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REVIEW

Non-crossbridge stiffness in active muscle fibres

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ABSTRACT

Stretching of an activated skeletal muscle induces a transient tension increase followed by a period during which the tension remains elevated well above the isometric level at an almost constant value. This excess of tension in response to stretching has been called 'static tension' and attributed to an increase in fibre stiffness above the resting value, named 'static stiffness'. This observation was originally made, by our group, in frog intact muscle fibres and has been confirmed more recently, by us, in mammalian intact fibres. Following stimulation, fibre stiffness starts to increase during the latent period well before crossbridge force generation and it is present throughout the whole contraction in both single twitches and tetani. Static stiffness is dependent on sarcomere length in a different way from crossbridge force and is independent of stretching amplitude and velocity. Static stiffness follows a time course which is distinct from that of active force and very similar to the myoplasmic calcium concentration time course. We therefore hypothesize that static stiffness is due to a calcium-dependent stiffening of a non-crossbridge sarcomere structure, such as the titin filament. According to this hypothesis, titin, in addition to its well-recognized role in determining the muscle passive tension, could have a role during muscle activity.

KEY WORDS: Static stiffness, Titin, Non-crossbridge stiffness, Intact muscle fibre, Mouse muscle

Introduction

The first mechanical change induced by stimulation of a skeletal muscle is an increase in the stiffness that occurs during the latent period when the muscle is developing zero active force. Muscle stiffness increases throughout the whole tension rise during a twitch as in a tetanic contraction due to the formation of crossbridges that cross-link the myosin and actin filaments in the overlap region of the sarcomere (Ford et al., 1981; Cecchi et al., 1982). However, as reported for the first time about 20 years ago in intact frog muscle fibres, a small portion of the stiffness increase is not due to crossbridge formation (Bagni et al., 1994, 2002, 2004) but to some other sarcomere structures whose stiffness increases upon stimulation. This stiffness is detected from the force response to stretches (and hold) applied to activated muscle fibres. The stretch produces a fast force transient followed by a period during which tension well exceeds isometric tension. This excess of tension is referred to as 'static tension' while the ratio between static tension and stretch amplitude is termed 'static stiffness'.

Later static stiffness was observed in intact mammalian skeletal muscle fibres (Roots et al., 2007; Colombini et al., 2009; Nocella et al., 2012, 2014) and in permeabilized fibres (Campbell and Moss, 2002; Cornachione and Rassier, 2012). More recently, it has also been shown in skeletal muscle myofibrils (Cornachione et al., 2015). Static stiffness starts to rise during the latent period, when active force has not yet developed, and reaches a maximum value after a few milliseconds when active tension is still near zero. The time course of static stiffness is significantly different with respect to that of active tension development, and is very similar to the myoplasmic [Ca^{2+}] time course (Baylor et al., 1984; Baylor and Hollingworth, 2003). Static stiffness persists almost unaltered in the presence of a crossbridge inhibitor such as 2,3-butanedione monoxime (BDM) or *N*-benzyl-*p*-toluene sulphonamide (BTS; Colombini et al., 2010), which greatly reduce active tension (Horiuti et al., 1988; Shaw et al., 2003; Colombini et al., 2010), but it decreases in the presence of dantrolene sodium, methoxyverapamil and deuterium oxide, which inhibit calcium release from the sarcoplasmic reticulum (Bagni et al., 2004). Static stiffness is independent of stretching amplitude and velocity and dependent on sarcomere length. It has been hypothesized that static stiffness is due to a Ca^{2+}-dependent stiffening of a non-crossbridge sarcomere structure, still unknown at present, which might be titin (Bagni et al., 1994, 2002, 2004; Rassier et al., 2005; Roots et al., 2007; Colombini et al., 2009; Nishikawa et al., 2012; Nocella et al., 2012, 2014; Rassier, 2012).

Titin structure and function

Titin is the largest protein (3–4 MDa) known at present. Titin filaments span the entire half-sarcomere (Fig. 1) and are anchored to the Z-line and to the M-line region of the sarcomere (Funatsu et al., 1993). Each titin molecule is associated with one thin filament in the I-band and one thick filament in the A-band. Titin preserves the precise sarcomere arrangement of thick and thin filaments and maintains myosin filaments centred in the middle of the sarcomere, avoiding overlap mismatch in the two adjacent overlap zones of the sarcomere. In the A-band, titin is tightly associated with the thick filament and is inextensible (Muhle-Goll et al., 2001). In the I-band region, all titin isoforms present two immunoglobulin (Ig)-like domains (proximal and distal Ig domain) and one PEVK segment (a sequence rich in proline, glutamate, valine and lysine residues). The PEVK region and the linked proximal Ig domain function as springs in series (Linke et al., 1998; Granzier and Labeit, 2007; Ottenheijm and Granzier, 2010).

The role of titin in muscle passive tension has long been known (Horowits and Podolsky, 1987). Many studies describe similar passive force–fibre length extension relationships both for myofibrils and for titin molecules (Linke et al., 1998; Labeit et al., 2003; Wang et al., 1991). At the shorter sarcomere lengths, stretching straightens the relatively compliant folded Ig domains of the I-band titin and there is only a little increase in passive force (Wang et al., 1991; Granzier and Labeit, 2004). At the longer sarcomere lengths, force increases steeply because the PEVK region elongates as a Hookean spring (Linke et al., 1998). The steep increase in passive force starts at different sarcomere lengths in muscles (Wang et al., 1991; Gautel and Goulding, 1996) with different titin isoforms and with PEVK segments of different size.

