Differential Expression of Cardiac Titin Isoforms and Modulation of Cellular Stiffness

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Abstract—Extension of the I-band segment of titin gives rise to part of the diastolic force of cardiac muscle. Previous studies of human cardiac titin transcripts suggested a series of differential splicing events in the I-band segment of titin leading to the so-called N2A and N2B isoform transcripts. Here we investigated titin expression at the protein level in a wide range of mammalian species. Results indicate that the myocardium coexpresses 2 distinct titin isoforms: a smaller isoform containing the N2B element only (N2B titin) and a larger isoform with both the N2B and N2A elements (N2BA titin). The expression ratio of large N2BA to small N2B titin isoforms was found to vary greatly in different species; e.g., in the left ventricle the ratio is ≈0.05 in mouse and ≈1.5 in pig. Differences in the expression ratio were also found between atria and ventricles and between different layers of the ventricular wall. Immunofluorescence experiments with isoform-specific antibodies suggest that coexpression of these isoforms takes place at the single-myocyte level. The diastolic properties of single cardiac myocytes isolated from various species expressing high levels of the small (rat and mouse) or large (pig) titin isoform were studied. On average, pig myocytes are significantly less stiff than mouse and rat myocytes. Gel analysis indicates that this result cannot be explained by varying amounts of titin in mouse and pig myocardium. Rather, low stiffness of pig myocytes can be explained by its high expression level of the large isoform: the longer extensible region of this isoform results in a lower fractional extension for a given sarcomere length and hence a lower force. Implications of our findings to cardiac function are discussed. (Circ Res. 2000;86:59-67.)

Key Words: compliance □ passive tension □ diastolic force □ mechanical properties □ myocyte □ connectin

During diastole, the myocardium stretches and passive force (F) is generated. A major contributor to this force is the giant protein titin, spanning the half-sarcomere from the Z-band to the M-line. When sarcomere length (SL) increases during diastole, the I-band region of titin extends and force develops. The shape of F-SL relation of titin is expected to influence ventricular filling during diastole and ventricular emptying during systole. In addition to influencing ventricular filling, titin also helps to maintain the structural integrity of the contracting sarcomere (for reviews, see References 1–5).

The force of titin arises from its extensible I-band region, which consists of 2 main segment types: (1) a segment rich in proline (P), glutamate (E), valine (V), and lysine (K) residues (the so-called PEVK segment) and (2) serially linked immunoglobulin (Ig)–like domains (tandem Ig segments) flanking this PEVK segment. Several titin isoforms are now known, all of which contain PEVK and tandem Ig segments. In addition to these common segments, the extensible region of the N2B isoform contains the N2B element (3 Ig domains and a 572-residue unique sequence), whereas the N2A class of isoforms contains the N2A element (4 Ig domains and a 106-residue unique sequence). N2B titin is found exclusively in cardiac muscle and contains within its central I-band region a 163-residue PEVK segment and tandem Ig segments with 37 Ig domains. N2A titins are found in skeletal muscles with PEVK and tandem Ig segments that differ in length in different muscles. Heart muscle expresses not only N2B transcripts but also N2A transcripts; the latter are associated with a larger PEVK segment than the N2B transcripts. Here we investigated cardiac titin isoform expression by high-resolution SDS-PAGE and by various immunolabeling techniques (Western blotting, immuneelectron microscopy [IEM], and immunofluorescence [IF]) with isoform-specific antibodies.

In slack sarcomeres, the extensible segment of titin has a short end-to-end length as a result of thermally induced bending motions that lead to a state of maximal entropy. Straightening of the extensible region by extending the sarcomere lowers the conformational entropy and results in a force, known as entropic force. The extensible region of titin does not straighten uniformly; rather, tandem Ig and PEVK
segments extend sequentially. In addition, it was recently found that the 572-residue unique sequence of the N2B element is extensible as well, which explains why the extensible region of N2B titin can be stretched to lengths much longer than the combined contour length (end-to-end length when completely straight) of the tandem Ig and PEVK segments. Variation in the length of the extensible region is predicted to influence the developed force on extension. Cardiac myocytes that express a larger isoform (referred to below as N2BA titin) have a longer extensible region and are predicted to develop less force than those that express N2B titin, as for a given SL the fractional extension (end-to-end length of extensible region divided by its contour length) of N2BA titin will be less than that of the N2B isoform (for details, see Discussion).

Here we investigated titin expression at the protein level. A number of mammalian species were surveyed for the expression of different cardiac titin isoforms. SDS-PAGE revealed that in most species both small and large isoforms are coexpressed but in widely varying ratios. Western blots studies with N2B and N2A antibodies suggest that the smaller isoform contains the N2B element (N2B titin), whereas the larger isoform contains both the N2B and N2A elements (N2BA titin). To test the hypothesis that the expression of the N2BA isoform leads to a decrease in cell stiffness, mechanical experiments were performed on single cardiac myocytes isolated from hearts showing a high- or low-N2BA expression level. The results indicate that expressing small and large titin isoforms at different ratios is a means to modulate cardiac myocyte stiffness.

Materials and Methods

Cardiac Myocyte Isolation

Ventricular and atrial myocytes were isolated from rat, mouse, and pig by digesting their extracellular matrix with collagenase and hyaluronidase. The protocol to isolate cells from rat has been explained earlier, and the protocol for mouse and pig was adapted from Wolkska and Solaro. For isolating pig atrial cells, we used the blending method developed by Hofmann et al. The myocytes were chemically skinned, as previously described. To prevent degradation, all solutions contained protease inhibitors (see Reference 14 and online Materials and Methods).

