Actomyosin Function in Left and Right Ventricles of Failing Human Hearts is Identical

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Abstract

Human systemic blood system offers larger resistance to the blood flow than pulmonary system: the Left Ventricle (LV) pumps blood more forcefully than the Right Ventricle (RV). The difference in pumping action may arise from the more efficient interaction between actin and myosin in the LV, from the more efficient operation of other sarcomeric proteins or from the morphological differences between ventricles. These questions cannot be answered by using whole ventricles or isolated myocytes because the number of molecules involved in tension generation is of the order of 1011. Averaging data from such large number of molecules makes unequivocal characterization of any differences impossible. Measurements must be taken from a few molecules. Moreover, data must be obtained in-situ to account for the molecular crowding effects that are likely to occur in crowded environment such as muscle. We measured kinetics of actomyosin cycle in contracting myocytes from failing ventricles by analyzing fluctuations in orientation of a few actin and myosin molecules in situ. Fluctuations in orientation were caused by ATP-induced repetitive cycles of binding-dissociation of myosin from actin. In both left and right failing ventricles the rate constants characterizing contraction were identical. We also measured distribution of spatial orientations of actin and myosin. The spatial distributions were identical in myocytes from both ventricles. These results show that there is no difference in the way actomyosin interacts with thin filaments in failing left and right ventricles, suggesting that the difference in pumping efficiencies are due either to muscle proteins other than actin and myosin, or that they are due to morphological differences between left and right ventricles.

Keywords: Cross-bridge orientation; Heart ventricles; Fluorescence polarization

Introduction

The Left Ventricle (LV) has to overcome a large resistance offered by a systemic system, while the Right Ventricle (RV) has to overcome a lesser resistance offered by a pulmonary system. However, it is not clear whether the ability to develop larger force by the LV is due to: 1. More efficient force generation by actin and myosin, 2. More efficient operation of other muscle proteins or 3. Dissimilarities of basic fiber structures of the two ventricles. The left ventricle is composed largely of oblique and circumferential fibers [1], which are known to be more mechanically efficient than the transverse fibers in the free wall of the right ventricle [2,3].

Orientation of the lever arm of myosin head is a defining parameter of force producing interaction between actin and myosin. In order to expose differences in this interaction between left and right ventricles, it is necessary to measure the rate constants governing the interaction, and the spatial distribution of myosin lever arm orientations associated with contraction of each ventricle. Whole ventricles or isolated myocytes cannot be used in such experiments because they contain millions of actomyosin molecules and data originating from so many molecules would get averaged out. All the rate constants of the mechanochemical cycle of actomyosin become unrecoverable, and the final distribution of orientations a large assembly will be a perfect Gaussian, irrespective of whether the data are taken from the left or right ventricle (Central Limit Theorem, [4]). In assessing the actin-myosin interaction, the contribution of individual molecules has to be measured [5,6]. Because of these technical difficulties the question whether individual actin and myosin molecules of LV and RV interact differently has never been asked.

We developed the ability to study few molecules out of millions present in a myocyte. This was possible by focusing on a minute section of a sarcomere. Force-producing interactions between actin and myosin take place the Overlap-band (O-band). We measured kinetics and spatial distribution of a few molecules of actin and myosin in the O-band of sarcomere in working failing myocytes. It is important that the measurements be carried in-situ because protein concentration in muscle is high, [7]. Consequently the molecular crowding effects may play a role in the operation of muscle [8,9]. We measured fluctuations of orientation of a few actomyosin

Abreviations

ACF: Auto Correlation Function; AP: Alexa633 Phalloidin; EDC: Ethyl-3-[3-(dimethylamino)-propyl]-Carbodiimide; FCS: Fluorescence Correlation Spectroscopy; LV: Left Ventricle; HF: Ventricles from the Failing Heart; NF: Ventricles from Non Failing Heart; OV: Observational Volume; PF: Polarization of Fluorescence; RV: Right Ventricle; SD: Standard Deviation; SSA: Steady State Anisotropy; UP: Unlabeled Phalloidin; XB: Myosin Cross-Bridge
molecules in situ during contraction. Fluctuations were reported by anisotropy of fluorescence, which is a convenient method to measure conformation changes [10-15]. From fluctuations we calculated the rate constants of myosin interacting with actin. We also compared the spatial distribution of actin and myosin molecules during contraction of both ventricles.