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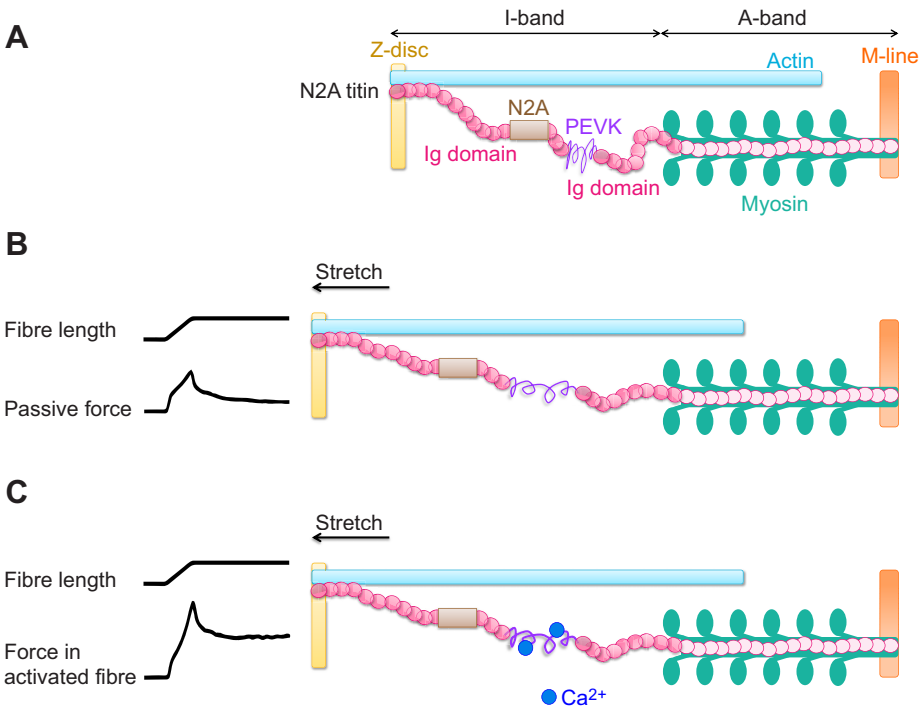

Fig. 1. Schematic diagram of a skeletal muscle half-sarcomere in three distinct titin states: at resting sarcomere length, and after a stretch applied to a passive fibre and to an activated fibre during the latent period. For clarity, only the three main filament systems of the sarcomere (actin, myosin and titin filaments) are shown. The titin filament, bound to the actin filament in the I-band and to the myosin filament in the A-band, is simply represented, highlighting the regions proposed to be involved in strain sensing (Ig domains, PEVK segment, N2A unique sequence). B and C also show (on the left) the length change applied and the consequent force response. (A) Resting sarcomere length. (B) Passive response due to sequential extension of titin elastic regions (Linke et al., 1998). (C) Force response to a stretch applied during the latent period, when the internal calcium concentration is increased but crossbridges are not yet attached. Note that, at the higher calcium concentration, the force response to stretch is higher than in the passive fibre, indicating a stiffening of the half-sarcomere. This could be due to titin stiffening following calcium binding to E-rich PEVK that decreases the persistent length of titin (Labeit et al., 2003). This titin stiffening may be responsible for the static stiffness.

Titin extensibility and therefore passive tension depend on the number and length of spring-like elements present in each isoform. In fact, passive tension is produced at shorter sarcomere lengths in the shorter titin isoform.

Cardiac muscles express two titin isoforms (Granzier and Labeit, 2004). One isoform, called N2B, is expressed with a single spring-like segment (N2B), whereas the other isoform presents two spring-like domains (N2B and N2A) and is called N2BA titin. Skeletal muscles express the N2A titin isoform, but fast muscles present a shorter N2A isoform than slow muscles (Neagoe et al., 2003).

The role of titin in active muscle is at present still debated (Pavlov et al., 2009). It is known that titin N2A is calcium sensitive (Neagoe et al., 2003) and becomes stiffer in the presence of calcium, but the mechanism by which calcium regulates titin stiffness is still unknown.

Aim

This review summarizes the current knowledge regarding static stiffness from the earliest experiments performed by our group with frog muscle fibres, which allowed us for the first time to determine its existence, to our more recent results in mouse skeletal muscle fibres, which confirm its presence and function. Through the analysis of the principal characteristics of static stiffness in activated muscle, this review affirms that static stiffness is independent of crossbridges. Our aim was also to evaluate the proposed mechanism through which titin stiffening, during the earliest activation phases when intracellular calcium concentration is increased but crossbridges are not yet formed, could be responsible for the higher force response to stretch compared with passive fibre.

Static stiffness measurements

Static stiffness was detected and measured by applying a rapid lengthening to the activated intact fibre during twitch or tetanic contractions (see Bagni et al., 1994, 2002, 2004, 2005; Colombini et al., 2009; Nocella et al., 2012, 2014).

Fibre dissection, mounting and stimulation

Fibres were dissected with intact membrane from frog (*Rana esculenta*) or mouse (C57/BL6) skeletal muscles. The dissection was performed manually under a stereo-microscope with a fine pair of scissors and needles. Single intact frog fibres were isolated from tibialis anterior or lumbricalis digiti IV muscles. Single intact mouse fibres or small bundles of up to 10 fibres were dissected from flexor digitorum brevis (FDB), extensor digitorum longus (EDL) and soleus muscles. Particular care was taken to avoid excessive stretching and to obtain fibres as clean as possible from connective tissue. Single fibres or bundles were mounted horizontally in an experimental chamber by means of aluminium foil microclips compressed onto the tendons, between the lever arms of a capacitance force transducer and a fast electromagnetic motor that could apply stretches of the desired amplitude and velocity. Stimuli of alternate polarity were applied transversely to the fibres by means of two platinum-plate electrodes placed parallel to a preparation. Experiments were performed at about 14°C on frog fibres (Bagni et al., 1994, 2002, 2004) and at 24, 30 or 35°C on mouse fibres (Colombini et al., 2009; Nocella et al., 2012, 2014).

