Extensible Behavior of Titin in the Miniswine Left Ventricle

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Background—The sarcomeric protein titin is a molecular spring responsible for passive tension and restoring forces of cardiomyocytes. Extension of titin as a function of sarcomere length (SL) has been studied in rodents, which predominantly express the smaller, stiffer N2B titin isoform. Large mammals coexpress roughly equal proportions of N2B and N2BA titin, the larger, more compliant isoform. We hypothesized that extension of titin in relation to SL differs in large mammals and that this difference is functionally important.

Methods and Results—We characterized the filling pressure–SL relation in diastolic-arrested miniswine left ventricles. SL was 2.15 to 2.25 μm at a filling pressure of ~0 mm Hg and reached a maximum of ~2.50 μm with overfilling. In the normal filling pressure range, SL ranged from ~2.32 to ~2.40 μm. We assessed titin extension as a function of SL using immunoelectron microscopy, which allowed delineation of the behavior of specific spring segments. The major isoform difference was that the N2B-Us segment extended ~4-fold more as a function of SL in N2B compared with N2BA titin. Using this segment, we estimated sarcomeric force development with a worm-like chain model and found that N2B develops markedly greater force than N2BA titin. The resulting force with coexpression of N2B and N2BA titin is intermediate.

Conclusions—In light of murine studies showing that operating SLs are shorter than in miniswine, our results indicate that coexpression of the 2 titin isoforms in large mammals allows longer SLs without the development of excessive diastolic tension. (Circulation. 2010;121:768-774.)

Key Words: diastole ▪ myocardium ▪ titin ▪ ventricles

The giant sarcomeric protein titin is anchored in the Z disk and bound to the thick filament.1 Its I-band region contains a molecular spring consisting of PEVK, Ig repeat, and N2B and N2A elements. In slack cardiomyocytes, the spring corresponds to a flexible chain with a mean square end-to-end distance much below its contour length.2 With lengthening, spring elements extend and passive tension develops.3,4 Across the physiological sarcomere length (SL) range, titin is the predominant source of cardiomyocyte passive tension. In the normal left ventricle (LV), it contributes at least as much as collagen to passive myocardial stiffness.5,6 At systolic SLs below slack, titin is an important source of the restoring force resulting in cardiomyocyte elastic recoil.7

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In rodent LV, immunoelectron microscopic studies reveal that with sarcomere extension the spring elements of titin lengthen differentially, with each dominating distinct portions of the cardiomyocyte length-tension relation.3,4 N2B titin, which contains the N2B but not the N2A element, predominates in rodent LV.8 In contrast, large mammals, including humans, express mixtures of the N2BA isoform, containing both N2B and N2A elements, and the N2B isoform.8–11 Both isoforms are coexpressed within the sarcomere.12 Because N2B titin is much stiffer than N2BA titin, the passive stiffness of rodent cardiomyocytes, myocardium, and LV exceeds that of large mammals.8,9 Titin isoform switching (increased N2BA:N2B ratio) is observed in patients with dilated cardiomyopathy.10,11 Moreover, considering the importance of clinical diastolic dysfunction,13 detailed information about how titin determines diastolic stiffness in large mammalian LVs is important to our understanding of heart failure.

In view of these isoform and stiffness differences, we hypothesized that SL range and titin behavior differ in large mammalian LVs compared with predominantly N2B-expressing species and that these differences serve a useful function. Accordingly, in the present study, we determined relations between transmural filling pressure (FP) and normalized diastolic segment length and SL in the intact miniswine LV arrested in diastole. Because turgor influences the LV FP-volume relation,14 experiments were performed with and without a physiological level of coronary perfusion.
pressure. We then used immunoelectron microscopy to characterize extension of the spring elements of titin and related them to SL and LV filling.