The results show that the kinetics and the steady-state distribution of actin and myosin were the same in contracting myocytes from left and right ventricles from failing human heart. It follows that the difference in ventricular function are caused either by non-tension generating muscle proteins or by morphological differences between ventricles.

**Materials and Methods**

**Chemicals and solutions**

All chemicals were from Sigma-Aldrich (St Louis, MO) except the fluorescent dye ScTau-647-mono-maleimide which was from SETA BioMedicals (Urbana, IL) and Alexa633 phalloidin (AP) and Unlabeled Phalloidin (UP) were from Molecular Probes (Eugene, OR). The glycerinating solution contained: 50% glycerol, 150 mM KCl, 10 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 5 mM EGTA, 5 mM ATP, 1 mM DTT, 2 mM PMSF and 0.1% β-mercaptoethanol. Cαrigor solution contained 50 mM KCl, 10 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 0.1 mM CaCl₂. Contracting solution contained in addition 5 mM ATP, 20 mM creatine phosphate and 10 units/ml of 1 mg/ml creatine kinase. EDTA-rigor solution contained 50 mM KCl, 10 mM Tris-HCl pH 7.5, 5 mM EDTA. Glycercination solution contained 50 mM KCl, 10 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 2 mM EGTA, 5 mM ATP, 20 mM creatine phosphate and 10 units/ml of 1 mg/ml creatine kinase.

**Preparation of ventricles**

Samples of human myocardium were collected at the University of Kentucky using procedures that were approved by the local Institutional Review Board. Failing myocardial samples were...
Procured from patients who received heart transplants. All samples were passed to a researcher as soon as they were removed from the patient and snap-frozen in liquid nitrogen within a few minutes. Samples were shipped to UNTHSC on dry ice. Immediately upon arrival in Fort Worth they placed for 24 hrs in glycerinating solution at 0°C. After 24 hrs, the glycerinating solution was replaced with a fresh solution and placed at -20°C. Myocytes (MF) were made from glycerinated hearts after a minimum of 2 weeks at -20°C.

**Preparation of myocytes**

2 weeks in a solution containing glycerine and EGTA caused creation of large holes in the myocyte membrane allowing direct access of MgATP to the O-bands. Myocytes are only ~0.5μm thick thus limiting the Observational Volume to AttoLiters (10^-12 L, see Figure 2A). Preparation of myocytes involved thorough washing of ventricles with ice-cold EDTA-rigor solution and incubating them for 1 hr in this solution in order to wash out ATP present in the glycerinating solution without causing contraction. They were then washed thoroughly with Ca-rigor solution and homogenized in the Cole-Palmer LabGen 125 homogenizer for 10s homogenization after a cool down period of 30s. Myocytes were made from ventricles which had spent at least 2 weeks in glycerinating solution at -20°C, and were used within 2 days of preparation.

**Actin labelling**

Long-wavelength probe (Alexa633-phalloidin, AP) was selected in order to minimize autofluorescence [16]. 1mg/ml myocytes (approx. 10μM actin) were incubated for 10min in the Ca rigor solution containing in addition 100nM AP+10μM unlabeled phalloidin at 10μM actin) were incubated for 10 min in the Ca rigor solution to facilitate adhering of myocytes to glass. Myocytes were placed on an ethanol cleaned #1 coverslip coated with polylysine and gently resuspended in Ca-rigor solution. A 50µL suspension was allowed to adhere to the glass for 10 min before a final wash with Ca-rigor solution (7.5) remained unchanged throughout the 20min reaction. The reaction was stopped by adding 20mM DTT. The pH of the solution (7.5) remained unchanged throughout the 20min reaction. The reaction is allowed to occur at 30°C for ten minutes. The reaction is inefficient because of short tissue preparation and low temperature. Thus only a small fraction of myosin carry fluorescent label. Since two SeTau molecules per molecule are employed the overall extinction coefficient of the peptide is extremely high (400,000 M^-1 cm^-1). This strategy permits obtaining data from only few molecules of myosin ex-vivo. The dyes are immobilized by myocytes and their combined transition dipoles have a well-defined orientation.