Gel Electrophoresis

Myocardial and skeletal muscle samples were quick-frozen in liquid nitrogen, pulverized, and then rapidly solubilized. The samples were analyzed with SDS-PAGE (2% to 9.5% acrylamide gradient gels) and stained with Coomassie blue. Titin and myosin heavy chain (MHC) bands were analyzed as previously described, with some adaptations (see online Materials and Methods).

Western Blotting

Western blotting was performed with affinity-purified anti-titin antibodies (raised in rabbit) specific to the N-terminal end of titin (Z1/Z2 and Zx), the C-terminal end of titin (T51), to Ig repeats at the C-terminal end of the PEVK segment (bx283/4), N2A (X105 to X106), and N2B (X150 to X151, X214 to X215, and X216 to X217) (for details, see Figure 3A and online Materials and Methods).

Results

Immunofluorescence

Single- or double-labeling experiments with PEVK (9D10) and N2B (X151 to X151) or N2A (X105 to X106) antibodies were performed on cardiac myocytes (for details, see online Materials and Methods).

Passive Tension–SL Measurement of Cardiac Myocytes

For technical details of force and online SL measurement, see References 12 and 14. For technical details of force and online SL measurement, see References 13 and 14. The F-SL relationship of the cells was measured by imposing a slow ramp stretch-release (0.1 length/s) on cells in relaxing solution.

Electron Microscopy

Electron microscopy was used to obtain cross sections of skinned cells to determine the myofibrillar fractional area. Immunoelectron microscopy was used to study rat and pig left ventricular cells labeled with N2A (X105 to X106) and N2B (X150 to X151) antibodies.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

SDS-PAGE Analysis

Comparative gel electrophoresis of human skeletal muscle (soleus) and rat, rabbit, bovine, and human myocardium confirmed earlier findings and provided novel results as well. Confirming earlier findings, skeletal muscle and rat myocardium contain a single major titin band at the top of the gel (T1 in Figure 1, left 2 lanes), representing the full-length titin molecule. T1 mobility greatly varies, reflecting the difference in molecular mass of the 3.7-MDa soleus titin and the 2.97-MDa rat cardiac titin. The samples also reveal a minor band with higher mobility (T2) derived from a large titin degradation product that contains the A-band segment of...
titin. The T2 band is barely detectable in samples carefully prepared to minimize degradation (Figure 1). Novel findings were obtained when using rabbit, bovine, and human myocardium. These species contain two T1 bands, a bottom band that comigrates with the T1 band of rat and a top band with clearly reduced mobility (Figure 1). The top T1 band is just visible in rabbit (Figure 1, inset) but is easily detectable in cow and humans. The reason that two T1 bands in cardiac muscle have gone unnoticed in our earlier works is that our previous studies focused on rat and rabbit cardiac titin, and in these species the top T1 band is minor or absent. Limited gel resolution is another explanation why two T1 bands may have been missed in our previous studies. Only large-pore gels with high spatial resolution allow the two T1 bands in cow and human to be clearly separated.

To follow up on these findings, we surveyed additional species, examined ventricular and atrial myocardium in some of them, and determined the ratio of the top to bottom T1 bands using quantitative densitometry. Two T1 bands were consistently found in many species, but the density of the bands varied greatly. Rat, rabbit, and mouse myocardium contain barely detectable levels of the top T1 band, in dog and human the 2 bands are present at similar levels, and in pig and cow the top T1 band dominates (Figures 2A and 2B).

Furthermore, in the 2 species in which we examined both left ventricular and left atrial myocardium, the expression ratio of the top to the bottom band is much higher in the atrium than in the ventricle (Figures 2A and 2B). For example, in the left ventricle of the cow, the top T1 band is 1.8 times more abundant than the bottom band, whereas in the left atrium the top band is 21 times more abundant. Finally, we also studied titin expression in the subendocardial, midwall, and subepicardial regions of the ventricular free wall of the pig. Significantly higher levels of the large isoform were found in the subendocardium (Figure 2C).

Immunolabeling Experiments

Using the mobility of human soleus T1 (3.7 MDa) and rat cardiac titin T1 (2.97 MDa) as standards suggests that the two T1 bands of pig differ by ~0.35 MDa in mass. To test whether both T1 bands represent full-length titin molecules (and not degradation products), Western blot experiments were performed with antibodies against the terminal ends of the molecule (Figure 3A). The presence of the N-terminal end of titin was investigated with Z1/Z2 and Zr antibodies (Z1/Z2 labels the 200 first N-terminal amino acid residues and Zr labels epitopes ~500 residues from the N terminus), and the presence of the C-terminal end of titin with T51 antibody (which labels ~200 residues from the C terminus). Western blot results show that all antibodies reacted with the two T1 bands (Figure 3B). Thus, it is likely that the two T1 bands represent full-length titin molecules and thus distinct isoforms.