Methods

LV FP-SL Relations

Protocol

Eleven male Yorkshire miniswine with an average weight of 28.4 kg (range, 26 to 33 kg) were used to delineate the LV transmural FP-SL relation across a range between ~0 mm Hg and markedly overdamped conditions. They were sedated with ketamine (20 mg/kg IM), anesthetized with isoflurane, and endotracheally intubated. Anesthesia was maintained with 2% to 3% isoflurane. A large-animal respirator was used for ventilation with 100% supplemental oxygen. A midline sternotomy was performed, the pericardium was incised, and the heart was suspended in a pericardial cradle. Ascending aortic pressure was measured through a fluid-filled catheter inserted from a femoral artery. A 14F, fluid-filled, stiff plastic cannula with multiple side holes was inserted in the LV through the apex and secured with a pursestring suture. LV pressure was measured through the cannula with a Becton Dickinson DTX Plus (DT-4812) transducer zeroed at the middle of the right atrium. A pair of ultrasonic crystals (1.0-mm diameter) was then inserted ~1 cm apart to track the motion of a circumferentially oriented midwall segment in the LV anterior free wall at the level of its minor axis. Midwall fibers at this site are oriented circumferentially in multiple species. After completion of the experiments, we confirmed that the crystals were midwall. Finally, inflatable cuffs were positioned around each caval vessel.

Once instrumentation was complete, warmed saline was administered intravenously as necessary to raise LV end-diastolic pressure (EDP) to the normal range (5 to 13 mm Hg). Ventilation was then suspended at end expiration, steady-state measurements of LV pressure and segment length were recorded for several beats, and a bivacual occlusion was performed. The occlusion was maintained until LVEDP reached a minimum value. Heparin sulfate 10 000 U IV was then administered; the azygous vein was ligated; and snares were positioned around the pulmonary hila and veins, caval vessels, and the aorta just above the aortic valve. The ultrasonic crystals were left in place. The heart was then arrested in diastole with an aortic root injection of 35 mL of 20% KCL with 3 mmol/L butanedioxone monoxime and all snares secured to isolate the heart from the rest of the circulation. A plastic cannula was inserted into the free wall of the right ventricle, which was vented to atmosphere. The isolated left heart was drained of blood by applying negative pressure through the LV cannula.

We then filled the LV by reinfusing warmed (37°C), heparinized blood to a predetermined level of pressure encompassing a range from ~0 to ~50 mm Hg. In 5 of the 11 experiments, coronary perfusion pressure was not varied after arrest. Aortic pressure was in the 5- to 15-mm Hg range under these conditions. In the other 6 experiments, coronary perfusion pressure was manipulated to a physiological level. To accomplish this, after the arrested LV was filled to the desired pressure, saline (37°C) was infused continuously into the aortic root below the snare at pressures ranging from 75 to 90 mm Hg. Consistent with turtor,14 pressurizing the coronary circulation invariably raised the LV pressure, which was then lowered to the desired level by removal of blood. The increase in pressure with coronary pressurization was dependent on the starting LV pressure. It was 1 to 2 mm Hg when the initial pressure was 0 or near 0 mm Hg to >10 mm Hg in the heart with the highest pressure (~50 mm Hg).

Once the desired FP was reached, the heart was rapidly fixed by injection of 100 mL of 6.25% glutaraldehyde in phosphate buffer (pH 7.4) at 40 mL/s into the aortic root with a power injector during continuous recording with a General Electric OEC900 digital fluoroscope. The glutaraldehyde injectate contained a small volume of radiopaque ultrasonic crystals, we determined that fixation did not change the distance between them. The ultrasonic crystal signal was monitored after cardiac arrest until the moment of fixation. Fixation resulted in an abrupt, upward shift of the signal. Because the directly visualized distance between the crystals did not change, it is likely that slowing the velocity of ultrasound by fixation caused this effect. After fixation, an additional 50 to 100 mL glutaraldehyde was flushed through the coronary circulation. The LV cannula and ultrasonic crystals were removed, and the fixed heart was removed from the chest and immersed in 6.25% glutaraldehyde in phosphate buffer for ~24 hours at room temperature.

