Extensibility of Isoforms of Cardiac Titin: Variation in Contour Length of Molecular Subsegments Provides a Basis for Cellular Passive Stiffness Diversity

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ABSTRACT Titin is a giant polypeptide that spans between the Z- and M-lines of the cardiac muscle sarcomere and that develops force when extended. This force arises from titin's extensible I-band region, which consists mainly of three segment types: serially linked immunoglobulin-like domains (Ig segments), interrupted by the PEVK segment, and the N2B unique sequence. Recently it was reported that the myocardium of large mammals co-expresses small (N2B) and large (N2BA) cardiac isoforms and that the passive stiffness of cardiac myocytes varies with the isoform expression ratio. To understand the molecular basis of the differences in passive stiffness we investigated titin's extensibility in bovine atrium, which expresses predominantly N2BA titin, and compared it to that of rat, which expresses predominantly N2B titin. Immunoelectron microscopy was used with antibodies that flank the Ig segments, the PEVK segment, and the unique sequence of the N2B element. The extension of the various segments was then determined as a function of sarcomere length (SL). When slack sarcomeres of bovine atrium were stretched, the PEVK segment extended much more steeply and the unique N2B sequence less steeply than in rat, while the Ig segments behaved similarly in both species. However, the extensions normalized with the segment's contour length (i.e., the fractional extensions) of Ig, PEVK, and unique sequence segments all increase less steeply with SL in cow than in rat. Considering that fractional extension determines the level of entropic force, these differences in fractional extension are expected to result in shallow and steep passive force-SL curves in myocytes that express high levels of N2BA and N2B titin, respectively. Thus, the findings provide a molecular basis for passive stiffness diversity.

INTRODUCTION

Titin is a giant elastic protein that generates the majority of the passive force of the cardiac myocyte and which, together with the extracellular passive force generator collagen, influences ventricular filling during diastole and ejection during systole. Titin also helps to maintain sarcomeric integrity during contraction and has been implicated in myofibrillogenesis as a scaffold and cell-signaling molecule. (For reviews and original citations see Gregorio et al., 1999; Trinick and Tskhovrebova, 1999; Labeit et al., 1997; Maruyama, 1997; Wang, 1996.)

Titin's force arises from its extensible I-band region, which consists (Labeit and Kolmerer, 1995) of three main segment types: 1) a type rich in proline (P), glutamate (E), valine (V), and lysine (K) residues (the so-called PEVK segment); 2) serially linked immunoglobulin-like domains (Ig segments); and 3) the N2B/N2A elements (N2B: 3 Ig domains and a 572-residue unique sequence; N2A: 4 Ig domains and a 106-residue unique sequence). The extensible region of cardiac N2B titins contains the N2B element, a 163-residue PEVK segment and Ig segments with 37 Ig domains (Labeit and Kolmerer, 1995). At physiological SL values the Ig segments, the PEVK, and the unique N2B sequence all extend (Linke et al., 1999; Trombitás et al., 1999). N2B cardiac titin may thus be viewed as a three-element molecular spring.

Recently it was found (Freiburg et al., 2000; Cazorla et al., 2000) that a class of cardiac titin isoforms (so-called N2BA titins) contain, in addition to the N2B element, the N2A element. N2BA titins contain a 600–800-residue PEVK segment and a varying number of Ig domains (12–25) that make up a "middle" Ig segment located between the proximal and distal Ig segments. Myocardium of large mammals (including human) expresses high levels of N2BA titins, and the N2BA expression level correlates inversely with passive stiffness of cardiac myocytes (Cazorla et al., 2000).

In this work we studied the extensibility of bovine atrium titin, which is predominately the N2BA isoform, and compared the results with those for rat ventricle titin, which is predominantly the N2B isoform. The findings indicate that differences in fractional extension of the PEVK and N2B unique sequence underlie the variation in the passive stiffness of cardiac myocytes that express different titin isoforms.

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MATERIALS AND METHODS

Antibodies

The bindings sites of the anti-titin antibodies that were used are shown in Fig. 1 *B*. Un was raised against the Ig repeats N2B-I24-I25 and Uc against