**Cross-linking**

Failing myocytes contract (shorten) after addition of contracting solution. Shortening would make it impossible to collect data for 20sec from a single spot. Myocytes must be prevented from any movement while preserving the ability to contract (and to develop force). To prevent shortening 1mg/ml myocytes were incubated for 20 min at room temperature with 20mM water-soluble cross-linker 1-Ethyl-3-[3-(Dimethylamino)-propyl]-Carboxidiimide (EDC) [19-26]. The reaction was stopped by adding 20mM DTT. The pH of the solution (7.5) remained unchanged throughout the 20min reaction. As a control we observed cross-linked myocytes under a Nomarski microscope after addition of contracting solution. Myocytes did not shorten or become dehydrated during at least 10min. To check whether cross-linking does not affect ATPase of myocytes, ATPase was measured independently by two researchers (Table 1). The

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### Table 1: The rates are in number of ATP molecules turned over by 1 myosin molecule per second. 1 mg/ml of myocytes, 5 mM ATP, samples ran in triplicate.

<table>
<thead>
<tr>
<th>Ventricle from</th>
<th>Type</th>
<th>Control</th>
<th>Cross-linked</th>
<th>Cross-linked and labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>6634F LV</td>
<td>Failing</td>
<td>0.040±0.004</td>
<td>0.034±0.004</td>
<td>0.037±0.005</td>
</tr>
<tr>
<td>6634F RV</td>
<td>Failing</td>
<td>0.033±0.003</td>
<td>0.037±0.004</td>
<td>0.043±0.005</td>
</tr>
</tbody>
</table>

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SeTauf647-maleimide was the dye chosen to label SH groups on ELC’s. It is excited in the far red and hence bypasses most contributions of autofluorescence [16]. SeTau is also resistant to photobleaching (the initial rate = 2.4 s^-1) because of nano-encapsulation of the squaraine moiety of the dye chromophore system in a mixed aliphatic-aromatic macrocycle. Very high extinction coefficient (200,000 M^-1 cm^-1), quantum yield (0.65) and large Stokes shift (44nm) makes it ideal for our purpose. Its overall fluorescence intensity was 4.2 times larger than fluorescence intensity of Alexa647. The dye is attached to ELC at positions 174 and 181. 5nM of labeled ELC is exchanged with the native ELC of ventricular myosin in the exchange solution containing 15mM KCl, 5mM EDTA, 10mM KH2PO4, 1mM Tri-Fluo-Perazine (TFP) and 10mM imidazole, pH 7 [18]. The reaction is allowed to occur at 30°C for ten minutes. The reaction is inefficient because of short tissue preparation and low temperature. Thus only a small fraction of myosin carry fluorescent label. Since two SeTau molecules per molecule are employed the overall extinction coefficient of the peptide is extremely high (400,000 M^-1 cm^-1). This strategy permits obtaining data from only few molecules of myosin ex-vivo. The dyes are immobilized by myocytes and their combined transition dipoles have a well-defined orientation.

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**Myosin labelling**

To measure myosin orientation, Essential Light Chain (ELC) (part of the lever arm of myosin) is labeled with a fluorescent dye. Human ELC contains one SH group, but to increase extinction coefficient another SH group was added by genetic manipulation. Series of steps to include additional thiol group is illustrated in Figure 1.

To genetically modify human fast Essential Light Chain (ELC), native LC is cloned into pQE60 vector to produce pQE-ELC expression vector [17]. The ELC cDNA insert in pQE60-ELC construct consists one Cys at amino acid position 181. We have introduced Cys in place of Gly at amino acid position 174 by site directed mutagenesis to generate pQE60-ELCG174C expression vector. The resulting pQE60-ELCG174C expression vector contained two Cys residues at amino acid positions 174 and 181. The pQE60-ELCG174C construct was generated by PCR-based site directed mutagenesis using the QuickChange kit from Stratagene (La Jolla, CA), pQE60-ELC template plasmid, and two complimentary primers F-hELCG174C: 5'-gaagttggggactccgtgcatgtca-aga-agacctctaggtctgc-3' and R-hELCG174C: 5'gcagccataggcttccttgcatgcagctccaagtccttcc-3'. The sequence of the plasmid pQE60-ELCG174C is confirmed by DNA-sequencing of both strands of the entire plasmid. Afterwards, the plasmid pQE60-ELCG174C is introduced into Escherichia coli M15 cells (Qiagen). The expressed recombinant proteins is purified on Ni-NTA-Agarose column and confirmed by immunoblotting with human ELC monoclonal antibody.

