A 95 % confidence interval for the difference of means was 13249 to 2979. The difference of 177 between means for B was significant at the level better than 5% ($t = -3.203$, $P = 0.002$ with 78 degrees of freedom. ($P = 0.002$). A 95 percent confidence interval for the difference of means was 287 to 67. There were no age-dependent differences in the kinetics measurements performed on myofibrils from 2.5, 5 and 13-month old male Tg-R58Q and age and gender matched control Tg-WT mice. No differences were also observed between myofibrils from left and right ventricles or papillary muscles.

Functional Studies in Skinned Cardiac Muscle Fibers

The functional consequences of the R58Q FHC mutation were also examined in glycerinated transgenic cardiac muscle fibers, the same which were used for the preparation of cardiac myofibrils for the single molecule studies (above). In accord with our earlier report on fresh (not glycerinated) skinned muscle fibers [14], a significant decrease in the maximal force and a significant increase in myofilament Ca^{2+} sensitivity were observed in Tg-R58Q fibers compared with Tg-WT ($P < 0.05$) (Fig. 7). Data are the average from n individual fibers $\pm$ SE. Fig. 7A shows a 2.5-fold decrease in maximal force measured in papillary muscle fibers from Tg-R58Q mice compared with Tg-WT ($P < 0.001$). The average fiber diameter was about 120 μm . Fig. 7B shows a mutation dependent change in EC_{50} , from $2.80 \pm 0.02 \mu\text{M}$ (Tg-WT) to $2.09 \pm 0.05 \mu\text{M}$ (Tg-R58Q). The Hill coefficients were $n_H = 2.47 \pm 0.10$ for Tg-WT and $n_H = 1.97 \pm 0.04$ for Tg-R58Q (Fig. 7B). Sixteen individual fibers from Tg-WT mice and thirty five from Tg-R58Q mice were examined, with ~ 4 fibers originating from the same mouse heart.

DISCUSSION

In order to elucidate the effect of the FHC-linked R58Q mutation on cardiac muscle performance at the molecular level, we compared polarized fluorescence originating from cardiac myofibrils prepared from Tg-R58Q *versus* Tg-WT mouse hearts. Kinetic parameters were extracted from the data by analysis of fluctuations. This has essential advantages over earlier method. Earlier, we have used a simple visual method to extract these parameters from muscle containing different FHC-causing mutation [17]. In the previous work we observed fluctuations visually by EMCCD camera and extracted kinetics by manually analyzing current traces. The fluctuation method makes this process much less laborious and thus allows statistical analysis of many experiments. It also allows more concentrated labeling, obviates the effects of photobleaching and reduces contribution of random noise.

We made two fundamental assumptions. First, the signals analyzed in the current work are the orthogonal intensities $I_{||}$ or $I_{\perp}$, rather than PF because polarization, being a ratio of two noisy intensities, was also very noisy. This is justified because over a short period of time the $I_{||}$ and $I_{\perp}$ behave similarly to PF [32]. Also, Barcellona et al. [48] have shown that the autocorrelation function of polarized fluorescence intensity approximates the autocorrelation function of PF. The second assumption was that fluctuations of actin reflect fluctuations of myosin. This is justified because we have shown earlier that fluctuations of actin were paralleled by fluctuations of myosin lever arm [49, 50].

It remains to relate the experimental constants extracted from experimental correlation functions with the theoretical constants reflecting cross-bridge kinetics. This is attempted below:

Derivation of the correlation function of a system that switches between a number of states and where the time between two switches is exponentially distributed

With reference to Fig. 8, the system has N states here designated a_n , $n=1 \dots N$. They switch like $a_n \rightarrow a_{n+1}$ and $a_N \rightarrow a_1$ in sequence. The time between 2 consecutive switches is exponentially distributed such that the time t in state a_n is distributed as $k_n \exp(-k_n t)$. Let a_n also designate the signal value at that state.

The correlation function is a sum of terms, each term corresponds to the correlation of a state a_n to a state a_m . So the correlation function is the sum of terms with all possible combinations of a_n and a_m . Choose arbitrarily the term that starts with a_1 at time 0 and ends with a_n at time t . All other terms are calculated in the same way which is seen by cyclic permutation of the indices. The shortest way to reach a_n in sequence from a_1 is by stopping when reaching a_n first time. Other ways is to pass a_n a number of times, i.e. to circle around the sequence a number of times before stopping at a_n . The time for reaching the state a_n is then the sum of the times in the switching sequence. The distribution of this time, which is the sum of exponentially distributed times, is calculated by convolution. The calculation of the probability that the system is in state a_n at time t if it started in state a_1 at time 0 is done by integrating the probability distribution of the time to reach state a_n minus the integration of the probability distribution to reach state a_{n+1} (or a_1 if $n=N$). The integrations and convolutions are much easier to do after Laplace transformations of the probability distributions. Convolutions are transformed to simple multiplications and integrations by multiplying with $1/s$ where s is the Laplace transformation parameter corresponding to t . The Laplace transform of $k_n \exp(-k_n t)$ is $k_n/(s+k_n)$. So the Laplace transform of the time probability distribution between states a_1 and a_n with R full rounds of switches is:

$$\frac{k_1}{s+k_1} \dots \frac{k_{n-1}}{s+k_{n-1}} \underbrace{\left(\frac{k_n}{s+k_n} \dots \right)^R}_{\text{A full round of swithes}}$$