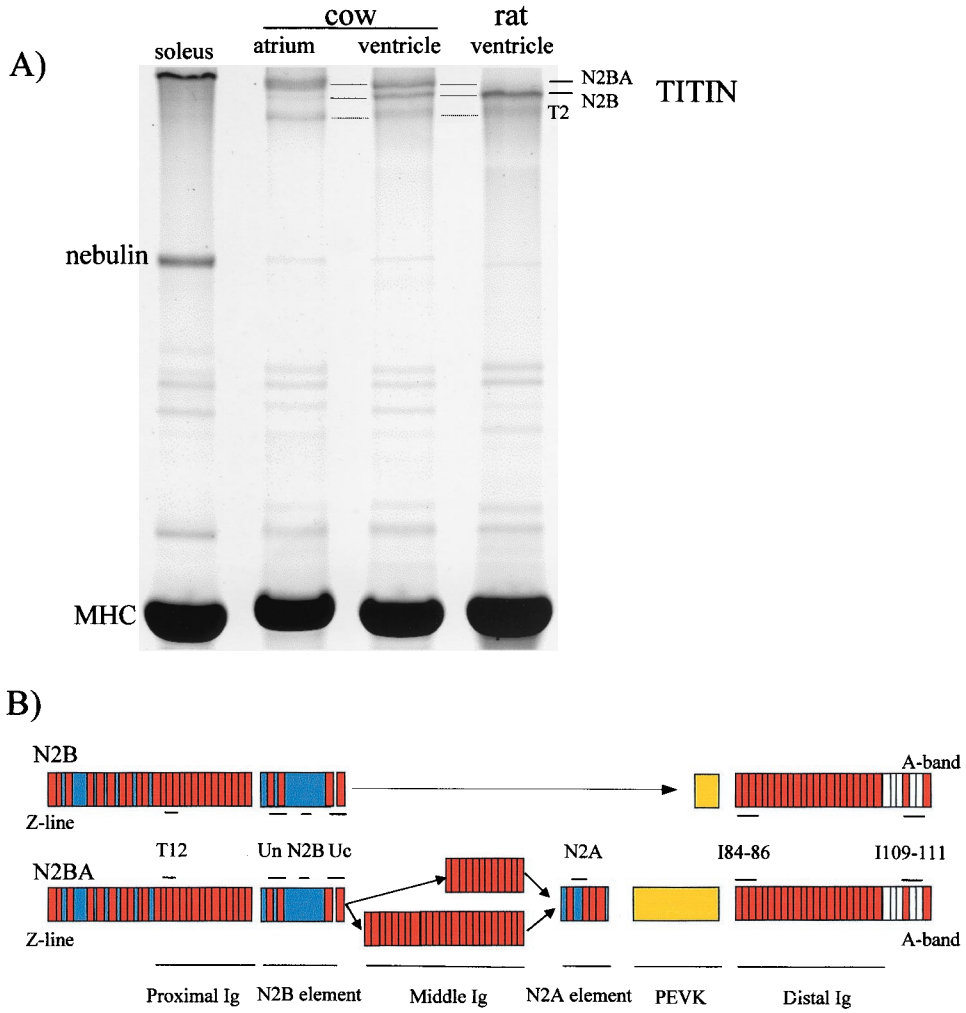

FIGURE 1 (*A*) SDS-PAGE of myocardial tissues from cow and rat. Rat ventricle and cow atrium contain predominantly N2B and N2BA titin, respectively. Cow ventricle expresses N2B and N2BA titin at similar levels. (Human soleus muscle was included to provide molecular mass standards. Soleus T1: ~3.7 MDa; nebulin: ~800 kDa.) MHC: myosin heavy chain. (*B*) Domain structure of I-band regions of cardiac N2B and N2BA titins (adapted from Labeit and Kolmerer; 1995 and Freiburg et al., 2000) and location of binding sites of antibodies used in this work (T12, Un, N2B, Uc, I84–86, I109–111). Two N2BA splice pathways are shown, representing the pathway with the lowest (12) and highest (25) number of Ig domains identified so far (from Freiburg et al., 2000). *Red:* Ig domains; *white:* fibronectin domains; *yellow:* PEVK segment; *blue:* unique sequence.

the Ig repeats N2B-I26-I27 (see Freiburg et al., 2000). The N2B antibody was raised against part of the unique N2B sequence (for details see Helmes et al., 1999). The N2A antibody was raised against two of the Ig domains that make up the N2A element (I80–81); antibody I84–86 was to the nucleotide positions 13978–14826 of the human cardiac titin data library entry (GenBank accession no. X90568). I109–111 was raised against Ig/FN3 repeats I109–111. For further details see Freiburg et al. (2000) and Trombitás et al. (1999).

Immunoelectron microscopy (IEM)

Muscle strips were dissected from the wall muscle of bovine left atrium and rat left ventricle. The strips were skinned overnight in relaxing solution with 1% Triton X-100 and were then extensively washed with relaxing solution. To prevent a reduction in passive tension as a result of titin-degradation, all solutions contained protease inhibitors (Granzier and Irving, 1995). The strips were stretched to different lengths, held in that state for 2 min, and then fixed, immunolabeled, embedded, and processed for EM as explained in Granzier et al. (1996) and Trombitás et al. (1999). The Z-line-to-epitope distances were measured according to Trombitás et al. (1999). In the case of the broad N2A-labeled region (see Results) the middle of the region was taken as the epitope location.

Gel electrophoresis

Muscle samples were quick-frozen in liquid nitrogen, pulverized, and then rapidly solubilized (Granzier and Irving, 1995). The samples were analyzed with SDS-PAGE (Granzier and Wang, 1993). After electrophoretic separation the gels were stained with 0.1% Coomassie Blue G250 (Neuhoff et al., 1988). Wet gels were scanned and analyzed as explained in Cazorla et al. (2000).

Calculations

Measured segment extensions (z) were divided by the segment's contour length (L) to obtain fractional extensions (z/L). The z-SL and z/L-SL relations were fit with an exponential equation: $A - Be^{-CxSL}$; where A, B, and C are constants and SL is sarcomere length (μm). The fitted z/L-SL relations were used to compute the entropic force-SL relation using the wormlike chain equation:

$$F(pN) = \frac{k_B T}{A}\left(\frac{z}{L} + \frac{1}{4(1 - z/L)^2} - \frac{1}{4}\right) \quad (1)$$

where k_B is Boltzmann's constant, T is absolute temperature, and A is the persistence length. The persistence length was assumed to be 15 nm for the

Ig segment and 1.5 nm for the PEVK segment (for details see Kellermayer et al., 1997).

