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Contribution of titin and extracellular matrix to passive pressure and measurement of sarcomere length in the mouse left ventricle

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Abstract

It remains to be established to what degree titin and the extracellular matrix (ECM) contribute to passive pressure in the left ventricle (LV). Thus, we aimed to elucidate the contribution of major molecular determinants of passive pressure in the normal mouse LV. Furthermore, we determined the working sarcomere length (SL) range of the LV to bridge our findings to earlier work in skinned muscle fibers. We utilized Frank-Starling type protocols to obtain diastolic pressure-volume relationships (PVR) in Langendorff perfused isolated LVs. To quantify the molecular contribution of titin and ECM, we innovated on methods of fiber mechanics to chemically permeabilize intact LVs and measure a fully passive PVR. To differentially dissect the contributions of the ECM and titin, we utilized myofilament extraction techniques in permeabilized LVs, measuring passive PVRs at each stage in the protocol. Myofilament extraction suggests that titin contributes ~80% of passive pressures in the heart. Langendorff perfusion was also used to chemically fix passive and BaCl₂ activated hearts at specific volumes to determine that the maximal working SL range of the midwall LV fibers is approximately 1.8-2.2 μm . A model of the passive SL-Volume relationship was then used to estimate the pressure-SL relationships, indicating that the ECM contribution does not exceed titin's contribution until large volumes with SLs >~2.2 μm . In conclusion, within physiological volumes titin is the dominant contributor to LV passive pressure, and ECM-based pressures dominates at larger volumes.

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Research Highlights:

- >We developed a method to determine the titin and collagen contribution to passive pressure in the whole left ventricle of the mouse.
- >The maximal working sarcomere length range was determined to be ~1.8-2.2 μm .
- >Titin is the major contributor to passive pressure in the normal working range and collagen the major contributor at larger volumes.

DISCLOSURES

None

Keywords

Titin/connectin; Collagen; Extracellular Matrix; Diastole; Skinned

INTRODUCTION

The systolic properties that underlie the pump function of the heart have been well studied from the whole organ-level down to the single myosin molecule [1]. In contrast, the diastolic properties, which underlie the filling characteristics of the heart cycle, are less well understood but no less important. The collagen-dominated extracellular matrix (ECM) has been viewed as the primary determinant of passive pressure in the left ventricle (LV) based on studies of fibrosis [2-5]. However, characterization of the intracellular protein titin (also known as connectin) has shown that titin contributes to passive tension of cardiac muscle [6-8] and is thus another candidate for generating diastolic pressures. Titin is a filament that reaches from the Z-disk to the M-line region of the sarcomere[9]; parts of the molecule are firmly anchored to either the Z-disk or thick filament and are therefore inextensible [10] whereas titin's I-band spanning region functions as a molecular spring that develops force as sarcomeres are stretched during diastole (for reviews with original references see [11-13]). Experiments in permeabilized (skinned) muscle fibers have shown that titin dominates the passive tension-sarcomere length (SL) relationship up to a SL of $\sim 2.2\mu\text{m}$ with the ECM playing a lesser role[6-8]. Although it is tempting to deduce that titin is also the dominant source of passive pressures of the heart, extending muscle fiber data to whole heart behavior is complicated by regional variation in muscle properties, including the complex weave of fibers in the LV with different orientations [14-15], and, importantly, the working SL range of the LV is not well known. Thus, extrapolating *in vitro* results with permeabilized muscle fibers to the *in-vivo* intact heart is not straightforward and, as a result, the specific contribution of titin and the ECM to passive diastolic LV pressure has remained obscure.

In this study, we utilized a mouse isolated heart system and, with a single beat Frank-Starling protocol, measured the LV systolic and diastolic pressure volume relationships (PVR). We innovated on methods developed for *in vitro* muscle fiber studies to obtain PVRs in 1) permeabilized the whole mouse heart, and 2) extracted the thin and thick filament from the permeabilized heart to separate the titin-based and ECM-based LV pressures. By chemically fixing the LV at predetermined volumes and then measuring the SL of muscle strips dissected from the LV wall with laser diffraction, we constructed the LV volume-SL relation and the titin-based passive pressure-SL and ECM-based passive pressure-SL relations.

MATERIALS AND METHODS

ANIMALS

Male C57BL/6 mice were used at 15 weeks of age. All experiments were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the IACUC of the University of Arizona.