To measure SL at the smallest possible LV volume, an additional experiment was performed without coronary pressurization. The LV was emptied by suction after cardiac arrest, the proximal left anterior descending coronary artery was cannulated, and the anterior wall was selectively fixed by injection of glutaraldehyde directly into the vessel. We selectively fixed the anterior wall because in preliminary attempts we found that the aortic valve was incompetent during glutaraldehyde injection into the aortic root when the LV was fully collapsed. This heart was also immersed in glutaraldehyde overnight.

SL Measurement

The next day, transmural sections (~1 cm²) were removed from the fixed LV at the site where the crystals were inserted and washed in PBS. Thin strips ~2 mm long were dissected from the middle 1 to 2 mm of the wall, taking care to avoid staining the tissue and using naturally occurring bundles of cardiomyocytes oriented in the same direction that were easy to separate from adjacent tissue. The strips were placed in a chamber with a glass bottom and top, and the average SL was measured by laser diffraction (~15 strips per heart).21 Thus, each heart yielded 1 SL-LVP data point. Variation in SL among the strips from each heart was ~10 nm.

Data Analysis

SLs and associated FPs were fitted to a monoeponential function for conditions with and without coronary perfusion. In addition, the relation between normalized diastolic segment length change and SL was estimated. For this analysis, we used LVEDP–segment length data obtained during bivacual occlusion as follows. During bivacual occlusion, minimum EDP usually reached values near 0 mm Hg; however, in 2 instances, the minimum value was in the 1- to 2-mm Hg range. Normalized diastolic segment length change for each heart was calculated as the segment length present just before fixation normalized to the value closest to 2 mm Hg during bivacual occlusion (thus, values with EDP <2 mm Hg were <1). In 2 hearts, values could not be calculated because crystal tracking was lost at low LVEDPs.

In addition to SL measured in the arrested heart, we calculated minimum systolic SLs during bivacual occlusion. To accomplish this, we matched the segment length present just before fixation to the SL measured in the midwall after fixation at the site of crystal placement. We assumed a linear relation between segment length and SL to calculate minimum systolic SL. As with normalized segment lengths, 2 hearts could not be used because of loss of crystal tracking.

Immuonelectron Microscopic Determination of Titin Extension

Because fixation precluded immunoelectron microscopic measurements, we used 4 additional KCl-arrested, unfixed hearts to dissect midwall strips (~10-mm length×1-mm diameter; ~10 strips per heart) at the same site where crystals were inserted in the previous experiments. Strips were placed in relaxing solution with Triton for 8 hours, washed with relaxing solution, and then stretched at ~0.1 µm per sarcomere to different lengths, where they were held, fixed in 3% formaldehyde/PBS solution, and washed and blocked in 1% BSA/PBS solution.15 They were then immunolabeled, embedded, and processed for electron microscopy.12 Antibodies were obtained from Eurogentec (Brussels, Belgium) and Biogenes (Berlin, Germany) and affinity purified. Titin monoclonal antibody T12 was purchased from Boehringer-Mannheim (Indianapolis, Ind). After
Figure 1. Top, FP-SL relations in arrested LV with and without physiological coronary perfusion pressure (CP). Bottom, Normalized diastolic segment length change–SL relations. See text for details. LVP indicates LV pressure.

dilation of antibodies to appropriate concentrations (typically ∼50 µg/mL), samples were first labeled with primary and then secondary antibodies for 48 hours. Tissue was then fixed in 3% glutaraldehyde/0.2% tannic acid and 1% osmium-tetroxide solutions and embedded in araldite. Samples were dehydrated in graded series of ethanol, with final rinses in 100% propylene oxide before infiltration and embedding. Sections were cut with a Leica microtome (Leica Microsystems, Wetzlar, Germany) and stained with 2% potassium permanganate and then 0.25% lead citrate. Images were obtained with a JEOL 1200 microscope (JEOL Ltd., Tokyo, Japan). Distances from the Z line to epitope were measured from negatives after high-resolution scanning (UMAX, UC-1260, UMAX Technologies, Taiwan) and digital image processing with custom-written macros for the NIH Image analysis program (version 1.6, Wayne Rasband, National Institutes of Health, Bethesda, Md). This entire procedure yielded ∼40 data points (10 strips from 4 hearts) at varying SLs per epitope. For additional details, see the article by Trombitas et al.12