RESULTS

Cardiac titin isoforms

SDS-PAGE of myocardium (Fig. 1 *A*) revealed two major T1 bands and a minor T2 band (a degradation product of T1). Freiburg et al. (2000) and Cazorla et al. (2000) recently identified the lower-molecular-weight T1 band as N2B titin (containing the N2B sequence element) and the higher-molecular-weight T1 band as N2BA titin (containing both the N2B and N2A elements). Based on studies of human and rabbit myocardial titin transcripts, N2BA titin contains a long PEVK segment (~600–800 residues) and a variable number of Ig domains that make up a middle Ig segment (Freiburg et al., 2000). The results of Fig. 1 *A* indicate that rat left ventricular (LV) myocardium expresses predominately N2B titin and bovine left atrium (LA) predominately N2BA titin. Consistent with this notion are EM results of rat LV and bovine LA doubly labeled with N2B and N2A antibodies. Rat LV reveals only one main epitope in the I-band region of the sarcomere, while the bovine LA reveals two epitopes (Fig. 2 *A*). Labeling with a single N2B or N2A antibody (results not shown) indicated that the rat LV epitope is derived from the N2B antibody, and the bovine near Z-line and near A-band epitopes from the N2B and N2A antibodies, respectively. It is also worth noting that the bovine experiments with the N2A antibody revealed in the I-band a relatively broadly labeled region that sometimes appeared as two closely spaced epitopes (data not shown). It is unlikely that the broadly labeled region results from nonspecific binding because the phenomenon has not been observed in human soleus fibers (Trombitás et al., 1998a). Rather, the bovine LA may co-express N2BA isoforms that differ slightly in size.

In summary, SDS-PAGE and IEM experiments suggest that rat LV and bovine LA express predominately N2B and N2BA titin, respectively, making these myocardial specimens ideal for a comparison of the extensibility of N2B and N2BA titins.

Marking titin's subsegments

Examples of immunolabeled sarcomeres are shown in Fig. 2. Although spotty labeling was sometimes seen, well-defined stripes were typically present. These stripes allowed us to measure the distance from each epitope to the center of the Z-line. Results of all epitopes are shown in Fig. 3. The lengths of the Ig segment, PEVK segment, and unique sequence were measured as the distance between epitopes that demarcates their ends. Considering that the exact binding sites of the antibodies within the antigen that was used to raise them are unknown, a slight uncertainty exists as to the exact molecular make-up of the segments marked by two antibodies. For both isoforms we assumed (Fig. 1 *B*) that the proximal and distal Ig segments are between T12 and Un, and between I84–86 and I109–111, respectively. The unique N2B sequence is demarcated by Un and Uc. We assumed that the PEVK segment is between Uc and I84–86 for the N2B isoform, and between N2A and I84–86 for the N2BA isoform. The middle Ig segment of the N2BA isoform is assumed to be between Uc and N2A.

Ig segments

In the rat, the length of the proximal Ig segment increased continuously as the slack sarcomere was extended, while the distal Ig segment showed an initial steep increase in length followed by a more gradual one (Fig. 4 *A*). In the cow, extension of the proximal Ig segment also increased continuously with SL (Fig. 4 *B*), but the increase was less steep than in the rat (Fig. 4 *A*, *solid* and *broken lines* for rat and cow, respectively). This study is the first to investigate extension of the middle Ig segment of the N2BA isoform. The inset of Fig. 4 *B* shows a bovine sarcomere labeled with Uc and N2A to demarcate this middle segment. The length of the middle Ig segment initially increased with SL from near zero at an SL of ~1.8 to 0.08 μm at an SL of ~2.5 μm, while at longer SLs the increase was minimal (Fig. 4 *B*). Note that the final length of the middle Ig segment is similar to that of the distal Ig segment. The combined lengths of the all-Ig segments of cow and rat are shown in Fig. 5 *A*. Results indicate that the total end-to-end length of the Ig segments is similar in rat and cow (the possible explanation for their similarity is discussed below).

The total Ig segment lengths were converted to fractional extensions by dividing the measured end-to-end lengths by the contour length (L). The value of L was calculated by assuming that Ig domains are folded and that the spacing of folded Ig domains in a fully extended segment is 5 nm (Trombitás et al., 1998a). For the rat the obtained total L of Ig segments is 185 nm (37 $\times$ 5). For the cow, we assumed that the middle Ig segment had an L of 120 nm (based on its extensible behavior that is similar to that of the distal Ig segment: Fig. 4 *B*) and the total Ig segment L was 305 nm (185 + 120). The obtained fractional extension versus SL relations of rat and cow are shown in Fig. 6 *A*. Along the full SL range studied, the fractional extension is less in the cow than in the rat.

Unique sequence of the N2B element

The end-to-end length of the unique N2B sequence is at a given SL much longer in rat than in cow (Figs. 2 *B* and 5 *B*). To obtain fractional extensions, end-to-end lengths were normalized with a contour length of 220 nm (572 residues $\times$ 0.38 nm), which assumes that the entire length of