Cardiac muscle differentially expresses transcripts, which code for the N2B and N2A elements, respectively. To determine the identity of the titin bands seen on SDS gels, Western blot studies were performed using a library of antibodies raised against sequences from the N2B and N2A elements (see Figure 3A). These antibodies were first tested on skeletal muscle titin (human soleus), which is known to contain only N2A titin, and, as expected, the N2A element–specific antibody reacted with skeletal muscle titin, whereas the N2B element–specific antibodies did not (Figure 3C). Western blot experiments were also performed using pig myocardium, because it contains high levels of both T1 bands (Figure 2). The top T1 band reacted with both N2A- and N2B-specific antibodies, whereas the bottom T1 band reacted only with the N2B antibody (Figure 3D). These results are consistent with the top T1 band representing a titin isoform containing both the N2B and N2A elements (referred to as N2BA cardiac titin) and the bottom band an isoform that contains only the N2B element (N2B cardiac titin).

To investigate titin in the sarcomere, immunoelectron microscopy was performed on pig and rat ventricular cells using N2A- and N2B-specific antibodies. Pig cells were positive for both N2B and N2A antibodies and rat cells only for the N2B-specific antibodies (Figure 3E). These results are consistent with the conclusion that pig myocardium expresses the N2BA isoform and rat myocardium predominately the N2B isoform. Furthermore, in the pig cells the N2B epitope was on average closer to the Z-line than the N2A epitope. For example, at a SL of 2.45 μm, the distance between the epitope and the middle of the Z-line was 175±21 nm (n=9).
for the N2B epitope and 279±15 nm (n=10) for the N2A epitope. Thus, in the N2BA isoform the N2B element is N-terminal of the N2A element. This result is consistent with recent sequencing work on cardiac titins (A. Freiburg and S. Labeit, unpublished results, 1999).

**Immunofluorescence**

The SDS-PAGE and Western blot results were performed at the tissue level, and the results may be explained either by coexpression of isoforms in cells or by discrete expression in cells (ie, cells either express N2B or N2BA titin, but not both) with species variation in the ratio of cells that are N2B or N2BA pure. To distinguish between coexpression and discrete expression, IF studies were carried out on single cardiac myocytes. Considering that only N2BA titin reacts with the N2A element–specific antibodies, discrete expression predicts that a certain fraction of cells (N2B cells) will not stain with this antibody. This fraction can be estimated from the expression ratio of N2B and N2BA titins determined by solubilizing a large number of cells from the cell suspension used for IF, followed by electrophoresis and quantitative densitometry. Results indicate that 60±5% of total titin in pig

**TABLE 1. IF of Cardiac Myocytes**

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Pig</th>
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<tbody>
<tr>
<td></td>
<td>No. Positive Cells</td>
<td>No. Negative Cells</td>
</tr>
<tr>
<td>N2B (X150–X151)</td>
<td>126 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>N2A (X105–X106)</td>
<td>20 (12%)</td>
<td>149 (88%)</td>
</tr>
<tr>
<td>PEVK (9D10)</td>
<td>51 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Predicted N2A results*</td>
<td>5%</td>
<td>95%</td>
</tr>
</tbody>
</table>

*By “discrete hypothesis.”
left ventricle is N2BA titin and 40±7% is N2B titin (Table 1). Thus, the discrete expression hypothesis predicts that only 60% of the pig cells stain with the N2A antibody. Antibodies specific for the N2B element are predicted to stain all pig cells. For a comparison, mouse myocytes were studied as well. Considering that mouse myocardium contains a barely detectable N2BA band (Figure 2), discrete expression predicts that only few mouse cells are positive for the N2A antibodies whereas all cells are predicted to be positive for the N2B element–specific antibodies.

Experiments with the N2B antibodies revealed that all pig and mouse cells were positive (Table 1; Figures 4A and 4B), consistent with the results of the above-discussed Western blot studies. When using the N2A antibodies, we obtained...
positive cells (Figures 4C and 4E) and cells that contained a faint background fluorescence level without a clear banding pattern (Figures 4D and 4F) similar to that obtained in control experiments in which the primary antibodies were omitted from the labeling protocol (no–primary antibody control). To test whether absence of labeling with the N2A antibodies may have resulted from titin degradation or inability of the N2A antibody to penetrate some of the cells, experiments with the anti-titin antibody 9D10 were performed. 9D10 labels the PEVK region of titin, a site that is sensitive to degradation. Furthermore, 9D10 is an IgM type antibody, which is the largest of all antibody types, and, therefore, 9D10 is expected to diffuse slowly into the cells. Results revealed that all cells were 9D10 positive (Figures 4G and 4I; Table 1), suggesting that antibodies can fully penetrate the cells and that all cells contain intact titin. Therefore, cells that gave rise to a fluorescence similar to that of no–primary antibody control cells were scored as N2A-negative cells.

It was found that 92% of the pig cells are positive for the N2A antibody (Table 1), a value much higher than the 60±5% positive cells predicted by the discrete expression hypothesis (cells express only one of the isoforms). On the other hand, the coexpression model (all cells coexpress isoforms at the same ratio) predicts that all pig cells would be N2A positive, whereas we found that 8% of pig cells are N2A negative. These findings may be explained by coexpression that varies from cell to cell, with a small fraction of the cells expressing predominantly one of the isoforms.