The factors corresponding to the full round of switches are:

$$\frac{k_1}{s+k_1} \dots \frac{k_N}{s+k_N}$$

The Laplace transform of the probability that the system ends up in state a_n after time t and R rounds when starting at state a_1 is then:

$$\frac{1}{s} \frac{k_1}{s+k_1} \dots \frac{k_{n-1}}{s+k_{n-1}} \left(\frac{k_1}{s+k_1} \dots \frac{k_N}{s+k_N} \right)^R - \frac{1}{s} \frac{k_1}{s+k_1} \dots \frac{k_n}{s+k_n} \left(\frac{k_1}{s+k_1} \dots \frac{k_N}{s+k_N} \right)^R$$

This can be simplified to

$$\frac{k_1}{s+k_1} \dots \frac{k_{n-1}}{s+k_{n-1}} \frac{1}{s+k_n} \left(\frac{k_1}{s+k_1} \dots \frac{k_N}{s+k_N} \right)^R$$

The Laplace transform of the probability that the system is in state a_n at time t when starting in state a_1 at time 0 is then the sum of all number of rounds.

$$\frac{k_1}{s+k_1} \dots \frac{k_{n-1}}{s+k_{n-1}} \frac{1}{s+k_n} \sum_{R=0}^{\infty} \left(\frac{k_1}{s+k_1} \dots \frac{k_N}{s+k_N} \right)^R$$

This is a geometric series that results in:

$$\frac{k_1}{s+k_1} \dots \frac{k_{n-1}}{s+k_{n-1}} \frac{1}{s+k_n} \frac{1}{1 - \frac{k_1}{s+k_1} \dots \frac{k_N}{s+k_N}}$$

This can be simplified to:

$$\frac{k_1}{s+k_1} \dots \frac{k_{n-1}}{s+k_{n-1}} \frac{1}{s+k_n} \frac{(s+k_1) \dots (s+k_N)}{(s+k_1) \dots (s+k_N) - k_1 \dots k_N}$$

This can be further simplified to:

$$\frac{k_1 \dots k_{n-1} (s+k_{n+1}) \dots (s+k_N)}{(s+k_1) \dots (s+k_N) - k_1 \dots k_N}$$

The correlation function is the sum by all such terms multiplied by their start and end state signals and the probabilities for being in the start states, which for a_1 is:

$$\frac{1/k_1}{1/k_1 + \dots + 1/k_N}$$

All terms in the Laplace transform of the correlation function have the same denominator which is a polynomial in s and can be written as:

$$(s+k_1) \dots (s+k_N) - k_1 \dots k_N$$

Call the roots K_n

$$(s+k_1) \dots (s+k_N) - k_1 \dots k_N = (s+K_1) \dots (s+K_N)$$

It is seen that one root is zero, because the constant term in the polynomial is subtracted away. Choose $K_N=0$.

Since the numerators have lower polynomial degrees than the denominators, the terms, and therefore also the Laplace transform of the correlation function, can be decomposed into simple terms of constants times $1/(s+K_n)$. By inverse Laplace transformation these terms correspond to constants times exponentials $\exp(-K_n t)$. This means that the correlation function is the sum of such exponentials. Since K_N is zero one term is constant.

The polynomial can also be written as:

$$s^N + p_1 s^{N-1} + \dots + p_{N-1} s + p_N$$

Here p_N is zero. Since the coefficients p_1 to p_{N-1} have the same expressions in the k_1 to k_N as in the K_1 to K_N this means by the theory of polynomials that

$$\sum_{n=1}^{N-1} K_n^m = \sum_{n=1}^N k_n^m$$

for all m less than N . But this does not hold for $m=N$ because p_N does not have the same expressions in the k_1 to k_N as the K_1 to K_N . The main points of this derivation are listed in the Supplementary Materials.

In our specific case changes of fluorescent intensity arise when a cross-bridge transits from low to high anisotropy. The simplest scheme illustrating how this occurs during contraction of muscle is shown as a 3-step process in Fig. 9. Although this mechano-chemical cycle is an oversimplification of the full scheme [51–53], it contains the main steps involved in the conformational changes. A is actin, M is myosin, AM is acto-myosin, D is ADP and T is ATP. The state of lowest anisotropy occurs when myosin is dissociated from actin, because then the cross-bridge is unable to inhibit bending motion of actin (we think that signal is produced by bending motion of a small segment of actin rather than by rotation of actin monomers, because there is no evidence that cross-bridge binding may prevent the rotation of G-actin [20, 21]). This state consists of two intermediates: MT (myosin containing ATP) which is then slowly hydrolyzed [54] to ADP. Hydrolysis step does not contribute to anisotropy change. A cross-bridge then binds weakly to actin with a rate k_3 , a state that is

assumed to be characterized by an intermediate anisotropy. The weakly bound AMDP cross-bridge then releases Pi and undergoes a power stroke at a rate k_1 , where it becomes strongly, stereospecifically bound to actin. This conformation is characterized by high anisotropy which arises because a cross-bridge binding is able to inhibit bending motions of the segment of the whole filament (rather than prevent the rotation of the actin monomers - there is no evidence that cross-bridge binding may prevent the rotation of G-actin [20, 21]). It then rapidly transforms to a “near rigor” AM [52] with no orientation change. This is followed by binding of ATP by AM and dissociation from actin at a rate k_2 , and the process is then repeated.