THE INTACT ISOLATED HEART

An isolated perfused mouse hearts system was utilized as previously described[16] and is detailed in the supplemental methods. After equilibrating approximately 10 minutes, the hearts were subject to repeated isovolumic Frank-Starling protocols, consisting of rapid volume changes imposed during the diastolic interval and measuring systolic pressure of the subsequent beat (interbeat interval 450ms) and diastolic pressure at the end of the following

diastole (Fig.1B). After each volume increment, the volume was returned to baseline volume. The volume changes (a ramp of 50ms duration, $\sim 0.5 \mu\text{L}$ increments) were to a volume large enough for V_{max} (volume that gives optimal developed pressure) to be determined and V_{max} was then used for volume normalization so that different hearts can be compared, while baseline volumes were near 70% V_{max} . All Frank-Starling volume protocols were generated and data acquired via a custom PC interface.

PERMEABILIZING THE HEART

To quantify the specific pressure contribution of titin and ECM the perfusate was changed to an oxygenated CaCl_2 -free Tyrode, which contained protease inhibitors ([in mM] 0.01 E64, 0.04 Leupeptin and 0.5 phenylmethylsulfonyl fluoride [PMSF]), 10mM EGTA to chelate free calcium, and 10mM BDM to prevent contraction upon permeabilization. The heart was washed for 5 minutes with this calcium free solution before being subjected for 30 min to a skinning solution (1% Triton X-100 in a pCa 9.0 relaxing solution ([in mM] 10 BES, 10 EGTA, 6.56 MgCl_2 , 5.88 Na-ATP, 1 DTT, 46.35 K-propionate, 15 creatine phosphate with 10 mg/mL bovine serum albumin (BSA)) with protease inhibitors (as above) to prevent protein degradation). The perfusate was then changed to triton-free relaxing solution with protease inhibitors and allowed to wash for 30 minutes. The exact same Frank-Starling protocols were then repeated to obtain permeabilized heart pressure-volume data.

In developing and validating the permeabilization protocol, we noted that the coronary flow during the intact constant-pressure perfusion indicates the tight-seal of the coronary tree and the potential quality of the permeabilization. Flow rates above 3.0 mL/min during the intact, paced portion of the study were typically indicative of a leak in the arterial side of the coronary perfusion, leading to poor permeabilization (Supplemental Fig.1) and these hearts were excluded from analysis.

PERFUSION PRESSURE IN THE INTACT ISOLATED HEART

We analyzed the potential effect of perfusion pressure on intact pressure-volume relationships (PVR) by using five perfusion pressures to determine a relationship between perfusion pressure and the diastolic pressure. Perfusion pressures of 90, 75, 60, 45, and 30 mmHg were initiated in random order with at least 1 minute of equilibration before Frank-Starling data was acquired for each perfusion pressure. Pressures below 30mmHg were avoided to prevent an ischemic response. Hearts were returned to 90mmHg, stabilized, and permeabilized as above. At each volume, a linear relationship was calculated between diastolic pressure and perfusion pressure and the zero perfusion pressure intercept was used to approximate the diastolic PVR at zero perfusion pressure (See Inset of Fig.3A).

MOLECULAR DISSECTION

To determine the contribution of titin to passive pressure of the permeabilized heart versus the contribution of the extracellular matrix (ECM) we used a myofilament extraction method consisting of perfusing with high potassium chloride and potassium iodide, a well established method in permeabilized muscle mechanics[6-7]. The permeabilized heart was first perfused for 90 min with 1.0M KCl in relaxing solution (flow rate of 1mL/min), to extract thick filaments. Thick filament extraction was followed by thin filament extraction by perfusing for 90 min with 0.6M KI in relaxing solution (1mL/min). The heart was then washed with an additional 30 mL of relaxing solution. Frank-Starling protocols were repeated as described for permeabilized hearts to obtain the extraction insensitive pressure, which we assumed was ECM-based. Subtracting the ECM-based pressure from the total pressure measure prior to myofilament extraction gave the extraction sensitive pressure, which we assumed was titin-based. (See Supplemental Methods/Results trypsin-based titin degradation experiments.)

ANALYSIS OF PASSIVE PRESSURE-VOLUME RELATIONSHIPS

Frank-Starling Protocol data were analyzed offline using a custom LabVIEW VI (National Instruments, Austin TX). All volumes were normalized relative to the volume at maximum developed pressure ($V_{\max}$). Because the balloon used in the study contributes modestly to pressures at the higher volumes that were used, Frank-Starling measurements were repeated on the balloons after the heart was removed and the obtained balloon characteristic was subtracted from the LV measurements. All diastolic and passive Frank-Starling data was fit to a monoexponential curve. We were unable to convert pressures to wall stress in this study because the heart weight (which is needed for the conversion from pressure to wall stress) changes during myofilament extraction. Thus we present balloon corrected PVR, not stress-strain measurements in this work.