Statistics
Data are reported as mean±SD. To test for slope and intercept differences between the FP-SL relations with and without physiological coronary perfusion pressure, a natural logarithm transformation of the SL data was performed to linearize pressure versus SL data on the basis of their hypothesized exponential relationship. Transformed data were subjected to a general linear model analysis to examine the relationship of log-normal SL with pressure and to test the equality of slopes between groups with high and low coronary perfusion pressure using a group-by-pressure statistical interaction term. A subsequent nested model was used to test for equality of intercepts between the groups after accepting the equality of the slopes.22

Results
LV FP-SL Relations
Relations between FP and anterior midwall SLs determined in the fixed LV are depicted in Figure 1 (top) for studies with (high coronary pressurization) and without (low coronary pressurization) coronary pressurization, along with fitted relations for each condition. A curvilinear relation is evident with and without coronary pressurization. The fitted relation appears shifted upward and to the left with coronary pressurization at FPs >4 to 5 mm Hg, again consistent with turgor. Although in each case LV pressure rose with coronary pressurization at constant LV volume, neither the slope nor the intercept of the curves was significantly different at P<0.05.

At FPs of ∼0 mm Hg, anterior midwall SL was in the 2.15- to 2.25-µm range. Because the slope of the relation is so flat across this range, it is difficult to be precise about SL at 0 mm Hg. In the completely collapsed LV, SL was 1.99 µm. Thus, the true SL at 0 mm Hg must be greater than this value. At FPs near the lower limit of normal (∼5 mm Hg), anterior midwall SL was in the 2.30- to 2.35-µm range. At the upper end of the normal range (12 to 13 mm Hg), on the basis of coronary pressurization data, SL was in the 2.45-µm range. At pressures greater than the normal range, SLs increased to a maximum of ∼2.50 µm. The relation between normalized diastolic segment length change and SL is shown in the bottom of Figure 1. This relation was linear and, in contrast to FP-SL relations, did not appear altered by coronary pressurization.

Typical recordings of LV pressure and segment length during bicaval occlusion are shown in Figure 2 (segment length is converted to SL, as described earlier). Baseline minimum systolic SL (the first 3 beats) was 1.90 to 1.95 µm. For the entire group, this value was 1.96±0.16 µm. During bicaval occlusion, minimum systolic SL progressively declined. The arrow indicates the first beat with estimated SL <1.85 µm; EDP was ∼5 mm Hg. The shortest SL in this example was ∼1.65 µm when EDP was ∼1 mm Hg. The lowest EDP during bicaval occlusion averaged 0.8±1.2 mm Hg for the entire group. Minimum systolic SL at this EDP was 1.68±0.14 µm. In all but 1 experiment, minimum systolic SL at the lowest EDP was <1.85 to 1.90 µm. By interpolating between data points for the 8 experiments in which SL was <1.85 µm, we estimate that an SL of 1.85 µm was reached at EDPs ranging from 3 to 7 mm Hg during bicaval occlusion.

Titin Extension by Immunoelectron Microscopy
To delineate the extensibility of the spring elements of titin, we stretched skinned strips to various SLs encompassing the
range determined in the FP-SL experiments and then immu-
nolabeled with antibodies to various epitopes, as shown in
Figure 3 (left). Examples of labeled sarcomeres are also
shown in Figure 3 (right). Each antibody was detected in a
single epitope per half-sarcomere except Uc, which is di-
rected toward an epitope C terminal of the N2B unique
sequence and labels 2 epitopes per half-sarcomere. We
previously showed that the N2BA isoform is labeled closer
to the Z disk (as a result of extra extensibility provided by
its middle tandem Ig and longer PEVK), whereas the N2B
isoform labels closer to the A band.12 The distance from
the epitope to mid Z disk is plotted as a function of SL in Figure 4
(top; segments demarcated by the epitopes shown in Figure 3,
left). End-to-end lengths of the N2B-unique sequence (N2B-Us)
are shown in Figure 4 (bottom). The lines of the linear fits
indicate that N2B-Us extends \( \times 4 \)-fold more as a function of SL
in the N2B than in the N2BA isoform.