**Labeling and exchange of ELC into myocytes**

SeTauf647-maleimide was the dye chosen to label SH groups on ELC’s. It is excited in the far red and hence bypasses most contributions of autofluorescence [16]. SeTau is also resistant to photobleaching (the initial rate = 2.4 s^-1) because of nano-encapsulation of the squaraine moiety of the dye chromophore system in a mixed aliphatic-aromatic macrocycle. Very high extinction coefficient (200,000 M^-1 cm^-1), quantum yield (0.65) and large Stokes shift (44nm) makes it ideal for our purpose. Its overall fluorescence intensity was 4.2 times larger than fluorescence intensity of Alexa647. The dye is attached to ELC at positions 174 and 181. 5nM of labeled ELC is exchanged with the native ELC of ventricular myosin in the exchange solution containing 15mM KCl, 5mM EDTA, 10mM K2HPO4, 5mM ATP, 1mM Tri-Fluo-Perazine (TFP) and 10mM imidazole, pH 7 [18]. The reaction is allowed to occur at 30°C for ten minutes. The reaction is inefficient because of short tissue preparation and low temperature. Thus only a small fraction of myosin carry fluorescent label. Since two SeTau molecules per molecule are employed the overall extinction coefficient of the peptide is extremely high (400,000 M^-1 cm^-1). This strategy permits obtaining data from only few molecules of myosin ex-vivo. The dyes are immobilized by myocytes and their combined transition dipoles have a well-defined orientation.
measurements were carried out using Anaspec (Fremont, Ca) Sensolyte MG Phosphate Colorimetric Assay at 30°C in a 96 well plate and read on a microplate reader at 650nm, with and without cross-linker EDC. Samples were ran in triplicate. Readings were taken for 30 minutes at 2 min. intervals. ATPase of unmodified myocytes was in agreement with [27]. The ATPase was slightly higher for the RV than the LV.

**Instrument**

The data was collected by Pico-Quant MT 200 instrument coupled to the Olympus IX 71 microscope. 10µW laser beam at 635nm modulated at 20 MHz was focused on the O-band by an Olympus x100, NA=1.2 objective. The experimental apparatus was the same as in [28].

**Data collection**

Before each experiment, the instrument was calibrated to ensure that 100% of emitted light was detected by a parallel or perpendicular channel. Fluorescence of an isotropic solution of a dye with a long fluorescence lifetime (rhodamine 700, excitation at 635nm) was measured. Such dye is practically isotropic because it has no time to reorient itself during ns short laser pulse. Its steady-state anisotropy is 0.400. First the amplifier controlling signal to the perpendicular channel was adjusted to a maximum. Next, the prism was rotated 90°and the amplifier controlling signal to the parallel channel was adjusted to be equal to the signal of parallel channel. Finally, the solution was replaced by a completely anisotropic solution and it was verified that signal reported by each channel was equal. The laser beam was focused by an Olympus x60, NA=1.2 water immersion objective on the O-band of a myocytes. The fluorescence intensity was collected for 20 sec. distributed onto 2,000,000 10µs bins yielding overall time resolution of 10 msec. Fluctuations of Polarization of Fluorescence (PF) and of steady-state anisotropy (r) were computed from the orthogonal components of fluorescent light. The Autocorrelation Function (ACF) was computed from these 2,000 measurements. Huxley at al. determined that a characteristic lifetime of one fluctuation is on the order of milliseconds, so during 20 s the orientation will change X=~20,000 times [29]. The precision of measurement is therefore approximately 1/√X = 0.7%. Approximately 20–30 myocytes were examined in each ventricle.