Diastolic Properties of Cardiac Myocytes
To investigate the functional significance of coexpressing titin isoforms, mechanical experiments were performed on single cardiac myocytes, and their F–SL relation was measured. The force generated by the intermediate filament network was determined by extracting the thin and thick filaments from the myocytes using KCl- and KI-containing relaxing solutions (see Reference 14). Extraction removes titin as a force generator as the extraction-induced depolymerization of actin and myosin removes the anchors of titin in the sarcomere. The force after extraction was subtracted from the force before extraction, resulting in data that represent the force produced by titin alone. This force was converted to tension (T) by dividing it by the cross-sectional area of the cell. The cells studied had been isolated by either blending myocardial tissue (cf Reference 16) or by enzymatic digestion of the heart (see figure legends). When ventricular cells isolated by blending or digestion were compared (data not shown), no statistically significant differences were found in the cellular content of titin or the titin-based stiffness of the cells.

Titin-based T–SL curves of 15 mouse and 10 pig ventricular cells are shown in Figure 5. Results indicate that the average stiffness of the mouse myocytes is much higher than that of the pig (Figure 5, inset). To test for statistical differences between mouse and pig cells, the tension developed at a SL of 2.4 μm was compared using a Mann-Whitney test (this test was used because results were not normally distributed). This revealed that mouse ventricular cells are significantly stiffer than pig myocytes (P<0.05). To determine whether the differences in stiffness may have resulted from differences in the fractional area of myofibrils, cells were prepared for electron microscopy and their cross sections were analyzed. Results revealed that this explanation is unlikely, because mouse and pig cells contain similar fractional areas of myofibrils (Table 2). We also studied whether differences in the cellular content of titin could explain the mechanical differences between pig and mouse cells. The ratio of titin relative to MHC was determined using quantitative densitometry (for details, see Materials and Methods). It was found that the titin/MHC ratios of mouse and pig cells are not significantly different (Table 2). The titin/MHC ratio was also used to determine the number of titin molecules per half-thick filament (for details, see Materials and Methods). Results indicate 6.0±0.3 and 6.5±0.4 titin molecules per half-thick filament in mouse and pig cells, respectively (Table 2). Thus, a difference in the titin content of mouse and pig cells is an unlikely explanation for their different passive properties.

**TABLE 2. Cross-Sectional Analysis and Titin Content of Cardiac Myocytes**

<table>
<thead>
<tr>
<th></th>
<th>Mouse</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross section, μm²</td>
<td>271±18 (n=16)</td>
<td>329±10 (n=10)</td>
</tr>
<tr>
<td>% Myofibrillar area</td>
<td>68±3 (n=7)</td>
<td>73±7 (n=3)</td>
</tr>
<tr>
<td>N2BA:N2B ratio</td>
<td>&lt;0.05 (n=6)</td>
<td>1.48±0.11 (n=8)</td>
</tr>
<tr>
<td>Titin/MHC ratio*</td>
<td>0.29±0.02 (n=5)</td>
<td>0.34±0.02 (n=5)</td>
</tr>
<tr>
<td>No. of titin molecules/half-thick filament†</td>
<td>6.0±0.3 (n=5)</td>
<td>6.5±0.4 (n=5)</td>
</tr>
</tbody>
</table>

Values mean ± SEM.

*To determine the amount of titin relative to that of MHC, gels were scanned and the total optical density of the MHC and of all titin peaks was determined for a range of loadings. The slope of the linear range of the optical density vs loading relation of titin and MHC was measured, and the slope ratio was taken as a measure of relative amount of titin in the samples.

†This relative amount was converted to the number of titin molecules per half-thick filament, assuming 150 myosin molecules per half-thick filament, a molecular mass of 0.205 MDa for MHC, 2.97 MDa for N2B titin, and 3.35 MDa for N2BA. For myocardium that coexpresses titin isoforms, the average molecular mass was calculated from the isoform expression ratio.
One of the isoforms contains the N2B element and the other component in cells isolated from tissue that coexpresses both titin isoforms.

It may be noted from Figure 5 that a few of the pig cells are as stiff as most of the mouse cells, whereas one of the mouse cells is as compliant as most pig cells. Although experimental error may underlie part of this variation, differences in cell behavior may also result from cell-to-cell variation in the expression ratio of N2B and N2BA titins. For example, the compliant mouse cells may express high levels of N2BA titin.

Results indicate that most of the pig atrial cells were compliant (ie, their T-SL curves are shallow) and that a few cells were rigid and behaved like the average mouse cell (Figure 6, red curves). In contrast, rat cardiac cells were all stiff (Figure 6). Considering that the rat expresses predominantly N2B titin, cell-to-cell variation appears more prominent in cells isolated from tissue that coexpresses both titin isoforms.

**Discussion**

We found that the myocardium coexpresses 2 titin isoforms. One of the isoforms contains the N2B element and the other both the N2B and N2A elements. The expression ratio of these isoforms varies in different species and in different locations within the heart. Furthermore, results suggest that coexpression of isoforms takes place at the level of the single cell. The mechanical properties of single cardiac myocytes were measured, and this revealed that on average, cells isolated from a species that contains high levels of N2BA titin (pig) are significantly less stiff than those from species that contain high levels of N2B titin (rat and mouse). Below, we discuss these findings and their functional significance.