For 3 states the analytical solution depends on the relationship between $A=k_1^2+k_2^2+k_3^2$ and $B=2k_2k_3+2k_1k_3+2k_1k_2$, where k 's are the theoretical kinetic constants. Taking $k_1 \ll k_2$ and $k_1 \ll k_3$ (because k_1 is the rate limiting dissociation of Pi) we get $A > B$. In this case the theoretical expression for correlation function is:

$$R_3(t) = \frac{(a_1k_2k_3+a_2k_3k_1+a_3k_1k_2)^2}{(k_2k_3+k_3k_1+k_1k_2)^2} + \frac{k_1k_2k_3(A\kappa+B)}{2\kappa(k_2k_3+k_3k_1+k_1k_2)^2} \exp\left(-\frac{1}{2}(k_1+k_2+k_3-\kappa)t\right) + \frac{k_1k_2k_3(A\kappa-B)}{2\kappa(k_2k_3+k_3k_1+k_1k_2)^2} \exp\left(-\frac{1}{2}(k_1+k_2+k_3+\kappa)t\right) \quad (1)$$

where

$$\kappa = \sqrt{k_1^2+k_2^2+k_3^2-2k_2k_3-2k_3k_1-2k_1k_2} \quad (2)$$

$$A = a_1^2(k_2+k_3) + a_2^2(k_3+k_1) + a_3^2(k_1+k_2) - 2(k_1a_2a_3+k_2a_3a_1+k_3a_1a_2) \quad (3)$$

$$B = a_1^2(k_2^2+k_3^2-k_1(k_2+k_3)) + a_2^2(k_3^2+k_1^2-k_2(k_3+k_1)) + a_3^2(k_1^2+k_2^2-k_3(k_1+k_2)) + 2(k_2k_3-k_1^2)a_2a_3 + 2(k_3k_1-k_2^2)a_3a_1 + 2(k_1k_2-k_3^2)a_1a_2 \quad (4)$$

From the fit of the experimental correlation functions to R_3 we obtain the experimental values of K_1 , K_2 and K_3 (for our specific case we call K_1 , K_2 and K_3 α , β and γ , respectively). The average values of α 's & β 's are given in Table 1 and the experimental α and β are related to theoretical values k_1 , k_2 and k_3 by:

$$\begin{aligned} k_1+k_2+k_3 &= \alpha+\beta \\ k_1^2+k_2^2+k_3^2 &= \alpha^2+\beta^2 \end{aligned} \quad (5)$$

The details of the derivation are given below:

We have simulated the signals by special random number generation according to the model and calculated the correlation function in order to test the fact that $k_1+k_2+k_3=\alpha+\beta$ and $k_1^2+k_2^2+k_3^2=\alpha^2+\beta^2$. The test showed a perfect agreement.

Therefore for WT myofibrils:

$$\begin{aligned}k_1+k_2+k_3 &= 20,470 \text{ s}^{-1} \\ k_1^2+k_2^2+k_3^2 &= 4.00 \times 10^8 \text{ s}^{-2}\end{aligned}$$

and for MUT myofibrils:

$$\begin{aligned}k_1+k_2+k_3 &= 12,178 \text{ s}^{-1} \\ k_1^2+k_2^2+k_3^2 &= 1.41 \times 10^8 \text{ s}^{-2}\end{aligned}$$

Fig. 10 shows that solutions of eq. 5 exist only for certain sets of k_1 , k_2 and k_3 . The physically relevant k 's are the small parts of the ellipse indicated by the red color and arrows. The reason that they are small is due to the fact that α and β are so different. This means that for WT muscle $k_1(\text{WT})$, the smallest of the k 's, must be $<446 \text{ s}^{-1}$. For mutated muscle, $k_1(\text{MUT}) < 250 \text{ s}^{-1}$. This also means that the largest of the three k 's must be close to the larger of α and β . For WT and mutated myofibrils it means that $k_2(\text{WT}) = \alpha(\text{WT}) = 19,993 \text{ s}^{-1}$ and $k_2(\text{MUT}) = \alpha(\text{MUT}) = 11,878 \text{ s}^{-1}$, respectively. Further, the fact that α and β are so different means that the sum of the two smaller k 's must be close to the smaller α or β . For WT and mutated myofibrils it means that $k_1(\text{WT})+k_3(\text{WT}) = \beta(\text{WT}) = 477 \text{ s}^{-1}$ and $k_1(\text{MUT})+k_3(\text{MUT}) = \beta(\text{MUT}) = 300 \text{ s}^{-1}$, respectively. Since $k_3 \gg k_1$ (k_1 is rate limiting) only a small error is made by stating that $k_3(\text{WT}) \approx 477 \text{ s}^{-1}$ and $k_3(\text{MUT}) \approx 300 \text{ s}^{-1}$. The values of experimental α 's and β 's and calculated k 's are summarized in Table 1. The solutions are symmetrical, i.e. it is possible that the values for k_2 and k_3 are reversed, but it does not change the fact that mutation always decreases the values.

