Passive force generation and titin isoforms in mammalian skeletal muscle

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ABSTRACT When relaxed striated muscle cells are stretched, a resting tension is produced which is thought to arise from stretching long, elastic filaments composed of titin (also called connectin). Here, I show that single skinned rabbit soleus muscle fibers produce resting tension that is several-fold lower than that found in rabbit psoas fibers. At sarcomere lengths where the slope of the resting tension-sarcomere length relation is low, electron microscopy of skinned fibers indicates that thick filaments move from the center to the side of the sarcomere during prolonged activation. As sarcomeres are stretched and the resting tension-sarcomere length relation becomes steeper, this movement is decreased. The sarcomere length range over which thick filament movement decreases is higher in soleus than in psoas fibers, paralleling the different lengths at which the slope of the resting tension-sarcomere length relations increase. These results indicate that the large differences in resting tension between single psoas and soleus fibers are due to different tensions exerted by the elastic elements linking the end of each thick filament to the nearest Z-disc, i.e., the titin filaments. Quantitative gel electrophoresis of proteins from single muscle fibers excludes the possibility that resting tension is less in soleus than in psoas fibers simply because they have fewer titin filaments. A small difference in the electrophoretic mobility of titin between psoas and soleus fibers suggests the alternate possibility that mammalian muscle cells use at least two titin isoforms with differing elastic properties to produce variations in resting tension.

INTRODUCTION

Active force production in skeletal muscle results from the interaction of thick filaments containing myosin with thin filaments containing actin. In addition, these cells produce passive forces that resist stretch independent of ATP hydrolysis and actomyosin interactions. Most of this passive force, or resting tension, is now thought to arise from stretching long, elastic filaments composed of titin (also called connectin) (Horowits et al., 1986; Yoshioka et al., 1986; Horowits and Podolsky, 1987; Funatsu et al., 1990).

With a subunit molecular weight of 2.5–3 million daltons (Maruyama et al., 1984; Kurzban and Wang, 1988), the titin molecule is unusually large. Antibody labeling studies indicate that each titin molecule extends from the Z-disc to the center of the sarcomere (Wang et al., 1985; Furst et al., 1988; Itoh et al., 1988; Whiting et al., 1989). These studies showed that when the sarcomere is stretched, the region of the titin molecule found in the A-band generally behaves as if it were rigidly bound to the thick filaments. In contrast, the region of the titin molecule that links the thick filaments to the Z-disc behaves elasticly (Wang et al., 1985; Furst et al., 1988; Itoh et al., 1988; Horowits et al., 1989; Whiting et al., 1989).

As elastic elements linking each end of the thick filament to the Z-disc, the titin filaments are in a position to produce resting tension as well as to provide a force which tends to center the thick filaments within the sarcomere. Several independent lines of evidence indicate that titin performs both of these functions. First, degradation of titin and nebulin by ionizing radiation (Horowits et al., 1986) or by enzymatic digestion (Yoshioka et al., 1986; Funatsu et al., 1990) decreases resting tension, and also leads to axial misalignment of thick filaments (Horowits et al., 1986). In the case of enzymatic digestion, the decrease in resting tension parallels the degradation of titin, but not of nebulin (Funatsu et al., 1990). Second, it has recently been shown that thick filaments move away from the center of the sarcomere during isometric contraction in a manner which depends on sarcomere length (Horowits and Podolsky, 1987). The experimentally observed dependence of the extent of thick filament movement on sarcomere length, the observed time course of the movement, and the observed tension output during the movement were all found to be in quantitative agreement with a mechanical model in which all of the measured resting tension originates in elastic elements linking the ends of the thick filaments to the Z-disc; these observations were not consistent with a model in which these structures were omitted (Horowits and Podolsky, 1988). Finally, titin is the only known sarcomeric protein which is organized as an elastic link between thick filaments and Z-discs. Although nebulin was once a putative component of such a structure, recent evidence suggests that nebulin forms an inelastic

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structure in parallel with thin filaments (Wang and Wright, 1988), and hence cannot account for the mechanical properties of relaxed muscle fibers.