Discussion

SLs and FP

In the present study, we first delineated the relation between
transmural FP and SL in the anterior midwall of the in situ,
diastolic-arrested LV. At a transmural FP of \( \approx 0 \) mm Hg, SLs
were 2.15 to 2.25 \( \mu m \). Because the FP-SL relation is so flat
around 0 mm Hg, we cannot be more precise about this value,
but the SL of 1.99 \( \mu m \) in the collapsed LV establishes an
approximate lower limit. In the closed-chest state, the normal
LV intracavitary FP is between \( \approx 5 \) and 12 to 13 mm Hg. SLs
were \( \approx 2.30 \) to \( \approx 2.45 \mu m \) for this range of FP; however, these
values for the normal intracavitary FP range slightly overes-
timate transmural FP because they do not include the external
pressure imposed on the LV by the pericardium. Under
completely physiological conditions with the pericardium
intact, at any intracavitary FP, SL would be lower than that
measured under the conditions of our experiments. We
previously found that the intact pericardium accounts for
\( \approx 2 \) mm Hg at a measured LV intracavitary pressure of \( \approx 12 \)
to 13 mm Hg.23 Thus, in our experiments, the upper limit of
normal SL is best approximated by that present under
conditions of coronary pressurization at a transmural pressure
of \( \approx 10 \) mm Hg or, as shown in Figure 1 (top), slightly
\( >2.40 \mu m \). At the lower end of the normal range, the

Figure 3. Left, Domain structure of extensible I-band region of cardiac N2B (top) and N2BA (bottom) isoforms. Red indicates Ig
domains; blue, unique sequences; yellow, PEVK sequence; and white, fibronectin domains. Antibodies used were raised against
epitopes indicated by the horizontal lines. T12 is directed to I2/I3; UN, to I24/I25; UC, to I26/I27; I84, to I84–I86; and MIR, to I109–
I111. These antibodies mark the proximal and distal tandem Ig segments, the N2B unique sequence (N2B-Us), PEVK (N2B isoform),
and PEVK plus middle tandem Ig (N2BA isoform). Right, Examples of sarcomeres labeled with anti-titin antibodies. All sarcomeres are
labeled with MIR (except for bottom micrograph of iii) and 1 additional antibody. Note from iii that Uc labels 2 epitopes, best seen in
the bottom micrograph. This has been studied in other species that coexpress titin isoforms and is due to the extra extensibility of the
middle tandem Ig and PEVK of N2BA titin. As a result, the UC epitope of N2BA titin [UC(A)] is closer to the Z line than the UC epitope
of N2B titin [UC(B)]. For details, see the work by Trombitas et al.12

Figure 4. Top, Scattergram of the distance from the epitope to
Z line vs SL. Labeling patterns are assumed to be derived from
coexpressed N2B and N2BA isoforms. The lengths of the vari-
ous I-band segments were determined as shown on the left of
Figure 3. Bottom, Lengths of N2B-Us for N2B and N2BA iso-
forms. The large number of data points reflects the fact that
\( \approx 10 \) strips were available per heart.
pericardial effect is reduced. If we assume that the pericardium accounts for \( \approx 1 \) mm Hg of pressure at an intracavitary pressure of 5 mm Hg, then the low end of the normal SL range would occur at a transmural pressure of 4 mm Hg, corresponding to an SL of \( \approx 2.32 \) \( \mu \)m. Thus, our results indicate that changes in diastolic SL over the normal FP range are modest.