**Cardiac Titin Isoforms**

Previous reverse transcriptase–polymerase chain reaction (RT-PCR) studies of human cardiac titin revealed the existence of N2A and N2B isoform transcripts. Here we studied isoform expression at the protein level. SDS-PAGE indicates that myocardium of many species contains two T1 bands, a top band that reacts with N2A- and N2B-element–specific antibodies and a bottom band with only N2B-specific antibodies. That the two T1 bands represent full-length titin molecules is supported by Western blot experiments with antibodies against the N-terminal end (Z1/Z2 and Zr) and the C-terminal end (T51) of titin. These experiments showed that both T1 bands of pig are full-length molecules and not degradation products. Absence of titin degradation is also supported by the immunoelectron microscopy data. Degradation results in retraction of titin toward the Z-line and the A-band, and no such retraction was found in the cells that we investigated. Hence, we conclude that the two T1 bands represent distinct isoforms. This conclusion is in accordance with recent RT-PCR studies using rabbit cardiac mRNA, which revealed that differential splicing gives rise to 2 main classes of cardiac titin isoforms, the N2B and N2BA titins, so named because they contain either the N2B element or both the N2B and N2A elements (A. Freiburg and S. Labeit, unpublished results, 1999). Cardiac N2B titin is the smallest isoform known to date and the small size results from skipping the I20 to I75 segment and splicing together the I19 to I76 encoding exons. The splicing of the exon coding for I19 to exons in the I47 to I60 segment leads to a family of isoforms, all including the N2A segment, and thus these isoforms are referred to as N2BA titins.

Because of the sequence differences, the molecular mass of the cardiac titin isoforms varies from ~3.3 MDa for the N2BA titin isoform to ~3.0 MDa for N2B titin (Reference 6 and A. Freiburg and S. Labeit, unpublished results, 1999). Our current work indicates that this mass difference is large enough to allow separation of N2B and N2BA titins on high-resolution gels (Figures 1 through 3). This enables studies of the expression levels of the isoforms in different species and in different locations within the heart. Results establish that coexpression of isoforms takes places in a wide range of species, but at widely varying ratios, and that atria express higher N2B levels than the ventricles. The quantitative densitometric study of titin in mouse and pig (expressing high levels of N2B and N2BA titin, respectively) reveals that while the expression ratio of titin isoforms varies greatly, the number of titin molecules per half-thick filament appears constant (Table 2). Thus, the stoichiometry of titin is well controlled, whereas the isoform expression ratio is variable.

**Coexpression of Titin Isoforms at the Single–Cardiac Myocyte Level**

The titin isoform coexpression results were obtained with SDS-PAGE and Western blot techniques using myocardial tissue as starting material. Coexpression of isoforms at the tissue level can be explained by assuming (1) that individual cells express only one of the isoforms (discrete expression hypothesis) and (2) that the ratio of N2B cells to N2BA cells determines the isoform expression ratio of the myocardial tissue. However, we found that the ratio of positive and negative cells is inconsistent with a discrete expression of isoforms (Table 1, see Results). Instead, results are consistent with a coexpression of titin isoforms within individual cells. The small fraction of negative cells can be explained by cell-to-cell variation in the coexpression ratios resulting in
some cells with predominantly one isoform. This “variable coexpression” hypothesis is also consistent with recent immunoelectron microscopy studies that revealed coexpression of isoforms within the same I-band and cell-to-cell variation in the coexpression level (K. Trombitás, S. Labeit, and H. Granzier, unpublished data, 1999). In summary, our findings indicate that the titin isoform coexpression seen at the myocardial level results from coexpression at the level of the single myocardial myocyte.

**Diastolic Properties of Cardiac Myocytes**

Whether diastolic properties of N2B cells are predicted to be different from those of N2BA cells can be ascertained from the molecular mechanism of titin-based F development. The molecular mechanism that underlies the force of titin has been investigated in dynamic light-scattering studies on titin in solution,23 mechanical studies on single titin molecules,24–27 and immunoelectron microscopic studies on skeletal and cardiac muscle titin.11,28,29 From these, a model has emerged in which the tandem-Ig segments (containing folded Ig domains) and the PEVK segment (acting largely as an unfolded polypeptide) behave as serially linked entropic springs. In short sarcomeres, these springs are in a contracted state with high entropy, and on sarcomere extension the springs straighten, lowering their conformational entropy and resulting in a force, known as entropic force. This force increases with the fractional extension of the segment (end-to-end length divided by the contour length) (see Reference 8).

The serially linked entropic-springs model of F development may be applied to size variants of the elastic segment of titin by adapting the entropic forces to the fractional extensions multiplied by the contour lengths of the size variant’s tandem Ig and PEVK segments. The contour lengths of tandem Ig and PEVK segments are ~100 and ~250 nm longer, respectively, in N2BA than in N2B titin (assuming a 5-nm repeat per Ig domain and 3.8 Å per PEVK residue; see References 11 and 12). It follows that at a given SL the fractional extension of tandem Ig and PEVK segments is considerably less for N2BA titin than for N2B titin and, therefore, F will be lower. This prediction is qualitatively unaffected by the extensibility of the unique N2B sequence,12 because both cardiac titin isoforms contain this sequence. In conclusion, the serially linked entropic-springs model of F development predicts that the F-SL relation will increase less steeply (ie, the compliance is higher) for N2BA containing cells than for N2B cells.