Experimental protocol

The experimental protocol used to determine the static stiffness was the same in frog and mouse fibres. The stretches applied for the measurements were ramp shaped with an amplitude of 2–6% fibre length (l_0) and a stretch time of 0.6–1.0 ms corresponding to a stretching velocity between 30 and 70 l_0 s^{-1}. The use of fast stretches reduced the crossbridge cycling during the stretch itself and the time necessary for the tension to reach a constant value after the stretch to a few milliseconds (<10 ms).

The measurements were made during twitch contraction or short (2–3 stimuli) or long tetani. The analysis was usually limited to the initial part of the tetanus rise as fibres developing a tension greater than 50% of plateau tetanic tension (P_0) were quickly damaged by the fast stretch used. To reduce crossbridge formation and to avoid

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fibre damage, BDM (Bagni et al., 2002, 2004) or BTS (Nocella et al., 2014) was added to the normal solution in some experiments.

Static tension and static stiffness measurements

We used the following method to measure static stiffness: one end of the activated fibre was rapidly stretched by an electromagnetic motor and held for a period longer than the stimulation period while the force response was measured at the other end of the fibre with a force transducer. Fig. 2 shows an example of the force response to a fast stretch applied during a short tetanus in a small bundle of fibres dissected from FDB mouse muscle. After an initial peak, synchronous with the stretch and mainly due to the crossbridge response to the stretch, tension settles in a few milliseconds to a constant value above the isometric level. Static tension is generated during the stretch, but it cannot be measured immediately after the stretch because of the presence of the initial force transient. Three force records were necessary for each measurement: the isometric response, the response of the active fibres to fast stretches and hold, and the response of the passive fibres to the same stretch. By subtracting the isometric and passive forces from the active response to fast stretch, we obtained the subtracted trace on which measurements were made (Fig. 2). The subtraction of the isometric record from the response of the active fibres to stretch allows the static tension measurements to be on a flat line even when the stretch is applied on tension rise or relaxation. By subtracting the passive response, we corrected for the resting tension and stiffness of the relaxed fibres. The subtracted trace represents the excess of force induced by the stretch. This excess of tension is the static tension. As the static tension is constant for some milliseconds, it is possible to read its value without significant error after the end of the fast transient (as indicated by the arrow in Fig. 2). The ratio between static tension and stretch amplitude, measured at the sarcomere or fibre length level, represents the static stiffness. In order to compare the results from different experiments and different fibre types, static stiffness data in this review are reported in the figures as the ratio between static tension and P_0 for 1% l_0 stretch amplitude.

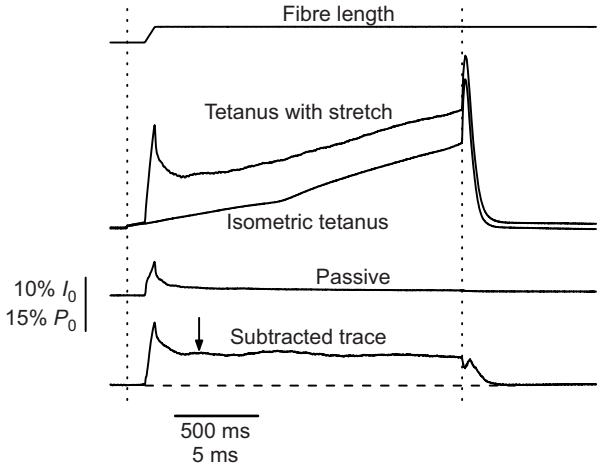

Fig. 2. Static stiffness measurement. Force response to a stretch applied with 3 ms delay after a stimulus at 1.65% plateau tetanic tension (P_0) on the rise of a short tetanus (three stimuli) in flexor digitorum brevis (FDB) mouse fibres. The subtracted trace is obtained by subtracting the passive force response and isometric tetanus from the tetanus with stretch, and represents the excess of tension induced by the stretch. The static tension is measured after the end of the fast transient, as indicated by the arrow. The ratio between static tension and stretch amplitude is the static stiffness. Stretch amplitude, 3.1% fibre length (l_0); stretch duration, 630 µs; sarcomere length, 2.65 µm. Sampling time is 10 µs between the vertical dotted lines (calibration bar value: 5 ms) and 1 ms outside the lines (calibration bar value: 500 ms). The horizontal dashed line indicates zero tension. Temperature, 30°C.

Static stiffness in frog muscle fibres

Static stiffness was detected for the first time by our group many years ago in single intact frog muscle fibres. The first paper was published in 1994 (Bagni et al., 1994) and in the following years all the principal characteristics of the static stiffness were identified (Bagni et al., 2002, 2004, 2005). In this review, static stiffness characteristics will be analysed more in detail by presenting the most recent data obtained in mouse skeletal muscle (Colombini et al., 2009; Nocella et al., 2012, 2014), but by taking into account the key role of the previous studies on frog muscle fibres which are briefly summarized below.