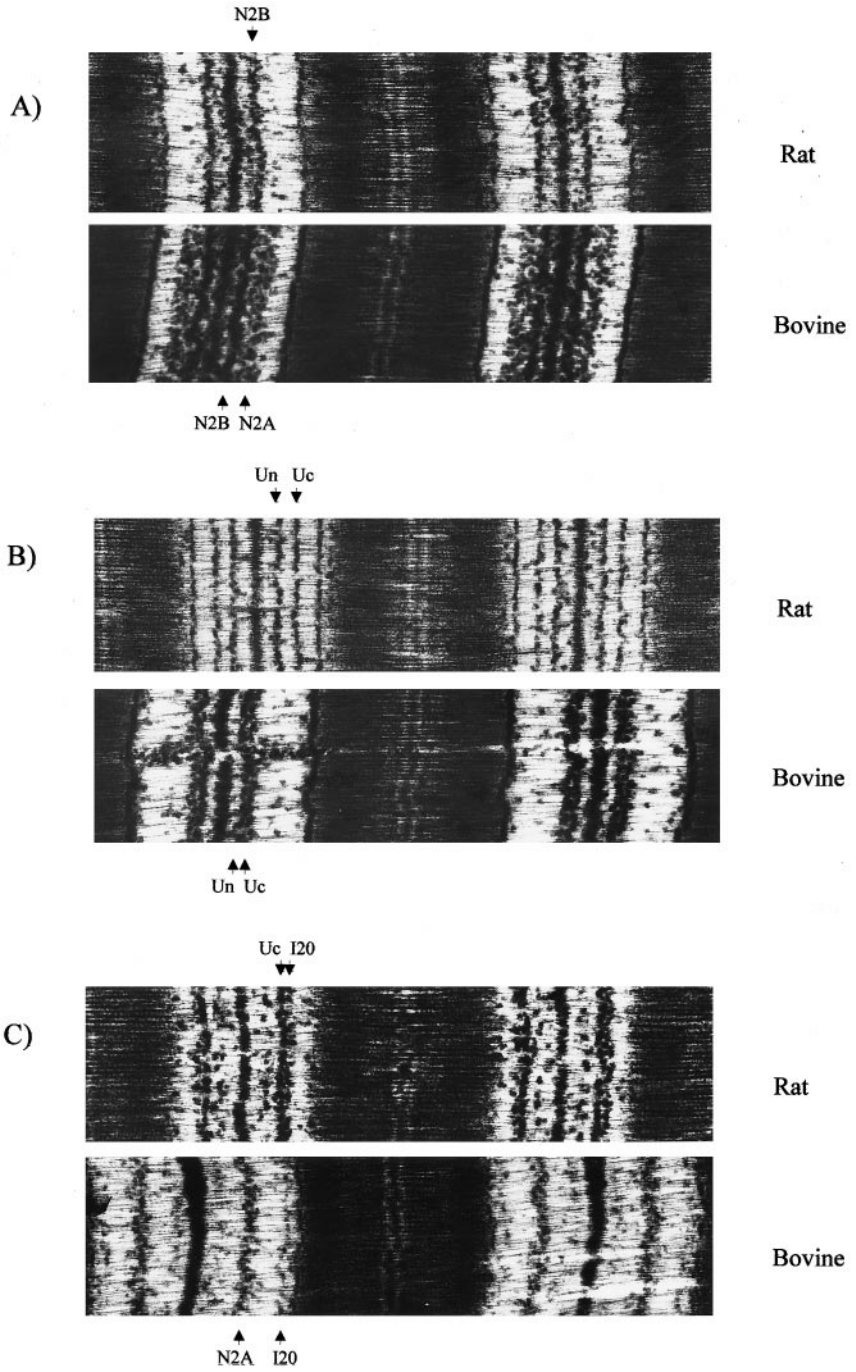

FIGURE 2 Examples of rat (LV) and cow (LA) cardiac sarcomeres labeled with anti-titin antibodies. (*A*) Sarcomeres labeled with both N2B and N2A antibodies. In rat only the N2B antibody labels, while in cow both the N2B and N2A antibodies label the sarcomere (as deduced from experiments with a single antibody). (*B*) Sarcomeres labeled with both Un and Uc antibodies. Un and Uc demarcate the unique N2B sequence. Results show that the unique sequence is much longer in rat than in cow. (*C*) Sarcomeres labeled with Uc and I84–86 (*top*) and N2A and I84–86 (*bottom*) to demarcate the PEVK segment length of rat and cow, respectively. The PEVK segment is much longer in the bovine sarcomere. (The near A-band labeling of some of the sarcomeres results from the I109–111 antibody that was used in some experiments.) Scale bar: 1.0 μm.

the N2B unique sequence is unfolded. Fig. 6 *B* indicates that the fractional extension increases with SL much more steeply in rat than in cow. For example, at an SL of 2.3 μm the segment reaches ~50% of its contour length in rat, but only 20% in cow.

PEVK segment

Upon stretching slack sarcomeres, the length of the bovine PEVK increases more steeply than that of the rat PEVK (Figs. 2 *C* and 5 *C*). The fractional extension of the PEVK segment was calculated by assuming that *L* is 60 nm in rat and 300 nm in cow (based on 163 PEVK residues in the rat and 800 residues in the cow, and assuming a 0.38 nm residue spacing). At a given SL the end-to-end length of the PEVK in the cow is much longer than in the rat (Fig. 5 *C*); however, due to the differences in *L* the fractional extension of the PEVK segment is less in the cow than in the rat (Fig. 6 *C*). For example, at an SL of 2.3 μm the segment reaches ~40% of its contour length in cow and ~90% in rat.

