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Gigantic variety: expression patterns of titin isoforms in striated muscles and consequences for myofibrillar passive stiffness

CIPRIAN NEAGOE[1], CHRISTIANE A. OPITZ[1], IRINA MAKARENKO[1,2] and WOLFGANG A. LINKE[1,*]
[1]*Institute of Physiology and Pathophysiology, University of Heidelberg, Im Neuenheimer Feld 326, D-69120 Heidelberg, Germany;* [2]*Permanent address: Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Russia*

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Abstract

The giant muscle protein titin has become a focus of research interests in the field of muscle mechanics due to its importance for passive muscle stiffness. Here we summarize research activities leading to current understanding of titin's mechanical role in the sarcomere. We then show how low-porosity polyacrylamide-gel electrophoresis, optimised for resolving megadalton proteins, can identify differences in titin-isoform expression in the hearts of 10 different vertebrate species and in several skeletal muscles of the rabbit. A large variety of titin-expression patterns is apparent, which is analysed in terms of its effect on the passive tension of isolated myofibrils obtained from selected muscle types. We show and discuss evidence indicating that vertebrate striated muscle cells are capable of adjusting their passive stiffness in the following ways: (1) Cardiomyocytes co-express long (N2BA) and short (N2B) titin isoform in the same half-sarcomeres and vary the N2BA:N2B ratio to adjust stiffness. Hearts from different mammalian species vary widely in their N2BA:N2B ratio; right ventricles show higher ratios than left ventricles. There is also a significant gradient of N2BA:N2B ratio in a given heart, from basal to apical; transmural ratio differences are less distinct. (2) Skeletal muscles can express longer or shorter I-band-titin (N2A-isoform) to achieve lower or higher titin-derived stiffness, respectively. (3) Some skeletal muscles co-express longer ($N2A_L$) and shorter ($N2A_S$) titin isoforms, also at the single-fibre level (e.g., rabbit psoas); variations in overall $N2A_L$:$N2A_S$ ratio may add to the fine-tuning of titin-based stiffness in the whole muscle. Whereas it is established that titin, together with extracellular collagen, determines the passive tension at physiological sarcomere lengths in cardiac muscle, it remains to be seen to which degree titin and/or extracellular structures are important for the physiological passive-tension generation of whole skeletal muscle.

Introduction

Towards understanding passive muscle elasticity

Already more than a century ago, vertebrate striated muscle was not only known to produce active forces during contraction but also to generate passive forces upon stretch (Roy, 1881; Brodie, 1895; Haycraft, 1904, and references therein). The passive length–tension curve of skeletal muscle was described as being nearly straight at low stretch forces but to increase in a highly nonlinear manner with greater loading (Roy, 1881; Haycraft, 1904). At the same time, it was already evident to contemporary physiologists that muscle in the non-activated (relaxed) state shows thermoelastic properties of the rubber-like kind (e.g. Roy, 1881, and references therein): the temperature of the relaxed muscle rises during stretch and falls during release. This behaviour contrasts that of activated muscle, which shows the kind of 'normal' elasticity that is associated with crystalline materials (Hill, 1938, 1952). Active muscle became the focus of many prominent scientists working in the field of muscle mechanics in the second half of the 20th century, but the last 10–20 years have seen a 'revival' of research interests in passive muscle elasticity – mainly thanks to the discovery of a 'third' set of intrasarcomeric filaments, apart from thin and thick filaments (Figure 1A). This 'third filament' was shown to be elastic and to consist (mainly of exclusively) of a protein initially described as connectin (Maruyama *et al.*, 1977a, b) and later named titin, because of its apparently gigantic size determined on polyacrylamide gels (Wang *et al.*, 1979). With the increasing acceptance of titin as a principal passive force-bearing element in the sarcomere it became necessary to re-evaluate earlier concepts (Hill, 1968; Fung, 1993) proposing weak actin–myosin interactions and extracellular elastic elements as sources of passive muscle elasticity.

In the first part of this paper we provide a short overview of past and present research related to titin

*To whom correspondence should be addressed: Tel.: +49-6221-544130; Fax: +49-6221-544049; E-mail: wolfgang.linke@urz.uni-heidelberg.de

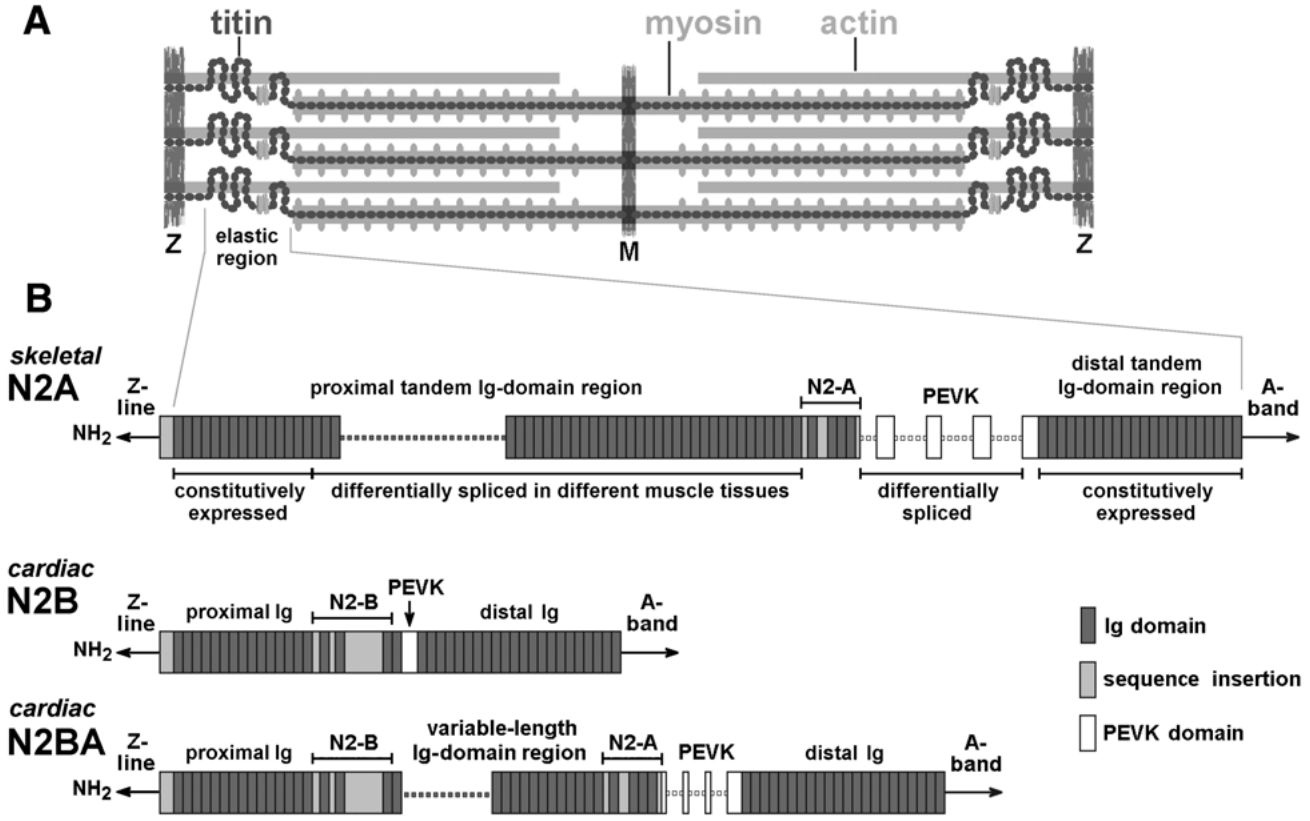

Fig. 1. Arrangement of titin filaments in the vertebrate sarcomere (A) and domain architecture of various isoforms of the elastic I-band titin section (B) according to Freiburg *et al.* (2000). Although single titin polypeptides (Mr ≥ 3 MDa) span the entire half of a sarcomere, only a molecular segment confined to the I-band is functionally elastic. The elastic region is made up of structurally distinct segments: stretches of tandem-Ig modules that adopt a stable beta-sheet fold, interrupted by unique sequences.

mechanics. The second, experimental part addresses the issue of titin isoform diversity in various heart and skeletal muscle samples; functional implications for passive muscle elasticity will be discussed.