Characteristics of static stiffness

Static stiffness is independent of the active tension in a twitch contraction (Bagni et al., 1994) or in a tetanus (Bagni et al., 2002); it is present during the latent period and increases as the overlap between myofilaments decreases. Static stiffness maintains a constant value for a long period after the force transient and is independent of stretch amplitude even when the stretch is great enough to forcibly detach the crossbridges. Its amplitude is independent of stretching velocity. Static stiffness is not significantly affected by BDM, which strongly inhibits crossbridge formation without affecting calcium release. Conversely, it is reduced by dantrolene sodium, methoxyverapamil (D600) and deuterium oxide, which depress force development by inhibiting calcium release (Bagni et al., 2004). The characteristics of static stiffness are equivalent to those expected from a linear elasticity located in parallel with the crossbridges, but not to a simple passive structure as its stiffness increases with activation independently of force.

Comparison of static and total fibre stiffness

By comparing static and total fibre stiffness (crossbridge plus static stiffness), it is clear (Fig. 3) that static stiffness contributes little to total stiffness, being present only during the latent period and the first phase of tension development when relatively few crossbridges are attached. However, static stiffness accounts for all the total stiffness increase during the latent period. In fact, after subtraction of static stiffness from total stiffness (Fig. 3B), the observed lead of stiffness over tension (Cecchi et al., 1982) during the latent period disappears, and the first increase in fibre stiffness becomes coincident with force.

Mechanisms responsible for static stiffness

All these data support the idea that static stiffness is not related to crossbridges but that its time course resembles that of intracellular calcium concentration, as measured by calcium indicators (Baylor et al., 1984). For this reason, it was speculated (Bagni et al., 1994, 2002) that static stiffness could be due to the stiffness increase of a sarcomere structure following the calcium concentration increase after fibre stimulation. Although the results obtained in frog muscle fibres did not provide direct information about the structure responsible for the static stiffness, they did show that Ca^{2+} not only promotes crossbridge formation but also increases the stiffness of an unidentified elastic structure of the sarcomere (Bagni et al., 1994, 2002). Based on this knowledge, titin was thought to be the best candidate and the increased stiffness was hypothesized as being due to a calcium-dependent change in titin elasticity (Tatsumi et al., 2001) or to a calcium-dependent titin–actin interaction (Kellermayer and Granzier, 1996).

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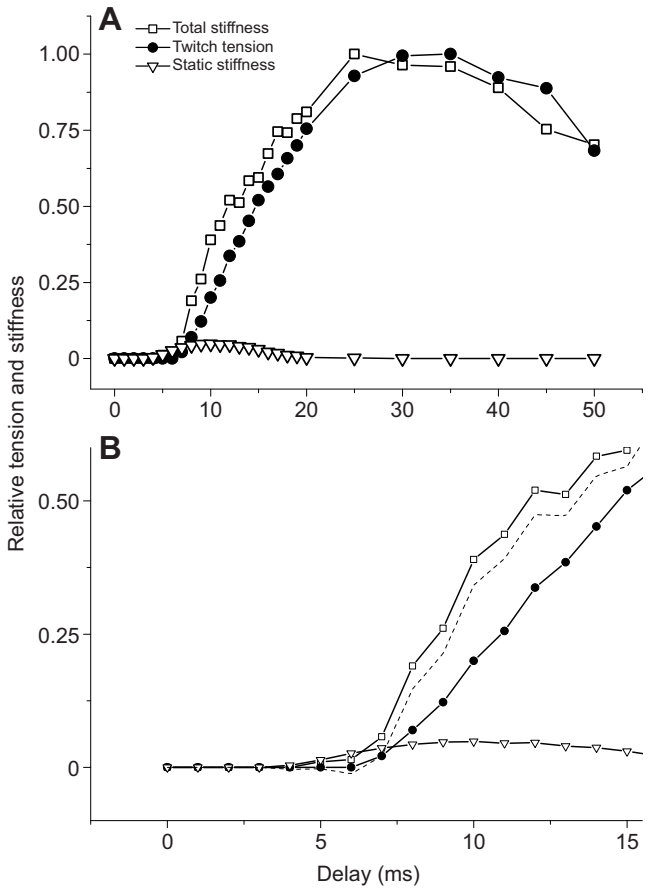

Fig. 3. Time course of static stiffness and total fibre stiffness during the latent period, and early twitch tension development in a single frog muscle fibre. (A) Total and static stiffness are expressed relative to the maximum total stiffness; twitch tension is expressed relative to its maximum value. (B) Expanded portion of A. The dashed line is the difference between total and static stiffness. Stimulus starts at 0 ms delay. Stretch amplitude, 2.7% sarcomere length (l_s); stretching velocity, 43 l_s s^{-1}. Temperature, 15°C.

Static stiffness in mouse muscle fibres

The results obtained from frog muscle fibres indicate that non-crossbridge calcium-dependent static stiffness is a small fraction of the total activated fibre stiffness. However, its physiological role could be vital for improving the mechanical stability of the sarcomere during the initial phases of muscle contraction when the tension developed is small and only a few crossbridges are attached. Considering the relevant function of static stiffness, we decided to ascertain whether the static stiffness is also present in mammalian skeletal muscle and whether it has the same characteristics as in frog fibres. The importance of extending the static stiffness measurements to mammalian fibres is that, whereas the titin isoform expressed in frog muscle is less certain, it is known that mouse skeletal muscles express the calcium-sensitive N2A titin isoform (Neagoe et al., 2003). Static stiffness was detected and characterized for the first time in single intact skeletal muscle fibres isolated from FDB mouse muscle (Colombini et al., 2009). Subsequently, the analysis was extended to fast and slow mouse muscles such as EDL and soleus (Nocella et al., 2012) that express a short and a long titin isoform, respectively, with different mechanical properties (Prado et al., 2005; Fukuda et al., 2008).