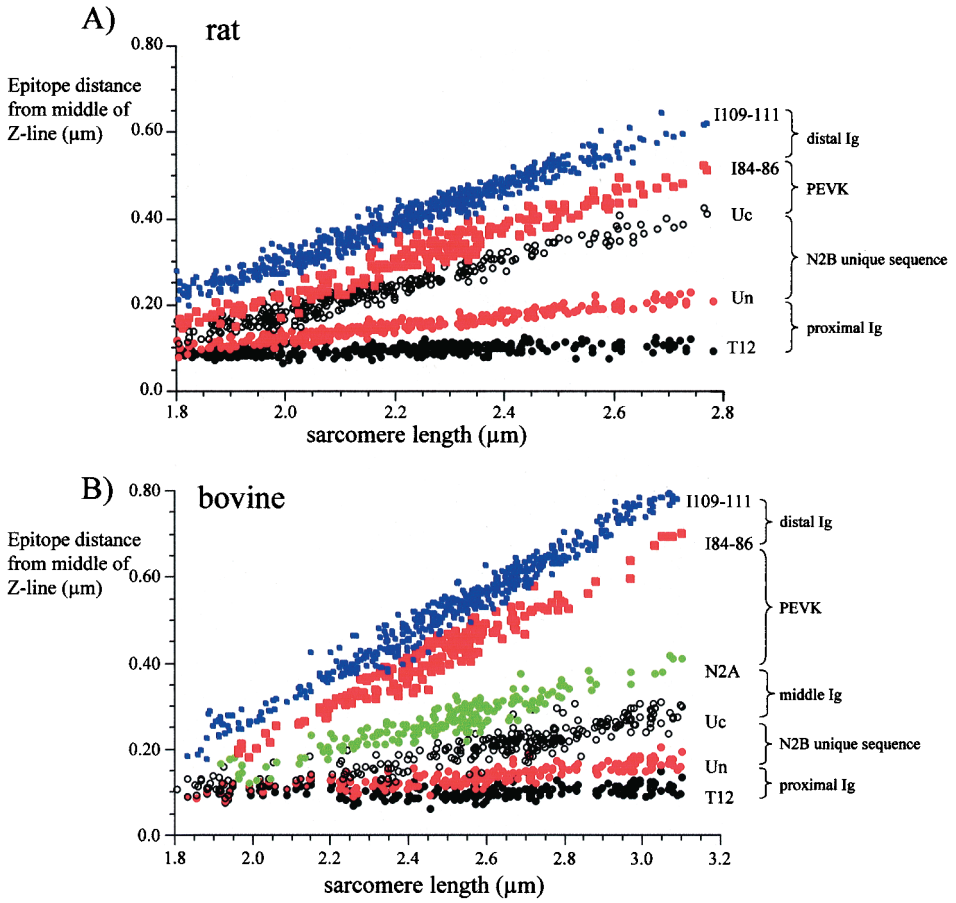

FIGURE 3 Scattergram of distances from epitopes to middle of Z-line versus SL.

DISCUSSION

It was recently reported that mammals co-express N2B and N2BA titins and that the N2BA expression level varies from low in myocardium of rodents to intermediate in dog, and high in pig, cow, and human (Cazorla et al., 2000). Furthermore, the expression level of N2BA titin was found to correlate inversely with the passive stiffness of the cardiac myocyte. In this work we investigated the extensibility of N2BA titin and compared it to that of N2B titin. Results indicate that the fractional extension-sarcomere length relations of Ig, PEVK, and N2B unique sequence all increase less steeply in N2BA titin than in N2B titin. Below we discuss how these findings may explain the variation in the passive stiffness of cardiac myocytes that express different titin isoforms.

I-band sequence of N2B and N2BA titins

The enormous size of titin has so far precluded sequencing of titins of many different species, and the I-band sequences of N2B and N2BA cardiac titins have mainly been studied in human and rabbit (Labeit and Kolmerer, 1995; Freiburg et al., 2000). Considering the finding that both human and rabbit myocardium do not represent pure isoform systems (Cazorla et al., 2000), we worked in this study with rat LV and cow LA because they express predominately N2B and N2BA titins, respectively (Fig. 1 *B*). We assumed that their titin sequences are identical to those reported for rabbit and human cardiac titins. Titin sequences are well conserved in human and rabbit (Freiburg et al., 2000) while a study that examined the N2B PEVK sequence in a wide range of species (Berri et al., 1997) also reported limited species variation. Thus, the assumption that the titin sequences of rat and bovine N2B and N2BA titins can be based on those of rabbit and human is reasonable.

Extensibility of N2B titin

It was found (Fig. 5) that all three segment types that make up the extensible region of N2B titin in rat (Ig segments, the unique N2B sequence, and the PEVK segment) extend along the physiological SL range (SLs $<\sim 2.3$), in agreement with recent findings in mouse and rabbit (Linke et al., 1999; Trombitás et al., 1999). At the maximal SL of this

