Role of Mutations in the Essential Light Chain (ELC) of Myosin in Familial Hypertrophic Cardiomyopathy (FHC)

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UNIVERSITY OF MIAMI

ROLE OF MUTATIONS IN THE ESSENTIAL LIGHT CHAIN (ELC) OF MYOSIN IN FAMILIAL HYPERTROPHIC CARDIOMYOPATHY (FHC)

By

Alexander Raytman

A THESIS

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Coral Gables, Florida

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ROLE OF MUTATIONS IN THE ESSENTIAL LIGHT CHAIN (ELC) OF MYOSIN IN FAMILIAL HYPERTROPHIC CARDIOMYOPATHY (FHC)

Alexander Raytman

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Force generation and the ability of the heart muscle to contract and correspondingly to beat depends upon multiple interactions between myosin and actin-tropomyosin-troponin, the key proteins of the contractile apparatus. The myosin molecule consists of two heavy chains and two types of light chains, two essential (ELC) and two regulatory (RLC) light chains. We hypothesize that mutations in myosin ELC may affect the ability of myosin to bind to actin, thus producing structurally and/or functionally abnormal sarcomeres effecting heart muscle contraction and relaxation. We believe that this pathological process underlies the basis of Familial Hypertrophic Cardiomyopathy (FHC), a genetic disorder caused by mutations in the genes encoding the major myofilament proteins, including the myosin ELC. I have investigated the effects of two FHC ELC mutations, A57G and E143K, on the actin-myosin interaction and generation of contractile force. Here, I show evidence that mutations in the ELC may cause disruptions in sarcomeric structure which then may cause abnormal muscle contraction and lead to compensatory hypertrophy.
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Familial Hypertrophic Cardiomyopathy (FHC) is an autosomal dominant disease that usually manifests itself by enlargement of the left ventricle and interventricular septum (IVS) (Fig. 1.1) (1). FHC results from the inability of heart to adequately pump blood which causes hypertension, premature fatigue and heart failure. FHC can result in sudden cardiac death (SCD), and is one of the leading causes of SCD among young athletes (2-4). Therefore, treatment of FHC should be made one of the priorities of cardiovascular research. However, little is known about molecular mechanisms of FHC. FHC is caused by mutations in genes that code for major contractile proteins of the heart, including myosin heavy chain (MHC), regulatory light chain (RLC) and essential light chain (ELC) (5,6). Our group proposes that the molecular disruption of the sarcomeric structure followed by...
an abnormal myofilament function could be the cause of FHC due to the altered interactions among all major sarcomeric proteins. Alternatively, development of histopathological changes suggests that the abnormal myofilament function precedes morphological abnormalities (5).

**Role of ELC in muscle contraction**

The sarcomere consists mainly of the thin filament, consisting of tropomyosin, troponin I, troponin C, troponin T and actin, and the thick filament, consisting of myosin heavy chain and myosin light chains. Hence, contractility of muscle is determined by the interactions between these proteins.

The muscle contraction is initiated when Ca$^{2+}$ binds to troponin C (7). This triggers a series of changes, involving tropomyosin, troponin I, troponin C and troponin T (8-12) which results in actin-myosin interaction.

Movement of actin against myosin filaments is what generates force and muscle contraction. Myosin consists of two heavy chains and four light chains: two regulatory light chains (RLC) and two essential light chains (ELC). N-terminal portions of the heavy chains together with the light chains form globular heads, so called cross-bridges or S1(13). These cross-bridges interact with actin molecules from the thin filaments moving them along(13). The cross-bridges are product-inhibited ATPases(14) and MgATP breakdown fuels the process. Actin is thought to be more passive partner, with the primary function of providing binding sites for the cross-bridges (13). In the absence of ATP, myosin is tightly bound to actin in the “rigor” state. Binding of ATP causes the cross-bridges to dissociate, which is followed by ATP hydrolysis and formation of myosin-products complex. This primes the cross-bridge to attach to the actin complex, which causes the cross-bridge to change its shape and move actin approximately 10nm –
“power stroke” state. Energy released with the breakdown of ATP allows for this movement. Then phosphate and ADP are released from the myosin molecule and the cycle starts again (13-14).

ELC is located in the lever arm domain of the myosin head (15). It belongs to the EF-hand superfamily of Ca$^{2+}$ binding proteins; however, the binding of Ca$^{2+}$ to striated muscle ELC has been evolutionarily lost (16-19). Both RLC and ELC seem to be involved in the force generation during muscle contraction, however it is unclear what roles they play (20-21). In particular little is known on how ELC in particular influences muscle contraction and how different isoforms of ELC participate in the actin-myosin interaction. There are reports of molecular contacts between ELC and actin during contraction (20). Therefore, it is possible that ELC may play a role the development of cardiomyopathy but it is not known how mutations in ELC can cause the disease. Thus, my work has addressed this understudied area of cardiac muscle biology and aimed at determining the basis for ELC induced cardiomyopathy.

**Myosin ELC isoforms**

In humans there have been identified multiple tissue specific isoforms of ELC that are expressed in ventricles, atria, skeletal and smooth muscle (22-25). Even though the sequences differ somewhat between these isoforms, they fall into two main types of a longer one and a shorter one. Both are expressed by the same gene but due to a single splicing event (26) they differ by about 45 amino acids at the N-terminus (20). Both longer and shorter isoforms are expressed in the skeletal muscle; however only the longer one is normally expressed in the heart. The smooth muscle expresses only the short ELC (for review see (27)). Previous studies have shown that the N-terminal ELC extension may be in contact with actin during muscle contraction (28-33). In fact, it is the first four
amino acids in the N-terminal extension (APKK), which are considered to form the actin binding site on ELC (34). If this N-terminal tail is removed, the properties of the “longer” ELC isoform without the N-terminal tail – so-called D43 mutant become similar to those of the shorter ELC isoform, expressed in the skeletal muscle (34). Hence our laboratory has been studying the importance of N-terminal tail of ELC in the ELC-actin interaction, force production and muscle contraction (34). For example, it is interesting to study whether ELC-mediated cardiomyopathy involves the N-terminus of ELC affecting the direct ELC-actin interaction. Our group is studying this using transgenic mice (Tg-D43) expressing a truncated form of the human cardiac ELC, missing 43 amino-acids from its N-terminal tale (34). Although D43 mutation is not part of this project it was used as a control in some experiments.