In summary, we demonstrate that the R58Q mutation leads to a decrease in the rate of cross-bridge binding to actin (k_3), dissociation from actin (k_2) and most likely decreases the rate at which a cross-bridge undergoes a power stroke (k_1). These changes must result in a decrease in force. As shown in Fig. 6A, a decrease in rate constants of the R58Q mutated cross-bridges parallels decreased force per cross-sectional area observed in Tg-R58Q vs. Tg-WT glycerinated skinned muscle fibers. Slower cross-bridge kinetics most likely underlines the mechanism by which Tg-R58Q fibers develop low force. In particular, lower power-stroke rate will lead to accumulation of cross-bridges in non-force producing AMDP state. These data suggest that the R58Q hearts may be subject to inefficient energy utilization by producing less contractile force than the WT hearts for the same supply of ATP. This would ultimately lead to metabolite accumulation in the R58Q mutated myocardium and compromised heart performance. Interestingly, an inefficient energy use by the R58Q mutant mice was also manifested in our earlier studies, where cardiac efficiency defined as a ratio of cardiac power to O_2 consumption was significantly compromised in Tg-R58Q mouse hearts compared with Tg-WT controls [15]. In addition, we observed an R58Q-dependent enhancement of ATPase activity at base (in pCa 8 solution) and incomplete relaxation ultimately leading to global diastolic dysfunction in Tg-R58Q animals [15]. Finally, we have previously used a specific model of muscle action utilizing the rates of cross-bridge association (f) and dissociation (g) [55] to extract kinetic parameters from global measurements, specifically to examine the effects of another RLC FHC mutation, D166V [56]. We showed that the D166V mutation drastically decreased g , which resulted in compromised contractility of the D166V murine hearts [56]. In parallel, using our recently developed SMD, we demonstrated a D166V-dependent decrease in myosin cross-bridge kinetics at the level of single molecules [17].

Implications for R58Q-induced FHC disease—It has long been known that sustained or progressive demands on the heart can result in a series of compensatory responses

including cardiac hypertrophy and myocardial remodeling. Elucidating the mechanisms of FHC-linked RLC mutations and how they might result in hypertrophic disease is a multilevel task and experiments have to be performed at the molecular, cellular and organ levels. The R58Q mutation of RLC is our most studied FHC mutation, which has been thoroughly characterized at the protein [57] [16], myofibrillar [58] [15], muscle fiber [14] and organ [15] levels in the RLC reconstituted cardiac muscle preparations [58] or in a transgenic mice [14] [15] [16]. The Glutamine for Arginine substitution (R58Q) eliminated the capability of the RLC to bind Ca^{2+} and only when the R58Q-RLC mutant was phosphorylated at Ser-15 with the Ca^{2+} -calmodulin activated MLCK, the Ca^{2+} binding was restored [57]. However, a rescue of the Ca^{2+} binding to RLC by MLCK-induced RLC phosphorylation could be largely compromised in the R58Q myocardium. In support of this notion, a significant downregulation of the phosphorylated form of RLC was recently observed in Tg-R58Q ventricular extracts [15]. These results suggested that the R58Q mutation may play a role in alterations of myofilament calcium signaling. As we observed earlier [14] and in this report, the R58Q mutation results in an increase in the Ca^{2+} sensitivity of contraction (Fig. 6B). The myofilament calcium sensitivity is a function of two components: 1) $k_{\text{on}}/k_{\text{off}}$ of Ca^{2+} binding to Troponin C (TnC), a major myofilament Ca^{2+} buffer, and 2) the kinetics of force generating myosin cross-bridges [59]. Therefore, the observed leftward shift in the force-pCa relationship in Tg-R58Q myocardium could be due to a slower R58Q cross-bridge kinetics or a decreased Ca^{2+} dissociation from TnC or both. The results of our current study clearly show a decrease in the rate of cross-bridge dissociation from actin (k_2) in R58Q myofibrils. As a consequence, prolonged force transients were measured in intact muscle fibers from Tg-R58Q mice [14]. These changes when placed *in vivo* would lead to diastolic dysfunction as previously confirmed [15]. Thus, the origin of these pathophysiological consequences was the slower cross-bridge kinetics of contracting R58Q hearts. The predicted functional outcome would be inefficient ATP utilization by the R58Q myocardium, as described above. The R58Q-dependent increase in calcium sensitivity would be expected to produce a hypercontractile phenotype with increased use of ATP. Thus, energy expenditure through inefficient energy use would lead to increased turnover of ATP, particularly during stress. From the clinical standpoint, these R58Q-induced multilevel changes in cardiac contractility would lead to severe FHC phenotype including SCD, cardiac abnormalities observed in patients harboring this FHC RLC mutation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH grants: R01AR048622 (to J.B.), R01HL071778 (to D.S.-C.), R01HL090786 (to J.B. and D.S.-C.), and by Texas ETF grant (CCFT). R.L. is the recipient of the Research Mobility program from the Polish Ministry of Science and Higher Education.

ABBREVIATIONS

FHC	Familial Hypertrophic Cardiomyopathy
RLC	Regulatory Light Chain of Myosin
SCD	Sudden Cardiac Death
SMD	Single Molecule Detection
PF	Polarization of Fluorescence

RP	Rhodamine-Phalloidin
AP	Alexa-Phalloidin
UP	Unlabeled-Phalloidin
DV	Detection Volume
MUT	Myofibrils carrying R58Q mutation at RLC
k₁k₂, and k₃	theoretical (model-dependent) rate constants of cross-bridge cycle
α, β and γ	experimental (model-independent) rate constants