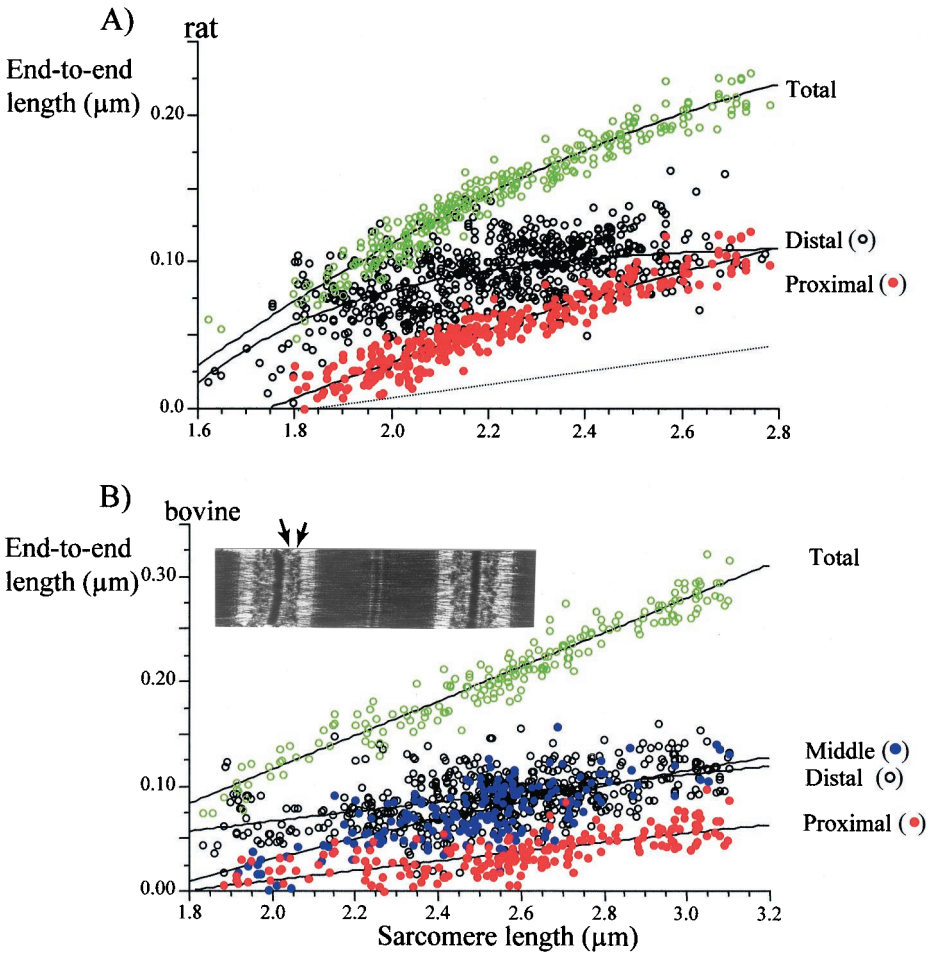

FIGURE 4 Extensible behavior of Ig segments. (*A*) Results obtained from rat LV. The length of the proximal Ig segment increases continuously along the full SL range studied. The solid curve represents the fit (see Methods) to the rat data; the broken line is the fit of the proximal Ig segment of cow (see below). The distal Ig segment length of rat initially increases rapidly, and then more slowly. Total: sum of the proximal and distal segments. (*B*) Results obtained from cow LA. The length of the proximal Ig segment increases continuously along the full SL range studied, while the lengths of the distal and middle Ig segment initially increase rapidly and then much more slowly. Total: sum of proximal, middle, and distal segments. The inset shows an example of a sarcomere labeled with Uc and N2A (near Z-line and near A-band epitopes, respectively) and reveals the length of the middle Ig segment. See text for additional details.

study (2.8 μm) the length of the distal Ig segment is ~110 nm in the rat. This value corresponds to an average domain spacing of ~4.5 nm, a distance that can likely be accommodated by a folded domain and its short linker sequence (Trombitás et al., 1998b). However, the proximal Ig segment (15 domains) extends continuously, and at an SL of 2.8 μm its length is ~110 nm, or ~7 nm per domain. This average spacing is significantly larger ($p < 0.05$) than the 5-nm spacing that can be accommodated by folded domains only. It is possible that the ~7 nm domain spacing results from the presence of a few unfolded domains in the segment (an unfolded domain spans ~30 nm). Alternatively, the long spacing may reflect extensibility of long linking sequences between domains in the proximal Ig segment or of unique sequences contained within the segment demarcated by the T12 and Un antibodies (Fig. 1 *C*). Recently, novel exons located 5′ of the N2B sequence that code for several Ig domains and novel sequence elements have been identified in the titin gene (Freiburg et al., 2000). Whether these novel exons are expressed in the heart and contribute to titin's extensibility remains to be established.

Extensibility of N2BA titin

The recently discovered N2BA isoform contains both the N2B and N2A splice elements (hence its name), a middle Ig segment, and a PEVK segment severalfold longer than in N2B titin (Fig. 1 *B*). Considering that this isoform is expressed at high levels in the myocardium of large mammals (including human), it is important to understand its extensible properties. The LA of the cow expresses predominantly N2BA titin (Fig. 1 *A*), making it ideal for investigating this class of titin isoforms.

The extensibility of the unique middle Ig segment of N2BA titin is similar to that of the distal Ig segment (Fig. 4 *B*): both segments extend in sarcomeres between slack and ~2.5 μm, but extension is limited at longer SLs. The limited extension at long lengths (where passive forces are high: see Cazorla et al., 2000) suggests that under our experimental conditions the Ig domains of both segments are stable and resist unfolding. Our finding that the middle Ig segment extends to a maximal length that is similar to that of the distal segment suggests that the middle segment contains ~25 Ig domains, similar to the distal Ig segment.