**Estimating the number of observed actin molecules**

To determine this number it is necessary to measure fluorescence intensity associated with one molecule of Alexa633-phalloidin. This number was determined by Fluorescence Correlation Spectroscopy (FCS). The autocorrelation function at delay time 0 of fluctuations of freely diffusing AP molecules entering and leaving the OV is equal to the inverse of the number of molecules contributing to the fluctuations N = 1/ACF(0) [30,31]. 15nM Alexa-phalloidin was illuminated with 1-70µW of laser power at 635nm. A calibration curve was constructed by plotting the power of the laser vs. the rate of photon arrival per molecule of the dye. From this curve it was determined that the number of photons/sec collected from a single AP molecule illuminated by a typical laser power used in each experiment (22µW) was ~400 photons/sec/mol. The number of observed actin molecules was estimated as ~16.

**Estimating the number of observed myosin molecules**

The procedure was identical to the one outlined above for actin, except that 15nM of SeTau dye was used for calibration. A calibration curve showed that that the number of photons/sec collected from a single SeTau molecule was ~900 photons/sec/mol. The number of observed myosin molecules was estimated as ~6.

**Time resolved anisotropy measurements**

The measurements were performed on FT300 fluorescence lifetime spectrometer (PicoQuant GmbH, Germany). The excitation source was Fianium super-continuum white light source (Fianium Ltd, Whitelase SC400-4). White light was passed through a monochromator to get 635nm light at 10MHz repetition rate. The emission was observed at 665nm through a long pass 650nm filter by fast microchannel photomultiplier tube. The resolution was kept at 4ps per channel, and the pulse width was less than 100 ps. For measuring anisotropy decays, the fluorescence intensity decays were collected while orienting the emission polarizer in vertical and horizontal respective to the vertically oriented excitation polarizer. The vertical (parallel) and horizontal (perpendicular) intensity decays were used to calculate the time dependent anisotropy using the equation:

\[ r(t) = (I_{Parallel}(t) - I_{Perpendicular}(t)) / (I_{Parallel}(t) + 2I_{Perpendicular}(t)) \]

The anisotropy decay was analyzed using Fluofit 4.0 program provided by PicoQuant and was fitted using formula:

\[ r(t) = \Sigma 2592R_i e^{(-t/\varphi_i)} \]

Where, r is the total anisotropy, Ri is the fractional anisotropy amplitude associated with component, φi is the rotation correlation time and t is the time in nanoseconds. Fluorescence lifetime was measured with the emission polarizer at 54.7°.

**Statistical analysis**

Autocorrelation function was calculated using Origin 2016 (OriginLab Corporation, Northampton, MA) or using R (version 3.3.1). The autocorrelation curve was fitted with a bi-exponential decay model in order to extract the rate constants using Origin 2016 or XPFIT (version 1.2.1) Alango Ltd. XPFIT employs the Inverse Laplace algorithm in order to numerically invert the decay time domain data. The goodness of fit was assessed by chi-squared. Comparisons between groups were performed using an unpaired Student’s t-test by Origin 8.5 program. The differences were deemed significant when P<0.05. Origin 2016 was also used to compute histograms and the autocorrelation functions.

**Experimental problems**

The difficulty in obtaining single molecule data in-situ is assuring adequate Signal/Noise (S/N) ratio. In-situ data contains significant contributions from autofluorescence and the background due to the dense environment [7]. The background signal consists of a constant fluorescence coming from myosin that is always present in the OV (observing single myosin molecule in-vitro e.g. [32-36] poses no such problem). In contrast to the regular FCS, actin and myosin molecules do not translate and fluorescent signal does not fluctuate between zero and maximum. In our experiments only anisotropy fluctuates around the mean. Moreover, to avoid photobleaching the laser beam...
cannot be focused on the same spot for much more than 20 sec.

**Measuring Orientation of Actin**

The rationale of using conformation of F-actin to monitor the kinetics of actomyosin cycle in ventricles is that actin anisotropy reflects the physiological state of a myocyte as illustrated in Figure 2. It shows the relation between the actomyosin cycle and changes of anisotropy of actin.