**FHC-associated ELC mutations**

Several mutations in ELC have been identified in patients with FHC: E56G, A57G, E143K, M149V and R154H (35-39). Fig. 1.2 (27), adapted from the crystal structure of the scallop regulatory domain by Houdusse and Cohen (1WDC) (40), depicts five ELC FHC mutations in the ELC molecule. The N-terminal ELC mutations, E56G and A57G, are predicted to be in the α-helix of the first EF-hand domain. (27) The E143K, M149V and R154H mutations are located in the third EF-hand motif. As shown schematically in Fig. 1.2, these three residues are located in the exposed loop of the EF3 structure of ELC (E143K), the β-sheet region of the C-terminus of the loop (M149V) and in the exiting helix of the EF3 (R154H) (27). Another recently identified mutation – M173V is not shown and it was not part of my project.
In humans, mutations in ELC have been shown to cause various phenotypes of FHC from asymmetric septal hypertrophy (A57G) (35), atypical left ventricular chamber thickening (M149V, R154H) to a restrictive cardiomyopathy-like phenotype (E143K) (36). While most of them have been associated with SCD at a young age (A57G, E143K, and M149V), some (E56G, R154H) have shown less severe symptoms (35-39). The A57G mutation was found in two unrelated Korean families and one Japanese patient. It was not found in the control group of unrelated individuals (35). While the mutation did not manifest clinically until early adulthood, the mutation usually resulted in asymmetrical septal hypertrophy by mid 30’s and in high incidence of sudden cardiac death among patients in 40’s and 50’s. E143K was characterized from a single patient, whose two siblings died at a young age (36). The patient was characterized with left ventricular
hypertrophy and the apex of his right ventricle was hypertrophied as well. Medical records for his deceased siblings indicated that they had cardiomyopathy. The patient was homozygous for the mutation, but his sister and parents who were heterozygous did not exhibit any symptoms.

At present, it is not clear how any of these ELC mutations cause FHC, we hypothesize that mutations in sarcomeric proteins may disrupt the structure of the protein and therefore affect sarcomere assembly leading to altered cardiac function. Abnormal interactions between these individual myofilament components due to their altered molecular composition may cause FHC. Sequence analysis shows that the residues which are affected by FHC are highly conserved among different species (27). In this work I have investigated the mechanisms by which two of these mutations A57G and E143K may cause FHC.

**Transgenic animal models**

Our lab has generated transgenic mouse models, expressing wild-type (WT) and D43 (34), as well as the A57G mutant of the human cardiac ELC. The A57G mice express ~85% of the transgene. Unpublished data from the lab show that Tg-A57G mice have enlarged hearts and fibrotic lesions.
CHAPTER 2

RESULTS

Question

In this work I have addressed the question of how the ELC mutations may cause Familial Hypertrophic Cardiomyopathy (FHC). In particular I have studied two ELC mutations A57G and to a lesser extent E143K. I have used D43 mutant as a control in some experiments.

Hypothesis

My working hypothesis was that ELC-mutations may lead to Familial Hypertrophic Cardiomyopathy by disrupting the ELC-MHC and actin-myosin interactions in the sarcomere. Disruption of these interactions may result in weaker muscle contractions. In order to compensate for these weaker contractions the left ventricular and septal walls of the heart enlarge in size ultimately causing hypertrophy. Thus, effects of the mutations in ELC would be visible on each level of muscle organization: molecular protein-protein interactions within the sarcomere, muscle fiber contraction and the organ level. In order to study this hypothesis I have conducted two sets of experiments: study of interaction at the molecular level between two major sarcomeric proteins and the experiments in the muscle fibers.

Research strategy

In order to investigate the effects of ELC mutations on the development of FHC I have pursued a two-fold approach. First I have done experiments at the molecular level such as studying interactions between ELC and the heavy chain of myosin (ELC exchange
studies), interactions between actin and mutant-myosin (fluorescence binding studies) and the ability of ELC alone to bind actin (co-sedimentation assay). Then I have done muscle fiber studies to investigate the effects of A57G mutation on fiber kinetics and myofilament Ca$^{2+}$ sensitivity. In this work I have not focused on the effects of ELC mutations on the organ level.

**Experiments at the molecular level**

Mutations in the ELC have been associated with malignant FHC phenotypes including SCD. However, little is known how exactly these mutations cause cardiomyopathy. My data suggest that ELC mutations, namely A57G might cause a decrease in binding of ELC to myosin and mutant myosin to actin.

I have used porcine cardiac myosin for my studies and exchanged endogenous porcine ELC for recombinant human ventricular ELC both wild type and the mutants. The myosin was obtained as described previously (41). I have expressed human ELC in bacteria. Strains of *E. coli*, carrying human ELC were previously developed by the lab. I have inoculated the media with bacteria carrying WT, A57G and E143K, then pelleted and froze the pellets according to the protocol established in the lab (42). Further, I have purified and lyophilized WT and A57G protein, according to the lab protocol(42). **Fig. 2.1** shows fractions containing A57G protein after the purification with Q-sepharose buffer, prior to lyophilization.
A57G Protein Purification with Q-Sepharose buffer

Figure 2.1 A57G protein expressed in bacteria after purification. Fractions eluted with Q-Sepharose buffer were collected and run on SDS-PAGE gel along with WT ELC standard, which was later stained with Coomassie blue stain. Fractions (numbered in order of elution) 1 – fraction 51, 2- fraction 55, 3- fraction 59, 4- ELC WT standard, 5- fraction 83, 6- fraction 86, 7- fraction 89, 8- fraction 92, 9- fraction 95, 10 - fraction 98, 11 - fraction 101, 12- fraction 104, 13- fraction 107, 14- ELC WT standard, 15 – fraction 110, 16- fraction -113, 17- fraction 117, 18 – fraction 120, 19- load, 20-wash. Fractions 83-107 were collected for lyophilization. High MW bands represent MHC impurity.