Earlier elastic filament research

The presence of a 'third' set of sarcomere filaments was already recognized ~50 years ago, when Huxley and Hanson (1954) showed that actin and myosin extraction does not disintegrate the sarcomere; it remains held together by a 'ghost-like' set of longitudinal elastic filaments. The existence of these filaments was reinforced when fine protein strands, 2–4 nm in diameter, were found to span the gap between thick and thin filaments in sarcomeres stretched beyond actin–myosin overlap (Huxley and Peachey, 1961; Carlsen *et al.*, 1961; Sjöstrand, 1962). The term 'gap filaments' proposed for these structures is still used by some researchers today. Another term still in use is 'connecting (C-) filaments', initially described as linking Z-lines and thick filaments in insect flight muscle (Garamvölgyi, 1966; White and Thorson, 1973; Trombitas and Tigyi-Sebes, 1974). A general 'problem' with these elastic filaments was that their proximity to the thick and thin filaments made it difficult to reach firm conclusions about their disposition and function. However, single titin molecules of about 1 μm size could be isolated from muscle and visualised in the electron microscope (Maruyama *et al.*, 1984; Trinick *et al.*, 1984; Wang *et al.*, 1984; Nave *et al.*, 1989), suggesting that the titin molecule may span across half a sarcomere. Furthermore, immunoelectron microscopy of sarcomeres using titin-specific antibodies revealed that the molecular segment of titin that runs along myosin is inextensible, whereas the I-band segment extends upon stretch (Fürst *et al.*, 1988; Itoh *et al.*, 1988; Whiting *et al.*, 1989). Evidence for titin's important role in muscle extensibility and elasticity accumulated (Horowits *et al.*, 1986; Funatsu *et al.*, 1990; Wang *et al.*, 1991; Granzier and Wang, 1993; Trombitas *et al.*, 1993). Titin filaments also were proposed to be responsible for centering the A-band in the sarcomere during active contraction (Horowits and Podolsky, 1988; Horowits *et al.*, 1989). The A-band segment of titin was suggested to be a 'molecular ruler' that helps define the precise length of myosin thick filaments in the sarcomere (Whiting *et al.*, 1989). The relative importance of titin filaments, compared to extracellular elastic structures, in determining passive tension was estimated for frog skeletal muscle (Magid and Law, 1985) and rabbit cardiac muscle (Linke *et al.*, 1994). Studies of passive stiffness of isolated myofibrils indicated that most of this stiffness is unrelated to weak actin–myosin interactions; the main contributor to stiffness again was considered to be titin (Bartoo *et al.*, 1997). Nevertheless,

even after many details about titin structure and function became available, researchers within the muscle community still remained sceptical about the identity of this protein; after all, titin was estimated to have a molecular mass of about 3 MDa (Maruyama *et al*., 1984; Kurzban and Wang, 1988), and the idea of the existence of a single molecule of this huge size remained foreign to many.

Molecular structure of titin

Following partial sequence information (Labeit *et al*., 1990; 1992), the ultimate evidence for the existence of a giant titin polypeptide chain came with the report of the full-length sequence of human titin (Labeit and Kolmerer, 1995). With a molecular mass of ~3 to nearly 4 MDa, it is the largest protein known to date. Titin is, after myosin and actin, the third most abundant protein in vertebrate striated muscle (Labeit *et al*., 1997). Titin molecules have a modular architecture (Labeit and Kolmerer, 1995; Freiburg *et al*., 2000) and mainly consist of up to 300 immunoglobulin-like and fibronectin type-3-like (FN3) domains, which adopt a beta-sheet fold with 7–8 strands (Pfuhl and Pastore, 1995; Politou *et al*., 1995; Improta *et al*., 1996; Mayans *et al*., 2001). The A-band and I-band regions of titin, especially the M-line and Z-disk regions, are involved in various protein–protein interactions (reviewed by Trinick and Tskhovrebova, 1999; Clark *et al*., 2002; Granzier and Labeit, 2002), which give rise to important novel functions of the 'third' sarcomere filament (see below).

The I-band portion of titin contains the elastic region (Figure 1), although a 100 nm-wide segment adjoining the Z-disk is inextensible due to tight association with the thin filaments (Linke *et al*., 1997; Trombitas *et al*., 1997). I-band titin has Ig-domains but no FN3-domains and contains unique sequence insertions, most notably a segment rich in proline (P), glutamate (E), valine (V) and lysine (K) (Figure 1B). This 'PEVK domain' has a length of a few hundred to a few thousand amino-acid residues, depending on muscle type (Freiburg *et al*., 2000; Bang *et al*., 2001). The PEVK domain is flanked by a 'proximal' and a 'distal' tandem-Ig region; the latter is constitutively expressed in mammalian muscles (22 Ig-modules), the former varies in length in different muscles (Figure 1B). The muscle type-specific expression of variable-length PEVK-domains and proximal Ig-regions gives rise to different titin isoforms (Labeit and Kolmerer, 1995; Freiburg *et al*., 2000), which are alternatively spliced from a single gene (chromosomal location in humans, 2q31). Skeletal-muscle sarcomeres contain so-called N2A-titin isoform (Figure 1B), which is, for instance, 3.7 MDa in human or rabbit soleus muscle, but ~3.4 MDa in psoas muscle (Freiburg *et al*., 2000). Cardiac cells co-express short N2B isoform and longer N2BA isoform, which has structural elements from both the N2A and N2B isoforms (Figure 1B). The terms 'N2B isoform' and 'N2A isoform' arose because there are N2-A and N2-B segments of titin contained within the central I-band region (Figure 1B), which were speculated to colocalize with the N2-line of the sarcomere – an electron-dense band in the I-band sometimes visible on electron micrographs. The N2-B segment of cardiac titin contains another sequence insertion of substantial length (Figure 1B): a 572-residue 'N2B-unique sequence' (uN2B). N2BA and N2B titin isoforms are co-expressed in cardiac muscles at the level of the half-sarcomere (Linke *et al*., 1996; Trombitas *et al*., 2001; Neagoe *et al*., 2002). Interestingly, different species show dramatically different cardiac N2BA:N2B titin-expression ratios (Cazorla *et al*., 2000). The N2BA:N2B titin ratio can be modified in diseased human myocardium (Neagoe *et al*., 2002). Finally, a short 600–700 kDa titin isoform was recently described to be expressed in relatively low amounts in all striated muscles, and was named Novex-3 (Bang *et al*., 2001).

Diverse functional roles of titin

An increasing variety of functional roles has been ascribed to titin, which can be summarized as follows. (1) Titin provides the sarcomere with a structural framework through association with many other myofibrillar and cytoskeletal proteins (see reviews by Trinick and Tskhovrebova, 1999; Clark *et al*., 2002; Granzier and Labeit, 2002). (2) Titin may also serve to link myofibrils to surrounding membrane systems, as it interacts at the N-terminus with small ankyrin-1, a transmembrane protein of the sarcoplasmic reticulum (Kontrogianni-Konstantopoulos and Bloch, 2003). (3) Titin most likely is involved in signalling processes, because it binds to various proteins with roles in sarcomere assembly and turnover (Sorimachi *et al*., 1995; Mayans *et al*., 1998; Centner *et al*., 2001; McElhinny *et al*., 2002; Ma and Wang, 2002; Pizon *et al*., 2002), associates indirectly with metabolic enzymes important for energy supply to the contractile apparatus (Lange *et al*., 2002), and is phosphorylatable at various sites (Granzier and Labeit, 2002). (4) Titin may modulate active muscle contraction through yet little understood effects on the contractile proteins, actin and myosin (Fukuda *et al*., 2001; Kulke *et al*., 2001a; Muhle-Goll *et al*., 2001; Granzier and Labeit, 2002). (5) Titin is a main player in determining passive muscle mechanics: the protein develops passive tension in stretched nonactivated muscle (see recent reviews by Granzier and Labeit, 2002; Linke and Fernandez, 2002; Tskhovrebova and Trinick, 2002), shows high passive elastic-recoil speed (Minajeva *et al*., 2002) and helps to smooth out muscle contractions by contributing to the muscle's viscous/viscoelastic properties (reviewed by Linke and Fernandez, 2002). The following considerations will center around the analysis of titin's elastic behavior.