References

1. Maron BJ. The young competitive athlete with cardiovascular abnormalities: causes of sudden death, detection by preparticipation screening, and standards for disqualification. *Card Electrophysiol Rev.* 2002; 6:100–3. [PubMed: 11984027]
2. Maron BJ. Hypertrophic cardiomyopathy - A systematic review. *Jama-Journal of the American Medical Association.* 2002; 287(10):1308–1320.
3. Tardiff JC. Sarcomeric proteins and familial hypertrophic cardiomyopathy: Linking mutations in structural proteins to complex cardiovascular phenotypes. *Heart Failure Reviews.* 2005; 10(3):237–248. [PubMed: 16416046]
4. Alcalai R, Seidman JG, Seidman CE. Genetic basis of hypertrophic cardiomyopathy: from bench to the clinics. *J Cardiovasc Electrophysiol.* 2008; 19(1):104–10. [PubMed: 17916152]
5. Poetter K, Jiang H, Hassanzadeh S, Master SR, Chang A, Dalakas MC, Rayment I, Sellers JR, Fananapazir L, Epstein ND. Mutations in either the essential or regulatory light chains of myosin are associated with a rare myopathy in human heart and skeletal muscle. *Nat Genet.* 1996; 13:63–9. [PubMed: 8673105]
6. Flavigny J, Richard P, Isnard R, Carrier L, Charron P, Bonne G, Forissier JF, Desnos M, Dubourg O, Komajda M, Schwartz K, Hainque B. Identification of two novel mutations in the ventricular regulatory myosin light chain gene (MYL2) associated with familial and classical forms of hypertrophic cardiomyopathy. *J Mol Med.* 1998; 76(3–4):208–14. [PubMed: 9535554]
7. Andersen PS, Havndrup O, Bundgaard H, Moolman-Smoock JC, Larsen LA, Mogensen J, Brink PA, Borglum AD, Corfield VA, Kjeldsen K, Vuust J, Christiansen M. Myosin light chain mutations in familial hypertrophic cardiomyopathy: phenotypic presentation and frequency in Danish and South African populations. *J Med Genet.* 2001; 38(12):E43. [PubMed: 11748309]
8. Kabaeva ZT, Perrot A, Wolter B, Dietz R, Cardim N, Correia JM, Schulte HD, Aldashev AA, Mirrakhimov MM, Osterziel KJ. Systematic analysis of the regulatory and essential myosin light chain genes: genetic variants and mutations in hypertrophic cardiomyopathy. *Eur J Hum Genet.* 2002; 10(11):741–8. [PubMed: 12404107]
9. Richard P, Charron P, Carrier L, Ledeuil C, Cheav T, Pichereau C, Benaiche A, Isnard R, Dubourg O, Burban M, Gueffet JP, Millaire A, Desnos M, Schwartz K, Hainque B, Komajda M. for the EUROGENE Heart Failure Project, . Hypertrophic Cardiomyopathy: Distribution of Disease Genes, Spectrum of Mutations, and Implications for a Molecular Diagnosis Strategy. *Circulation.* 2003; 107:2227–2232. [PubMed: 12707239]
10. Morner S, Richard P, Kazzam E, Hellman U, Hainque B, Schwartz K, Waldenstrom A. Identification of the genotypes causing hypertrophic cardiomyopathy in northern Sweden. *J Mol Cell Cardiol.* 2003; 35(7):841–9. [PubMed: 12818575]
11. Hougs L, Havndrup O, Bundgaard H, Kober L, Vuust J, Larsen LA, Christiansen M, Andersen PS. One third of Danish hypertrophic cardiomyopathy patients have mutations in MYH7 rod region. *Eur J Hum Genet.* 2005; 13:161–165. [PubMed: 15483641]
12. Lee WH, Hwang TH, Kimura A, Park SW, Satoh M, Nishi H, Harada H, Toyama J, Park JE. Different expressivity of a ventricular essential myosin light chain gene Ala57Gly mutation in