Static stiffness time course

One of the most important results obtained from the static stiffness studies in frog muscle fibres in support of the independence of this stiffness from crossbridge formation was the evidence that the time course of static stiffness development was significantly different from that of isometric tension (Fig. 3).

To describe static stiffness development after activation, we applied stretches in fibres at different times following the beginning of stimulation (Colombini et al., 2009; Nocella et al., 2012, 2014).

In fact, static tension remains almost constant for the whole tension record, as expected from the stretching of a pure elastic structure, while active tension increases (Fig. 2). This means that the parallel stiffness of the sarcomeres is increased by the activation, but sarcomere length is not changed and, therefore, sarcomere stiffening does not affect the force and can be detected only by stretching, as we did. If sarcomere stiffness increases further after the stretch, again no changes in force will occur and a new stretch would be needed to detect it.

Fig. 4 shows the average time course of static stiffness and isometric tension during the rise of a series of short tetani determined in single intact FDB muscle fibres by applying stretches at rest and at various delays from the start of stimulation. The amplitude of static stiffness is strongly dependent on the duration of the delay between the stimulation and the stretch application time. As in frog fibres, the static stiffness time course is completely different from that of active tension. The development of static stiffness clearly precedes the active tension, as it is already detectable in the latent period 1–2 ms after the stimulus, when active tension has not yet developed. Static stiffness reaches the first peak at about 4 ms after the first stimulus when the isometric tension has just started to rise. Static stiffness starts to fall while isometric tension is still growing. Note that the static stiffness time course is strongly modulated by the stimulation frequency while the tension rise is relatively smooth. The frequency dependence of static stiffness is clearly shown in Fig. 5. Here, measurements were taken during a short tetanus at stimulation frequencies of 70 and 150 Hz. At low frequency, there are two separated peaks corresponding to the effect of the individual stimuli. At higher frequency, only a small drop shows the separation between stimuli.

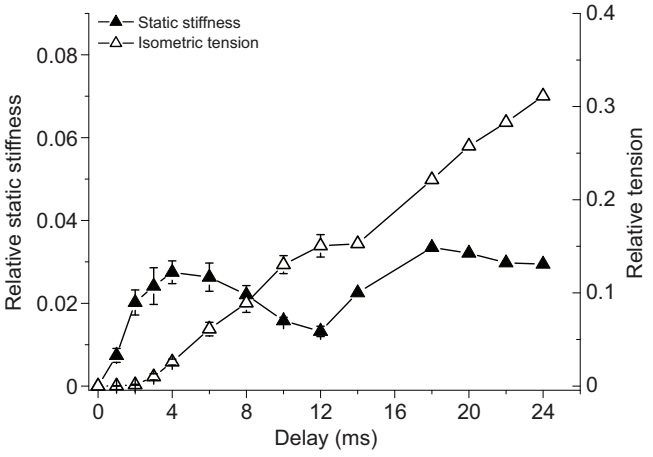

Fig. 4. Average time course of static stiffness and isometric tension during the initial rise of a short tetanus in FDB mouse fibres. Tension values are expressed relative to P_0. Static stiffness values are reported as the ratio between static tension and P_0 for 1% l_0 stretch amplitude. Stimulus starts at 0 ms delay. Stimulation frequency, 80 Hz. Mean values are presented ±s.e.m. (N=5). Temperature, 24°C.

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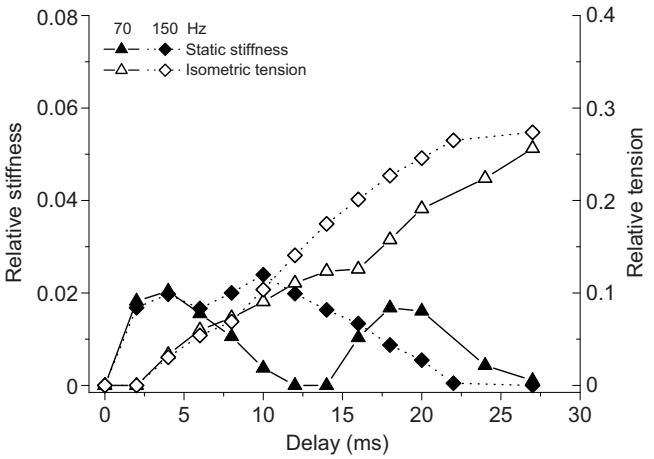

Fig. 5. Effects of stimulation frequency (70 and 150 Hz) on static stiffness and isometric tension development in a FDB fibre. Short tetani comprised three stimuli at 70 Hz and two stimuli at 150 Hz. Tension and static stiffness are expressed as in Fig. 4. Stretch amplitude, 3.1% l_0; stretch duration, 630 µs. Temperature, 24°C.