Head (blue) Lower 50KD domain (gray) are separate. The lever arm (green coil) is up. Steady State Anisotropy (SSA) of F-actin-phalloidin is low (SSA=0.220±0.006). Next, myosin containing products of hydrolysis binds weakly to thin filaments with a rate constant k\text{ON} (the red arrow indicates the direction of the dipole moment of the fluorophore attached to a single actin molecule) (C). Dissociation of Pi ADP and formation of strong binding Actin+Myosin+ADP state (D) is associated with the closing of Upper and Lower 50KD domains and causes increase of anisotropy of F-actin (SSA=0.248±0.007. At the same time, transition from weak to strong binding causes power stroke (indicated by the black arrow). System then undergoes transition to rigor state (D→E), without change of anisotropy. Finally, binding of ATP to the head in rigor state (E) causes dissociation of myosin with a rate constant k\text{OFF}. During dissociation the lever arm (green coil) repositions itself from the orientation pointing down to the orientation pointing up. The changes in anisotropy of a single Alexa-phalloidin dipole during contraction are shown schematically as a red line. Anisotropy measurements taken in vitro, with 1µM actin labeled at 10% with phalloidin 488, 0.5µM S1, 2 mM ADP, 5 mM ATP.

The main reason for using anisotropy of F-actin to assess the kinetics of actomyosin cycle is that labeling ventricular actin in situ is simple, reproducible and causes no ATPase alteration. Labeling actin with fluorescent phalloidin involves simply gentle irrigation of myocytes, because phalloidin binds to F-actin extremely strongly. Moreover, the orientation of phalloidin transition dipole reflects orientation of actin [14,15] and is easy to measure [10-15]. Finally, the number of actin molecules that are labeled can be easily controlled [37,38]. The instrument used to measure anisotropy is the same as in [28].

In summary: the ATP-induced interactions of myosin cross-bridges with actin result in a rapid momentum transfer from myosin to actin. The force resulting from this transfer is reflected in the alteration of conformation of actin [40,41]. Anisotropy of F-actin is low when it is free or weakly bound to myosin head, or high when it is strongly bound to the myosin head containing ADP or to nucleotide free myosin head (rigor). The cycle repeats itself with the period T (equal to the inverse of ATPase activity). The net result is that anisotropy of phalloidin fluctuates during contraction between low and high values with the rate constants k\text{ON} and k\text{OFF}.
We minimized the number of observed actin molecules by carrying out experiments on the O-band of contracting myocytes isolated from a failing human ventricle. The experimental arrangement is shown in Figure 3.

Kinetics of orientation changes of actin

The first parameter that we studied to reveal characteristics of human ventricles was kinetics of orientation changes of actin as illustrated in Figure 3C. Figure 4 highlights again the conformational transitions under study:

Failing ventricular myocytes contracted after addition of a solution containing ATP [28]. They are cross-linked so do not shorten, but retain full ATPase activity. As noted before, changes of orientation of actin during contraction occur because momentum is rapidly transferred from myosin cross-bridges to actin. Red line in Fig. 1 illustrates the changes of anisotropy of a single Alexa-phalloidin molecule during one ATPase cycle of contraction. It is ON-OFF transition in which the ON transition is the rate with which myosin head binds strongly to thin filaments, and OFF transition is the rate of head dissociation from thin filaments.

Experimentally we were able to observe changes in 16 actin molecules (see Materials and Methods). The method introduced by Magde and Elson [31,43,44] was used to extract the rate constants from data: Autocorrelation Function (ACF) of fluctuations of anisotropy is calculated. It is an average of the sum of products of the instantaneous values of PF and the values of PF delayed by delay time $\tau$ from 0 to 2 sec. An example of ACF is shown in Figure 5.

ACF reveals existence of two different states. The theoretical ACF of the ON-OFF process is shown in Eq. 1 [45].

$$ACF= \frac{(a_{OFF} k_{ON} + a_{ON} k_{OFF})^2}{(k_{OFF} + k_{ON})^2} + \frac{k_{OFF} k_{ON}}{(k_{OFF} + k_{ON})} e^{-\frac{k_{OFF} + k_{ON}}{\tau}}$$  \hspace{1cm} Eq. 1

where $k_{ON}$ and $k_{OFF}$ are the ON and OFF rate constants and $a_{ON}$, $a_{OFF}$ is the magnitude of anisotropy change (determined by the fitting program). Experimental ACF is fitted to eq. 1 by the non-linear fit from which the rates $k_{ON}$ and $k_{OFF}$ are obtained. The data is summarized in Table 2.

Spatial distribution of actin in contracting failing myocytes

An additional parameter characterizing actin is the distribution of orientations during contraction. The distribution was Gaussian. It was characterized by a Full Width at Half Maximum (FWHM). It indicates the width of the distribution of orientations. Examples of measurements of spatial distribution from 148 experiments from LVs and 153 experiments from RVs are shown in Figure 6. All the data is summarized in Table 3.