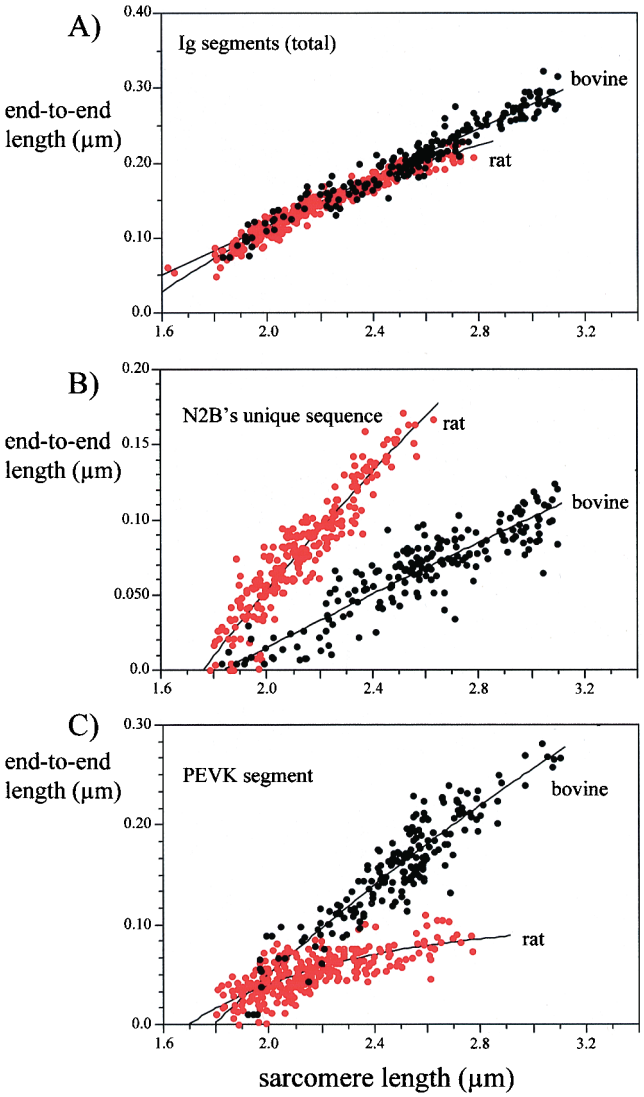

FIGURE 5 Sarcomere length dependence of end-to-end lengths of Ig segments (*A*), N2B's unique sequence (*B*), and PEVK segment (*C*). Data from rat are in red and those from cow are in black. See text for details.

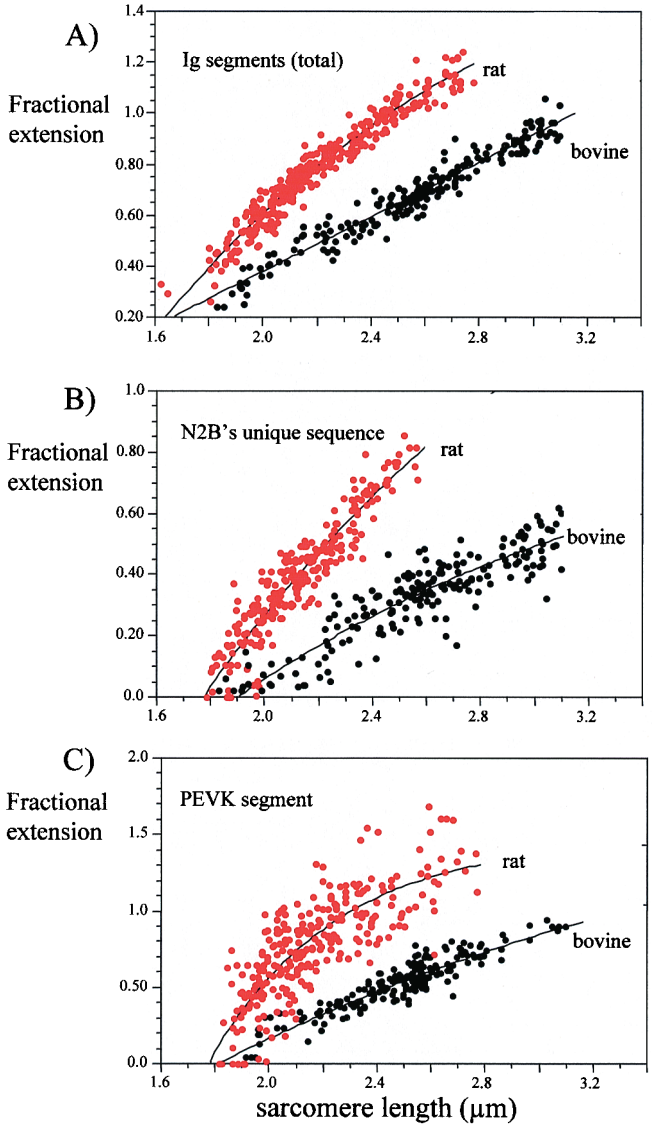

FIGURE 6 Sarcomere length dependence of fractional extension of Ig segments (*A*), N2B's unique sequence (*B*), and PEVK segment (*C*). Data from rat are in red and those from cow are in black. Note that for all three segment types the fractional extension is less for cow than for rat. See text for additional details. (Note: fractional extensions that exceed 1.0 may reflect an underestimation of the contour length due to imperfect demarcation of the segments with the available antibodies. In (*A*) these values may also reflect Ig domain unfolding. See text for details.)

Thus, the N2BA isoform expressed by the cow is likely to be one of the largest of the various N2BA titins identified so far (Fig. 1 *B*). The large number of additional Ig domains and the much longer PEVK segment of N2BA titin results in a much higher molecular mass (~300 kDa) than that of N2B titin, consistent with the lower mobility of N2BA titin on SDS-PAGE gels (Fig. 1 *A*).

The PEVK segment of N2BA titin continuously extends along the studied SL range and reaches ~280 nm at a SL of 3.2 μm (Fig. 5 *C*). This length is much longer that that of the N2B isoform (~60 nm), consistent with the higher number of residues contained within the PEVK segment of N2BA versus N2B titin (~800 vs. 163). Interestingly, the recently established PEVK behavior of rabbit N2BA titin (Linke et al., 1999) deviates from that of the cow. In the rabbit the PEVK length reaches a plateau of ~90 nm at an SL of ~2.6 μm with little extension at longer SLs (Fig. 5 *B* of Linke et al., 1999) while in the cow the PEVK continues to extend and is ~280 nm long at 3.2 μm SL (Fig. 5 *C*). The source for this discrepancy remains to be established. An explanation may be provided by the finding that the cow atrium expresses predominately N2BA titin (Fig 1 *A*), while in the rabbit myocardium the N2B isoform dominates (Cazorla et al., 2000). Possibly the extensible properties of N2BA molecules in the rabbit sarcomere may be influenced by the abundant N2B titins within its vicinity.