Then native porcine ELC was exchanged for human ELC expressed in bacteria. Although we have transgenic animal models it is difficult to obtain enough myosin for fluorescence experiments from murine hearts due to their small size and impracticability of going through the entire myosin purification process to conduct just one fluorescence experiment. However, one porcine cardiac myosin preparation allows for several weeks’ worth of myosin supply for fluorescence experiments. Additionally heavy chain of myosin differs in mice and humans: mice express α-myosin heavy chain, while adult humans express mostly β-myosin heavy chain. Porcine myosin is mostly β- MHC, therefore it is a more suitable model of human cardiac myosin. The α and β myosins differ in their kinetics and force development properties. The exchange process is described in the Methods section. I have followed the protocol previously developed in the lab (unpublished data) by adopting it to my experimental conditions.
Fluorescence and light scattering measurements

In order to investigate the consequences of these ELC mutations on the interaction of myosin with actin, I monitored the association of the ELC-exchanged myosin with rabbit skeletal F-actin, fluorescently labeled with pyrene. To label actin, I followed the protocol of Kouyama and Mihashi (43). Labeling was measured by comparing the relative absorbance of pyrene and actin: [pyrene]_{344} = (Abs_{344} - Abs_{500})/22000 x dilution factor. [actin]_{280} = (Abs_{280} - Abs_{500})/1.1 x dilution factor. [pyrene]_{344} / [actin]_{280} x 100 = % labeling.

For convenience and due to high homology between different actin isoforms, rabbit skeletal actin was used in all binding assays. Fluorescence and light scattering were measured using JASCO 6500 Fluorometer. Light scattering was recorded at 341.5 nm with excitation at 340nm. Fluorescence was recorded at 408 nm with excitation at 366 nm. (Full protocol is provided in the Methods section). At the end of each experiment 5 mM MgATP was added to dissociate actin from myosin and the fluorescence spectra were recorded and compared to the pyrene-actin spectrum obtained before titration in the absence of myosin.

Next, I wanted to test whether there is a direct association of the human ELC (not bound to MHC) with actin and whether the mutations in ELC affect this ELC-actin interaction. I have used the same protocol as above for fluorescence measurements except that instead of exchanged myosin I have titrated actin with human recombinant ELC directly. For this experiment I have used actin buffer as a control. I have not used buffer as a control in any previous experiment.
Co-sedimentation

Since fluorescence experiments could not provide me with an answer of whether ELC mutations affect actin-myosin binding I have turned to co-sedimentation experiments – measuring ELC–actin binding by examining density bands of the centrifuged mixture using SDS-PAGE and densitometry. It is not clear what role ELC plays in actin-myosin interaction and I have looked to test whether ELC participates directly in this interaction. My working hypothesis was that ELC participates in actin-myosin interactions and binds directly to actin during muscle contraction. In order to test that my first step was to look whether ELC is capable of binding to actin. To do that I have performed co-sedimentation experiments – I let actin interact with ELC in F-actin buffer. I have used the same recombinant human ELC as in the experiments above. After allowing ELC to react with rabbit skeletal F-actin for 30 min at the room temperature I have centrifuged the mixture. Filamentous actin would precipitate out while ELC would remain in the solution. If ELC were to bind to F-actin it would be found in the pellet as well. Then both pellet and supernatant were analyzed with ELC- specific antibodies, provided by our collaborator Dr. Epstein’s lab, to determine what amount if any of ELC bound to actin. (The full protocol may be found in the methods section). D43 ELC which is not expected to bind actin was used as a negative control. Additionally ELC solution with no actin was used as another control to determine extent of ELC precipitation upon centrifugation.

Muscle fiber studies

In order to investigate effects of these interactions at the muscle fiber level, I have studied the functional consequences of the A57G mutation in the papillary muscles from
the hearts of transgenic mice expressing human ELC-A57G protein compared to WT. **Fig. 2.2** shows the location of the papillary muscle in the heart.

![Location of the papillary muscle.](image)

**Figure 2.2** Location of the papillary muscle.

Transgenic mice carrying human WT and A57G-mutant ELC were generated by our group prior to my joining of the lab. Both models express approximately 85% of the transgene and were readily available for my studies. I have conducted a series of experiments determining fiber kinetics and Ca^{2+} sensitivity in these transgenic muscle fibers. For all the experiments below I have used age and gender matched mice – 3 ½ month old male mice expressing WT or A57G respectively.

**Fiber Kinetics**

Maximum force development and the force-pCa relationship were measured using the Guth Force Transducer (5, 42). **(Fig. 2.3)**. Muscle fibers ~100 µm thick were isolated and processed as described in Szczesna-Cordary et al (42). They were attached to the arms of force transducer and immersed in pCa8 buffer.
**Force Transducer apparatus**

![Schematic force transducer apparatus diagram](image)

**Figure 2.3** Schematic force transducer apparatus. The fiber is mounted between two clips, one of which is connected to the amplifier and chart recorder. As the fiber is placed in the series of Ca\(^{2+}\) containing solutions it contracts and relaxes which causes the pen to move on the chart recorder. This can be used to measure relative force of contraction. David Dweck 2005.

pCa refers to a negative log on Ca\(^{2+}\) concentration. Hence, pCa8 buffer has Ca\(^{2+}\) concentration of 10\(^{-8}\). (full description of the buffer can be found in the Methods section).

Ca\(^{2+}\) initiates muscle contraction. Fiber contraction/relaxation depends on Ca\(^{2+}\) concentration. At pCa8 muscle fibers are considered to be fully relaxed and at pCa4 fully contracted. It is possible to achieve intermediate levels of contraction/relaxation by immersing the fiber in solutions with pCa intermediate between pCa8 and pCa4. I have measured contraction using pCa7, pCa6, pCa5.8, pCa5.6, pCa5.4, pCa5.2, pCa5.0 and pCa4.5 buffers to establish pCa\(_{50}\) at which half of maximal force is observed.

Force was recorded as movement of the transducer’s pen in mm and then converted to KN/ m\(^2\) according to the following equation: pen movement (mm) \* 0.2757985 (mg/mm) = weight (mg); weight (mg) / \(\pi r^2\) = weight per cross section area (mg/mm\(^2\)); weight per area (mg / mm\(^2\)) \* g = Force per cross section area (N/m\(^2\)); Force per cross section area (N/m\(^2\)) \* 0.001 \* gain of the machine = Force per cross section area (KN/ m\(^2\)) where: r
=radius of the fiber and g is gravity; 1 mg exerts force of 9.8 µN. (Note: I assume that the fiber is circular and its width is an appropriate approximation of the diameter) pCa$_{50}$ value calcium concentration required for half maximum force was measured from the recording chart as pCa concentration corresponding to the movement of the pen to the half maximum distance.