PROTEIN QUANTIFICATION

Lateral wall segments from all experimental conditions were frozen in liquid nitrogen and stored at -80°C . Tissue samples were weighed, solubilized and electrophoresed on 2-7% SDS-PAGE gradient gels, stained with Coomassie Blue and integrated optical density (IOD) for titin (T1), titin degradation products (T2), myosin, and actin quantified as previously described [17].

SARCOMERE LENGTH (SL) MEASUREMENT IN ISOLATED HEARTS

To determine the working SL range of the LV, we used glutaraldehyde fixation and laser diffraction for SL measurement. Following the intact heart isolation, we first obtained $V_{\max}$. For passive measurements, 16 hearts were set at a given volume relative to $V_{\max}$ (60% 70%, 85% and 100% of $V_{\max}$ with 4 hearts per group) and perfusion was changed to Ca^{2+} free Tyrode and pacing was stopped. Fixation was performed by changing the perfusate to Tyrode with 2% glutaraldehyde for 30 minutes. Constant pressure perfusion was used throughout this period. Hearts were removed and post-fixed in 2% glutaraldehyde for at least 2 hrs, then subsequently washed and stored at 4°C with PBS (in mM: 2.7 KCl, 1.5 KH_2PO_4 , 137 NaCl, 8 Na_2HPO_4 , pH 7.2 at 24°C).

To examine the minimum SL in contracted hearts we used barium contractures. Barium contracture has previously been utilized in intact heart and muscle preparations in large animals to provide a stable (tetanus-like) contracted state in the heart [18-19]. In 4 hearts, volume was held at 70% of $V_{\max}$ and 4 hearts were fixed preload-free (without balloon). First perfusion was changed to Ca^{2+} free Tyrode solution. After transient beating had ceased, Tyrode solution with 0.5 mM BaCl_2 was used to cause a sustained ventricular contraction. Fixation was completed in Tyrode solution with 0.5 mM BaCl_2 and 2% glutaraldehyde and then fixed and stored as above.

Hearts in PBS solution were cut into 2mm thick equatorial rings (See Fig. 5A shows several examples). Lateral wall sections of the full wall thickness and approximately 2 mm wide along the circumference were dissected. Thin ~2mm long muscle strips that were easy to separate were carefully dissected from the midwall and epicardial and endocardial regions, for SL measurement for both passive and barium activated hearts. Fiber preparations were placed in a small chamber and SL was measured via laser diffraction with a minimum of 20 fiber measurements average per heart. The midwall SL-volume relationship was modeled using a thick-walled cylinder[14]. The model is detailed in the Supplemental Methods.

STATISTICS

Statistical analysis performed using Student t-Test in MS Excel (Microsoft Corporation, Redmond WA) with $p < 0.05$ was considered significant. All data shown as mean $\pm$ SE.

RESULTS

THE INTACT AND PERMEABILIZED MOUSE LV

The goal of this study was to determine the source(s) of diastolic pressure in the mouse left ventricle (LV). A small balloon with a pressure sensor inside was guided into the LV, providing the ability to examine the relationships between volume and systolic and diastolic pressures in the beating heart (Fig. 1A). A Frank-Starling (FS) protocol was used with volume changes imposed during the diastolic interval and measurement of systolic pressure during the subsequent beat and of diastolic pressure at the end of the following diastole (Fig. 1B). A wide range of volumes was used to determine the volume with maximal pressure (V_{max}) and then use V_{max} to normalize results from different hearts from the developed pressure (systolic pressure minus diastolic pressure). Although large diastolic pressures were generated near V_{max} , repeating protocols gave nearly identical results (thin lines in Fig. 1C indicate results from a previous FS protocol imposed on the LV), indicating that the protocol did not cause significant damage. Thus the experimental protocol made it possible to determine developed and diastolic pressures of the mouse LV at a range of volumes, including V_{max} .