The extension-SL relations of the N2B unique sequence is shallower in N2BA than in N2B titin (Fig. 5 *B*). It is doubtful that this difference reflects differences in the contour length of the unique N2B sequence, as the maximal length to which this sequence extends is similar in cow and rat (~200 nm; results not shown). Instead, the longer contour length of the PEVK segment in N2BA titin may explain the results. Considering that the PEVK segment is more compliant than the N2B unique sequence (Trombitás et al., 1999) the additional extensibility provided by the longer PEVK contour length results in less stretch of the stiff unique sequence, explaining its more shallow increase with SL in bovine atrium. The extra extensibility of the long PEVK segment of N2BA titin also results in a lower fractional extension of the tandem Ig segments, explaining why their total extension is similar in N2A and N2BA titin (Fig. 5 *A*), despite the additional Ig domains of N2BA titin.

In sum, our data indicate that a major difference between N2BA and N2B titins is the extra source of extensibility in N2BA titin provided by its much longer PEVK segment. The long PEVK segment reduces the need to extend the stiffer unique N2B sequence. It will also increase the upper limit of the SL range within which Ig segments can operate without requiring Ig domain unfolding. The latter may reflect a longer end-diastolic SL in cow than in rat. The end-diastolic SL of the rat heart is likely to be ~2.3 μm (Grimm et al., 1980), consistent with the SL range along which rat trabeculae can be repeatedly stretch-released without inflicting damage to collagen (Kentish et al., 1986; Wu and Granzier, unpublished observation). The end-diastolic SL of the bovine heart has not been studied. Our preliminary studies indicate that bovine atrial muscle can be stretch-released to a SL of ~2.5 μm without inducing damage (Wu and Granzier, unpublished observation), suggesting that the in vivo working range extends to longer SLs in cow than in rat. The degree to which heart function is stable at SLs that exceed 2.3 μm (where thin and thick filament overlap is suboptimal) remains to be established. This issue, as well as the relation between differential expression of titin isoforms and end-diastolic SL, warrants further study.

Fractional extension and passive stiffness

An important conclusion of this study is that the fractional extensions of titin's subsegments are considerably lower in N2BA than in N2B titin (Fig. 6). These differences in fractional extension may explain the previously reported low passive stiffness of cardiac myocytes that express high levels of N2BA titin (Cazorla et al., 2000). The relation between factional extension and passive stiffness can be evaluated by considering the molecular mechanism of passive force generation. A passive force model has emerged in which the 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 high-entropy state (PEVK and Ig segments are "contracted") and upon sarcomere extension the springs straighten, lowering their conformational entropy and resulting in a passive force, known as entropic force. This passive (or entropic) force increases in a nonlinear fashion with the segment's fractional extension (see Eq. 1). It follows from the higher fractional extension in rat (Fig. 6) that at identical SLs passive forces are predicted to be higher in rat than in cow. This conclusion is further highlighted in Fig. 7, in which experimentally determined fractional extensions were used to derive predicted passive force-SL relations. Predicted titin-based passive force increases much more steeply with SL (i.e., passive stiffness is higher) in N2B titin than N2BA titin. These stiffness differences are consistent with those reported by Fabiato and Fabiato (1978) for myocytes from rat and dog, and by Cazorla et al. (2000) for myocytes from rat and pig, species that express high levels of N2B and N2BA titin, respectively. It is important to emphasize that titin is not the only element responsible for passive stiffness of cardiac muscle as collagen contributes as well, especially at long SLs (Kentish et al., 1986; Granzier and Irving, 1995). The degree to which differential expression of titin isoforms modulates overall muscle stiffness remains to be established.

In sum, this work provides a molecular basis for understanding the diversity in passive mechanical properties of cardiac myocytes. Plasticity in splicing of Ig and PEVK sequences results in isoforms with Ig and PEVK segments that vary in contour length. As a result, the fractional extension-sarcomere length relation of titin's extensible subsegments varies in different isoforms, and this gives rise to variation in passive stiffness.

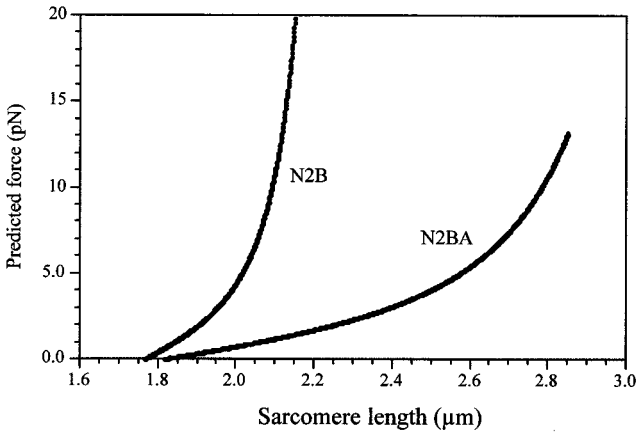

FIGURE 7 The predicted passive force-SL relation of a single titin molecule increases more steeply in N2B titin (rat) than in N2BA titin (cow). (The fits of the fractional extension-SL relations of Fig. 6 *C* were used to calculate the entropic force-SL relations of a single N2B or N2BA titin molecule. See Methods for additional details.)

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