**Kinetics measurements**

Next I have measured rates of the relaxation of the muscle fibers from transgenic mice using a Diazo-2, Ca$^{2+}$ chelator. Upon UV-photolysis it changes conformation from a low affinity for Ca$^{2+}$ ($K_d = 2.2$ µM) to high affinity ($K_d = 0.073$ µM), thus absorbing Ca$^{2+}$ from the solution. (46). In absence of Ca$^{2+}$ the fibers relax. Using Labview by National Instruments it is possible to measure $k_{rel}$ - the rate at which the fibers relax in this experiment and $T1/2$ - time at which half of relaxation takes place. The fibers for these experiments were the same as those used in maximum force and pCa$_{50}$ measurements.

**Experiments at the molecular level**

**ELC exchange**

The human cardiac ELC has a lower molecular weight than porcine cardiac ELC and migrates faster on the SDS-PAGE, which makes it possible to distinguish between the endogenous and exchanged ELC. **Fig. 2.4** demonstrates the representative SDS-PAGE stained with Coomassie Blue of ELC-mutant exchanged porcine myosin and relative percent exchanges of different mutants.
Porcine cardiac myosin after ELC exchange

Figure 2.4 Representative ELC mutant exchanged porcine myosin. Lane 1, Control –no exchange; lane 2, WT-exchanged myosin; lane 3- D43( D43 ELC migrates further down, below RLC band due to the lower molecular weight); lane 4 -A57G; lane 5-E143K. Percent exchange is determined as exchanged ELC/total ELCx100%.

I have evaluated relative intensity of the bands using Odyssey software. Table 2.1 shows all exchange reactions expressed as ratios exchanged ELC to total ELC content (exchanged plus native ELC) with average values and standard deviations.

**ELC exchange results**

<table>
<thead>
<tr>
<th></th>
<th>Average percent exchange</th>
<th>Standard deviation</th>
<th>N</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>55.69</td>
<td>18.41</td>
<td>6</td>
</tr>
<tr>
<td>D43</td>
<td>57.84</td>
<td>11.61</td>
<td>6</td>
</tr>
<tr>
<td>A57G</td>
<td>40.96</td>
<td>27.96</td>
<td>6</td>
</tr>
<tr>
<td>E143K</td>
<td>52.27</td>
<td>9.68</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2.1 ELC exchange results. Averages and standard deviations.

At this step, I could see that there was a lower % exchange of A57G (41%) compared to WT (56%). E143K and D43 exchanged at about the same level as WT (52% and 58% respectively). I have done t-test using Sigma Plot software to determine the significance of these results. I have compared independently WT with A57G, WT with E143K and WT with D43. T-test showed that the difference between each two groups is not great enough to exclude possibility that the difference is due to the random sampling
variability. Additionally the power of the test was lower than desired value, because of the low sample size indicating that I am less likely to detect the difference if the difference actually exists. With my results it is not possible to determine whether there is a significant difference or not. Similarly One-Way ANOVA testing showed that the differences between mutations are not great enough to exclude the possibility of random sampling variability and indicated that with my results it is not possible to determine whether or not there is a difference between exchange rates of the mutations I have studied. Hence I cannot determine whether A57G ELC mutant has a lower binding affinity to MHC potentially affecting the assembly of myosin within the sarcomere in furtherance of my hypothesis or A57G uses some other way inflict FHC

**Fluorescence and Light scattering measurements**

**Fig. 2.5** shows the excitation spectra of pyrene-actin before and after titration with myosin and treatment with MgATP.
Excitation spectra of pyrene-actin after titration with myosin, containing WT human ELC

Figure 2.5 Excitation spectra of pyrene-actin before and after titration with myosin and treatment with MgATP. Emission was fixed at 407nm and excitation spectra was recorded between 306 and 390 nm. Note peaks at 345 and 366nm. Black – spectra before myosin titration. Red – spectra after myosin titration and Blue – spectra upon addition of MgATP.

Emission was fixed at 407nm whereas excitation spectra was recorded between 306 and 390nm. Note peaks at 345nm and 366nm in excitation spectra before titration. Titration causes disappearance of these peaks, while addition of MgATP causes dissociation of myosin from actin and partial restoration of the peaks.

Fig. 2.6 shows normalized titration curves with standard deviations comparing WT Fluorescence and Light Scattering of A57G- and E143K-exchanged myosins while binding to actin. The graphs represent percent change in the original fluorescence/light scattering vs. ratio of myosin concentration to actin concentration in the sample.
Fluorescence and Light scattering measurements of actin-myosin binding

Figure 2.6 Normalized titration curves with standard deviations of A. Fluorescence and B Light Scattering. Black-WT, green- A57G and blue – E143K curves. The graph shows percent change in the original fluorescence/light scattering vs. ratio of myosin concentration to actin concentration in the sample. Fluorescence was measured in arbitrary units and then normalized to percent change from initial reading with initial being 100%. Light scattering was measured in arbitrary units and then normalized to percent change from initial reading with light scattering at the point where actin to myosin concentration ratio in the solution was 1:1 being 100%. Total volume of the solution has changed from 2.0 ml to approximately 3.5 ml depending on the concentration of myosin added.

There were six experiments done for fluorescence – four where actin concentration was 2µM and two where actin concentration was 0.5µM. The fluorescence was measured in arbitrary units and then normalized to percent change from initial reading with initial being 100%. There were eight experiments done for light scattering – six where actin concentration was 2µM and two where actin concentration was 0.5µM. The light scattering was measured in arbitrary units and then normalized to percent change from initial reading with light scattering at the point where actin to myosin concentration ratio in the solution was 1:1 being 100%. The volume of the solution has changed during titration from 2.0 ml to approximately 3.5 ml depending on the concentration of myosin added.
I have additionally plotted each individual set of data as change in fluorescence (arbitrary units) vs. myosin concentration (not shown). I have used the equation derived in our lab to fit the data and determine $K_d$ of actin-myosin binding, using SigmaPlot software. For fluorescence: $f = \frac{m_1 - m_2*(K_d + n*a + x - \sqrt{(K_d + n*a + x)^2 - 4*n*a*x})}{2*n*a}$ and for Light scattering $f = \frac{m_1 + m_2*(K_d + n*a + x - \sqrt{(K_d + n*a + x)^2 - 4*n*a*x})}{2*n*a}$, where $m_1$ and $m_2$ are initial and final readings, $a$ - concentration of actin, $K_d$ is the binding constant, $x$ – myosin concentration and $n$ is a stochiometry. The average $K_d$ and standard deviations are presented in the Table 2.2