Titin-extension model

To understand the molecular events taking place upon stretching titin *in situ*, the pattern of extension of titin's molecular subsegments was analysed by immunolabeling and by force measurements on single cells or single

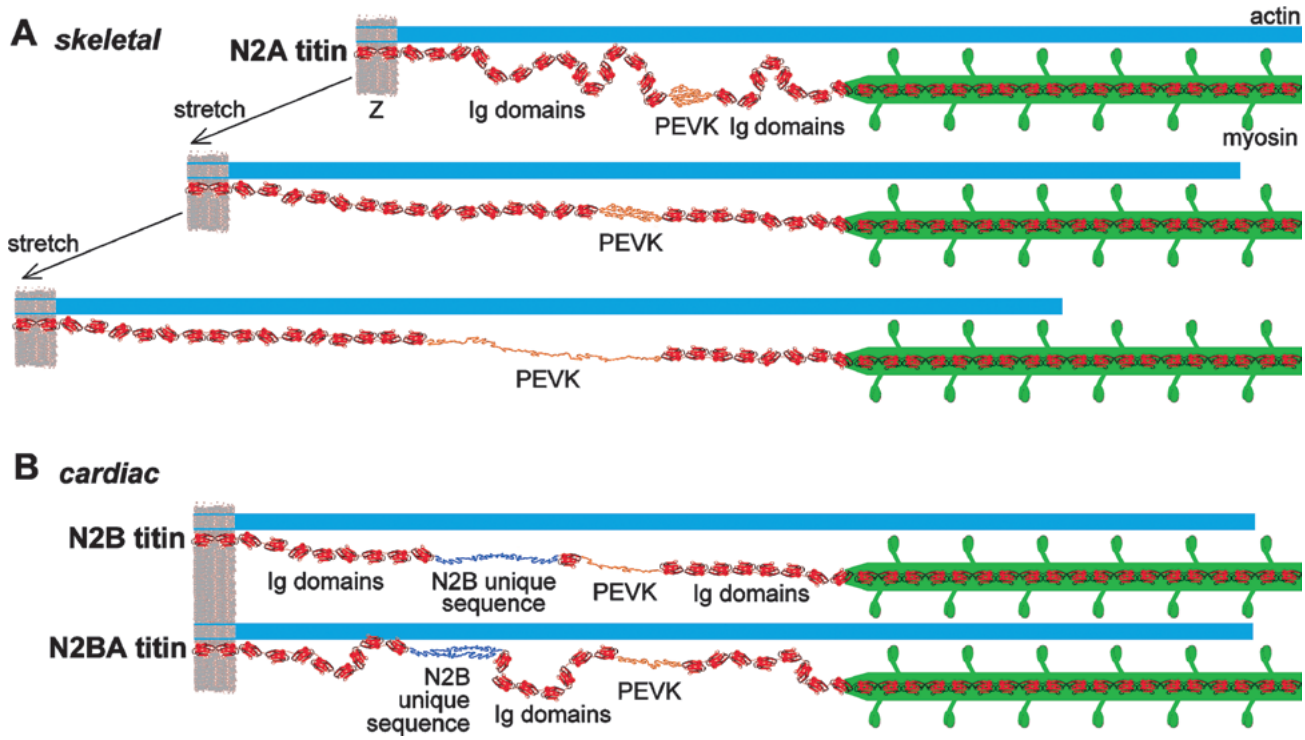

Fig. 2. Models for the extensibility of structurally distinct titin regions in the half-sarcomere. (A) Sequential extension of Ig-domain regions and the PEVK-domain in N2A-titin isoform of skeletal muscle (adapted from Linke *et al.*, 1996). Low stretch forces straighten the Ig-regions, higher forces extend the PEVK-domain. (B) In cardiac muscle, N2B-titin and N2BA-titin are co-expressed in the same half-sarcomere and are thought to extend independently. At a given SL, the shorter N2B-isoform is strained to a higher degree than the longer N2BA-isoform. Note that cardiac titin contains, in addition to Ig-regions and the PEVK domain, the extensible N2B unique sequence (after Linke *et al.*, 1999). The two cardiac titin isoforms are distinguished by the length of the PEVK domain (N2BA-PEVK has a lot more amino-acid residues than N2B-PEVK) and by the presence/absence of a differentially spliced block of Ig-domains in the middle I-band. For clarity, the models do not show the real number of titin modules known from sequence studies (cf., Figure 1).

myofibrils (Linke and Granzier, 1998). It was demonstrated that the tandem-Ig-domain regions extend mainly at low stretch forces, whereas the PEVK domain stretches predominantly at higher (physiological) forces (Figure 2A; adapted from Linke *et al.*, 1996). This kind of sequential titin-extension model for skeletal muscle was reported in a number of independent studies (Gautel and Goulding, 1996; Linke *et al.*, 1996; 1998a, b; Trombitas *et al.*, 1998). In overstretched muscle, titin may recruit more molecular segments to the elastic region: segments normally bound to the thick filament (Wang *et al.*, 1991; 1993) and the thin filament adjacent to the Z-disk. In cardiac muscle, the uN2B was shown to act as a third spring element that extends at higher physiological sarcomere lengths (SLs) (Linke *et al.*, 1999; Trombitas *et al.*, 1999). Figure 2B schematically shows a possible arrangement of parallel titin strands in a stretched cardiac half-sarcomere, based on immunolabeling data of Linke *et al.* (1999). Because of the co-expression of short and long titin isoforms in cardiac half-sarcomeres, the three spring elements (Ig-domain regions, PEVK domain, uN2B) may be strained to a higher degree in the N2B isoform than in the N2BA isoform (Figure 2B), at a given SL.

A still debated question is whether or not titin's Ig domains unfold when a sarcomere is stretched (Minajeva *et al.*, 2001). A likely scenario is that the proximal tandem-Ig-domain regions may see a very few of their modules unfold under physiological conditions, especially toward the high end of the physiological SL range, but the vast majority of Ig-domains will remain folded (Minajeva *et al.*, 2001; Li *et al.*, 2002; Linke and Fernandez, 2002). Unfolding–refolding of Ig-domains would be useful in that it can act as a shock absorber to handle damaging high forces acting on a muscle cell. In any case, it is evident that the interplay between the structurally distinct molecular segments within I-band titin provide striated muscle cells with unique elastic properties. Titins are finely tuned molecular springs capable of adjusting their mechanical properties to the particular requirements of the muscle.

Molecular basis of titin elasticity

The determination of titin's amino-acid sequence (Labeit and Kolmerer, 1995) paved the way for novel approaches to understanding the molecular basis of titin elasticity. State-of-the-art techniques are now used to explore titin elasticity at the single-molecule level. Studies using laser tweezers or the atomic force microscope (AFM) revealed a nonlinear force response upon stretching titin, which was explained in terms of polymer-elasticity theory (Kellermayer *et al.*, 1997; Rief *et al.*, 1997; Tskhovrebova *et al.*, 1997). Polymer elasticity in the low-force regime as occurs in a physiological

environment results predominantly from an entropic source. That is, a polymer chain has a tendency to retract inwards in order to maximize the number of permitted spatial conformations it can adopt, driven by thermal fluctuations of the surrounding solvent molecules. As for the titin molecules, the force–extension relationship could be described well with purely entropic models, such as the freely jointed chain (Flory, 1969) or wormlike chain (Marko and Siggia, 1995). These models were also used successfully to describe the elasticity of titin in the environment of the sarcomere (Granzier *et al*., 1997; Linke *et al*., 1998a, b, Trombitas *et al*., 1998; Helmes *et al*., 1999). Titin appears to show evidence for additional sources of elasticity, e.g. electrostatic enthalpy (Linke *et al*., 1998a), which may be incorporated into entropic models by the addition of a bulk modulus factor (Smith *et al*., 1996). The predominantly entropic spring-like behaviour of titin can explain, in principle, the century-old notion of rubber-like thermoelastic properties of passive muscle (Roy, 1881; Hill, 1952); stretching an entropic spring at higher temperature requires a slightly larger force than stretching the same spring at lower temperature. Although it seems valid to relate the macroscopic thermoelastic behaviour of passive muscle to the entropic-spring characteristics of titin, we note that technical obstacles have so far prevented direct experimental tests of the temperature dependency of the elasticity of isolated titin molecules.