We studied the passive tension–SL relation of single cardiac myocytes isolated from mouse, rat, and pig myocardium. The mouse and rat were chosen because their cardiac myocytes are predicted to be stiff (they express high levels of N2B titin), and the pig because its cells are predicted to be compliant (high levels of N2BA titin). Consistent with the predicted tension differences, pig myocytes are on average significantly more compliant than rat and mouse cells (Figures 5 and 6). Considering the similar fractional cell areas of myofibrils and the similar number of titin molecules per half–thick filament (Table 2), it is unlikely that these mechanical results can be explained by differences in the amount of titin per unit cross-sectional cell area. Rather, it is likely that these mechanical differences result from within titin itself. It is also worthwhile to point out that studies performed more than 20 years ago by Fabiato and Fabiato50 are consistent with our findings. In this earlier work, it was shown that dog myocytes are more compliant than rat myocytes, consistent with the high levels of N2BA titin that we found in dog myocardium and high levels of N2B titin in rat (Figure 2). Finally, Brady31 has reported the passive stiffness modulus (normalized to the cross-sectional area) of myocytes from several species, and this revealed lower stiffness in rabbit than in rat. This finding is in agreement with the higher N2BA levels of rabbit (Figure 3).

Mechanical results of cells isolated from the same ventricle often showed considerable variation (Figures 5 and 6). Although some of this variation may result from experimental error (such as errors in the cross-sectional area measurement), it seems more likely that additional sources of variation exist as well. It is also worth noting that variation in mechanical properties has been reported in intact guinea pig ventricular cells by Gannier et al13 and Cazorla et al,33 with a tendency of the cells to separate into stiff and compliant subpopulations. Variation in cellular stiffness is consistent with the variable coexpression hypothesis that was derived from the IF study (see above). Variation in the titin isoform coexpression ratio of cardiac myocytes is expected to give rise to variation in the level of passive tension for a given SL, with an increase in cell compliance as the N2BA expression level increases.

In summary, our mechanical studies reveal cell-to-cell variation in cell stiffness, but on average cells isolated from myocardium that expresses high levels of N2B titin are stiff and those from myocardium that expresses N2BA titin are more compliant. Thus, the diastolic properties of cardiac myocytes isolated from different species are not the same, but instead they vary with the expression ratio of the titin isoforms.

**Functional Significance**

Work on rat cardiac muscle has shown that both titin and collagen are major contributors to passive stiffness of the myocardium, with the contribution of titin dominating at shorter lengths to midrange of the physiological SL range and with collagen contributing more at longer lengths.14 Although comparative muscle studies in different species remain to be done, it is likely that variation in titin-based stiffness of the myocytes will translate into variation in stiffness at the level of the myocardium. Thus, we speculate that as the expression level of N2BA titin increases and cell stiffness decreases, myocardial stiffness will decrease as well. Variation in myocardial stiffness is expected to influence filling of the heart. For example, lower myocardial stiffness will allow for faster filling and larger end-diastolic volumes for a given filling pressure. The variation in expression levels of titin isoforms in different species may thus be related to variation in filling rate and/or filling volume of the heart. We speculate that the cell-to-cell stiffness variation within species is related to strain equalization of muscle fibers in different layers of the wall. Consistent with this explanation is the variation in the N2BA/N2B isoform expression ratio that was found in different layers of the ventricular wall (Figure 2C).
In conclusion, this work revealed that the myocardium coexpresses titin isoforms and that the expression ratio of the isoforms varies between and within species in different locations of the heart. Coexpression of titin isoforms at different levels modulates cellular stiffness, and we hypothesize that this influences filling of the heart. Research at the multicellular and organ levels will be required to test this hypothesis and to fully elucidate the functional significance of the present findings.

Acknowledgments

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Differential Expression of Cardiac Titin Isoforms and Modulation of Cellular Stiffness
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Material and Methods online

Cardiac myocyte isolation. In this work cardiac myocytes were isolated from various sources. (The protocols conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, 1985). Myocytes were isolated from the left ventricle of rat (Sprague Dawley) as explained earlier. Mouse myocytes were isolated from 10-12 week old FVB-Taconic mice, using an isolation procedure adapted from Wolska. Briefly, the coronary arteries were perfused with oxygenated physiological saline solution (PSS) containing (in mmol/L): 133.5 NaCl, 4 KCl, 1.2 NaH₂PO₄, 1.2 MgSO₄, 10 HEPES, 11 glucose, pH 7.4. Solutions were preheated to achieve a perfusion temperature of 37°C. During perfusion the heart was immersed in a tissue-organ bath kept at 37°C. The hearts were first perfused for 5 min with Ca²⁺-free PSS containing 0.1% bovine serum albumin (BSA) at a 1.3 ml/min flow rate, and subsequently for 10-20 min with PSS containing 25 μmol/L CaCl₂, collagenase (317 U/mg, type II Worthington) and hyaluronidase (1000 U/mg, Sigma) using a 1.6 ml/min flow rate. Then the heart was washed with Ca²⁺-free PSS containing 15 mmol/L 2,3-butanedione monoxime (BDM). The atria were then removed and quick-frozen in liquid nitrogen for gel electrophoresis. Ventricles were cut into small pieces that were repeatedly drawn through a plastic pipette tip to release isolated cells. The majority of myocytes obtained this way had a normal, rod-like shape. The cells were skinned for 50-minutes with 1% Triton X-100 in relaxing solution containing in mmol/L: 40 imidazole; 10 EGTA; 6.4 Mg-Acetate; 5.9 NaATP; 10 creatine-phosphate; 80 K-propionate; 1.0 DTT; pH 7.0 at 21°C. The detergent was removed through extensive rinsing with relaxing solution. To prevent degradation, all solutions contained protease inhibitors (PMSF: 0.5 mmol/L; leupeptin: 0.04 mmol/L and E64: 0.01 mmol/L).