<table>
<thead>
<tr>
<th></th>
<th>Average $K_d$ (µM – fluorescence)</th>
<th>Standard deviation</th>
<th>$n$</th>
<th>Average $K_d$ (Light scattering)</th>
<th>Standard Deviation</th>
<th>$n$</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>0.42</td>
<td>0.26</td>
<td>6</td>
<td>0.23</td>
<td>0.09</td>
<td>8</td>
</tr>
<tr>
<td>A57G</td>
<td>0.34</td>
<td>0.28</td>
<td>6</td>
<td>0.38</td>
<td>0.22</td>
<td>8</td>
</tr>
<tr>
<td>E143K</td>
<td>0.73</td>
<td>0.34</td>
<td>6</td>
<td>0.16</td>
<td>0.06</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 2.2 Average $K_d$ of exchanged myosin with standard deviations. $n$ is a sample size.

Fluorescence data seems to indicate E143K myosin has a lower affinity towards actin with A57G myosin having even higher affinity than WT, while light scattering data shows otherwise with A57G myosin having higher lower affinity for actin with E143K myosin being about same level as WT. I have done ANOVA testing using Sigma Plot software to compare the $K_d$ values but differences between the sets were found to be sufficient to reject the possibility that the differences between the sets are due to the random sampling variability. Hence my data is not sufficient to conclude whether either mutation affects actin myosin interactions or they cause FHC by some other mechanism.

Next I have attempted to test whether there is a direct association of the human ELC (not bound to MHC) with actin. This experiment is illustrated on the Fig. 2.7. Note: it depicts percent change in fluorescence, not an absolute change. An absolute change in
fluorescence units for these experiments is significantly smaller than the change during myosin-actin binding studies shown in Fig. 2.6.

ELC-actin binding studies

**Figure 2.7** Titration of actin with human recombinant WT ELC and with actin buffer gives essentially the same results. Change in fluorescence is measured against number of times ELC or buffer was added to the mixture. In the end of the experiment total volume changes from 2ml to approximately 2.5 ml (depending on the concentration of ELC added). Fluorescence was measured in arbitrary units and then normalized to per cent change from initial reading with initial being 100%. For WT n=5, for buffer n=3. Note: this is percent change in fluorescence, not an absolute change. An absolute change in fluorescence units is significantly smaller than during myosin-actin binding studies.

Unfortunately, it turned out that titrating with buffer gives about the same decrease in fluorescence as titrating with WT ELC. This suggests that pyrene fluorescence bleaches overtime as the probe is exposed to light during titration. Additional experiments (not shown indicated that if I simply leave pyrene-labeled actin in the fluorometer the fluorescence decreases but not as much as it decreases during titration. It is possible that the bleaching occurs both overtime and with light exposure or dilution of the sample. Therefore, no ELC-actin binding was monitored with this method. Perhaps the binding is too weak to change the conformation of the probe and change fluorescence intensity.
**Cosedimentation study**

While I have only done a few of these experiments, I could observe that ELC does bind to actin and that the binding can be monitored by Western blots of the ELC bands present in the pelleted F-actin in co-sedimentation assay. **Fig.2.8** illustrates co-sedimentation of WT and D43 ELC mutants together with actin. D43 mutant is used as a negative control as it lacks the actin binding sequence: so this ELC mutant is not expected to bind to actin. The protein bands on the graph are labeled according to the actin concentration and the numbers below the lanes represent the relative band intensity determined by the Odyssey software. This experiment had double controls – first ELC with no actin was subjected to the same conditions as ELC-actin mixture to see how much ELC for each concentration would appear in the pellet regardless of actin bound and second – D43 experiment, where no ELC was expected to bind to the actin.
Figure 2.8 The ELC at concentrations 0.2µM-20µM was allowed to react with 4 µM F-actin for 30 min in 500 µl actin buffer: 40 mM KCl, 10 mM MOPS, pH 7.0, then ultracentrifuged for 1.5
hours at 200k x g. Pellets were separated from sups and run for WB using a MAB ELC as primary antibody (1:2000) and GAM IR 800 dye as secondary (1:4000). A- WT ELC, B- D43 mutant. The columns are labeled according to the actin concentration and the numbers below the lines represent the relative band intensity of each band, determined by the Odyssey software. D43 migrates further than Wt because of the lower molecular weight.

Table 2.3 represents differences between the band intensities of the WT pellet (ELC-actin mixture minus ELC of the same concentration span down by itself with no actin), corresponding to the ELC bound to actin. The data show that at higher concentrations more WT ELC is bound to actin, then D43. This is in accord with my hypothesis that ELC can bind to actin independently from MHC and affect the actin-myosin interaction.

<table>
<thead>
<tr>
<th>ELC concentration</th>
<th>WT</th>
<th>D43</th>
</tr>
</thead>
<tbody>
<tr>
<td>20µM</td>
<td>46.22</td>
<td>3.19</td>
</tr>
<tr>
<td>10µM</td>
<td>2.45</td>
<td>2.42</td>
</tr>
<tr>
<td>6µM</td>
<td>2.35</td>
<td>1.19</td>
</tr>
<tr>
<td>3µM</td>
<td>0</td>
<td>0.28</td>
</tr>
<tr>
<td>1µM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.2µM</td>
<td>2.35</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.3 Difference in pellet band intensities (ELC-actin-mixture minus ELC of the same concentration span down by itself) from Fig. 2.8 representing ELC bound to actin.

I have made efforts to quantify the data and determine if the ELC binding to WT can be fit by relating the band intensities to the amount of ELC loaded into each sample. I have also attempted to create a standard curve by doing a Western Blot of WT ELC of known concentrations and then by comparing the band intensities to the actual concentrations. Unfortunately method of evaluating band intensities is too unreliable and too dependent on the precision of the experiment and on variability of the band intensities. However, I anticipate that it will be possible in the future to evaluate if the binding is different for the mutants compared to WT given an adequate standard curve.
Muscle fiber studies

Fiber Kinetics

In Table 2.4 I present average maximum force and pCa$_{50}$ values for WT and A57G fibers. N=10 for each experiment. I have done t-test to determine statistical significance but the differences between pCa$_{50}$ values for WT and A57G and differences in average maximum force for WT and A57G were not sufficient to exclude possibility of random sample variance. Consequently, I cannot tell whether the A57G mutation causes significant difference in either maximum force or the pCa50 value. Again, as with ELC exchange and light scattering data the sample size is not large enough to conclusively establish whether there is a difference or not.