On the basis of variations in electrophoretic mobility (Hu et al., 1986; Wang and Wright, 1988; Akster et al., 1989) and antibody reactivity (Hill and Weber, 1986), several investigators have demonstrated that different striated muscles of a single animal can contain different isoforms of titin. Developmental variations in titin isoforms have also been observed (Yoshidomi et al., 1985). Meanwhile, previous studies from this laboratory have indicated that fibers taken from human biceps or quadriceps muscles (Horowits et al., 1990) exhibit much lower resting tension than rabbit psoas fibers (Horowits et al., 1986; Horowits and Podolsky, 1987). In this study, I show that rabbit soleus fibers also produce much lower resting tension that rabbit psoas fibers. I also present evidence that this difference in resting tension is accompanied by a difference in the form, but not in the amount, of titin found in psoas and soleus fibers.

METHODS

Fiber preparation and mechanical measurements

Strips of rabbit psoas and soleus muscle were chemically skinned (Wood et al., 1975) in a solution containing 150 mM potassium propionate, 5 mM KH_2PO_4, 3 mM magnesium acetate, 5 mM EGTA, and 3 mM Na_2ATP, pH 7.0, at 4°C. Isometric tension was measured from 1 to 6 mm long segments of single fibers at 7°C as previously described (Horowits et al., 1986; Horowits and Podolsky, 1987). The single fibers were connected to a leafspring force transducer (Hellam and Podolsky, 1969) and a fixed end using T-shaped aluminum foil clips (Goldman and Simmons, 1984). The solution bathing the fiber could be rapidly changed and its temperature thermoelectrically controlled using a bath similar to that described by Hellam and Podolsky (1969). Sarcomere length was monitored by laser light diffraction.

Resting tensions were measured 5 min after stretch in a relaxing solution containing 100 mM potassium propionate, 3 mM EGTA, 25 mM imidazole, 7 mM $MgCl_2$, 5 mM Na_2ATP, 15 mM Na_2-creatine phosphate, and 20 U/ml creatine kinase, pH 7.0. Active tension was measured at a sarcomere length of 2.4 μm in an activating solution containing 3.5 mM $CaCl_2$ added to the relaxing solution, yielding a final pCa of 4.5. The length and diameter of each fiber were measured by light microscopy while the fiber was immersed in skinning solution at a sarcomere length of 2.4 μm. Fiber diameter was taken as the mean of measurements made before and after rotating the fiber along its long axis by 90°. Fiber cross-section and volume were calculated assuming a cylindrical shape.

Gel electrophoresis

After measuring its mechanical properties and dimensions, each single fiber was dissolved in 4,000 volumes of buffer containing 10 mM Tris, 1 mM EDTA, 1% SDS, 10% glycerol, 70 mM β-mercaptoethanol, and 0.05% bromophenol blue, pH 8.0. Samples were boiled for 1-2 min and electrophoresed on a 2–12% gradient of polyacrylamide as described by Somerville and Wang (1981). Gels were silver stained using a commercially produced kit (DuPont, Boston, MA), and the protein bands quantitated using a Beckman DU-8 scanning densitometer (Beckman Instruments, Irvine, CA). For purposes of normalizing data obtained from different gels, actin peaks were calibrated by comparison with varying amounts of purified actin run on the same gel; all other peaks were calibrated by comparison with varying amounts of purified myosin run on the same gel. Because the intensity and coloration of silver staining varies between proteins, it should be noted that this method can yield an accurate absolute measure only for those proteins which are compared to purified standards of the same protein, in this case actin and myosin; measurements of other protein bands only yield relative values for comparison between samples (Merril et al., 1982; Nielsen and Brown, 1984). Therefore, the concentration of each protein found in both psoas and soleus fibers was expressed as a fraction of the mean value found in psoas fibers for that particular protein.