- familial hypertrophic cardiomyopathy. *American Heart Journal*. 2001; 141(2):184–189. [PubMed: 11174330]
13. Olson TM, Karst ML, Whitby FG, Driscoll DJ. Myosin light chain mutation causes autosomal recessive cardiomyopathy with mid-cavitary hypertrophy and restrictive physiology. *Circulation*. 2002; 105(20):2337–2340. [PubMed: 12021217]
 14. Wang Y, Xu Y, Kerrick WGL, Wang Y, Guzman G, Diaz-Perez Z, Szczesna-Cordary D. Prolonged Ca²⁺ and Force Transients in Myosin RLC Transgenic Mouse Fibers Expressing Malignant and Benign FHC Mutations. *J Mol Biol*. 2006; 361:286–299. [PubMed: 16837010]
 15. Abraham TP, Jones M, Kazmierczak K, Liang HY, Pinheiro AC, Wagg CS, Lopaschuk GD, Szczesna-Cordary D. Diastolic dysfunction in familial hypertrophic cardiomyopathy transgenic model mice. *Cardiovasc Res*. 2009; 82:84–92. [PubMed: 19150977]
 16. Greenberg MJ, Watt JD, Jones M, Kazmierczak K, Szczesna-Cordary D, Moore JR. Regulatory light chain mutations associated with cardiomyopathy affect myosin mechanics and kinetics. *J Mol Cell Cardiol*. 2009; 46(1):108–15. Epub 2008 Sep 27. [PubMed: 18929571]
 17. Muthu P, Mettikolla P, Calander N, Luchowski R, Gryczynski I, Gryczynski I, Szczesna-Cordary D, Borejdo J. Single Molecule Kinetics in the Familial Hypertrophic Cardiomyopathy D166V Mutant Mouse Heart. *Journal of Molecular and Cellular Cardiology*. 2009; 48(6):1264–71.
 18. Bukatina AE, Fuchs F, Watkins SC. A study on the mechanism of phalloidin-induced tension changes in skinned rabbit psoas muscle fibres. *J Muscle Res Cell Motil*. 1996; 17(3):365–71. [PubMed: 8814556]
 19. Prochniewicz-Nakayama E, Yanagida T, Oosawa F. Studies on conformation of F-actin in muscle fibers in the relaxed state, rigor, and during contraction using fluorescent phalloidin. *J Cell Biol*. 1983; 97:1663–1667. [PubMed: 6417144]
 20. Yanagida T, Oosawa F. Polarized fluorescence from epsilon-ADP incorporated into F-actin in a myosin-free single fiber: conformation of F-actin and changes induced in it by heavy meromyosin. *J Mol Biol*. 1978; 126(3):507–24. [PubMed: 370406]
 21. Yanagida T, Oosawa F. Conformational changes of F-actin-epsilon-ADP in thin filaments in myosin-free muscle fibers induced by Ca²⁺. *J Mol Biol*. 1980; 140(2):313–20. [PubMed: 6893608]
 22. Ando T. Propagation of Acto-S-1 ATPase reaction-coupled conformational change in actin along the filament. *J Biochem (Tokyo)*. 1989; 105(5):818–22. [PubMed: 2526809]
 23. Oplatka A. On the mechanochemistry of muscular contraction. *J Theor Biol*. 1972; 34(2):379–403. [PubMed: 4259393]
 24. Dos Remedios CG, Millikan RG, Morales MF. Polarization of tryptophan fluorescence from single striated muscle fibers. A molecular probe of contractile state. *J Gen Physiol*. 1972; 59:103–120. [PubMed: 4332133]
 25. Dos Remedios CG, Yount RG, Morales MF. Individual states in the cycle of muscle contraction. *Proc Natl Acad Sci U S A*. 1972; 69:2542–2546. [PubMed: 4341699]
 26. Nihei T, Mendelson RA, Botts J. Use of fluorescence polarization to observe changes in attitude of S1 moieties in muscle fibers. *Biophys J*. 1974; 14:236–242. [PubMed: 4132695]
 27. Tregear RT, Mendelson RA. Polarization from a helix of fluorophores and its relation to that obtained from muscle. *Biophys J*. 1975; 15:455–467. [PubMed: 19211017]
 28. Morales MF. Calculation of the polarized fluorescence from a labeled muscle fiber. *Proc Nat Acad Sci USA*. 1984; 81:145–9. [PubMed: 6582471]
 29. Sabido-David C, Hopkins SC, Saraswat LD, Lowey S, Goldman YE, Irving M. Orientation changes of fluorescent probes at five sites on the myosin regulatory light chain during contraction of single skeletal muscle fibres. *J Mol Biol*. 1998; 279(2):387–402. [PubMed: 9642045]
 30. Hopkins SC, Sabido-David C, Corrie JE, Irving M, Goldman YE. Fluorescence polarization transients from rhodamine isomers on the myosin regulatory light chain in skeletal muscle fibers. *Biophys J*. 1998; 74(6):3093–110. [PubMed: 9635763]
 31. Hopkins SC, Sabido-David C, van der Heide UA, Ferguson RE, Brandmeier BD, Dale RE, Kendrick-Jones J, Corrie JE, Trentham DR, Irving M, Goldman YE. Orientation changes of the myosin light chain domain during filament sliding in active and rigor muscle. *J Mol Biol*. 2002; 318(5):1275–91. [PubMed: 12083517]