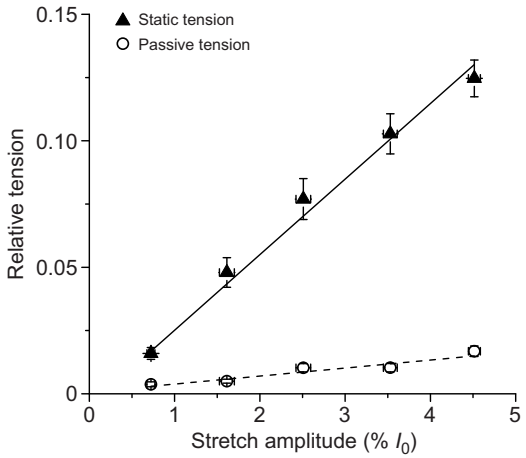

Fig. 6. Effect of stretch amplitude on static and passive tension responses. Stretches of the same duration (~770 µs) and different amplitude were applied to single FDB fibres during the tetanus rise 4 ms after the start of the stimulation and at rest. Static and passive tension, measured as described in Fig. 2, are expressed relative to P_0. Static and passive tension increase almost linearly with stretch amplitude. The slope of the regression line fitted to the experimental data (active fibres, continuous line; passive fibres, dashed line) represents static and passive stiffness. Note that stiffness in passive fibres was much smaller than in activated fibres. Mean values are presented ±s.e.m. (N=7). Mean initial sarcomere length, 2.38±0.01 µm. Temperature, 24°C.

All these results affirm that static stiffness is independent of active tension development and show that its time course is very similar to the internal calcium transient time course. In fact, as reported by Baylor and Hollingworth (2003), during a tetanus at 67 Hz stimulation frequency, the internal calcium concentration measured with fast calcium indicators showed separated peaks for each individual stimulus, similar to the static stiffness time course (Figs 4, 5).

Mouse fibres static stiffness was greater and faster than that of frog fibres. FDB static stiffness peak value was about 3% P_0 for 1% l_0 stretch amplitude at 4 ms after the stimulus (Colombini et al., 2009), while in frog fibres the highest value was about one-half of that and was reached about 6 ms later.

Static stiffness dependence on amplitude and stretch velocity

The properties of static stiffness were investigated by analysing the dependence on static tension of stretching amplitude and velocity. As in frog muscle fibres (Bagni et al., 1994, 2002, 2004), the data obtained in single intact FDB fibres (Colombini et al., 2009) during tetanic contraction by applying stretches of different amplitude and the same duration show that static stiffness is independent of stretching amplitude. Static tension increases almost linearly with the stretch amplitude, while the mean static stiffness, represented by the slope of the line fitted to the experimental data, is constant for the whole range used (Fig. 6). Also, the passive response increases linearly with the stretch amplitude but the slope of the regression line is much smaller than in activated fibres, indicating that stimulation increases passive stiffness of the fibre in a significant way (Fig. 6).

The dependence of static stiffness on stretching velocity was studied by applying stretches of different velocity but the same amplitude. The results indicate that stretching velocity does not affect static stiffness and that, within the limits of our experiments, the elasticity of the structure responsible for the static stiffness is Hookean and undamped.

Effects of temperature on static stiffness

We studied static stiffness in mouse muscle initially at room temperature (21–24°C) (Colombini et al., 2009). Considering that the *in situ* normal temperature is higher, we also performed successive studies at 30–35°C (Nocella et al., 2012, 2014). The presence of static stiffness at body temperature means that this non-crossbridge calcium-dependent stiffening is a general physiological characteristic of skeletal muscle.

Static stiffness measured at a higher temperature maintains the same characteristics as at lower temperature. Its amplitude is not affected by the temperature, but the time course is faster as for active force development and calcium kinetics. In FDB fibres, static stiffness peaks at 4–6 ms after the stimulus at 24°C and at 2–3 ms at physiological temperature.

Static stiffness dependence on sarcomere length

One of the most important characteristics of static stiffness determined in frog muscle fibres is that its value depends on the overlap between myofilaments differently from isometric twitch or tetanic tension. Recently, the complete relationship between static stiffness and sarcomere length has been reported in FDB mouse fibres (Nocella et al., 2014). The static stiffness–sarcomere length relationship and the active tension–sarcomere length relationship were studied over a sarcomere range between 2.6 and 4.4 µm, beyond the theoretical zero overlap length. Fig. 7A shows that the tension–sarcomere length curve reaches a maximum at about 3.3 µm and falls to zero at about 4 µm sarcomere length. The static stiffness–sarcomere length relationship is different (Fig. 7B): static stiffness increases from 2.6 µm to about 3.5 µm, and then starts to decrease. Static stiffness is almost 50% of the maximum at 4 µm and reaches zero at about 4.5 µm (extrapolated value), well above the zero overlap length. This confirms the non-crossbridge nature of static stiffness. The active-tension relationship illustrated in Fig. 7A differs from that expected on the overlap between myofilaments (which is maximum at about 2.6 µm), because tension is measured on tetanus rise (3 ms after the stimulus). Compared with the tetanus plateau, the force development measured during tetanus rise is modulated both by myofilament overlap and by changes to myofibrillar calcium sensitivity with sarcomere length (Williams et al., 2013).