<table>
<thead>
<tr>
<th></th>
<th>pCa50</th>
<th>F max, KN/m$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>WT</td>
<td>5.75</td>
<td>0.027</td>
</tr>
<tr>
<td>A57G</td>
<td>5.74</td>
<td>0.075</td>
</tr>
</tbody>
</table>

Table 2.4 pCa$_{50}$ and maximum force values. n=10 fibers for each mouse.

Kinetics measurements

Fig. 2.9 shows average values of relaxation rates of WT and A57G fibers together with the standard deviations. Percent change in the force exerted is measured vs. the time it takes the fiber to relax. Number of experiments is 10 for both WT and A57G fibers.
Relaxation rates measured by Diazo-2

Figure 2.9 Relaxation curves of WT and A57G fibers together with the standard deviations. Percent change in the force exerted is measured vs. the time it takes the fiber to relax. (Initial force reading is 100% and final relaxed state is 0%. Number of experiments is 10 for both WT and A57G mice. Blue is WT and red is A57G.

Additionally, each set of data was plotted independently (not shown) and fit to an exponential decay equation via Sigma Plot Software. $K_{rel}$ was determined from the exponential decay equation $f=a\exp(-b*x)+c\exp(-d*x)$ where $b$ or $d$ depict relaxation rates, $x$ is the time of relaxation. For single parameter equation, parameter $b$ is $K_{rel}$, while in double parameter equation where both $b$ and $d$ depict $K_{rel}$ with faster and slower components, $d$ parameter was used to determine $K_{rel}$. T1/2 was recorded directly from the graphs as the time to reach 50% of maximal force. I have done t-tests using Sigma Plot software on both $K_{rel}$ and T1/2. A significant (P<0.05) change in the relaxation (Table 2.5) was observed between Tg-A57G and Tg-WT fibers. The T1/2 of relaxation was 43.7±8.7 ms for Tg-A57G fibers compared to 34.6±2.8 ms for Tg-WT while relaxation rate was 19.6 ± 2.8 s⁻¹ for A57G and 26 ± 2.0 for WT fibers (n=10 fibers for each mouse
Therefore the muscle fibers containing A57G mutation relax slower than those containing WT human ELC.

<table>
<thead>
<tr>
<th></th>
<th>Relaxation Time (T¼) [ms]</th>
<th>Relaxation rate [s⁻¹]</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT ELC</td>
<td>34.6 ± 2.8</td>
<td>26 ± 2.0</td>
<td>10</td>
</tr>
<tr>
<td>A57G</td>
<td>43.7 ± 8.7</td>
<td>19.6 ± 2.8 (P=0.046)</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.5 Relaxation time and rate for skinned papillary muscle fibers of Tg WT and A57G ELC mutant mice. Presented are averages with standard deviations. Note for the fibers fit to the single parameter equation parameter b from the equation above represents the rate constant used in this table, while for the fibers fit to the double parameter equation parameter d represents the rate constant.
CHAPTER 3

DISCUSSION

Mutations involved in the current study, A57G and E143K were originally identified in the patients affected with Familial Hypertrophic Cardiomyopathy. The researchers who have discovered these mutations are clinical geneticists and did not determine the connection between these mutations and FHC. In this project I have investigated role of the above named ELC mutations in the development of FHC. This work encompasses the interaction at the molecular level between actin and myosin containing A57G and E143K mutations in ELC, between ELC and MHC and between ELC and actin and the effects of A57G ELC mutation on the muscle fibers.

**Experiments at the molecular level**

**ELC-MHC Interaction**

I started the experiments by exchanging human recombinant ELC for the endogenous porcine ELC. Initially I did these experiments in order to obtain myosin containing ELC mutations for the binding assays by fluorescence due to difficulties of expressing myosin heavy chain in vitro. Consequently, I have anticipated that the exchange would go until completion. Surprisingly, the exchange never went to completion despite 10 times excess of human recombinant ELC over the concentration of myosin. Even more surprisingly it seems that A57G ELC is incorporated to a lesser extent than either WT or E143K. (Average of 41% for A57G compared to 56% for WT, 52% for E143K and 58% for D43 mutations.) However t-tests and one-way ANOVA showed that the difference was not great enough to reject a possibility of random sample variance. Therefore, my data is not sufficient to establish whether A57G mutation causes FHC by lower incorporation into the sarcomere with a disruption of sarcomeric structure or it utilizes some other
mechanism for FHC development. The experiment was done only six times and ANOVA results indicated that due to the small sample size I’m less likely to detect a difference where the difference actually exists.

The major difficulty with this experiment is that the extent of exchange is measured as intensity bands by densitometry software; therefore precise loading of the gel is crucial as the densitometry results are imprecise. This resulted in band intensities varying significantly between individual experiments. In order to solve that problem more experiments are needed so that it may be possible to determine whether the binding affinity of A57G mutant to MHC is significantly different from the WT. At this time it is not possible to say whether A57G mutation decreases binding affinity of ELC to the MHC or this mutation results in FHC via a different mechanism. Another difficulty is that during the exchange the myosin was found to lose regulatory light chain (RLC). This interferes with the goal of creating myosin as close as possible to native state for actin-myosin binding studies. To solve this problem 5 times excess of porcine RLC was added to the reaction mixture during the exchange.

There is a possibility that ELC with A57G has lower affinity towards MHC. It is known that ELC facilitates inter domain interaction within the myosin head (27). This in turn leads to a fine-tuned ability of myosin to interact with actin and for the muscle to contract. Diminished ability of ELC carrying A57G mutation to bind to the myosin head may translate into inadequate protein formation and sarcomere assembly in vivo. This could then translate in diminished ability of myosin head to interact with actin and consequently for the muscle to contract.
Mutant myosin-actin interaction

As Fig 2.6 and Table 2.2 illustrate, no significant differences were found between binding affinities of the exchanged myosin, carrying WT and A57G and E143K mutations. Fluorescence shows that E143K myosin has a lower affinity towards actin than WT; A57G had the highest affinity. The light scattering data seems to indicate that there might be differences between A57G-exchanged myosin and the others, with A57G having a lower affinity for actin and E143K’s affinity being essentially the same as the WT.