The past years have seen great progress in single-molecule approaches to studying titin elasticity. Many details of titin's elastic behaviour have been revealed by AFM mechanics (e.g., Rief *et al*., 1998; Marszalek *et al*., 1999; Carrion-Vazquez *et al*., 1999; Oberhauser *et al*., 2001; Li *et al*., 2002; Linke *et al*., 2002; Watanabe *et al*., 2002), force measurements using optical tweezers (Kellermayer *et al*., 2001), electron microscopy of single titin molecules (Tskhovrebova and Trinick, 1997, 2001), and steered molecular dynamics simulations (e.g., Lu *et al*., 1998; Lu and Schulten, 2000; Gao *et al*., 2002; Fowler *et al*., 2002). A detailed description of these kind of experiments, which will not be discussed here, can be found in a special issue of this journal entitled '*Mechanics of Elastic Biomolecules*' (vol. 23, issue 5–6, 2002).

Titin isoform diversity and muscle stiffness

A main goal of this paper was to study an interesting aspect of titin structure/function: the variety of isoform expression in different muscle tissues and vertebrate species. We tested cardiac-muscle samples from altogether 10 different species for titin expression by low-porosity gel electrophoresis – a method we have optimized to increase the separation of protein bands in the high molecular-weight range (Kulke *et al*., 2001b; Anderson *et al*., 2002; Neagoe *et al*., 2002). We also investigated how titin-isoform composition (i.e., N2BA:N2B expression ratio) varies in the ventricles of goat and rabbit heart, from basal to apical, as well as across the ventricular wall. Further, we took a closer look at titin expression in several skeletal muscles of rabbit and report the presence of two distinct N2A-titin isoforms in some muscles (*psoas*, *tibialis anterior*). We show that two isoforms appear also in single *psoas* muscle fibres. Finally, we summarize results of mechanical experiments on single isolated myofibrils from *psoas*, *soleus*, and cardiac muscles and compare the passive length–tension curves of these samples against the background of titin-isoform expression. Although titin is now accepted to be a principal determinant of passive muscle stiffness, it is obvious that extracellular connective-tissue elements should not be ignored in their possible contribution to this stiffness. We argue that it is necessary to have a fresh look at the relative importance of cellular (titin) vs. extracellular (collagen, etc.) structures in determining passive stiffness in various mammalian skeletal muscle types.

Materials and methods

Sodium dodecyl sulphate polyacrylamide-gel electrophoresis

Low-percentage SDS-PAGE was employed (Linke *et al*., 1997; Neagoe *et al*., 2002) to investigate titin expression in cardiac muscles from various species and in different rabbit skeletal muscle tissues. Animal tissue was obtained from local slaughterhouses or, in the case of mouse, rat, rabbit, and frog (*Xenopus*), from the university's animal house, in accordance with institutional guidelines. Cat heart tissue was kindly provided by Dr Walter Herzog (University of Calgary, Canada). Human heart tissue was kindly provided by Dr Roger Hajjar and Dr Federica del Monte (Massachusetts General Hospital, Boston, USA; see Neagoe *et al*., 2002). SDS-PAGE was usually performed on muscle samples previously stored for various time periods at −80°C. Sometimes we also examined freshly excised muscle tissue.

Samples were solubilized by quickly homogenizing 30–60 mg of frozen tissue in 100 µl ice-cold relaxing buffer supplemented with 40 µg/ml leupeptin. Samples were centrifuged briefly and the pellet fraction was used for further analysis. Solubilisation buffer (1% SDS, 1% 2-mercaptoethanol, 10% glycerol, 8 µg/ml leupeptin, 6 µM bromphenol blue, 4.3 mM Tris–HCl, pH 8.8, 4.3 mM EDTA) was immediately added to the pellet, samples were incubated for 5 min on ice and then boiled (95°C) for 3 min. Total protein content in solubilised samples was determined spectrophotometrically. Lanes on gels were loaded with similar amounts of solubilised protein (usually, ~70–80 µg). We note that charging similar amounts of protein to each lane is important, because the loading of a lane can affect the apparent mobility of titin on these gels.

To detect high molecular-weight proteins, we used agarose-strengthened 2.0% polyacrylamide gels with a Laemmli buffer system (Tatsumi and Hattori, 1995).

Protein bands were visualised with Coomassie brilliant blue R. Gels were digitised and analysed for their optical density using TotalLab software (Phoretix, Newcastle upon Tyne, UK). 'Calibration gels' were prepared as described elsewhere (Anderson *et al.*, 2002; Neagoe *et al.*, 2002) to obtain calibration curves (Neuhoff *et al.*, 1990), which were then used to normalise the optical density per protein on each gel.

Myofibril mechanics

Cardiac or skeletal myofibrils to be used for force measurements were prepared as described (Linke *et al.*, 1996, 1997, 1999; Kulke *et al.*, 2001a; Neagoe *et al.*, 2002). Briefly, thin muscle strips were dissected in a solution containing (in mM): NaCl 132; KCl 5; EGTA 5; $MgCl_2$ 1; glucose 7; pH 7.1. The muscle strips were tied to thin glass rods and skinned in icecold rigor solution, containing (in mM): KCl 75; TRIS 10; EGTA 2; $MgCl_2$ 1 (pH 7.1) in the presence of 0.5% Triton X-100 for ≥4 h. To obtain individual myofibrils, the skinned strips were minced and homogenized in rigor solution. All solutions were supplemented with 40 μg/ml protease inhibitor leupeptin to minimize titin degradation.

Experimental apparatus

A setup for myofibrillar force measurements has been described (Linke *et al.*, 1997). Briefly, under a Zeiss Axiovert 135 microscope, a myofibril is suspended between micromanipulator-positioned glass needles attached to a piezoelectric micromotor (Physik Instrumente, Waldbronn, Germany) and a fibre optic-based force transducer (homebuilt) with nanonewton resolution. Attachment of the myofibrils is aided by coating the needle tips with a water-curing silicone adhesive (2:1 (v/v) mixture of Dow Corning 3145 RTV and 3140 RTV). Data collection and motor control were done with a PC, DAQ board, and custom-written LabView software (National Instruments, Austin, TX). Myofibril images were recorded with a colour-CCD camera (Sony), frame grabber, and Scion Image software (NIH, Bethesda, MD) or with a linear photodiode array, PC, DAQ board, and LabView algorithms (Minajeva *et al.*, 2002). Force data were obtained from preparations containing a maximum of three parallel myofibrils and were related to cross-sectional area inferred from the diameter of the specimens (Linke *et al.*, 1994, 1997).

Protocol

Passive force of nonactivated myofibrils was recorded (sampling rate, 1 kHz) in stretch protocols in which specimens were extended step-wise from slack SL. The stretch amplitude per step was 0.1–0.2 μm/sarcomere; a given stretch step was completed in less than 5 s, frequently within a few tens of milliseconds. Following each step, the specimen was held at a constant SL for 5–10 s, sometimes up to 2–3 min, to wait for stress relaxation. To test for possible shifts of force baseline, myofibrils were released back to slack SL.