In order to dissect specific molecular contributors to diastolic pressures, we permeabilized the intact heart by innovating on established methods used in isolated cardiac muscle fiber experiments. To prevent calcium-induced proteolysis of intracellular proteins during permeabilization, particularly titin (which is highly sensitive to proteolysis) hearts were first perfused with a calcium-free Tyrode perfusate that contained 10 mM EGTA and protease inhibitors (see Methods). This was followed by perfusion with skinning solution consisting of 1% Triton X100 added to relaxing solution, followed by washing with relaxing solution only (all solutions contained high concentrations of inhibitors). In previous work we and others have permeabilized ventricular wall muscle by submerging the preparations in skinning solutions with effective skinning as short as 30 minutes with rapid flow through [20] but typically requiring hours without flow through, even in small trabeculae[21], due to the slow diffusion of the skinning solution into the densely packed tissue. However, Langendorff perfusion provides fast access to all cells via the coronary circulation, allowing rapid permeabilization of the entire heart within minutes as indicated by the pale and opaque appearance due to the washout of myoglobin from the permeabilized cells (compare Figs. 2Aa and 2Ab)[22]. It is also interesting to note that perfusing the hearts with activating solution (pCa 4.5) causes activation that is rapidly reversed upon perfusion with relaxing solution (Fig. 2Ad) showing that the contractile machinery is functional. SDS-PAGE analysis of permeabilized LVs that were quick frozen showed that the permeabilization method left titin intact (Fig. 2B, lanes 1 and 2) with no statistically significant changes compared to intact LVs (Table 1, first two columns).

The permeabilized LV develops passive pressures that increase steeply as the volume increases (Fig. 3A). Thus, perfusing the coronary system of intact hearts with Triton X-100 is a highly effective method for abolishing the calcium handling system of the cardiac myocytes while controlling the ionic milieu of the myofilaments and maintaining the structure of the LV. When the permeabilized heart was perfused with relaxing solution, the measured passive pressure volume relation was shifted down to lower pressures, when compared to the diastolic pressure-volume relation of the intact LV (see example in Fig. 3A). Because the intact hearts were continuously perfused to maintain oxygenation of the tissue, whereas the permeabilized LV was not perfused during the Frank-Starling protocols (we only washed intermittently with fresh relaxing solution; continuous perfusion is prohibitively expensive), we examined if the large difference between diastolic pressures of the intact LV and permeabilized LV is due to perfusion-based coronary turgor and fluid based heart wall edema pressures [23-24]. To examine this turgor/edema, intact hearts were

perfused at the following perfusion pressures: 90, 75, 60, 45, and 30 mmHg. (Perfusion pressures lower than ~30 mmHg were avoided because they resulted in ischemic hearts.) Within the experimental conditions, the measured diastolic pressures increased linearly with perfusion pressure (see both insets of Fig.3A). By extrapolating the obtained linear relationship to zero perfusion pressure at each volume, we estimated the passive pressure that would be measured with zero pressure perfusion (Fig.3A). The estimated diastolic pressures with zero perfusion were similar to permeabilized passive pressure values (Fig. 3B). One might also consider myofilament swelling that occurs with skinning [25]. However, we had no indication that the heart itself swelled (consistent with [26]), which is potentially due to the fact that myofilament swelling is compensated by a reduced volume fraction of the mitochondria [27]. Thus, it is likely that in constant pressure perfused isolated hearts, turgor or edema accounts for much of the diastolic pressures above that in permeabilized hearts (see also Discussion).

TITIN-BASED CONTRIBUTION TO PASSIVE PRESSURE OF MOUSE LV—To determine the role of titin and the extracellular matrix (ECM) in passive pressure generation of the mouse LV, we adapted a method that has previously been used to determine the contribution of titin to passive stiffness of permeabilized cardiac muscle: myofilament extraction via perfusion with 0.6M KCl and 1.0M KI (added to the relaxing solution) to remove titin's anchors in the sarcomere (see Methods for details). The KCl/KI-based myofilament extraction protocol provides a significant loss of proteins causing a slightly transparent appearance of the heart (Fig.2Ac) without any change in the shape of the heart, consistent with previous work that showed that the extraction protocol does not affect the extracellular matrix (ECM) [7]. Protein quantification shows that more than 90% of myosin and actin that anchor titin is lost and that nearly 60% of titin itself is washed out (Fig.2B lane 3; Table 1). Myofilament extraction drastically reduces the passive pressure (Fig.4A) leading to an average 86% reduction in passive pressure from 80 to 100% of V_{max} volumes (Fig.4B). This suggests that at these volumes the ECM provides approximately 14% of the passive tension in the heart and that titin contributes 86% of the passive pressure in the heart. To verify these results with an independent method, a trypsin-based proteolysis protocol was used for abolishing titin-based passive pressure. A mild treatment with low concentrations of trypsin has previously been shown to proteolyse titin while keeping the other muscle proteins intact (titin's high proteolytic sensitivity is caused by its large size, the open confirmation of titin spring elements, and abundance of lysine residues [28]). We evaluated the time-dependence of trypsin treatment and found that 6 minutes provides a significant level of titin degradation, without any noticeable degradation of myosin or other proteins and that under those conditions passive pressure is estimated to be reduced by 74% (Supplemental Figs.2 and 3), supporting our results based on myofilament extraction. Thus within the volume range that was used in the present study (60-100% V_{max}) titin is likely to be responsible for the majority of the passive pressure.