The problem with establishing significance of these results is the large differences between the sample data. ANOVA indicated that the difference between the data is not sufficient to exclude a possibility of random sample variance. This high variance between the data could be due the fact that some experiments possibly were not carried out to completion. In the beginning I worked with actin concentration of 2 µM and I only had enough exchanged myosin to titrate the sample to 1:1 ratio of actin to myosin. Although theoretically this should be sufficient to complete the binding and quench the fluorescence of pyrene-actin, in practice that might not have been sufficient and the curve might not have been complete. As a consequence the fitting of the curve to establish K_d might have been jeopardized. Later I switched to actin concentrations of 0.5 µM and was able to titrate until 3:1 ration of myosin to actin in the sample was achieved. This allowed experiments in this way which is not sufficient to reach a conclusion.

Another problem that might have affected my experiments is illustrated in Fig. 2.7: addition of actin buffer itself quenches fluorescence as well. The only plausible explanation that I have for that data is that pyrene fluorescence quenches relatively fast
upon light exposure and simple opening of the lid to add myosin/ELC may be sufficient to trigger quenching. However, I think that if there were differences in binding between ELC mutants they would still be observable since fluorescence quenching upon light exposure would have affected all the samples equally. (In theory pyrene-actin fluorescence may actually increase upon the binding of ELC, but I suspect that it ELC will behave the same way as myosin which quenches fluorescence. Additional experiments are required to determine if there is a fluorescence quenching upon light exposure and if so another fluorescent chemical instead of pyrene maybe better suited for these experiments.

My results indicated a difference although not significant between binding affinities to actin of A57G and E143K myosin and WT. Fluorescence seems to indicate that E143K has a higher $K_d$ i.e. lower affinity for actin than WT or A57G, while light scattering data shows that A57G might have a lower affinity for actin than either WT or E143K. One-way ANOVA indicated that the difference is not sufficient to exclude a possibility of random sample variance. Hence, it is possible that there are no differences between the binding affinities of the exchanged myosins to actin and either the percent of exchange is not high enough or the ELC mutations lead to FHC by some other mechanism.

If E143K mutation did not result in a similar decrease in affinity it is consistent with clinical data(36). The E143K mutation causes disease only among homozygous individuals; while heterozygous do not exhibit any symptoms. E143K was exchanged to about 56% (see myosin exchange data); this is similar to the amount of E143K ELC present in a heterozygous individual. Therefore, it is possible that in order to determine
an effect of E143K mutation on myosin-actin interaction a better exchange or a different experimental model is needed.

**Direct interaction of ELC with actin**

While I have only done a few initial experiments in this study Fig. 2.8 shows that WT ELC alone can bind F-actin. More importantly, the amount of WT ELC bound increases with the increase in total WT ELC concentration in the solution: experiments with WT ELC show that amount of ELC found in the pellet together with actin increases with increase in the loading ELC concentration. D43 mutant, which is not expected to bind to actin, displays no change in the amount of ELC found in the pellet with increase in the loading concentration: Table 2.3. This suggests that ELC may directly bind actin during actin-myosin interaction and this binding may vary between WT and ELC mutants.

The fundamental difficulty with this experiment is that the ELC binding is estimated from densitometry units of the gel bands. The band intensity may vary greatly between different gels therefore any estimates are limited to the bands on the same gel. Comparison between the gels is very limited. Additionally, amount of ELC present in supernatant (and the corresponding band intensities) greatly exceeds those of the pellet. The pellet and supernatant gels cannot be done on the same scale. My efforts to create a standard curve of ELC concentration from band intensities have not achieved significant results. Thus, while co-sedimentation can establish ELC-actin binding, any studies aimed at difference between ELC mutants in binding to actin would depend on improving the ability to translate band intensities into amount of ELC bound to actin.
Muscle fiber studies

Next goal of my study was to investigate the effects of the ELC mutations on the muscle fiber contraction and relaxation. Our lab has generated transgenic mouse models carrying human WT ELC and A57G mutation. The mice involved in the study were age and gender-matched. Both WT and A57G transgenic mice expressed about 80% of the human ELC.

Maximum force and pCa\textsubscript{50}

In these experiments papillary muscles (Fig. 2.2) were isolated from two mice: WT and A57G). Table 2.5 shows that there was no significant difference found between either maximum force exerted or the calcium concentration required for the half-maximum force response for A57G and WT fibers. This suggests that my data is not sufficient to determine whether the A57G mutation cause significant difference either in force generated or in calcium concentration required. Although maximum force per square area exerted by WT fibers was higher than the one exerted by A57G fibers, the difference was not found to be statistically significant to conclude on the difference (49.88 KN/m\textsuperscript{2} for WT vs. 42.24 KN/m\textsuperscript{2} for A57G). Thus mutations in ELC must utilize some other mechanism to affect muscle contraction or relaxation in order to cause FHC. The problem with this experiment is that although there were 10 fibers examined for each set of data all of them came from just one mouse for either WT or A57G. Thus it is possible that the differences or similarities between WT and A57G were rather the individual animals studied than between the phenotypes.
Relaxation rate and time

Fig. 2.9 and Table 2.5 showed that in the presence of a calcium chelator the A57G fibers displayed lower relaxation rate (more time required to relax) than the WT fibers. (19.6 ± 2.8 s⁻¹ for A57G and 26 ± 2.0 for WT fibers). Hence it is possible that although A57G exert the same maximum force and require the same amount of calcium to contract, nevertheless it takes them longer to relax. In order to compensate for this slower relaxation the heart would hypertrophy and eventually this would lead to FHC. This is in accord with clinical data showing the A57G mutation resulted in a sudden cardiac death in at least one case. It is possible that at times of stress the heart of patients with A57G mutation just is not able to relax/contract as fast as otherwise would be necessary.

Taken together with the findings from experiments on molecular interactions, this shows that a possible mechanism for FHC development in patients with A57G mutation is as follows: lower affinity of A57G ELC to MHC causes weaker binding of myosin to actin which in turn results in slower muscle relaxation. Slower relaxation may lead to diastolic dysfunction, which in turn may lead to hypertrophy and FHC.