Electron microscopy

Small bundles of skinned fibers ranging from 0.1 to 0.3 mm in diameter or single skinned fibers were attached to T-shaped aluminum foil clips (Goldman and Simmons, 1984). For ease of manipulation, the single fibers or bundles were then pinned, via the foil clips, to small pieces of styrofoam. Each piece of styrofoam was notched so that a laser beam could be passed through the pinned fibers and their sarcomere lengths measured by laser light diffraction. The relaxed preparations were pinned in skinning solution at sarcomere lengths ranging from 2.6 to 3.2 μm, and then incubated in relaxing solution at 4°C for several minutes. They were then incubated in activating solution at 4°C for 20 min. Following this prolonged activation, the fibers were fixed for 2 h at 4°C in activating solution containing 2.5% glutaraldehyde. The fibers were postfixed for 30 min with 1% osmium tetroxide, dehydrated with ethanol, and embedded in Spurr (Polysciences, Inc., Warrington, PA).

Gold or silver longitudinal sections were cut with a diamond knife edge oriented parallel to the long axis of the fibers. The sections were stained with uranyl acetate and lead citrate, and viewed and photographed using a Philips EM400 electron microscope (Philips Electronic Instruments, Mahwah, NJ). Micrographs were calibrated by assuming that the mean A-band width was 1.6 μm (Huxley, 1963; Page and Huxley, 1963; Trinick and Elliot, 1979; Horowits and Podolsky, 1987). Previous work indicates that the relative dimensions of the A-band and I-band regions are well preserved by the chemical fixation technique used (Horowits et al., 1989). The extent of A-band movement away from the center of the sarcomere, expressed as a percentage of its maximum possible movement, was measured from electron micrographs as previously described (Horowits and Podolsky, 1987).

RESULTS

Mechanical properties of single skinned fibers and positional stability of thick filaments

Active and resting tensions were measured in single skinned fibers, as illustrated by the tension records in Fig. 1. The active tensions measured in rabbit psoas and soleus fibers at a sarcomere length of 2.4 μm were similar, averaging 0.96 ± 0.05 and 0.93 ± 0.08 kg/cm^2, respectively (means ± s.e.m.). In contrast, the relaxed psoas and soleus fibers exhibited dramatically different

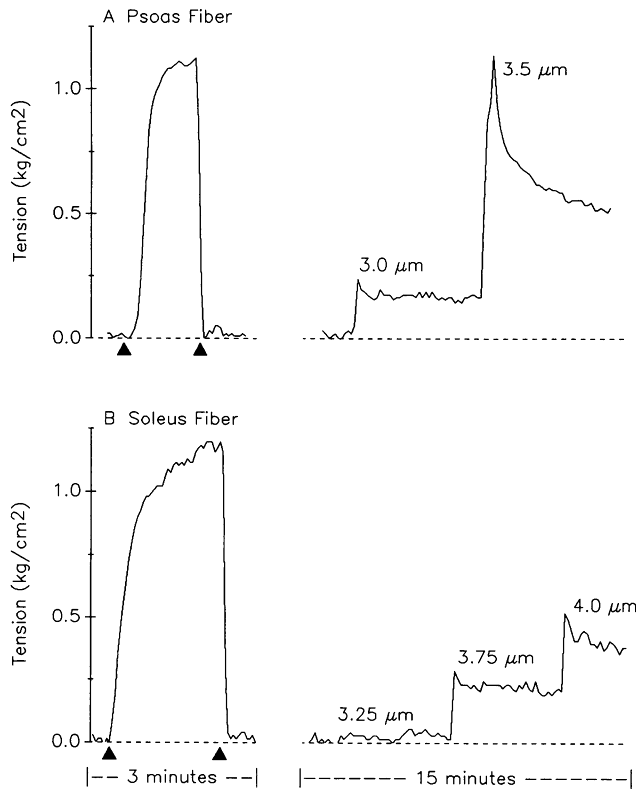

FIGURE 1 Typical tension records from single psoas (*A*) and soleus (*B*) fibers. The fibers were immersed in activating solution between the times indicated by closed triangles. After being returned to relaxing solution, the fibers were stretched to longer sarcomere lengths as indicated above the tension records. Original tension records were sampled every 3 or 9 s (left and right hand portions of the graphs, respectively) and redrawn by computer.