Results and discussion

Species differences and disease-related variability of cardiac titin-isoform expression

Mammalian cardiac muscle co-expresses both short (N2B) and long (N2BA) titin isoforms in the same sarcomere (Linke *et al.*, 1996; Cazorla *et al.*, 2000; Trombitas *et al.*, 2001; Neagoe *et al.*, 2002). The N2BA:N2B ratios vary widely in different species, with a tendency for small mammals to have a low ratio and larger mammals to have a high ratio (Cazorla *et al.*, 2000). Another more general pattern is that the atrium has a much higher relative N2BA-titin content than the left ventricle (LV) and that sub-epicard tissue exhibits a somewhat lower N2BA:N2B ratio than sub-endocard tissue (Bell *et al.*, 2000; Cazorla *et al.*, 2000). Here we analysed the N2BA:N2B titin-expression ratios in different locations within the hearts of ten different vertebrate species. Figures 3 and 4 show the results of 2% SDS-PAGE analyses, which we have optimised to detect the 3–4 MDa isoforms of titin with an estimated resolution of 80–100 kDa (Neagoe *et al.*, 2002). Since there are no molecular-weight markers available in this range, we always loaded one or more lanes on each gel with rabbit soleus-muscle tissue (Figure 3A, left lane), for which titin's molecular weight is known from sequencing to be 3700 kDa (Labeit and Kolmerer, 1995). In comparison, the human N2B-titin isoform is predicted to have a molecular weight of 2970 kDa. Skeletal muscles also express the 700–800 kDa nebulin, which was used as an additional 'marker' for molecular-weight detection. The identity of the titin bands was verified, at least in rat and human heart, by Western blotting using isoform-specific antibodies (Figure 3B; also see Neagoe *et al.*, 2002).

Titin-isoform expression in the different tissues was quantified by densitometry and the percentage of N2BA-titin isoform, relative to N2BA + N2B isoforms, was determined. The numbers above the lanes in Figure 3A indicate the relative N2BA-titin content measured for each tissue type. The following conclusions could be drawn:

(1) The molecular size of the N2B cardiac-titin isoform is similar in all mammalian species investigated, around 3000 kDa.
(2) The molecular size of the N2BA isoform(s) is between ~3250 and ~3400 kDa, which is quite consistent with the range of molecular weights known from sequence analysis (3200–3350 kDa; Freiburg *et al.*, 2000). These results also confirm that the bands assigned N2B and N2BA are likely to represent full-length titin isoforms.

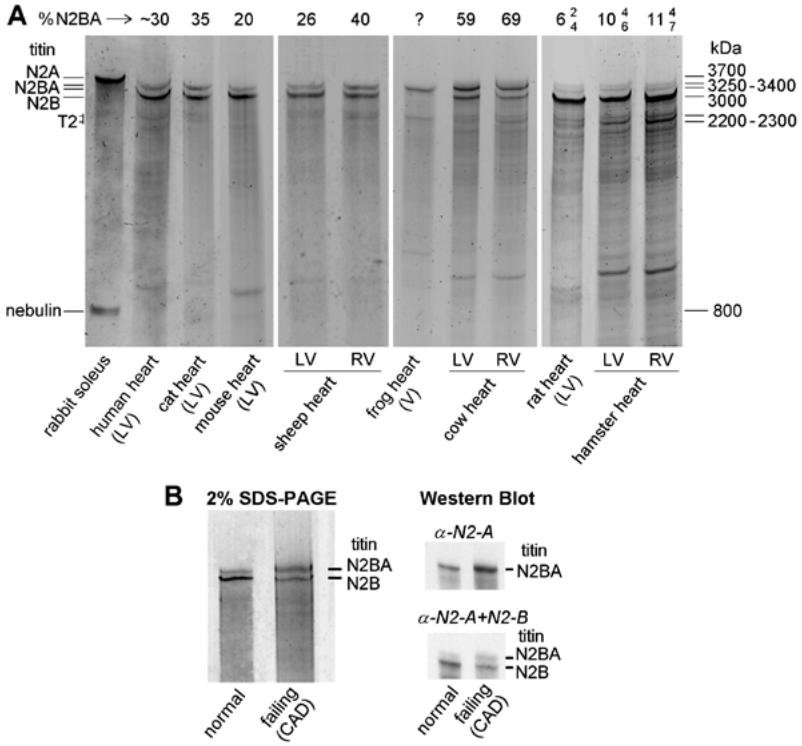

Fig. 3. 2% SDS-polyacrylamide gel electrophoresis to separate high molecular-weight proteins in heart tissue obtained from various species. (A) Different species express different ratios of N2BA:N2B cardiac titin. The percentage of N2BA titin-isoform content (N2BA + N2B isoforms = 100%) was calculated for each tissue type by densitometric analysis; percentage values are shown above each lane. For comparison of molecular weight, gels were also loaded with rabbit soleus protein, which has N2A-titin isoform of 3700 kDa (left lane). Note that hearts from rat and hamster show a faint doublet N2BA-titin band; the top band was of lower intensity than the bottom band. LV, left ventricle; RV, right ventricle; V, ventricle; T2, truncated forms of titin with a molecular mass of 2200–2300 kDa. (B) Examples for the differences in N2BA:N2B titin ratio observed between normal human hearts and long-term ischemic failing hearts from CAD patients (Neagoe *et al.*, 2002). The N2BA:N2B ratio shifts from ~30:70 in normal to ~50:50 in CAD hearts. Western blotting with sequence-assigned antibodies to N2-A titin or to all titin isoforms confirmed the shift.

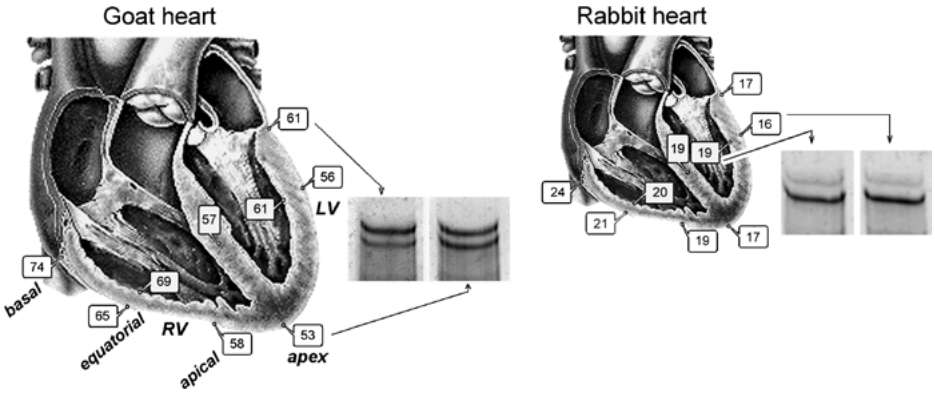

Fig. 4. Distribution of N2BA:N2B titin-isoform ratios in the LV and RV of goat and rabbit. Numbers show the average percentage of N2BA titin-isoform content (N2BA + N2B isoforms = 100%) for the particular locations indicated. For goat heart, a statistical comparison of values (presented as means ± SD) is given in Appendix A. Note the general gradient of decreasing relative N2BA-titin content from basal to equatorial to apical section planes. Sub-epicard tissue from the apex usually showed the lowest percentage of N2BA titin. RV has higher N2BA:N2B ratio than LV. Differences between sub-endocard and sub-epicard (equatorial plane) are less distinct. Rabbit heart shows much less variability than goat heart.

(3) The N2BA:N2B titin ratio varies greatly in these species; the percentage of N2BA-titin content in the LV increased in the following order (also see Figure 4): rat < hamster < rabbit < mouse < sheep < human < cat < goat < cow.

(4) The ratio between N2BA and N2B titin varies between LV and right ventricle (RV) of the same species (this comparison was made in hamster, rabbit, sheep, goat, and cow heart); RV was always found to have higher ratios than LV.