In order to conclusively establish that A57G causes slower fiber relaxation, more experiments are needed as the data presented comes from just two mice – one WT and one A57G. Thus it is possible that the differences are just differences between individual animals. Additionally to prove or disprove the hypothesis that the A57G mutation can cause changes in sarcomeric structure which result in slower muscle relaxation and could lead to hypertrophy more experiments are needed on muscle contraction. Namely, another method should be employed here which can assess the force transients in A57G
fibers to conclude on the effects of A57G mutation on cross-bridge kinetics in these fibers.

**Further Studies**

Additional experiments performed by the lab, independently of my work have shown that the A57G mutation can cause a phenotype of hypertrophy with severe fibrotic lesions in transgenic mice. The A57G mutation results in enlarged hearts and enlarged septal and left ventricular walls (unpublished data). These results are in line with my findings that A57G may alter fibers’ relaxation.

This work adds plausibility to a mechanism by which ELC mutations such as A57G may cause FHC. These mutations result in disruption of actin-myosin interactions at the molecular level. This in turn results in slower fiber relaxation (and possibly contraction as well). In order to compensate for the slower muscle contraction the heart hypertrophies which results in FHC and possibly a sudden cardiac death.

More experiments are needed to prove or disprove the hypothesis proposed above. All the experiments discussed in this work should be repeated to achieve statistically significant results. Alternatively different experimental approaches could be undertaken to move these studies forward.
CHAPTER 4

METHODS

ELC Exchange

Porcine myosin was obtained according to the protocol established in the lab (41) and stored in 50% glycerol at -20°C. Then myosin was precipitated with 13 volumes of 1mM DTT and dissolved in the buffer A, containing 0.4 M KCl, 20 mM MOPS and 1mM DTT pH 6.8. Human ELC was expressed in *E. coli* and purified according to the protocol established in the lab (42). The lyophilized ELC was dissolved in the buffer B, containing 20 mM MOPS and 1mM DTT, pH 6.8. Then porcine myosin was allowed to react with 5-10 fold molar excess of ELC mutant in the presence of 5 fold molar excess of RLC (as we observe lower levels of RLC after ELC-exchange) for 30 min constantly shaken. All reactions were done in presence of 10 mM MgCl$_2$, 0.2 M KCl, 20 mM MOPS, 0.5 mM TFP and 1mM DTT, pH 6.8 at the room temperature. After the reaction samples were precipitated with 13 volumes of ice-cold 1mM DTT for 15 min, dissolved in the buffer A (see above) and dialyzed overnight in the same buffer.

Fluorescence and light scattering measurements

Rabbit skeletal actin was obtained according to the protocol established in the lab (44, 45) and then labeled with Pyrene Iodoacetamide (PIA) (43) as follows: F-actin was dissolved in the F-actin buffer, containing 10 mM MOPS, 1 mM MgCl$_2$ and 40 mM KCl, pH 7, to the concentration of ~2 mg/ml and allowed to react with 1.5 molar excess of PIA, dissolved in DMF, for 16 hours in the dark at the room temperature constantly rotating. Then the reaction was quenched with 1mM DTT and centrifuged at 200k x g for 1.5 hours. Then F-actin was dialyzed against G-actin buffer (5 mM Tris-HCl pH 8.0, 0.2 mM CaCl$_2$, 0.2 mM ATP) to remove excess PIA and DTT and polymerized overnight to
F-actin. For our studies we used 1µM pyrene-actin and was titrated it with ELC-mutant exchanged myosins. Fluorescence and light scattering was measured using JASCO 6500 Fluorometer. Light scattering was recorded at 341.5 nm with excitation at 340nm. Fluorescence was recorded at 408 nm with excitation at 366 nm. At the end of each experiment 5 mM MgATP was added to dissociate actin from myosin and the fluorescence spectra was recorded and compared to the pyrene-actin spectrum obtained before titration in the absence of myosin.

**Cosedimentation assay**

Human cardiac ELC mutant proteins were expressed and purified as above and incubated at concentrations 0.2µM-20µM with 4 µM F-actin for 30 min in 500 µl of F-actin buffer at the room temperature, then ultracentrifuged for 1.5 hours x 54,000rpm. Pellets were separated from the supernatants and both run for WB using a MAB ELC as primary antibody (1:2000) and GAM IR 800 dye as secondary (1:4000). Then densitometry images of the gels were analyzed to determine % free ELC and % ELC bound to F-actin to determine whether mutant proteins bind to actin and with what affinity. The Δ43 ELC mutant lacking 43 amino acids from its N-terminus was used as a control. This mutant is not supposed to be able to bind to actin due to the absence of the N-terminal actin binding region. The assay is illustrated in Fig. 2.8.

**Steady-state force measurements**

Maximum force development and force-pCa relationship were measured using the Guth Force Transducer (5). Papillary muscle fibers for this experiment were isolated from the transgenic murine hearts as described previously (5, 34). Muscle fibers ~100 nm thick were isolated and processed as described in Szczesna-Cordary et al. (42). They were
attached to the arms of force transducer, immersed in pCa8 buffer (10^{-8} M [Ca^{2+}], 1mM [Mg^{2+}], 7mM EGTA, 2.5 mM [MgATP^{2-}], 20 mM MOPS, pH 7.0, 15 mM creatine phosphate, ionic strength = 150 mM adjusted with potassium propionate). After rinsing with pCa 8 buffer maximal force was established, in pCa 4 buffer (same as pCa 8 except [Ca^{2+}]=10^{-4} M). Then force was measured in different pCa solutions from pCa 8 to pCa 4 to establish pCa_{50} at which half of maximal force is observed.

**Rates of contraction and relaxation**

Once the maximum force of contraction is established a solution containing enough Ca^{2+} to generate 80% maximal force was mixed with chelator of Ca^{2+}, Diazo-2. Upon UV-photolysis it changes conformation from a low affinity for Ca^{2+} (K_d = 2.2 \mu M) to high affinity (K_d = 0.073 \mu M) (46). The fibers were exposed to a UV flash from Xenon lamp for ~3sec. The rate of relaxation was recorded with the software and the constants were calculated according to the equation: \( y = Ae^{-k_1 t} + Be^{-k_2 t} + C \), where \( k_1 \) and \( k_2 \) are the rate constants and \( A \) and \( B \) are the amplitudes of the force transient. T1/2 was determined at which half of relaxation takes place.
LITERATURE CITED