32. Muthu P, Talent JM, Gryczynski I, Borejdo J. Cross-bridge duty cycle in isometric contraction of skeletal myofibrils. *Biochemistry*. 2008; 47:5657–5667. [PubMed: 18426224]
33. Elson EL, Magde D. Fluorescence Correlation Spectroscopy: Conceptual Basis and Theory. *Biopolymers*. 1974; 13:1–28.
34. Elson EL. Fluorescence correlation spectroscopy and photobleaching recovery. *Annu Rev Phys Chem*. 1985; 36:379–406.
35. Elson EL. Quick tour of fluorescence correlation spectroscopy from its inception. *J Biomed Opt*. 2004; 9(5):857–64. [PubMed: 15447006]
36. Bagshaw, CR. *Muscle Contraction*. Chapman & Hall; London: 1982.
37. Minton AP, Wilf J. Effect of macromolecular crowding upon the structure and function of an enzyme: glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry*. 1981; 20(17):4821–4826. [PubMed: 7295652]
38. Minton AP. Excluded volume as a determinant of macromolecular structure and reactivity. *Biopolymers*. 1981; 20:2093–2120.
39. Eisenberg E, Hill TL, Chen Y. Cross-bridge model of muscle contraction. Quantitative analysis. *Biophys J*. 1980; 29(2):195–227. [PubMed: 6455168]
40. Mettikolla P, Luchowski R, Gryczynski I, Gryczynski Z, Szczesna-Cordary D, Borejdo J. Fluorescence Lifetime of Actin in the FHC Transgenic Heart. *Biochemistry*. 2009; 48(6):1264–1271. [PubMed: 19159226]
41. Herrmann C, Lionne C, Travers F, Barman T. Correlation of ActoS1, myofibrillar, and muscle fiber ATPases. *Biochemistry*. 1994; 33(14):4148–54. [PubMed: 8155632]
42. Borejdo J, Muthu P, Talent J, Akopova I, Burghardt TP. Rotation of Actin Monomers during Isometric Contraction of Skeletal Muscle. *J Biomed Optics*. 2007; 12(1):014013.
43. Midde K, Luchowski R, Das HK, Fedorick J, Dumka V, Gryczynski I, Gryczynski Z, Borejdo J. Evidence for pre-and post-power stroke of cross-bridges of contracting skeletal myofibrils. *Biophys J*. 2011; 100(4):1024–1033. [PubMed: 21320447]
44. Szczesna D, Lehrer SS. The binding of fluorescent phallotoxins to actin in myofibrils. *J Muscle Res Cell Motil*. 1993; 14(6):594–7. [PubMed: 8126219]
45. Ao X, Lehrer SS. Phalloidin unzips nebulin from thin filaments in skeletal myofibrils. *J Cell Sci*. 1995; 108(Pt 11):3397–403. [PubMed: 8586652]
46. Borovikov YS, Chernogriadskaia NA. Studies on conformational changes in F-actin of glycerinated muscle fibers during relaxation by means of polarized ultraviolet fluorescence microscopy. *Microsc Acta*. 1979; 81(5):383–92. [PubMed: 470613]
47. Qian H, Saffarian S, Elson EL. Concentration fluctuations in a mesoscopic oscillating chemical reaction system. *Proc Natl Acad Sci U S A*. 2002; 99(16):10376–81. Epub 2002 Jul 17. [PubMed: 12124397]
48. Barcellona ML, Gammon S, Hazlett T, Digman MA, Gratton E. Polarized fluorescence correlation spectroscopy of DNA-DAPI complexes. *Microsc Res Tech*. 2004; 65(4–5):205–17. [PubMed: 15630690]
49. Shepard AA, Dumka D, Akopova I, Talent J, Borejdo J. Simultaneous measurement of rotations of myosin, actin and ADP in contracting skeletal muscle fiber. *J Muscl Res Cell Mot*. 2004; 25:549–557.
50. Borejdo J, Shepard A, Dumka D, Akopova I, Talent J, Malka A, Burghardt TP. Changes in orientation of actin during contraction of muscle. *Biophys J*. 2004; 86:2308–2317. [PubMed: 15041669]
51. Houdusse A, Sweeney HL. Myosin motors: missing structures and hidden springs. *Curr Opin Struct Biol*. 2001; 11(2):182–94. [PubMed: 11297926]
52. Coureux PD, Sweeney HL, Houdusse A. Three myosin V structures delineate essential features of chemo-mechanical transduction. *Embo J*. 2004; 23(23):4527–37. [PubMed: 15510214]
53. Ferenczi MA, Bershtitsky SY, Koubassova N, Siththanandan V, Helsby WI, Panine P, Roessle M, Narayanan T, Tsaturyan AK. The “roll and lock” mechanism of force generation in muscle. *Structure*. 2005; 13(1):131–41. [PubMed: 15642268]

54. Hilber K, Sun YB, Irving M. Effects of sarcomere length and temperature on the rate of ATP utilisation by rabbit psoas muscle fibres. *J Physiol.* 2001; 531(Pt 3):771–80. [PubMed: 11251057]
55. Huxley AF. A hypothesis for the mechanism of contraction of muscle. *Prog Biophys Biophys Chem.* 1957; 7:255–318. [PubMed: 13485191]
56. Kerrick WG, Kazmierczak K, Xu Y, Wang Y, Szczesna-Cordary D. Malignant familial hypertrophic cardiomyopathy D166V mutation in the ventricular myosin regulatory light chain causes profound effects in skinned and intact papillary muscle fibers from transgenic mice. *Faseb J.* 2009; 23(3):855–65. Epub 2008 Nov 5. [PubMed: 18987303]
57. Szczesna D, Ghosh D, Li Q, Gomes AV, Guzman G, Arana C, Zhi G, Stull JT, Potter JD. Familial hypertrophic cardiomyopathy mutations in the regulatory light chains of myosin affect their structure, Ca²⁺ binding, and phosphorylation. *J Biol Chem.* 2001; 276(10):7086–92. Epub 2000 Dec 01. [PubMed: 11102452]
58. Szczesna-Cordary D, Guzman G, Ng SS, Zhao J. Familial hypertrophic cardiomyopathy-linked alterations in Ca²⁺ binding of human cardiac myosin regulatory light chain affect cardiac muscle contraction. *J Biol Chem.* 2004; 279(5):3535–42. [PubMed: 14594949]
59. Robinson JM, Wang Y, Kerrick WG, Kawai R, Cheung HC. Activation of striated muscle: nearest-neighbor regulatory-unit and cross-bridge influence on myofilament kinetics. *J Mol Biol.* 2002; 322:1065–1088. [PubMed: 12367529]

Highlights

- We studied the effect of the FHC-linked mutation on the kinetics of transgenic cardiac myofibrils
- Kinetics was determined from the decay of autocorrelation function of fluctuations in polarization
- the kinetic rates were significantly smaller in the contracting cardiac myofibrils from Tg mutated mice than in control

Fig. 1.

Origin of changes of orientation. Actin monomers are labeled with fluorescent phalloidin (red). Changes of orientation of actin result from interactions of actins with myosin cross-bridges within the area indicated by colored monomers. A small volume of a myofibril is illuminated with 532 nm light (white cone). The detector sees only the volume equivalent to ellipsoid-of-revolution indicated in green by the dashed line. This volume contains both actin and myosin, because sarcomere length is short and therefore the I-band (area where actin is alone) is nearly absent. In rigor there are no fluctuations of the transition dipole (red arrow). In rigor (top) autocorrelation is flat and fluctuations arise only from random fluctuations in the background signal and the instrumental noise. During isometric contraction (bottom panel) myosin cross-bridges are cycling and disorganize actin filaments. The orientation of the rhodamine dipole fluctuates in time. We record parallel ($\parallel$) component of fluorescent light.