SARCOMERE LENGTHS (SLs) IN THE ISOLATED MOUSE LV

In order to relate the results of the isolated heart to the known SL dependence of titin-based and ECM-based passive tension measured in muscle strips we determined the volume-SL relationship of the passive LV, utilizing tissue fixation and laser diffraction to measure SLs. Similar to the rapid triton-based permeabilization, coronary perfusion is an efficient method of distributing a 2% glutaraldehyde fixative solution to all cells in the LV wall. We first determined the developed pressure-volume relation of the LV and then the volume was set at a predetermined fraction of V_{max} , followed by rapid switching to a perfusate that contained 2% glutaraldehyde. Fixation appears to hold hearts at their preloaded volumes (Fig.5A) and muscle fibers can readily be dissected from the LV walls of these fixed hearts. Circumferentially oriented mid-wall fibers were carefully dissected and SL was measured

via laser diffraction. Mid-wall fibers dissected from passively fixed hearts had SLs of $1.96 \pm 0.08 \mu\text{m}$ at 60% V_{max} , $2.04 \pm 0.04 \mu\text{m}$ at 70% V_{max} , $2.07 \pm 0.05 \mu\text{m}$ at 85% V_{max} and reached $2.16 \pm 0.13 \mu\text{m}$ at V_{max} (Fig.5B). We also measured the SL in endocardial and epicardial fibers (Fig.5B). This data indicates that the SL range is smaller in the epicardium (2.01 ± 0.10 - $2.15 \pm 0.05 \mu\text{m}$ from 60% V_{max} to V_{max}) and larger in the endocardium (1.87 ± 0.06 - $2.09 \pm 0.04 \mu\text{m}$ from 60% V_{max} to V_{max}), a result consistent with previously reported transmural SL variation [29]. To identify the working SL range, we also determined SL in the contracted (activated) heart utilizing a barium chloride contraction; at 70% V_{max} , we obtained a midwall SL of $2.04 \pm 0.01 \mu\text{m}$ and $1.80 \pm 0.01 \mu\text{m}$ with no balloon preload. Epicardial SLs measured 2.08 ± 0.01 and $1.86 \pm 0.01 \mu\text{m}$ while endocardial SLs measured $1.98 \pm 0.02 \mu\text{m}$ and $1.73 \pm 0.02 \mu\text{m}$ at 70% V_{max} and no balloon preload, respectively.

We modeled the SL-volume relationship using a thick-walled cylindrical model. The SL at the mid-volume range (70% V_{max}) was set $2.03 \mu\text{m}$ to produce maximum overlap with our data. The model derived SL-volume relation shows good overlap with the measured SL values (Fig.5B, curve in gray). The SL-volume relationship was used to develop pressure-SL relationships of the LV before and after myofilament extraction (Fig. 5C). The obtained diastolic/passive pressure-SL relationships are nearly exponential within the 60-100% volume range, corresponding to a ~ 2.00 - $2.15 \mu\text{m}$ SL range. In a subset of hearts, we increased volumes to beyond V_{max} (Fig.6A) and although SLs were not measured in these hearts we used the model fit shown in Fig.5A to convert volumes to SLs (see also Supplemental Fig.5) and from this established the ECM- and titin-based pressure-SL relations. Findings indicate that the ECM-based PVR increases steeply at SLs $> \sim 2.15 \mu\text{m}$ and exceeds titin-based passive pressure at SLs larger than $\sim 2.2 \mu\text{m}$ (Fig.6B), a conclusion consistent with that measured on permeabilized muscle [7].

DISCUSSION

We determined the contributions of titin and the ECM to diastolic pressure in the mouse by performing experiments at the level of the whole LV. Although the passive tension contributions of titin and the ECM have been addressed in permeabilized muscle, including their SL dependencies, it is uncertain how properties measured at the level of the permeabilized muscle extrapolated to the level of the whole heart. This is due in large part to the complex fiber arrangements in the LV wall [19,29-30], and the unknown SL range along which the LV operates. In this work, we overcame these issues by measuring the contribution of the ECM and titin to the diastolic properties of the mouse LV and by measuring the working SL range of the LV. The obtained results show that both ECM and titin contribute to LV pressure, that titin is the dominant partner at physiological filling volumes, and that the ECM dominates at larger volumes.