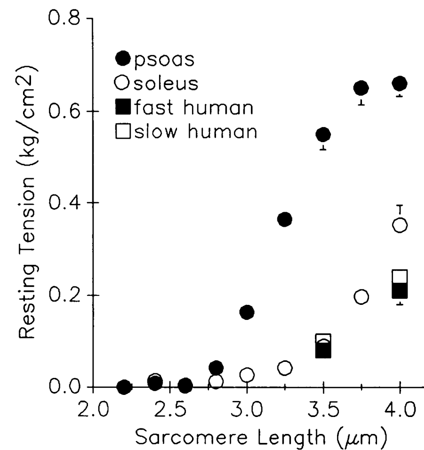

FIGURE 2 Resting tension vs sarcomere length. Each point represents the mean value from 4 to 6 single skinned rabbit psoas (●) or soleus (○) fibers. Error bars indicate one s.e.m. Data from single fast-twitch (■) and slow-twitch (□) human fibers is taken from Horowits et al. (1990).

levels of resting tension upon stretch to longer sarcomere lengths. At a sarcomere length of 3.0 μm, the psoas fiber in Fig. 1 *A* produced a resting tension that was ~15% of the maximum active force. In contrast, the soleus fiber in Fig. 1 *B* produced this level of resting tension only when stretched to much greater sarcomere lengths. The relationship between resting tension and sarcomere length is shown for both psoas and soleus fibers in Fig. 2. Although both psoas and soleus fibers exhibited an unrestrained, slack sarcomere length of 2.2 μm, psoas fibers produced several fold greater levels of resting tension at sarcomere lengths between 3 and 4 μm. For comparison, Fig. 2 also shows published values for resting tension in single skinned human fast and slow twitch muscle fibers (Horowits et al., 1990). The fast and slow twitch fibers from humans both exhibit levels of resting tension much lower than those found in the fast twitch rabbit psoas fibers, and similar to those found in the slow twitch rabbit soleus fibers.

Previous work has shown that the titin filaments are arranged as elastic links between the thick filaments and the Z-discs (Wang et al., 1985; Furst et al., 1988; Itoh et al., 1988; Horowits et al., 1989; Whiting et al., 1989), and hence provide a force which tends to position the thick filaments at the center of the sarcomere (Horowits and Podolsky, 1987, 1988; Horowits et al., 1989). The influence of titin on thick filament position is critical for the proper functioning of the sarcomere, because a sarcomere composed of only actin and myosin filaments is inherently unstable when activated. This is because during contraction, the amount of force on each half of the thick filament is proportional to the fraction of its cross-bridge bearing length which overlaps with thin filaments (Gordon et al., 1966). Therefore, any initial imbalance in force (which might be caused by imprecise centering of the thick filament or by the unsynchronized production of force by many independent cross-bridges) would be amplified as the thick filament is actually pulled toward one end of the sarcomere. The effectiveness of the titin filaments in preventing this instability can be easily observed by electron microscopy of skinned muscle fibers fixed after prolonged activation. Fig. 3 shows that after prolonged maximum activation at a sarcomere length of ~2.6 μm, soleus (*A*) and psoas (*B*) sarcomeres exhibit thick filaments that have moved from the middle to one side of the sarcomere. Fig. 3 also demonstrates that significant movement of thick filaments occurs in soleus sarcomeres that are up to 3.4 μm long (*C*), but that this movement is abolished at a sarcomere length of ~3.0 μm in psoas fibers (*D*). Fig. 4 illustrates the relationship between the extent of thick filament movement during prolonged activation and