(5) Some species (rat, hamster) exhibit not a single but a doublet N2BA-titin band; in these cases, the lower N2BA band is stronger than the upper N2BA band (Figure 3A, right three lanes). The two faint N2BA bands were consistently detectable only when the gels were loaded with sufficient amount of protein and were destained modestly. Until now, a doublet N2BA-titin band had been reported only for bovine left atrium (Trombitas *et al*., 2001).
(6) Frog ventricle expresses only a single titin isoform, the identity of which (N2B, N2BA, N2A) remains to be determined.

Finally, titin 'T2' bands, which are usually considered to be proteolytic products of intact titin, were quite faint on these gels, indicating that our tissue preparation procedure caused little titin degradation.

These results confirm that the N2BA:N2B titin-isoform ratio is very different in the hearts of different species. Although there appeared to be a trend in that the relative N2BA-titin content increases with the size of the heart (or the size of the animal), a clear exception to this trend was the relatively high percentage of N2BA titin in mouse heart. Others (Cazorla *et al*., 2000) have also reported a slightly higher relative N2BA-titin content in mouse heart than in rat heart. A possibility is that the specially bred laboratory mice studied here show 'unusual' titin-expression patterns, compared to wild mice, but further investigation would be needed to resolve this issue.

Recent results from our laboratory demonstrated that titin, together with collagen, can be modified in response to chronic human heart disease (Neagoe *et al*., 2002). We found that end-stage failing hearts from coronary artery disease (CAD) patients exhibit N2BA:N2B titin ratios of nearly 50:50, which compares to an average ratio of ~30:70 in normal donor hearts (Figure 3B). A rat model of ischemia-induced heart failure (LAD clip) confirmed a shift toward higher relative N2BA-titin content (Neagoe *et al*., 2002). In human CAD hearts, the increased N2BA:N2B ratio caused decreased titin-derived myofibrillar passive stiffness, although whole hearts were globally stiffened due to upregulation of collagen and desmin expression. We hypothesize that the fibrosis-induced elevated global stiffness is partially counteracted by the titin modifications leading to greater compliance of myofibrils. Reduced myofibrillar passive stiffness could affect important mechanical functions of the heart, such as elastic recoil of stretched myofibrils (Minajeva *et al*., 2002) and stretch activation (Vemuri *et al*., 1999), and even compromise the Frank-Starling mechanism (Cazorla *et al*., 2001; Fukuda *et al*., 2001). In comparison, a canine model of pacing-induced heart failure showed a minor shift toward higher-than-normal N2BA:N2B titin ratios after 2 weeks of pacing (Bell *et al*., 2000), but a slight yet statistically significant shift in the opposite direction after 4 weeks of pacing (Wu *et al*., 2002). At this point, we thus conclude that higher (sometimes perhaps lower) than normal or unchanged (Neagoe *et al*., 2002) N2BA:N2B titin ratios may be found under different myocardial disease conditions. Follow-up studies are necessary to establish with higher certainty which disease condition causes what kind of titin modification.

Gradients of N2BA:N2B titin-expression ratio across the mammalian heart

In the present work, we took a closer look at the distribution of N2BA:N2B titin ratios in the ventricles of two species, goat and rabbit (Figure 4; Appendix A). This analysis revealed three main results:

(1) The percentage of N2BA titin (relative to N2BA + N2B) decreases in both the left and right free ventricular walls, from basal to apical. Such a gradient is less obvious in the smaller rabbit heart than in the larger goat heart (Figure 4). Basal-to-apical gradients were not investigated for the intraventricular septum. Appendix A lists mean values (±error estimates) for the percentage of N2BA titin in different regions of goat heart. Statistically significant differences between locations were determined by Student's *t*-test.

(2) Within the same section plane (basal, equatorial), RV always shows higher N2BA:N2B ratios than LV, thus confirming and extending the novel result of differential titin-isoform expression in left and right heart mentioned above (point 4.).

(3) Tests for possible transmural differences (equatorial plane) in N2BA:N2B ratio revealed a trend showing a slightly lower percentage of N2BA-titin in sub-epicard tissue than in sub-endocard tissue, in both RV and LV of goat heart (Figure 4, left). However, differences between sub-epicard and sub-endocard did not reach statistical significance (Appendix A). Almost no transmural differences were found in rabbit heart (Figure 4, right).

In summary, our detailed analysis of the distribution of N2BA:N2B titin-expression ratios in mammalian heart confirms minor transmural differences, at least in larger mammalian species (also see Bell *et al*., 2000), but points out much larger gradients from base to apex, in both LV and RV. These differences in titin isoform-expression ratio are probably related to differences in wall stress experienced by the respective locations. It should be interesting to correlate the distribution of titin isoforms in the different regions of the heart with that of stiff collagen fibres and extracellular connective-tissue material.

Some skeletal muscles express more than a single isoform of titin

Current concepts on titin-isoform expression in vertebrate muscles assume that heart tissue co-expresses N2B and N2BA isoforms, whereas a given skeletal muscle expresses a single N2A-titin isoform (Granzier and Labeit, 2002). We are aware of a few scattered reports about 'more than a single titin band in skeletal muscles' detectable by low-percentage SDS-PAGE (Wang *et al*.,

1991; Fry *et al*., 1997; Lindstedt *et al*., 2002). However, Wang *et al*. (1991) and Fry *et al*. (1997) assigned a second (lower) titin band on the gels 'T2' – a truncated form of the full-length polypeptide (e.g., Kawamura *et al*., 1995). Here we estimate the apparent molecular mass of T2-titin bands at 2200–2300 kDa (Figure 3). Because there is no full-length titin isoform of this size expressed by alternative splicing (Freiburg *et al*., 2000; Bang *et al*., 2001), T2 bands most likely represent proteolytic products of intact T1. In our hands, SDS-PAGE on adult striated muscle tissue generally shows only very faint T2 bands, unless titin is purposely degraded before or during the biochemical analysis. Lindstedt *et al*. (2002) reported two greatly different titin sizes in quadriceps (vastus) muscle of cow, dog, rabbit, and mouse, but did not address the issue of possible titin degradation during the preparation procedure. Their lower titin band probably corresponds to the T2-titin form, although a final conclusion cannot be made, since no comparison was made with titins of known size; titin molecular weight thus could not be determined. If titin-isoform sizes are to be compared on low-porosity polyacrylamide gels, we recommend – because of the lack of molecular-weight markers in this size range – to load a few lanes on each gel with tissue in which titin has been sequenced (rabbit or human soleus, rabbit or human heart). This is useful also because the skeletal nebulin band, plus the known cardiac N2B-titin and skeletal N2A-titin bands, can be used to generate calibration curves for molecular weight analysis (Kulke *et al*., 2001b; Anderson *et al*., 2002; Neagoe *et al*., 2002).

Here we studied a number of different skeletal muscles from rabbit – along with LV tissue – for titin-isoform expression by 2% SDS-PAGE (Figure 5A). Our analysis confirms and extends previous evidence (Wang *et al*., 1991) indicating that the titin isoforms of these muscles have a different size: *m. soleus* and *m. longissimus dorsi* express long 3700 kDa N2A-isoform, *m. tibialis anterior* and *m. psoas* express shorter (~3300–3500 kDa) N2A-isoforms; the latter compares well with the molecular weight predicted from sequencing, ~3400 kDa (Granzier and Labeit, 2002). However, our improved method of separating megadalton-sized protein bands (Neagoe *et al*., 2002) also revealed a surprise in that the previously reported single rabbit psoas-titin band (e.g., Freiburg *et al*., 2000) could be separated into two closely spaced single bands (Figure 5A, lanes 3–5). The difference in molecular weight between the two bands was estimated to be approximately 100 kDa. This seems to be too small to be resolved by 2.8% SDS-PAGE (Minajeva *et al*., 2002) or on the 2–7% SDS-polyacrylamide gradient gels prepared by others (Freiburg *et al*., 2000). The lower N2A-titin band is of higher intensity than the upper N2A-band in *m. psoa*s, whereas the opposite is true for rabbit *m. tibialis anterio*r, which also revealed two closely spaced N2A-titin bands (Figure 5A, lane 7). We append that two T1-titin bands with various ratios of long vs. short N2A-isoform appeared also in some skeletal muscles of other species, e.g., *m. psoas* of mouse and *m. extensor digitorum longus* (EDL) of *Xenopus* (data not shown). Thus, we conclude that expression of more than a single isoform of titin is not unique to cardiac muscle, but is also a property of some skeletal muscles.