Measuring Orientation of Myosin

The direct way to report on conformation changes within the direct way to report on conformation changes within the heart is by measuring the orientation of myosin. This can be done using techniques such as cryo-electron microscopy. The figure shows an example of such an analysis, where the orientation of myosin is measured in different states of contraction.

Table 2: Summary of 153 experiments on RV and 148 experiments on LV from 5 failing hearts. The difference between LV and RV for the rate constant $k_{ON}$ was statistically significant ($P=0.049$, $t=0.794$) and 95 degrees of freedom. The difference between LV and RV for the rate constant $k_{OFF}$ was statistically significant ($P=0.383$, $t=0.127$) and 99 degrees of freedom. The errors are SEM. The error is larger for $k_{OFF}$ because there is less experimental points for faster process.

<table>
<thead>
<tr>
<th>Contraction of myocytes from failing heart</th>
<th>$k_{ON}$ (s$^{-1}$)</th>
<th>$k_{OFF}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV</td>
<td>0.98±0.02</td>
<td>0.28±0.02</td>
</tr>
<tr>
<td>RV</td>
<td>1.37±0.42</td>
<td>0.13±0.11</td>
</tr>
</tbody>
</table>
ventricular myocytes is to follow changes of orientation myosin. However, following orientation of myosin in-situ is not easy: it involves genetically engineering Essential Light Chain 1 (LC; see Materials and Methods), labeling it with the long-wavelength probe and replacing original LC with fluorescent one without affecting ATPase of a ventricle. The anisotropy of fluorescence reflects conformational changes of fluorecently labeled myosin of skeletal muscle fiber or myocytes [10-13]. The instrument used to measure anisotropy was the same as in [28].

How the anisotropy of labeled LC reflects the kinetics of a myosin head seen in Figure 7.

In order to avoid problems associated with averaging data from numerous molecules, just like in the case of actin, the number of observed myosin molecules needs to be minimized. It was done by measuring orientation of myosin in the A-band of contracting myocytes isolated from a human ventricle. The interaction between actin and myosin occur only in the part of an A-band where thick and thin filament overlap (O-band). An A-band contains ~104 myosin molecules, still too many to obtain kinetic information. To reduce this number, myosin was intentionally labeled inefficiently with fluorescent probe. Only a single myosin molecule out of 1000 was labeled. Myocytes were cross-linked with zero-length cross-linker to eliminate any movement during contraction while preserving ATPase activity, Figure 8 explains how the experiments were carried out.

**Kinetics of myosin orientation changes**

Myocytes were obtained from failing hearts. They do not shorten, but retain full ATPase activity: ATPases of control and cross-linked LV myocytes were 0.040±0.004 s⁻¹/mol; 0.034±0.004 s⁻¹/mol for respectively. ATPases of control and cross-linked RV myocytes were and 0.033±0.003 s⁻¹/mol; 0.037±0.004 s⁻¹/mol respectively. The experiment begins by placing isolated myocytes on an ethanol cleaned cover slip. The conformational transitions are shown as a Figure 9. The cycle begins when XB is dissociated from a thin filament where anisotropy is low. Binding to a thin filament causes a small increase in anisotropy (transition A). Dissociation of PI and assumption of actin-myosin-ADP complex (transition from apoenzyme to holoenzyme (transition B) causes further increase in anisotropy in accordance with [51]. Finally, dissociation of myosin from actin (transition C) occurs with the rate $k_{\text{Diss}}^{-1}$.

The repetitive changes in anisotropy cause intensity fluctuations. The ACF of fluctuations is calculated by the method first introduced by Magde and Elson [31,43,44]. The rate constants are computed from ACF by non-linear fit. The analytical form of ACF of the 3-state process is very complex and is fully described in [45].

Examples of the ACF are shown in [28]. Like ACF shown in Figure 5, the ACF reflects the two state process, even though anisotropy changes occur in three steps. XB binding to actin (rate kB) is too fast to be reflected in the ACF. This is due to the limited time resolution of the instrument. It collects photons every 10µsec, but in order to decrease the noise, the 2 M data points are packed into 2,000 bins, 1,000 points per bin. Thus the instrumental response time is 10 msc. We cannot detect processes faster than 100 Hz (s⁻¹). The rate constants were calculated from ACF’s by non-linear fit to analytical solution, described in [45].