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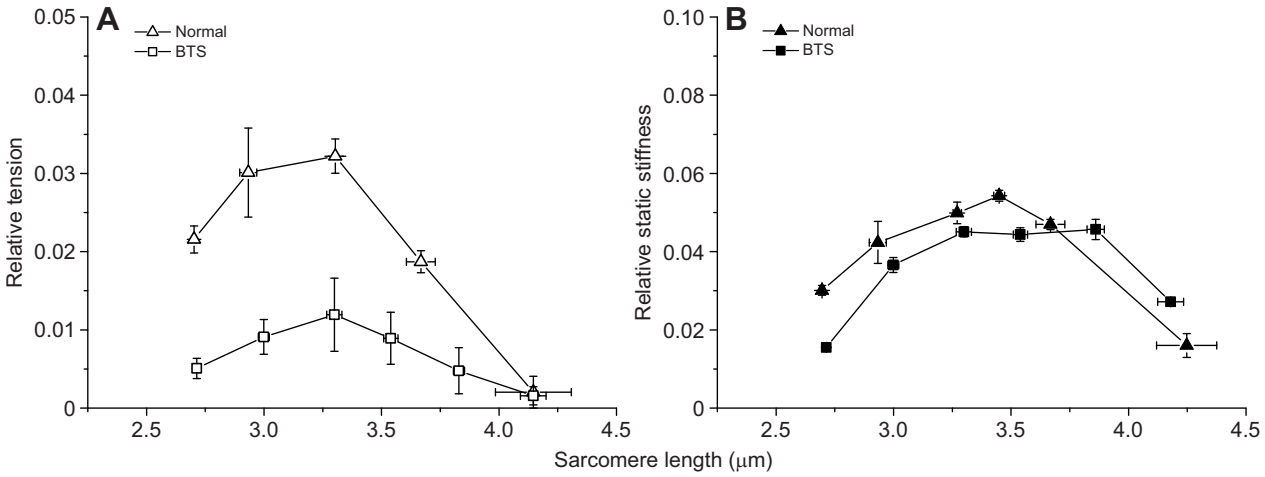

Fig. 7. Dependence of isometric tension and static stiffness on sarcomere length in FDB fibres. (A) Active tension–sarcomere length relationship in normal solution and with the crossbridge inhibitor BTS. (B) Static stiffness–sarcomere length relationship in normal and BTS solution. Tension and static stiffness were measured 3 ms after the first stimulus and were expressed as in Fig. 4. Mean relative values are presented ±s.e.m. (*N*=10). Temperature, 30°C.

To test for any interaction between crossbridges and static stiffness, the above-mentioned relationships were further studied by adding BTS, a crossbridge inhibitor that reduces active tension without affecting calcium release, to the normal solution (Pinniger et al., 2006). Fig. 7 shows that BTS reduces the active tension by about 3.5 times at any sarcomere length, but does not significantly decrease the static stiffness.

All these data support the idea that static stiffness is calcium-dependent and crossbridge-independent.

Static stiffness in slow and fast skeletal muscles

The presence of static stiffness in FDB fibres (Colombini et al., 2009; Nocella et al., 2014) expressing the N2A Ca^{2+}-sensitive titin isoform (Neagoe et al., 2003) supports our hypothesis that this stiffness could be due to a calcium-induced stiffening of titin. To better investigate the role of titin, we measured static stiffness in soleus and in EDL (Nocella et al., 2012), which express the N2A titin isoform with long and short PEVK segments and lower and higher proportions of E-rich motifs, respectively (Neagoe et al., 2003; Prado et al., 2005; Fukuda et al., 2008). It is well known that the I-band portion of titin, especially the PEVK segment, has elastic properties that act as a molecular spring whose bending stiffness increases upon Ca^{2+} binding to E-rich motifs in the PEVK segment (Labeit et al., 2003). After birth, progressive muscle development includes titin modification. In adulthood, titin is shorter and stiffer compared with soon after birth. The rearrangement is principally due to a reduction in the PEVK region length, suggesting that this leads to an increase in the calcium responsiveness of titin (Ottenheijm et al., 2009).

As in FDB muscle fibres, both in the EDL and soleus, static tension or stiffness and active tension have completely different time courses (Fig. 8A). However, there are some differences in the static stiffness characteristics of fast and slow muscles. In the EDL, the static stiffness time course has a sharp peak at 4 ms after the stimulus and then rapidly decays. In the soleus, in contrast, there is no clear peak. The static stiffness amplitude is 5 times greater in EDL than in soleus muscle and 2 times greater than in FDB (Fig. 8B). This is in agreement with a previous study on rat EDL and