For isolating myocytes from pig, Yorkshire type swine (20-30 kg) were obtained from the Washington State University Swine Center. Pigs were anaesthetized by intramuscular injection of Xylazine (4 mg/kg) mixed with Telazol (8 mg/kg) and the incision site received a local injection of Lidocaine (2 mg/kg). The method for isolating left ventricular cells was similar to the one described for mouse, with the following modifications. After excision the whole heart was perfused using a Langendorff perfusion system with a 0.2 mmol/L CaCl₂ in PSS, at a flow rate of 60 ml/min. The coronary arteries that supply the right atrium and ventricle were ligated and the right ventricle and atrium were removed for cell isolation via the blending method (see below). The remaining heart was then perfused for 20-30 min with a digestion solution
containing 75 μmol/L CaCl₂, collagenase (317 U/mg, type II Worthington) and hyaluronidase (1000 U/mg, Sigma) in PSS, followed by cell isolation as explained above for the mouse.

Most of the cells were isolated by the enzymatic procedure which was unsuccessful with the pig atrium. An alternative technique was tried with success using a blending method (cf. 3). Briefly, small pieces (~1 cm long, ~3 mm I diamter) of myocardial tissue were pre-skinned by placing them in relaxing solution containing 1% Triton X-100 for 50 min at 4°C. The pieces were then placed in fresh relaxing solution and homogenized in a blender (Ultra-Turrax TP18 with a S25N-10G shaft, Ika Works, Inc.) for 15 sec at 12,500 rpm, resulting in a suspension of small clumps of myocyte-sized preparations and cell fragments. Myocytes were skinned in 1% Triton X-100 solution for 50 min at 4°C. To prevent degradation, all solutions contained protease inhibitors (see above). Some cells were used immediately after cell isolation for mechanical experiments, other were solubilized for gel electrophoresis or stored at ~20°C in 50% glycerol in relaxing solution for immunolabeling experiments (see below).

**Gel electrophoresis.** Myocardial samples consisted of full wall-thickness samples and of sub-regions of the wall. Using fine scissors, a ~0.2mm layer of inner free wall (sub-endocardium) and outer free wall (sub-epicardium) were dissected as well as samples from the mid-region of the wall. Myocardial and skeletal muscle samples were quick-frozen in liquid nitrogen, pulverized and then rapidly solubilized.¹ The samples were analyzed with SDS-PAGE (2-9.5% acrylamide gradient gels). After electrophoretic separation the gels were stained with 0.1% Coomassie Blue G250.⁴

Wet gels were scanned at 300 dpi using a UMAX model UC1260 flatbed scanner operated in transmission mode. An optical filter was used to limit the light wavelength to that of the absorption peak of Coomassie blue. Scanned images were analyzed using National Institutes of Health Image (v 1.62, W. Rasband, NIMH, National Institutes of Health). Images were calibrated with a 34-step (0.1-3.4 OD) density filter (#ST-34, Kodak) scanned simultaneously with the gel. Typically, various overlapping titin peaks were obtained (two T1 peaks and one T2 peak, see Results) and Gaussian curve fits were used (One-D scan software, Scanalytics Inc., Fairfax, Va) to separate the different peaks and determine their ‘OD area.’

To determine the amount of titin relative to that of myosin heavy chain (MHC), gels were scanned and the total OD (optical density) of the MHC peak was determined as well as the total
OD of all titin peaks (T1 and T2 bands). In addition to T1 and T2 some samples also contained a band that barely entered the gel and western blotting with titin-specific antibodies indicated that this band is an aggregate of titin. The band was therefore included in the total titin OD. To ensure that results were obtained in the linear range of the SDS-PAGE system and scanner, for each sample a range of loadings were electrophoresed on the same gel. The OD of titin and MHC were determined for each lane and results of all lanes were plotted vs. their loadings. The slope of the linear range of this relation was determined using linear regression analysis and the slope ratios of titin and MHC were taken as a measure of relative amount of titin in the samples. This relative amount was converted to the number of titin molecules per half thick filament assuming 300 MHCs (150 myosin molecules) per half-thick filament and a MHC molecular mass of 0.205 MDa (For details: see 5). The molecular mass of N2B titin was taken as 2.97 MDa and that of N2BA at 3.35 MDa (6; Freiburg et al., unpublished data, 1999). For myocardium that co-expresses titin isoforms, the average molecular mass was calculated from the isoform expression ratio.