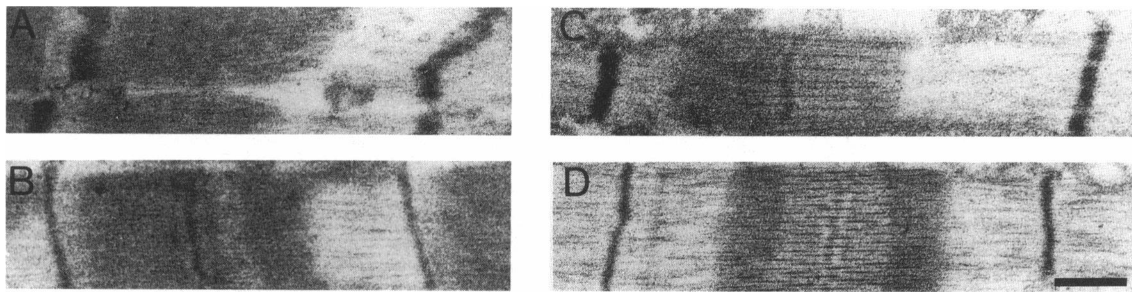

FIGURE 3 Electron micrographs of sarcomeres fixed after prolonged activation. (*A and C*) soleus fibers. (*B and D*) psoas fibers. Calibration bar in *D*: 0.5 μm.

sarcomere length for both psoas and soleus fibers. In psoas fibers, maximum motion occurs in sarcomeres <2.6 μm long, but decreases to almost zero as sarcomere length increases to 3.0 μm. The increased effectiveness of the titin filaments in holding the thick filaments at the center of the sarcomere during activation at longer sarcomere lengths indicates that the titin filaments are more resistant to stretch at the longer lengths. The range of lengths over which thick filament movement decreases (Fig. 4) corresponds to the range of lengths over which the sarcomere's resistance to stretch, as indicated by the slope of the resting tension-sarcomere length relation, increases (Fig. 2). As previously noted (Horowits and Podolsky, 1987), when one considers the magnitude of the active forces that tend to destabilize the position of the thick filament at the center of the sarcomere, this correspondence suggests that practically all of the resting tension in psoas fibers originates in the elastic links between the thick filaments and the Z-discs, i.e., the titin filaments. Relative to that

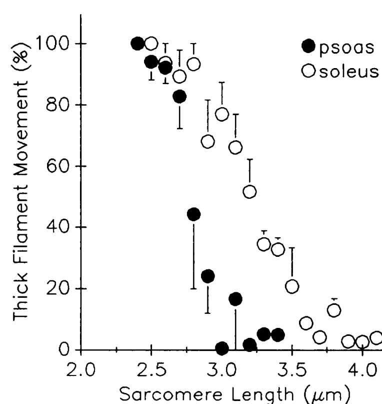

FIGURE 4 Thick filament movement vs sarcomere length. Each point represents the mean value from 2 to 12 psoas (●) or soleus (○) sarcomeres. Error bars indicate one s.e.m. Results are from electron micrographs of four psoas and four soleus preparations.

found in psoas fibers, the relation between thick filament movement and sarcomere length in soleus fibers is shifted towards higher lengths, with significant movement observed in sarcomeres up to 3.5 μm long (Fig. 4). As with psoas fibers, the range of lengths over which thick filament movement decreases in soleus fibers corresponds to the range over which the slope of the resting tension-length relation is increasing (Fig. 2). These results suggest that, as in psoas fibers, the resting tension in soleus fibers originates in the titin filaments, and that the large differences in resting tension observed between rabbit psoas and soleus fibers is caused by a difference in the amount of tension produced by stretch of the titin filaments.

Myofibrillar protein composition of single skinned fibers

Rabbit psoas and soleus muscle cells may produce different levels of resting tension when titin filaments are stretched by simply varying the number of titin filaments arranged in parallel between the end of each thick filament and the Z-disc. Alternately, the force produced by each titin filament may differ in the two muscles. To distinguish between these possibilities, each single fiber used in the mechanical experiments (Fig. 2) was dissolved in SDS and its proteins analyzed by gel electrophoresis (Fig. 5 and Table 1). Fig. 5 *A* illustrates that the major myofibrillar proteins are present in similar concentrations in single psoas and soleus fibers. Quantitation of gels by scanning densitometry revealed no significant differences in the concentrations of titin, nebulin, myosin, and actin found in single psoas and soleus fibers (Table 1). This clearly shows that the observed differences in resting tension are not due to differences in the number of titin filaments. Upon prolonged electrophoresis, a subtle difference in mobility between psoas and soleus titin becomes apparent (Fig. 5 *B*). Close examination reveals that soleus titin