PERMEABILIZATION OF THE ISOLATED HEART

While permeabilizing muscle preparations is slow, often taking hours for papillary muscles or trabeculae, the Langedorff perfusion of the heart provides direct access to all cells via the coronary tree. We found that whole hearts are rapidly permeabilized in less than 30 minutes, with no statistically significant change in myofilament protein content. Comparing the diastolic pressure-volume relation before and after permeabilization revealed a higher pressure in the intact heart (Fig. 3A). Because this higher pressure scales with perfusion pressure, we consider it likely that it is due to a turgor effect of the coronary vasculature at high coronary flows and due to fluid-buildup that is well known to occur in crystalloid perfused hearts [23,31]). This conclusion is consistent with the work of others that has shown the presence of turgor/edema in various species [23-24,31]. Because we noted that the heart becomes ischemic at perfusion pressures less than ~ 30 mmHg, we extrapolated the measured linear relationship between diastolic pressure and perfusion pressure to zero

perfusion pressure (inset of Fig. 3A). This results in a predicted turgor-free diastolic pressure that is close to that of the permeabilized LV (Fig. 3B). Due to the large variation in the data (likely caused by the linear extrapolation below the measured perfusion pressure range), we are unable to exclude alternate causes of small differences between turgor-free predicted PVR and permeabilized PVR, for example, due to a low level of actomyosin interaction [32]. However, it is clear that the largest difference in diastolic pressure between the intact and permeabilized LV is due to a perfusion induced turgor/edema effect. In summary, Langendorff perfusion with 1% Triton X-100 solution provides a stable, high quality permeabilized LV with functional myofilaments and with pressure-volume characteristics similar to that of the turgor/edema-free intact LV.

CONTRIBUTION OF TITIN AND ECM TO PASSIVE PRESSURE

It is well established that passive myocardial tension is derived from both cardiac myocytes (mainly titin) and the ECM (mainly collagen) but their relative importance to pressure generation at the level of the whole heart has not been studied. Here we adapted the myofilament extraction technique developed to eliminate titin-based tension from permeabilized myocardial muscle[7] to abolish titin as a source of passive pressure in the permeabilized LV. Following extraction, the titin protein level is reduced to ~30% and actin (thin filament) and myosin (thick filament), both of which function as anchors for titin in the sarcomere, to ~10% of control levels. Thus titin-based pressure is effectively abolished by the extraction technique. The ensuing reduction in pressure at low volumes was somewhat variable especially when baseline pressures were low, but the mean reduction of 84% at volumes 0.8-1.0 V_{max} identified titin as a main contributor to pressure of the passive LV. To verify these results with an independent method, we also used a titin proteolysis protocol, which had previously been shown to abolish titin-based passive tension without affecting the ECM[7]. Perfusing the hearts for 6 min did not cause a noticeable degradation of myosin or other proteins, but it resulted in a 64% degradation of titin. Following titin proteolysis, passive pressures were reduced by an average of 45%, and after correcting for the remaining titin, 74% of the pressure is eliminated (Supplemental Fig.4). Although this is slightly less than what was found with the myofilament extraction method (a possible explanation is the presence of cleavage products of titin that remain attached at one end to the Z-disk/A-band and that at their other end interact with actin and that bear force in extended sarcomeres[6]), overall the results of the two studies support the conclusion that titin is responsible for the majority of the pressure of the passive LV.

To provide a bridge from LV-based to muscle fiber-based findings, we measured the SL range of the working heart and showed that increasing LV volume from 60-100% of V_{max} result in only a 10% increase of SL, in the midwall region from 1.96 μ m to 2.16 μ m (Fig.5). It is possible that these values are slightly underestimated due to tissue shrinkage during fixation. However, such shrinkage is likely to have been small because hearts were isovolumic while fixed with glutaraldehyde and were never dehydrated, conditions that have previously been shown to only lead to shrinkage of at most 1-2% [33] and, furthermore, no rise in pressures were observed in our experiments during fixation. Taking the possibility of a few percent shrinkage into account provides an upper SL estimate of 2.2 μ m at V_{max}. Modeling our diastolic SL-volume relationship using a simple thick-walled cylinder (see Methods) results in a good fit of the measured SL-volume relation (Fig.5A) and this fit was used to convert pressure-volume relations into pressure-SL relation (Fig.5B). Considering the high passive pressure at V_{max} (~20 mmHg) and the steepness of the pressure-SL relation beyond V_{max} we consider it likely that V_{max} is an upper limit that may be reached only under conditions in which the heart is maximally filled. Under more normal baseline conditions the physiological end-diastolic volume is likely to be less than V_{max} because normal physiological end-diastolic pressures are typically <5 mmHg [34]. These lower