Two N2A-titin isoforms are expressed in single rabbit psoas fibres

We wanted to know whether single fibres of rabbit psoas muscle still show the same doublet N2A-titin band. Fifteen different single psoas fibres were investigated by 2% SDS-PAGE; a representative result is shown in Figure 5A, lane 5. Clearly, at the single-fibre level, titin still appeared as a doublet band. The ratio between longer isoform ($N2A_L$) and shorter isoform ($N2A_S$) was analysed by densitometry in all single fibres studied, and was compared to that measured in chunks of psoas muscle tissue (mix of many fibres). Figure 5B shows that the $N2A_L$:$N2A_S$ expression ratio was indistinguishable in the two types of preparation. The relative content of $N2A_S$-isoform ($N2A_L + N2A_S = 100\%$) was 72.4 ± 3.0% (mean ± SD; $n = 5$) in tissue chunks and 70.5 ± 4.4% ($n = 15$) in single fibres. This result indicates that individual psoas fibres may have more or less the same isoform-expression ratio. Whether this result can be extended to other skeletal muscle types showing doublet N2A-titin bands, is a matter of future investigation. Also, it will be of interest to study whether or not there is a correlation between $N2A_L$:$N2A_S$ expression ratio and fibre type (fast, slow, etc.) composition.

Consequences of titin isoform expression for myofibrillar passive-tension development

Previous studies showed that there is a correlation between titin-isoform size and passive tension of a skeletal muscle, in single fibres (Wang *et al*., 1991; Horowits, 1992; Freiburg *et al*., 2000) and at the single-myofibril level (Linke *et al*., 1996; Linke, 2000; Kulke *et al*., 2001b). Differences in passive tension between cardiac, psoas and soleus myofibrils of rabbit are illustrated in Figure 6B. These data sets represent a summary of measurements of the force response of isolated myofibrils to stepwise stretch in relaxing buffer, recorded under quasi steady-state conditions (Figure 6A). Figure 6B compares steady-state passive tension of the different myofibril types over a wide range of SLs. Note that both types of skeletal myofibrils show a relatively long initial range of low passive tension, before the curves begin to increase more steeply. This is interesting in light of early reports describing two distinct phases of passive-tension development in whole skeletal muscle – a low force, nearly linear phase followed by a highly nonlinear rise in force (Roy, 1881; Haycraft, 1904). Apparently, these passive-tension characteristics can be found also at the level of the single myofibril.

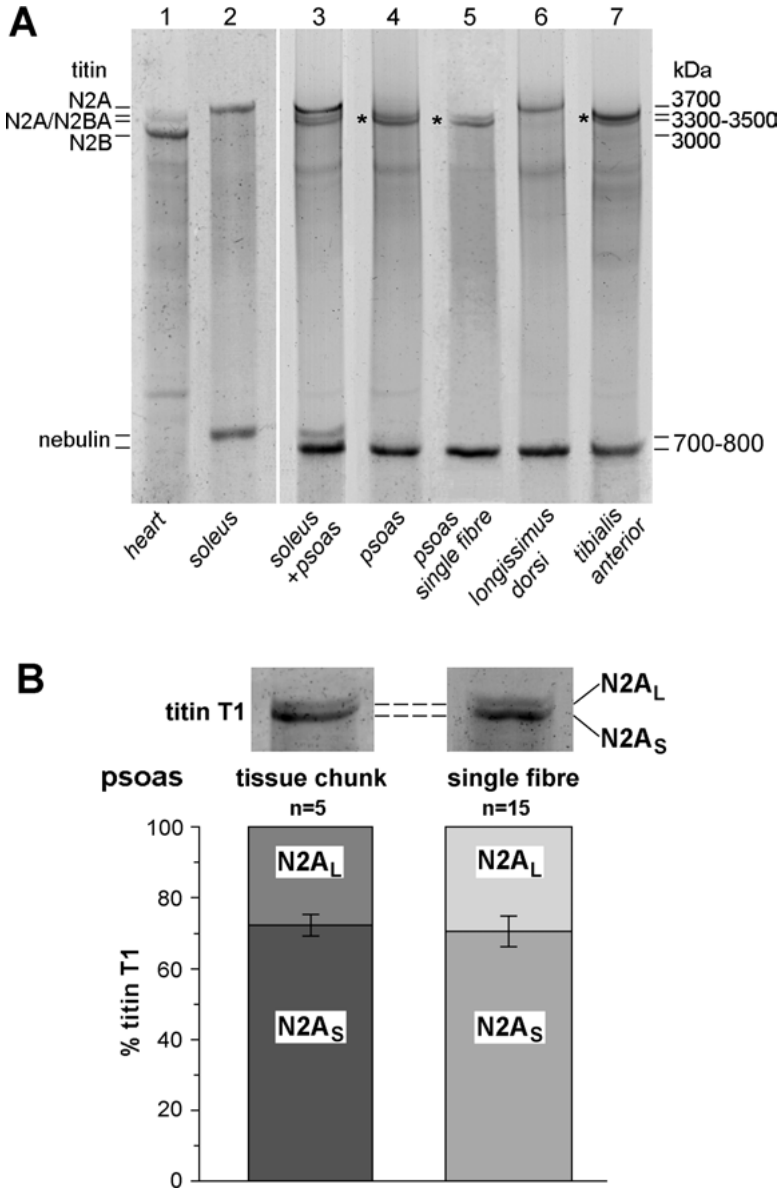

Fig. 5. 2% SDS-PAGE to analyse titin isoforms in different muscle types of rabbit. (A) The smallest isoform is N2B-titin in heart (left lane), ~3000 kDa , the largest titins are N2A-isoforms in *soleus* and *longissimus dorsi*, ~3700 kDa. Tissue obtained from *psoas* or *tibialis anterior* shows two titin isoforms of intermediate size (asterisks). The lower titin band is stronger than the upper band in *psoa*s, whereas the opposite is seen in *tibialis anterior*. Single *psoas* fibre (lane 5) contains a similar mix of N2A-titin isoforms as whole *psoa*s. Note that there is a larger nebulin isoform in soleus than in other skeletal muscles. (B) Analysis of N2A titin-isoform composition in chunks of *psoas* muscle (mix of fibres) and in single *psoas* fibres. $N2A_L$, longer N2A-isoform; $N2A_S$, shorter N2A-isoform. Results are mean ± SD. No statistically significant difference in $N2A_S$-titin content (relative to $N2A_L$ + $N2A_S$ content) was found between the the two types of preparation ($P = 0.39$, Student's *t*-test).

Relatively lower passive tension is seen in soleus myofibrils expressing 3700 kDa titin isoform than in psoas myofibrils expressing 3300–3500 kDa titin (Figure 6B). Thus, skeletal muscles can achieve lower or higher myofibrillar stiffness by expressing longer or shorter I-band-titin, respectively. The number of titin molecules in a sarcomere (which could also affect stiffness) is unlikely to vary greatly, due to stoichiometric constraints; there is evidence that the number of titin molecules per half thick filament is six (Liversage *et al*., 2001; Knupp *et al*., 2002). Finally, a novel potential mechanism to adjust titin-based stiffness to some degree follows from our finding that rabbit *m. psoas* (and other muscles) co-expresses shorter and longer N2A-titin isoforms (Figure 5); we tentatively propose that some skeletal muscles may be able to fine-tune their passive stiffness by varying the $N2A_L$:$N2A_S$ titin-expression ratio.