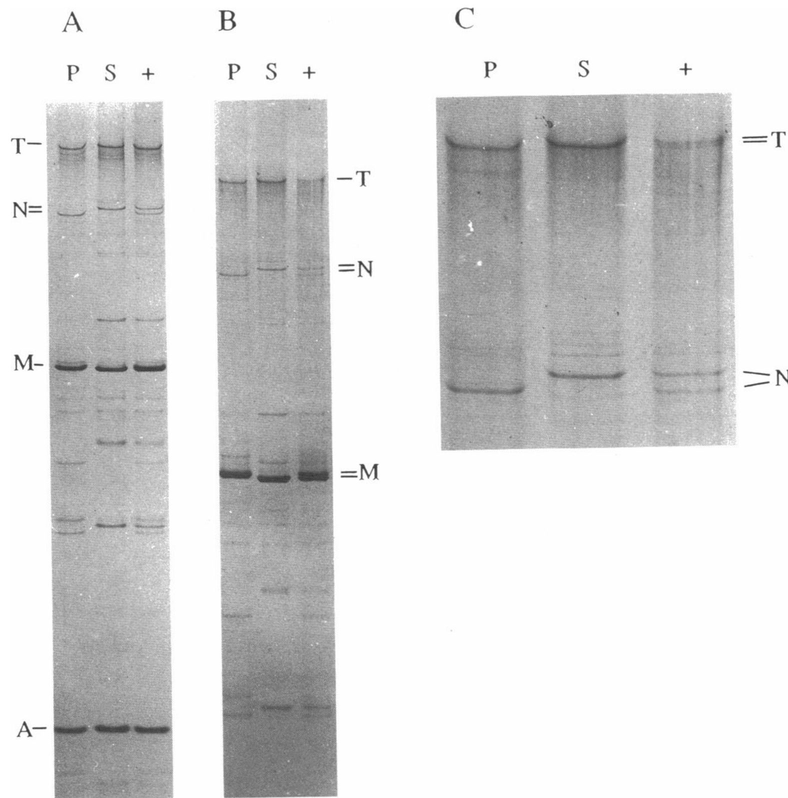

FIGURE 5 SDS polyacrylamide gel electrophoresis of solubilized single skinned fibers. Lanes were loaded with equal volumes of single psoas (*P*) or soleus (*S*) fibers, or a 1:1 mixture of the two (+). The separation of titin (*T*), nebulin (*N*), myosin heavy chain (*M*), and actin (*A*) is shown. *A* and *B* show samples electrophoresed for 4 and 8 h, respectively. In *B*, actin has been eluted from the gel. *C* is an enlargement of the upper portion of *B*, and shows the separation of psoas and soleus titin into two closely spaced but distinct bands. Note that psoas and soleus fibers also contain different forms of nebulin and myosin heavy chain.

migrates slightly slower than psoas titin (Fig. 5 *C*). Soleus fibers also appear to have a heavier form of nebulin than do psoas fibers (Fig. 5). These results reproduce the small but detectable difference in mobility between rabbit psoas and soleus titin that was recently demonstrated by Wang and Wright (1988) using a similar gel system. The finding that psoas and soleus muscle fibers differ in the form, but not in the amount, of titin that they contain suggests that the large differences in resting tension between psoas and soleus fibers may be due to differences within each titin molecule. However, definitive proof of this hypothesis must await a comparative study of the elastic properties of isolated titin.

TABLE 1 **Protein content of single skinned fibers**

	Relative protein concentration			
	Titin	Nebulin	Myosin	Actin
Psoas	1.00 ± 0.13	1.00 ± 0.13	1.00 ± 0.20	1.00 ± 0.05
Soleus	0.97 ± 0.14	0.99 ± 0.14	0.94 ± 0.22	0.91 ± 0.05

For each protein, values are expressed relative to the mean value found in psoas fibers. Data are means ± s.e.m. for the same eight single fibers used for mechanical measurements in Fig. 2.