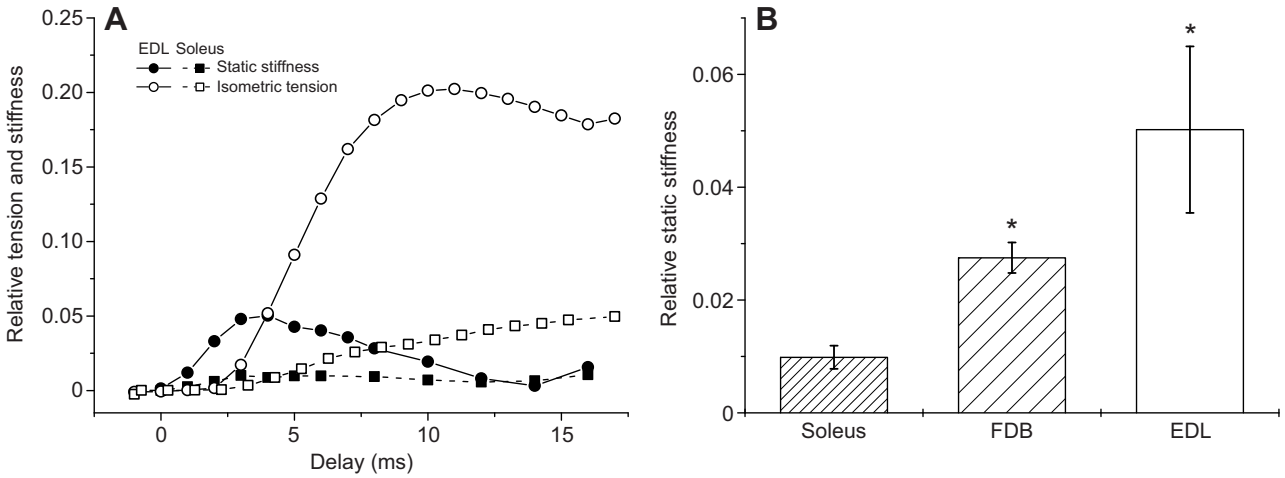

Fig. 8. Static stiffness in extensor digitorum longus (EDL), soleus and FDB fibres. (A) Comparison between static stiffness and active tension time course in EDL and soleus fibres during a short tetanus. Static stiffness in EDL fibres peaks at 4 ms after the start of stimulation and its amplitude is 5 times higher than that in soleus fibres. In soleus fibres, the peak is much broader. (B) Mean peak values ±s.e.m. of static stiffness in soleus (*N*=7), FDB (*N*=5) and EDL (*N*=6) fibres. The asterisks indicate a statistically significant difference compared with soleus (*P*<0.05). Temperature, 24°C.

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soleus muscles (Ramsey et al., 2010). The higher amplitude of static stiffness in fast muscles has been recently confirmed in experiments on psoas and soleus rabbit myofibrils (Cornachione et al., 2015).

All these data point out that static stiffness is a physiological characteristic of skeletal muscle but with different properties in slow and fast fibres. This is in agreement with different calcium kinetics (Baylor and Hollingworth, 2003) and different PEVK segment length (Neagoe et al., 2003; Prado et al., 2005; Fukuda et al., 2008).

Physiological role of static stiffness

Studies carried out on frog muscle fibre and slow and fast mouse muscle fibre at various experimental as well as physiological temperatures have demonstrated that static stiffness is a general characteristic of skeletal muscle and plays a fundamental role in its function. Static stiffness is only a small percentage of the total crossbridge fibre stiffness at tetanus plateau. However, it has important effects mainly during the latent period and the initial phases of muscle contraction when only a few crossbridges are attached.

All data reviewed here underline that static stiffness is a calcium-dependent stiffening of the activated fibres independent of crossbridge formation. Considering that the titin filament is mechanically distributed in parallel with the crossbridges (Fig. 1) and that calcium modulates the bending stiffness of the PEVK fragments (Labeit et al., 2003), titin seems to have all the characteristics required to explain the static stiffness properties. There are two possible mechanisms: the first is a direct effect of calcium on titin stiffness via calcium binding to the E-rich motifs (Tatsumi et al., 2001; Labeit et al., 2003; Cornachione and Rassier, 2012; Rassier et al., 2015), which could stabilize the PEVK segment, making it stiffer (Fig. 1C); the second involves an effect of calcium on titin–actin interactions that induces an increase in the binding between thin filament and titin, increasing sarcomere stiffness (Kellermayer and Granzier, 1996; Linke et al., 1997, 2002; Bianco et al., 2007; Leonard and Herzog, 2010; Nishikawa et al., 2012). The first mechanism could lead to a reduction in the persistent length of titin and a potential increase in passive force and stiffness. In this case, calcium binding to titin could be the only mechanism necessary for the increase in force induced by titin (Labeit et al., 2003). In support of the second mechanism, some *in vitro* motility assays have shown that in the presence of calcium, titin strongly inhibits the sliding of the actin filaments, suggesting an increase in titin binding to actin (Kellermayer and Granzier, 1996). However, more recently, other studies using recombinant titin fragments have failed to detect binding between actin and PEVK segments of titin (Kulke et al., 2001; Yamasaki et al., 2001). Another study has reported that an increase in calcium concentration reduced rather than increased titin–actin interaction (Stuyvers et al., 1998). In agreement with these results, Cornachione and Rassier (2012) observed that static tension was preserved after depletion with gelsolin of actin filaments, suggesting that titin–actin binding is not involved.

These hypotheses, however do not exclude other possible mechanisms, involving C-protein, nebulin or other known and unknown sarcomeric structures.

On the basis of the experimental data and evidence in the literature, our hypothesis is that during activation, Ca^{2+} binding to the glutamate-rich PEVK region of titin decreases the persistent length of protein (Labeit et al., 2003), potentially increasing its stiffness. When activated muscle fibres are stretched, the stiffer titin contributes to an increased tension (Fig. 1C) that persists for as long as intracellular calcium remains elevated. This could explain the calcium dependence of static stiffness. The greater peak amplitude of the calcium transient in EDL muscle and/or a higher sensitivity to calcium of EDL titin could justify the higher static stiffness increase upon stimulation in EDL compared with soleus or FDB fibres. This could occur because EDL has a greater proportion of the calcium-sensitive E-rich motifs in the PEVK segment, compared with the other muscles.

Conclusions

Although not directly demonstrated, we have shown that static stiffness reflects the Ca^{2+}-dependent stiffening of the titin filament following stimulation. In skeletal muscle, the titin filament not only is fundamental for the regulation of passive force but also may play an important physiological role during activation. The increased stiffening of titin and the consequent increase in fibre stiffness could be important to stabilize the ordered arrangement of myofilaments in the sarcomere during the latent period and during the initial phases of force development, when fibre activation is not uniform among different sarcomeres. The higher static stiffness in EDL suggests that a stiffer titin is better suited to maintain sarcomere organization during rapid force development in the fast muscle fibres, confirming the important physiological and protective role of this non-crossbridge stiffness in skeletal muscle.

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Competing interests

The authors declare no competing or financial interests.

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