pressures are reached at ~85% Vmax, or a SL of ~2.1 μm . Considering that the LV ejection fraction is typically greater than 60%, 35% Vmax is a reasonable estimate of the end systolic volume. At this volume the predicted SL (Supplemental Fig.5) is ~1.84 μm , a value that is close to the value measured in the unloaded barium contracted LV. Thus we consider it likely that the maximal physiological SL range in the mouse LV is from ~1.8 μm to ~2.2 μm . This estimate compares favorably to the only other mouse SL range that has been published. Using whole animal X-ray diffraction techniques Toh et al found a diastolic SL range of 1.9 to 2.1 μm for the *in situ* beating mouse heart [35]. Considering that in this study mice were anesthetized, which depresses contractility, it seems reasonable that the SL range of unanesthetized mice that are maximally filled might be slightly larger with a limit of ~1.8 to 2.2 μm . It is interesting to note that the mechanical slack length of mouse intact myocytes was recently determined at ~1.95 μm [32] and, thus, during a normal cardiac cycle the sarcomeres oscillate around the slack length with titin generating restoring force during systole (SL<1.95 μm) and passive force during diastole (SL>1.95 μm) .

The SL-volume relation was used to convert pressure-volume relations into pressure-SL relations, for the permeabilized heart, the myofilament extracted heart (ECM), and extraction sensitive (titin) pressures. This reveals that within the SL range of 1.95 to 2.15 μm titin is the main contributor to passive pressure (Fig.5C). This is consistent with previous work on permeabilized mouse myocardium that also showed that within this SL range titin is the most dominant contributor to passive muscle tension [6-8]. This finding is also supported by studies in which isolated rat hearts were perfused with collagenase to disrupt collagen fibrils, and in which it was found that the relation between passive pressure and wall strain (measured with piezoelectric crystals) was not significantly altered [36]. Furthermore, our findings suggest that collagen does not dominate until non-physiologic volumes are obtained (Fig.6). Thus our work shows that, in normal healthy mouse hearts, titin has the major impact and only at non physiologic volumes does collagen dominate the passive pressure generation.

Considering that titin is a primary source of passive pressure at physiological filling volumes, short-term changes in titin's compliance that are known to occur via phosphorylation of titin are expected to alter diastolic characteristics. Titin's spring region consists of three sequence types (tandem Ig elements, N2B element and the PEVK element), of which the N2B and PEVK elements can be phosphorylated—PKA [37-38] and PKG [39] phosphorylation of the N2B element lowers stiffness and PKC phosphorylation of the PEVK region increases passive stiffness [40-41]. Recent work by the Paulus group [42] has provided evidence that hypophosphorylation of titin contributes to the increased myocardial stiffness of patients with HFpEF (heart failure with preserved ejection fraction, also known as diastolic heart failure). In addition to rapid changes in post-translational modification, more long-term adaptations in titin arise from alternative splicing that give rise to two main classes of cardiac titin isoforms, the N2B and N2BA isoforms with the latter being more compliant [43-44]. These two isoforms are coexpressed at the level of the half sarcomere [45]. In chronic disease states the isoform expression ratio is known to change with increases in N2BA expression in human ischemic heart disease [46] and dilated cardiomyopathy [47-48], in pressure-overload induced hypertrophy in the mouse [BD Hudson, et al, unpublished data] and in long-term hypothyroidism in the rat [49]. Thus changes in diastolic pressure are expected from changes in titin phosphorylation and the titin isoform expression ratio that occurs in heart disease.

Composed predominantly of type I and III fibrillar collagens, the ECM has traditionally been seen as the main determinant of myocardial diastolic stiffness [2-4]. However, this view is founded to a large extent on work performed prior to the discovery of titin and its importance to diastolic stiffness. The present work suggests that the ECM is not the main

determinant of diastolic pressure at physiological volumes but that it dominates at larger volumes. However, this does not make the ECM less important. ECM remodeling is well known to occur in a wide range of heart diseases with changes in collagen location and magnitude, fiber crosslinking, and relative abundance of type I and III collagens [2]. The present work reveals that understanding the molecular changes that underlie altered filling pressure requires detailed knowledge of both ECM and titin, for which the organ level approach developed in this work will be invaluable.

Supplementary Material

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Figure 1.