Fig. 2.

Lifetime (**A**) and polarization (**B, C**,) images of rigor Tg-WT myofibril from mouse ventricular muscle fibers. The color bar at right of lifetime image is the lifetime scale, with red corresponding to 6 ns and blue to 2 ns. The red circle is the projection of the confocal aperture on the sample plane. Its diameter (1.2 μm) is equal to the diameter of the confocal aperture (50 μm) divided by the magnification of the objective (40). The B/W intensity scale is 0–255, with 255 corresponding to white and 0 to black. Blue and red arrows indicate the direction of polarization of exciting and fluorescent light, respectively. The exciting light is polarized vertically. In this experiment only, myofibrils were labeled with 1 μM Alexa488-phalloidin. **D.** Decay of anisotropy of a typical I-band. Blue: experimental decay; red line: fit to the function $r(t) = R_{\infty} + a \cdot \exp(-t/\theta)$, where $R_{\infty} = 0.29$ is the value of anisotropy at infinite time, $a = 0.07$, is the amplitude of the anisotropy change, and $\theta = 1.3$ ns, is the rotational correlation time. Data collected for 60 s. Data acquired with the PicoQuant Micro Time 200 confocal lifetime microscope. Excitation with a 470 nm pulse of light, emission through LP500 filter.

Fig. 3.

Comparison of the pattern of arrival of fluorescent photons in rigor (A) and contracting (B) myofibrils. Contracting muscle shows the evidence of sudden bursts of fluorescence caused by instantaneous near-alignment of absorption/emission dipole of rhodamine with the direction of polarization of the laser. No such bursts are evident in rigor pattern.

Fig. 4.

A. The time course of polarized intensity of a rigor myofibril from the Tg-WT heart myofibril. The original data was collected every 10 μ s, 1000 points were binned together to give time resolution of 10 ms. The vertical scale is the number of counts during 10 ms. Fluorescence intensities polarized parallel ($I_{||}$) to the myofibrillar axis. Myofibrillar axis is vertical on the microscope stage. **B.** The corresponding correlation function. Laser polarization is vertical on the microscope stage.

Fig. 5.

A. The time course of polarized intensity of contracting myofibril from the Tg-WT heart myofibril. The vertical scale is the number of counts during 10 ms in $I_{||}$. Myofibrillar axis and laser polarization are parallel on the microscope stage. **B.** The corresponding correlation functions. The solid line is the triple exponential fit $y = ae^{-\alpha\tau} + be^{-\beta\tau} + ce^{-\gamma\tau}$ to the cross-correlation function. $\alpha = 16,415$ Hz, $\beta = 428$ Hz, $\gamma = 18$ Hz.

Fig. 6. The time course of polarized intensity (**A**) and correlation functions (**B**) of contracting R58Q mutated Tg-myofibril. The solid line is the triple exponential fit $y = ae^{-\alpha\tau} + be^{-\beta\tau} + ce^{-\gamma\tau}$ to the cross-correlation function. $\alpha = 5214$ Hz, $\beta = 71$ Hz, $\gamma = 0$ Hz.

Fig. 7. The effect of the R58QV mutation in myosin RLC on maximal force generation (**A**) and Ca^{2+} sensitivity of force (**B**) determined in Tg-R58Q glycerinated skinned papillary muscle fibers compared to Tg-WT. Data are expressed as mean of n experiments (n fibers isolated from mouse hearts) $\pm$ SE.

Fig. 8.

The experimental system. It has N states here designated a_n , $n=1\dots N$. They switch like $a_n \rightarrow a_{n+1}$ and $a_N \rightarrow a_1$ in sequence. Each state has different anisotropy [19–21]. The time between 2 consecutive switches is exponentially distributed.

Fig. 9.

Simplified scheme of a cross-bridge cycle. Weakly binding AMDP cross-bridge goes through a power stroke at a rate k_1 , assumes strong binding rigor conformation AMD and rapidly transforms to a “near rigor” AM. No orientation change takes place during AMD-AM transition. This is followed by binding of ATP and dissociation from actin (k_2) followed by hydrolysis of ATP and a weak binding to actin (k_3). Solid and broken arrows indicate change and no change in anisotropy of the cross-bridge, respectively.

Fig. 10.

A: Possible solutions of eq. 5 are shown as solid colored lines. The dotted lines are impossible solutions (one of the rate constants is negative). The thin black lines are at A and B, and the midpoint of the ellipse is at $(A+B)/3$ for both x and y-coordinate. The ellipse is tilted 45 degrees and the ratio between the long and short axes is the square root of 3. $K_1=\alpha$, $K_2=\beta$, $K_3=\gamma$.

The effect of the R58Q mutation on the rate of decay of the correlation function (A, B) and calculated rates (k_1 , k_2). N is the number of myofibrils tested.

Table 1

Muscle	Mean α (sec ⁻¹)	SD α (sec ⁻¹)	Mean β (sec ⁻¹)	SD β (sec ⁻¹)	N	k_1 (sec ⁻¹)	k_2 (sec ⁻¹)	k_3 (sec ⁻¹)
Tg-WT	19993	14243	477	284	35	<466	19,993	477
Tg-R58Q	11878	8684	300	211	46	<250	11,878	300