As the LV was filled to pressures above normal, SL reached a maximum slightly in excess of \( \approx 2.50 \) \( \mu \)m. Pressurization of the coronary bed resulted in turgor,\(^\text{14}\) manifested by an increase in FP. (Although the FP-SL curves with and without pressurization did not differ statistically, we believe this was due to the small number of data points.) The relation between normalized diastolic segment length and SL was unaffected by coronary pressurization. Thus, physiological perfusion resulted in a predominantly radial as opposed to a circumferentially oriented stress on the myofibers, consistent with previous work.\(^\text{24}\)

Our SLs are longer than those reported by Sonnenblick et al\(^\text{25}\) in their study of anterior midwall SLs in the normal canine LV. They also used KCl arrest followed by glutaraldehyde fixation and measured SLs at FPs between 2 and 12 mm Hg (in addition to overfilled conditions). In their report, an SL averaging 2.07 \( \mu \)m was present at a transmural pressure of \( =8 \) mm Hg. With overfilling above the normal range, SL reached a maximum value of \( =2.25 \) \( \mu \)m. Sonnenblick et al did not attempt to identify SL at 0 mm Hg transmural pressure, nor did they systematically analyze the relation between SL and FP in the normal range. One explanation for this difference in results is that Sonnenblick et al did not simulate physiological coronary perfusion. Our finding that the normalized diastolic segment length–SL relation is unaffected by coronary pressurization makes this unlikely. Another possibility is that the EDP-SL relation differs substantially in canine and miniswine LVs. Although this seems unlikely, we cannot exclude it. We believe the most likely explanation is tissue shrinkage. This may have occurred as a result of the electron microscopy methods used by Sonnenblick et al,\(^\text{25}\) which can result in \( \approx 10\%\) reduction in SL.\(^\text{26}\) After fixing the tissue in situ, a procedure that by itself does not cause shrinkage as indicated by lack of change in the distance between the crystals, we used laser diffraction to measure SL without any further tissue preparation. Our methods thus minimize the possibility of shrinkage.

Rodriguez et al\(^\text{27}\) published the only other report relating SLs to FP in the normal LV of a large mammal (closed-chest dogs). Their measurements were made by calibrating SLs in vitro and in vivo using implanted radiopaque markers. They focused on systolic SL and did not attempt to systematically determine the relation between FP and diastolic SL. They did, however, estimate SL at \( =0\)-mm Hg transmural pressure and found it to be 2.00 to 2.05 \( \mu \)m, somewhat less than our estimate. Although Sonnenblick et al\(^\text{25}\) did not measure SL at 0 mm Hg, by extrapolation, their value appears to be considerably less than those reported here and by Rodriguez et al.\(^\text{27}\) Both the present study and that of Rodriguez et al\(^\text{27}\) indicate that SL at 0 mm Hg is greater than slack length (1.85 to 1.90 \( \mu \)m) measured in muscle strips or isolated cardiomyocytes. One possible explanation for this difference is that residual forces\(^\text{28}\) present at zero distending pressure significantly lengthen SL above slack length. Rodriguez et al\(^\text{29}\) showed in cross-sectional rings of LV tissue that such stresses do not affect unloaded SL. This suggests that there are residual stresses inherent to the 3-dimensional architecture of the fully intact LV that influence SL at zero transmural pressure.

Under baseline conditions in our experiments, estimated minimum systolic SLs were above the \( \approx 1.85\)- to 1.90- \( \mu \)m slack length value; however, as volume declined during bicaval occlusion, minimum SLs reached values below slack in all but 1 experiment. At below slack length, titin is largely responsible for cardiomyocyte diastolic recoil. The mechanism is thought to be reverse extension of the extensible region of titin during contraction below slack length.\(^\text{7}\) Thus, our results suggest that in the intact LV titin is responsible for recoil mainly at volumes below the normal range.