Compared to skeletal myofibrils, passive tension is much higher at any given SL in myofibrils from rabbit LV (Figure 6B). A somewhat shallower passive SL-tension curve is found in myofibrils from human LV. This result correlates well with the differences in N2BA:N2B titin-isoform ratio, ~17:83 in rabbit LV, but ~30:70 in normal human LV (see Figures 3 and 4, and Neagoe *et al*., 2002). The higher percentage of compliant N2BA titin in the myofibrils of human heart,

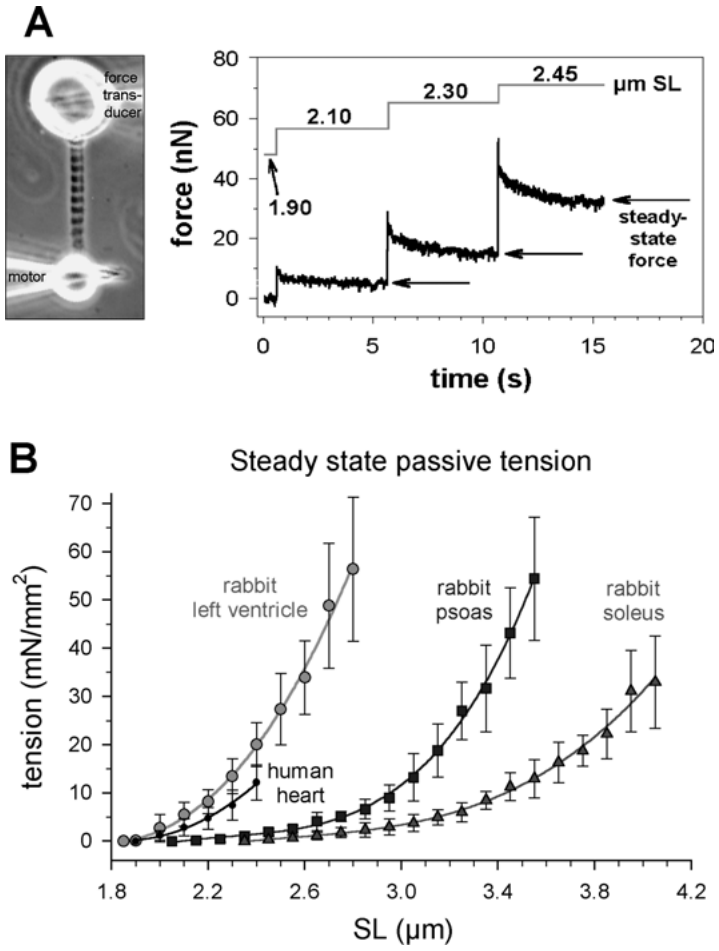

Fig. 6. Comparison of passive length–tension relationships of isolated myofibrils obtained from different tissue types. (A) Phase-contrast image of a small bundle of human heart myofibrils suspended between two glue-coated needle tips, one connected to a force transducer, the other to a micromotor (left). The diagram on the right illustrates the experimental protocol. For analysis we plotted the passive forces reached in a quasisteady-state, following stress relaxation. (B) Summary of results of passive-tension measurements on three different myofibril types from rabbit and on human heart myofibrils. The particular titin isoform(s) expressed determine the slope of these curves. For additional explanation, see text.

compared to rabbit heart, will shift passive tension to lower values. Again, this scenario works if the total number of titin molecules in the sarcomere (or the titin:myosin heavy chain ratio) is similar in the different heart tissues – a situation that has been confirmed experimentally (Cazorla *et al.*, 2000; Neagoe *et al.*, 2002). Thus, it can be generalized that cardiac muscles are capable of adjusting their titin-derived passive stiffness by co-expressing N2BA and N2B titin isoforms in the same half-sarcomere and varying the N2BA:N2B ratio. Because hearts from different mammalian species vary widely in their N2BA:N2B ratio (Figure 3), it can be expected that also the myofibrillar passive stiffness of these hearts exhibits large variation.

How does titin-based passive stiffness compare with the passive stiffness of whole muscles?

It is now established that in cardiac muscle, passive tension is determined principally by titin at short to intermediate physiological SLs, whereas at higher SLs it is the stiffness of extracellular collagen fibres that dominates passive-tension development (Linke *et al.*, 1994; Granzier and Irving, 1995; Wu *et al.*, 2000; Linke and Fernandez, 2002). Therefore, the passive SL-tension curves of single cardiac myofibrils increase less steeply than those of intact papillary muscles or intact trabeculae of the same species, at the high end of the physiological SL range (Linke *et al.*, 1994; Linke and Fernandez, 2002).

For whole skeletal muscle it is much less clear to what degree titin is responsible for the overall passive-tension level and to what degree extracellular stiff structures. A 'classical' paper by Magid and Law (1985) showed that the passive-force levels observed in single fibres are similar to those found in whole muscles. However, this study was performed on frog muscle preparations and it remains to be seen whether the authors' conclusion that the structures responsible for passive muscle stiffness reside mainly within the myofibrils, can be extended to mammalian skeletal muscle. Some puzzling result clearly indicating that the issue requires further investigation, comes from the observation that intact resting fibre bundles from slow-twitch soleus muscle (rat) have

higher passive stiffness than those from fast-twitch EDL muscle (Mutungi and Ranatunga, 1996). In contrast, isolated nonactivated soleus myofibrils are more compliant than any other myofibril type studied so far, including fast-twitch psoas myofibrils (Figure 6B). Therefore, expression of long I-band titin causing low myofibrillar passive stiffness (as in soleus) may not necessarily mean low stiffness of resting intact muscle. A more likely scenario is that the relative importance of intracellular (titin) vs. extracellular (collagen, connective tissue, etc.) structures in determining the passive-tension level of whole muscle varies greatly between different muscles. Further, there is evidence that under unloading conditions, rat soleus muscle may exhibit changes in titin structure and/or elasticity (Kasper and Xun, 2000; Toursel *et al*., 2002; Goto *et al*., 2002). On the other hand, connective-tissue accumulation in skeletal muscles can be influenced by passive stretch and muscle activation, and immobilized soleus muscles show increased accumulation (Williams *et al*., 1988; Gajdosik, 2001). Thus, there might be a dynamic adjustment of the relative contribution of titin and extracellular material to passive stiffness under various physiological and pathological conditions (Reich *et al*., 2000; Gajdosik, 2001). Experimental proof of this hypothetical mechanism will require mechanical studies both at the single-cell/single-myofibril level and whole-muscle level. Understanding the proposed interplay between muscle cells and extracellular connective tissue will ultimately be important for improving training strategies and therapeutic interventions designed to optimise muscle function.

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Appendix A

Results of gel electrophoretic analyses (2% SDS-PAGE) of goat heart tissue. Data indicate percentage of N2BA titin (relative to N2B + N2BA, which is 100%) and are presented as mean ± SD (number of lanes analysed). No/yes indicates statistically significant differences between the different tissue locations at the $P < 0.05$ level (unpaired Student's *t*-test). LV: left ventricle; RV: right ventricle; Epi: sub-epicard; Endo: sub-endocard; mid: mid-wall.

	LV, epi, basal	LV, epi, equatorial	LV, endo, equatorial	Septum, mid, equatorial	RV, epi, basal	RV, epi, equatorial	RV, endo, equatorial	RV, epi, apical	Apex, epi
LV, epi, basal	61 ± 2(6)								
LV, epi, equatorial	No	56 ± 3(5)							
LV, endo, equatorial	No	No	61 ± 1(4)						
Septum, mid, equat.	No	No	Yes	57 ± 1(4)					
RV, epi, basal	Yes	Yes	No	Yes	74 ± 4(5)				
RV, epi, equatorial	No	Yes	No	Yes	Yes	65 ± 2(8)			
RV, endo, equatorial	Yes	Yes	Yes	Yes	No	No	69 ± 1(6)		
RV, epi, apical	No	No	No	No	Yes	Yes	Yes	58 ± 1(7)	
Apex, epi	Yes	No	Yes	No	Yes	Yes	Yes	No	53 ± 3(5)

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