Measurement of Frank-Starling mechanism in mouse isolated heart. A) Schematic of isolated heart setup. A volume-controlled balloon is inserted into the left ventricle of a Langendorff perfused isolated heart; pressure is measured with a sensor inserted in the balloon. The heart is activated via two platinum electrodes at a beat-to-beat interval of 300ms. B) Example of single beat Frank-Starling protocol. The volume is changed in between two beats and systolic pressure is measured at the peak of the subsequent beat and diastolic pressures at the end of the subsequent diastolic phase. C) Example pressure-volume plot. Diastolic pressures (thick black) are fit via a monoexponential curve. Developed pressures (thick blue) are derived by the difference of systolic (thick red) and diastolic pressures. Volumes normalized to the volume with peak developed pressure (V_{max}). Thin lines show fit to measurements taken earlier in the experiment and the similarity of the two data sets indicate that the protocol does not induce major damage.

Figure 2.

Visual appearance of heart (A) and protein composition of LV wall (B). A) Example images of intact (a), permeabilized (b), and myofilament extracted (c) hearts. Permeabilization removes the red color from the heart, due to washout of myoglobin, but the heart remains opaque with a whitish color (b) and hearts rapidly activate using a pCa 4.5 solution and return to low pressure when washed with relaxing solution (Rx) (d). Myofilament extraction results in a translucent/transparent appearance (c) presumably due to extraction of myofilament proteins. B) 2-7% SDS-PAGE of hearts in the three experimental conditions. Permeabilized hearts have little change compared to intact heart samples except for loss of low molecular weight soluble proteins. Myofilament extraction shows a large loss of myosin and actin along with significant degradation and loss of titin.

Figure 3.

Effect of perfusion pressure on the passive pressure volume relationship (PVR). A) Example diastolic pressure-volume curves from a single heart perfused at 5 different perfusion pressures: (black to gray 90, 75, 60, 45, 30 mmHg). Estimating the resultant pressure at zero perfusion pressure (two examples in insets) creates a PVR curve (red) that is close to the PVR of the permeabilized heart (blue). B) The ratio between the estimated zero perfusion pressure and the permeabilized pressure ($n=6$, data shown as mean $\pm$ SE). (See text for details)

Figure 4.

Effect of myofilament extraction on passive pressure-volume relationship (PVR) of permeabilized LV. A) PVR of permeabilized hearts (light gray line), after myofilament extraction (broken line) showing extraction insensitive pressure (attributed to the ECM), and the extraction sensitive pressure PVR (dark line, attributed to titin). B) Percentage of myofilament based pressure (gray line is 86%). ($n=6$, data shown as mean $\pm$ SE)

Figure 5.

Sarcomere lengths (SL) in the isolated heart. A) Cross-sectional images of hearts fixed with glutaraldehyde. Top: Hearts fixed in passive (calcium-free) solutions at 60 and 70% of Vmax volume. Bottom: Hearts fixed under barium contracture with no preload and at 70% of Vmax volume. B) SL measured in fixed hearts at a range of volumes. Gray line indicates a model based estimate of midwall SL as a function of volume (see Methods for details). C) Pressure-SL relationship (same pressure data as Fig.4A with volumes converted to SL as per relationship shown in 5A). Data shown as mean $\pm$ SE.

Figure 6.

Example pressure volume relationship with volumes that exceed V_{max} . A) Pressure-volume relationships for intact, permeabilized, myofilament extraction insensitive (ECM) and calculated extraction insensitive (titin) pressures in a sample heart. Note the crossover of titin and ECM at $\sim 1.25 V_{max}$ (normalized volume). B) The pressure-SL relationship of the midwall indicates that ECM-based pressure exceeds that of titin at large volumes with SLs $> \sim 2.2 \mu m$.

Table 1

Analysis of SDS-PAGE Data of intact, permeabilized and myofilament extracted mouse LV.

IOD per mg	Intact n=4		Permeabilized n=3		Extracted n=6	
	IOD or Ratio	IOD or Ratio	IOD or Ratio	% of CTRL	IOD or Ratio	% of CTRL
T1	5.9±0.6	5.7±1.0	96±18%	1.9±0.4 [§]	32±7%	
T2	1.0±0.1	1.4±0.3	140±31%	2.3±0.6	222±58%	
MHC	42.6±2.3	38.3±6.3	90±15%	1.8±0.7 [§]	4±2%	
Actin	19.9±1.8	18.0±1.4	90±7%	1.3±0.4 [§]	6±2%	
IOD Ratios						
T2:T1	0.18±0.01	0.25±0.03 [#]	144±14%	1.44±0.32 [#]	823±186%	
Total Titin:MHC	0.17±0.02	0.19±0.02	109±10%	4.70±2.40 [†]	2500±1300% [†]	

IOD: integrated optical density; T1: intact titin; T2: titin degradation product(s); CTRL: control value

All data mean ± SE.

[†] two hearts had zero MHC and were not included in ratio calculations

[#] p<0.05

[§] p<0.01.