**Western blotting.** Western blotting with affinity purified anti-titin antibodies specific to the N-terminus end of titin (Z1/Z2 and Zr), the C-terminus of the PEVK (bk283/4), the C-terminus end of titin (T51; kindly provided to us by Dr. Furst) and the N2A and N2B elements was performed as explained in our earlier work.7 The bk283/4 antibodies were used earlier by Linke et al 8 using the name I20. Antibodies were raised in rabbits using as antigen titin fragments expressed in E. coli (Biogenes, Berlin, Germany). To raise the N2A X105-X106 antibody the titin sequence from the N2A splice pathway (base pairs 15307-16257 of human skeletal cDNA entry, EMBL data library accession X90569) was expressed in E.coli. For the N2B antibodies X150-X151, X214-X215 and X216-X217 the base pairs 11551 to 11928, 10,489 to 11,145, and 12,850 to 13,416 of the human cardiac titin cDNA sequence (EMBL data library accession X90568) were expressed. The X150-X151 sequence locates within the large unique sequence of the N2B element, whereas the X214-X215 and X216-X217 fragments correspond to the Ig repeats N2B-I16/I17 and N2B-I18/I19, which are contained within the N2B element. Thus, the X214-X215 and X216-X217 epitopes demarcate the unique sequence contained within the N2B segment at the N-terminal end and C-terminal end, respectively. Protein expression of antigens, purification and antibody production were essentially as described earlier.9
**Immunofluorescence.** Cardiac myocytes that had been stored in 50% glycerol in relaxing solution were allowed to settle (overnight at 4°C) in small wells with a glass slide as bottom (chamber slide, Lab-Tek®, Nunc Inc.). Cells were then washed followed by fixing with freshly made 3.7% formaldehyde (formaldehyde in relaxing solution for 5 min followed by formaldehyde in phosphate buffered saline (PBS) solution for 10 min). After extensive washing with PBS the slide was blocked with 1% BSA and 0.1% Triton X-100 in PBS for 30 min at room temperature. Cells were then incubated overnight at 4°C with the primary antibodies N2B_{X150-}

X151, PEVK_{9D10} and N2A_{X105-X106}. Antibodies were diluted with PBS containing 0.1% Triton X-100 using experimentally determined dilution factors that resulted in clear labeling and minimal background staining (dilution ranged from 1:50 to 1:200). Double labeling experiments with 9D10 (mouse monoclonal antibody, see 10) and N2B or N2A antibodies (rabbit polyclonal antibodies) were also performed. Control experiments were performed in which the primary antibodies were omitted from the labeling protocol (no-primary antibody control). Cells were then washed with PBS for 60-90 min to remove the excess of antibodies and were incubated with secondary antibody for 6 hours at 4°C. For 9D10 the secondary antibody was anti-mouse IgM conjugated with fluorescein isothiocyanate (FITC) (ref. F9259, Sigma) and for the other primary antibodies the secondary antibody was anti-rabbit IgG conjugated with tetramethylrhodamine isothiocyanate (TRITC) (ref. T6778, Sigma). The secondary antibodies were diluted (1:400) in 0.1% Triton X-100 / PBS. Following washing, cells were mounted in a fluoromount-G aqueous mounting medium (catalog number: 17984-25, Electron Microscopy Sciences). Cells were studied using a conventional epifluorescence microscope (Diaphot 300, Nikon) as well as a laser scanning confocal microscope (Nikon Eclipse TE300/Biorad MRC 1024 laser confocal, Hercules, Ca).

**Force – SL measurement of cardiac myocytes.** The instrumentation used and the single-myocyte mechanical protocols employed have been previously described 1. Briefly, myocytes were added to a chamber (volume ~300 µl) mounted on the stage of a phase-contrast microscope. First, one end of a single cell was glued to a motor (Cambridge Technology, type 6800). The free end was then positioned with a micromanipulator so that the myocyte axis aligned with the microscope optical axis, and the myocyte cross-sectional area was measured. Finally, the free end of the myocyte was glued to a force transducer (model 406A Cambridge Technology, Inc., resonance frequency ~75 Hz). To minimize the disturbing effect of meniscus forces, the force
transducer entered the chamber below the solution surface through the side of the chamber. A
gravity-based perfusion system was used to replace solutions in the chamber. Experiments were
performed at room temperature (20-22°C).

The sarcomere length (SL) was measured on-line at a frequency of 60 Hz, using a commercially
available sarcomere length detection system (Ionoptix Corp., Milton Ma). Measurements were
based on Fourier analysis of optical density traces of the striation pattern obtained by digitizing
images of the cells. The SL was measured in different regions of the cell and the average length
was determined. SL prior to the stretch-release protocol was measured, as was at the maximum
length reached during the protocol by holding the cell at these lengths for 10 sec. The SL during
the stretch and release was assumed to vary linearly between the start and end SLs.

The passive force – SL relationship of the cells was measured during a slow ramp stretch-
release (0.1 length/s) while cells were in relaxing solution. The first stretch-release was used to
determine the stretch amplitude required to reach the desired SL of 2.5 μm. To obtain
reproducible results, cells were rested at slack length for 15 min between stretch-release cycles.

Titin, collagen and intermediate filament-based force. In addition to titin, intermediate
filaments (IFs) also develop force in stretched myocytes.¹ To determine the force produced by
IFs, myocytes were incubated for 10 min with relaxing solution that contained 0.6 mol/L KCl
solution followed by a 30 min incubation in relaxing solution with 1.0 mol/L KI (method
according to ¹). These solutions depolymerize the thick and thin filaments and thereby remove
titin’s anchors in the sarcomere. The force decrease that resulted from extraction was assumed to
be titin based. The F-SL length relation following extraction was assumed to represent the IF
component in myocytes. Force was converted to tension by dividing measured forces by the cross-
sectional area of the myocyte.

Electron microscopy. Considering that only part of the cross-sectional area of cardiac myocytes
contains myofibrils, skinned cells were prepared for electron microscopy (cf. 7). Cross-sections
were made, photographed and the myofibrillar fractional area was determined using National
Institutes of Health Image (v 1.62, W. Rasband, NIMH, National Institutes of Health).
Immunoelectron microscopy was also used on rat and pig ventricular cells for labeling of
sections with N2Aₙ₁₀₅-₁₀₅ and N2Bₙ₁₅₀-₁₅₁ according to a protocol previously published ¹¹).
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