We analyzed myocytes from 5 heart failure ventricles. Table 4
shows averages from 5 ventricles. There was no statistical difference between LV and RV for either rate constant.

**Spatial distribution of the lever arm in contracting failing myocytes**

The distribution of orientations of lever arms was Gaussian. Examples of measurements of 27 experiments from LVs and 26 experiments from RVs are shown in Figure 10.

All data is summarized in Table 5. The distributions show no differences in the value of FWHM. AR² values indicate how well the fitted curve (red) matches a perfect Gaussian (a perfect fit has an AR² value of 1).

**Discussion**

The main conclusion of this paper is the kinetic rates of conformational transitions and the distribution of myosin and actin were the same in contracting in-situ actin and myosin from myocytes from failing LV and RV. Consistent with our data, earlier work...
showed failing RV and LV myocytes displayed similar decrease in development of maximal force [52]. However, this work also revealed interventricular differences in myocyte function in experimental congestive heart failure of rats. LV myocytes were less Ca²⁺-sensitive than RV myocytes. There were also differences in expression and activation of PKC-α and in phosphorylation of cTnI and cTnT. We did not measure either activation of PKC-α or phosphorylation of cTnI and cTnT, possibly better indicators of myocytes function. Other reasons for the difference between this work and ref [52] is that different species were examined and it is possible that the difference arose because we examined individual molecules while Belin et al. looked at whole myocytes. Individual molecules approach allows data collection under in situ conditions thus taking into account molecular crowding and packing of myosin in thick filaments, which may play an important role in crowded systems such as muscle.

Our results are consistent with the fact that actin is expressed from the same genes [53] in both ventricles and that there is no evidence of differential expression of β-myosin heavy chain in ventricles, although ventricular-specific expression was seen during chamber specification in the zebrafish embryo [54].

We were able to examine molecular function of individual molecules because we worked on mesoscopic - not macroscopic - samples. Observing individual molecules in-situ is technically challenging because myosin and actin are always present in the OV. They do not translate but remain fixed in the OV. Unlike in the regular Fluorescence Correlation Spectroscopy (FCS), the fluorescent signal does not fluctuate between zero and maximum; instead it fluctuates around non-zero mean. A further complication is that, to avoid photobleaching, the laser beam cannot be focused on the same spot for much more than 20 sec to. It must also be emphasized that the orientation of myosin the lever arm was measured using recombinant light chain exchanged with endogenous LC. This leaves open the possibility that the data may not reflect exactly on the interactions of endogenous LC which carries no fluorophores. Post translational modifications are unlikely to affect the results because in the mutated clone G has been shown to have Cys at amino acid position 147 by DNA sequencing. Therefore, expression of this mutated plasmid is expected to produce G147C isoform of the protein. The most obvious post translational modification, formation of Cys-Cys link between Cys 147 and 181, is impossible because sample was pre-washed with DTT (ll. 265). The isoform shift will occur upon substituting Gly for Cys and the overall negative charge will decrease.

The data from failing ventricles should be supplemented by data from non-failing ventricles. In view of the fact that human non-failing ventricles are difficult to obtain, we propose that large animal model of heart failure should be exploited to examine the differences between healthy and diseased ventricles.

Conclusions

- We looked for the differences between kinetics of orientation changes and spatial distributions of actin molecules in actomyosin complex during contraction of myocytes from failing LV and RV.
- We looked for the differences between kineticsof orientation changes and spatial distribution of lever arms of myosin during contraction of myocytes from failing LV and RV.
- Experiments were done in-situ thus accounting for molecular crowding.
- Actin kinetics was identical in contracting myocytes from failing LV and RV.
- Actin molecules in contracting myocytes from failing LV and RV were identically distributed in space.
• Myosin lever arm kinetics was identical in contracting myocytes from failing LV and RV.
• Myosin lever arms in contracting myocytes from LV and RV were identically distributed in space.
• We conclude that the difference in pumping efficiencies are due either to muscle proteins other than actin and myosin, or that they are due to morphological differences between left and right ventricles.

References