To estimate systolic SLs, we assumed that the crystals were tethered to the myocardium and that there is a linear relation between segment length measured with the crystals and SL. This relation was “calibrated” by equating the segment length at fixation with the measured SL. We inserted the crystals into the LV anterior wall at the level of the minor axis by means of a stop that positions them at a depth close to the middle of the wall and used external landmarks to orient them circumferentially.\(^\text{15}\) It is not possible to perfectly position the crystals circumferentially in the midwall, however. To the extent that the crystals differed in depth within the wall and/or were not perfectly aligned with the fibers, our method will overestimate systolic SL as the fibers shorten along their long axis and underestimate the contribution of titin to generation of a restoring force with contraction below slack length. Although our results suggest that a titin-dependent restoring force is present in the intact LV, it is important to be cautious about extrapolating to normal conditions because, in the open-chest, anesthetized state, contractility is invariably depressed and loading conditions are nonphysiological. Nonetheless, our systolic SL results are consistent with those of Rodriguez et al,\(^\text{27}\) who estimated SLs in the 1.70 range at end systole in their intact preparation with more physiological loading conditions.

**Titin Extension**

Our previous immunolabeling studies\(^\text{2,4}\) revealed that the segments that make up the spring region of titin attain a zero end-to-end length at an SL of \( \approx 1.8 \) \( \mu \)m and that the segments are slightly extended at an SL of \( \approx 1.9 \) \( \mu \)m, approximately the SL of isolated cardiomyocytes under slack conditions. This slightly extended state in slack sarcomeres is expected from statistical polymer chain analysis, which shows that the mean square end-to-end distance of a flexible chain at zero external force is not zero but is a function of the contour length of the chain multiplied by its persistence length.\(^\text{30}\) Furthermore, in a recently developed knockout model\(^\text{11}\) in which the N2B element was excised (reducing the contour length of the extensible region of titin by \( \approx 50\%\), slack length was reduced from \( \approx 1.9 \) to 1.85 \( \mu \)m, supporting the idea that the end-to-end length of titin in slack cardiomyocytes is nonzero and is a function of contour length. Thus, the zero force state of
wild-type titin is attained at a SL of \( \approx 1.9 \mu m \), where the extensible region is slightly extended.

We found that in miniswine diastolic SL was in the 2.15- to 2.25-\( \mu m \) range at a transmural LV FP of 0 mm Hg and increased slightly to \( > 2.50 \mu m \) at maximal distension, a range over which titin develops considerable passive force.\(^3\)\(^-\)\(^5\)

Passive force developed by titin is a function of fractional extension of the spring region, which varies for the N2B and N2BA isoforms.\(^4\) N2B titin has a shorter contour length than N2BA titin; thus, the fractional extension (end-to-end length divided by contour length) for a given sarcomere stretch will be higher in N2B than N2BA when both are present within the same sarcomere. Because it is a function of contour length (see the equation below), force is much higher for N2B than N2BA titin. We calculated the force-SL relations of the 2 titin isoforms from the extension of the N2B-Us segment measured in miniswine LV (Figure 4C). Although other segments could be used for such a calculation, it is convenient to focus on N2B-Us because its large size results in the most pronounced isoform-dependent differences in extension and its persistence length is well established. Linear fits to the measured end-to-end length of N2B-Us were used to calculate the force-SL relation of single N2B and N2BA molecules with the worm-like chain equation:\(^2\)

\[
[Fx(PL)]/k_B T = (z/L) + \left\{ 1/[4(1-(z/L)^2)] \right\} - (1/4)
\]

where \( F \) is force (in pN), \( k_B \) is the Boltzmann constant, \( T \) is absolute temperature, and PL and L are persistence and contour lengths. With an assumed PL 0.65 nm and L of 210 nm\(^2\), the predicted titin force increases much more steeply with SL for N2B than N2BA titin (Figure 5).

Because segments within the extensible region of titin are in series and thus experience identical forces, the force-SL relations in Figure 5 reflect the difference between N2B and N2BA titin molecules. Miniswine coexpress N2B and N2BA at roughly similar levels; thus, the resulting titin force will be intermediate between that of the 2 isoforms and lower than that of rods expressing high levels of N2B titin. The working diastolic SL range of mouse LV has recently been estimated to be 1.9 to 2.1 \( \mu m \),\(^3\)\(^2\) considerably less than what we observed in miniswine. These observations, combined with our own, suggest that coexpression of the 2 isoforms in large mammals serves an important physiological purpose.