DISCUSSION

Molecular basis of isoform specific differences in titin elasticity

Monoclonal antibody labeling indicates that individual titin molecules extend from the Z-disc to the center of the sarcomere, and are divided into an inelastic region that is rigidly bound along the length of the thick filament and an elastic region linking the end of the thick filament to the Z-disc (Wang et al., 1985; Furst et al., 1988; Itoh et al., 1988; Horowits et al., 1989; Whiting et al., 1989). Given that an unrestrained, slack sarcomere is 2.2-μm long and that thick filaments are 1.6-μm long, the fraction of titin mass in the elastic region of the molecule can be calculated if the following assumptions are made: first, that in a slack sarcomere the elastic

region of titin is unstrained; second, that the portion of titin bound along the length of the thick filament is unstrained; and third, that the unstrained titin molecule has a mass density that is uniform along its entire length. These assumptions are reasonable considering the recent observation that, except for a small globular head at one end, isolated oriented titin filaments appear to be micrometer long rods of uniform diameter (Nave et al., 1989). From these considerations, ~25% of the titin molecule may be found in the elastic region between the thick filaments and Z-disc. Fig. 2 indicates that, at similar levels of resting tension, the elastic region of titin between the thick filaments and Z-disc is ~40% longer in soleus than in psoas fibers. Therefore, the difference in resting tension between the two types of fibers is consistent with a 40% longer, and hence 40% more massive, elastic region of titin in soleus fibers. Such a change would lead to a 10% greater molecular weight of soleus titin relative to psoas titin. The difference in electrophoretic mobility shown in Fig. 5 *C* indicates that soleus titin is at least 5% larger than psoas titin. Therefore, the observed difference in electrophoretic mobility of the entire titin molecule is consistent with the estimate of mass difference in the elastic region of psoas and soleus titin derived from the physiological data (Fig. 2). This suggests a simple model in which muscle cells regulate resting tension by choosing between titin isoforms that differ in the number of elastic domains that are linked end to end to form the connection between thick filaments and Z-discs. An alternate possibility is that the less stiff titin isoform found in soleus fibers is formed by substituting domains which are more elastic for domains found in psoas titin which are less elastic. The two types of repeating domains deduced from titin cDNA sequences (Labeit et al., 1990) could play a role in such a mechanism. Further studies using monoclonal antibodies to explore structural differences between titin isoforms, as well as elucidation of the organization and function of the titin gene(s), may clarify the mechanisms by which resting tension is regulated; in particular, such studies should provide critical tests of the hypothesis, strongly suggested by the present work, that titin polymorphism accounts for the observed variations in resting tension.

Physiological implications

As previously noted, the resting tension orginating in the titin filaments may in principle vary over a wide range without risk of impairing the ability of the titin filaments to recenter the thick filaments between active contractions in vivo (Horowits and Podolsky, 1988). However, the physiological advantage of having muscle fibers with varying levels of resting tension is unclear.

Rabbit psoas and soleus muscles are relatively homogeneous, containing almost exclusively fast twitch and slow twitch fibers, respectively (Julian et al., 1981). However, Fig. 2 shows that human fast and slow twitch fibers which coexist in the same muscles produce similar levels of resting tension. Therefore, the level of resting tension observed in mammalian muscle fibers is not strictly a function of fiber type. Instead, these results suggest that the level of resting tension exhibited by a muscle fiber, and hence the isoform of titin it contains, is related to its anatomical location within the animal. It is in this context that the observed variations in resting tension must be considered. For example, in arthropods, resting tension of opposing muscles is thought to provide postural support (Yox et al., 1982). In higher animals, production of resting tension during passive lengthening may limit the extent of motion produced by an opposing muscle during active shortening (Freehafer et al., 1979; Brand, 1985). This effect could place a clinically relevant limit on mobility, especially following tendon transfer surgery (Freehafer et al., 1979; Brand, 1985). In addition, production of resting tension may help protect muscles from damage due to overextension (Brand, 1985). Further investigation of these possibilities may lead to an understanding of the function of the observed anatomical (this report; Hill and Weber, 1986; Hu et al., 1986; Wang and Wright, 1988; Akster et al., 1989) and developmental (Yoshidomi et al., 1985) variations in titin isoforms.

Note: another paper dealing with passive force generation and titin isoforms appeared while this manuscript was under review (Wang et al., 1991).

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