Greater expression of N2BA titin decreases the steepness of the force-SL relation, making it possible to achieve longer operating SLs without developing excessive tension and pressure. Put another way, for miniswine to operate in their normal range of diastolic volume and SL, titin isoform distribution must differ from rodents in a way that decreases stiffness.

**Conclusions**

Our results indicate that in miniswine diastolic SLs range from 2.15 to 2.25 to \( \approx 2.50 \mu m \) at transmural LV FPs between 0 mm Hg and overfilled conditions. In the normal operating transmural EDP range, SLs extend from \( \approx 2.32 \) to \( \approx 2.40 \mu m \). On the basis of the observed extension of titin in these ranges, the presence of substantial amounts of N2BA titin prevents excessive diastolic pressure elevation in miniswine and presumably other large mammals. These results are relevant to the effects of titin isoform shifting on diastolic stiffness in cardiomyopathy.\(^1\)\(^0\)\(^1\)\(^1\)

An increase in the ratio of N2B to N2B by itself would decrease stiffness. The magnitude of this effect will depend on the relation between SL and FP and the operating SL range. In a follow-up study to their original report, Ross et al\(^3\) measured SLs in the arteriovenous fistula model of high-output failure with chronic cardiac dilation. They found that the operating SL range in failing animals was similar to normal (ie, that SLs were not overstretched). This result suggests that a change in titin isoforms would have the same effect on myocardial stiffness in the chronically dilated LV as in the normal LV.

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**Disclosures**

None.

**References**

The giant sarcomeric protein titin is a multisegment molecular spring responsible for cardiomyocyte passive tension. In adults, titin occurs as N2B and N2BA isoforms, which are coexpressed within the sarcomere. N2B is smaller and stiffer than N2BA titin. Detailed extension of titin in relation to sarcomere length (SL) has been elucidated in rodents, which predominantly express N2B titin. Larger mammals, including humans, express isoform mixtures. This study was undertaken to delineate the relation between filling pressure, SL, and titin extension in a large mammal, the miniswine. The left ventricular filling pressure–SL relation was determined after diastolic arrest and fixation at various filling pressures. Undertaken to delineate the relation between filling pressure, SL, and titin extension in a large mammal, the miniswine. The left ventricular filling pressure–SL relation was determined after diastolic arrest and fixation at various filling pressures. Determination of the operating range of rodent SLs is considerably smaller than those of large mammals, our results imply that coexpression of both titin isoforms in large mammals allows longer SLs without the development of excessive diastolic tension. These results provide insights into the functional significance of titin isoform shifts reported in dilated cardiomyopathy.

CLINICAL PERSPECTIVE

The giant sarcomeric protein titin is a multisegment molecular spring responsible for cardiomyocyte passive tension. In adults, titin occurs as N2B and N2BA isoforms, which are coexpressed within the sarcomere. N2B is smaller and stiffer than N2BA titin. Detailed extension of titin in relation to sarcomere length (SL) has been elucidated in rodents, which predominantly express N2B titin. Larger mammals, including humans, express isoform mixtures. This study was undertaken to delineate the relation between filling pressure, SL, and titin extension in a large mammal, the miniswine. The left ventricular filling pressure–SL relation was determined after diastolic arrest and fixation at various filling pressures. Extension of various segments of titin in relation to SL was assessed in demembranated strips by immunoelectron microscopy using antibodies to selected epitopes. We found that the operating SL range between a filling pressure of 0 and markedly overdistended conditions ranged from 2.15 to 2.25 to \( \approx 2.50 \mu m \). The main difference between the 2 isoforms in this range was extension of the N2B-US segment, which was \( \approx 4 \) fold greater in N2B than N2BA titin. On the basis of the extension of this segment, we estimated that N2B titin develops much greater passive force than N2BA titin. Because the operating range of rodent SLs is considerably smaller than those of large mammals, our results imply that coexpression of both titin isoforms in large mammals allows longer SLs without the development of excessive diastolic tension. These results provide insights into the functional significance of titin isoform shifts reported in dilated cardiomyopathy.
Extensible Behavior of Titin in the Miniswine Left Ventricle
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