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Non-linear myofilament elasticity in frog intact muscle fibres

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SUMMARY

The aim of the present investigation was to elucidate the elastic properties of the myofilaments during tetanic activity in striated muscle. The study was carried out on intact single muscle fibres from the anterior tibialis muscle of *Rana temporaria* (2.0–2.5°C). The instantaneous stiffness was measured as the change in force that occurred in response to a high-frequency (2–4 kHz) length oscillation while the fibre was released to shorten against a pre-set constant load that ranged between 40 and 70% of maximum tetanic force in different experiments. Measurements of fibre stiffness were carried out, at a given load, both at 2.20 μm sarcomere length ($S_{2.20}$), i.e. at full overlap between the thick and thin filaments, and at 2.60 μm sarcomere length ($S_{2.60}$). The fact that the load on the fibre was constant during the stiffness measurements at the two sarcomere lengths implies that the stiffness of elastic elements, acting in series with the myofilaments, was constant at the two sarcomere lengths. The fibre stiffness was consistently lower at the extended sarcomere length, the $S_{2.60}/S_{2.20}$ ratio ranging from 0.83 to 0.97 at the different loads investigated. Based on the $S_{2.60}/S_{2.20}$ ratio, the compliance of the free portions of the thick and thin filaments could be calculated. The myofilament stiffness was found to increase progressively as the load was raised from 40 to 70% of maximum tetanic force. At 2.20 μm sarcomere length and at 40% of maximum load on the fibre, the calculated myofilament stiffness was approximately 2.5 times the maximum cross-bridge stiffness.

Key words: muscle fibre, striated muscle, myofilament elasticity, muscle mechanics, stiffness measurement.

INTRODUCTION

There is evidence that the thick and thin filaments in striated muscle, contrary to earlier assumptions, have a finite stiffness comparable with that existing in the myosin cross-bridges during maximal activity at optimal sarcomere length. These conclusions were drawn in two parallel studies in which the spacings of actin and myosin reflections in X-ray diffraction patterns were measured at high resolution at rest and during maximal activation (Huxley et al., 1994; Wakabayashi et al., 1994). In line with these findings, it has been demonstrated in both intact (Julian and Morgan, 1981; Bagni et al., 1990) and skinned (Higuchi et al., 1995) muscle fibres that the instantaneous stiffness of the fibre during activity is steadily increased as the sarcomere length is reduced within the plateau region of the length–tension relation, i.e. under conditions where the number of active cross-bridges can be presumed to remain constant while the free portions of the thick and thin filaments are reduced.

There is still a lack of information concerning the nature of the filament elasticity. Thus, it is still unknown whether the filament elasticity is Hookean or not, i.e. whether the strain of the filaments is proportional to the applied stress or whether there is a non-linear elasticity like that present in the tendons (Cleworth and Edman, 1972; Edman and Josephson, 2007). In a true Hookean spring, the stiffness, i.e. the force response to a given length change, remains the same at different loads applied to the spring. The simplest assumption, and so far the standpoint generally taken when account has been made of filament compliance in mechanical measurements (e.g. Piazzesi et al., 2007), is postulating that the myofilaments behave as a Hookean spring. Departure from this hypothesis is bound to complicate any modelling of muscle contraction as pointed out by Colombini and colleagues (Colombini et al., 2007).

The present study was undertaken to further elucidate the nature of the elasticity that acts in series with the myosin motors. The instantaneous stiffness was recorded by applying a high-frequency length oscillation to isolated muscle fibres, using an approach by which the compound stiffness signal from the fibre could be read out online (Edman and Lou, 1990). The stiffness was measured after the fibre was released to shorten against a pre-set load starting at two selected sarcomere lengths (2.20 and 2.60 μm). By keeping the load constant during the shortening phase at the two sarcomere lengths, both the cross-bridge stiffness, i.e. the stiffness emanating from the active cross-bridges, and the external series elasticity can be presumed to stay constant. Any difference in stiffness recorded between the two sarcomere lengths can therefore be presumed to reflect a change in filament compliance in consequence of altered filament overlap. Evidence will be presented to show that over a wide range of loads the filament elasticity has the characteristics of a non-Hookean spring.

MATERIALS AND METHODS

Preparation and mounting of muscle fibres

Measurements were made from single fibres dissected from the anterior tibialis muscle of *Rana temporaria* Linnaeus. The experiments were performed according to procedures approved by the Animal Ethics Committee of the University of Lund. The frogs were killed by decapitation followed by destruction of the spinal cord. The dissection was carried out by means of a fine pair of scissors; care being taken to avoid undue stretching of the fibres during the dissection procedure. Slips of tendon were left on each end of a fibre to be used as attachment points. Only fibres that had an insignificant amount of connective tissue were selected for this study. The fibres were dissected the afternoon before the experiment and were kept at +4°C overnight.

For an experiment, the muscle fibre was mounted between a force transducer and an arm extending from the moving coil of a fast electromagnetic puller (motor No. 1), which was the motor used to achieve low-amplitude length oscillations for stiffness measurements. The force transducer was mounted on the arm extending from a second electromagnetic puller (motor No. 2) that served to produce larger fibre movements during load-clamp recordings. The slip of tendon attached to the force transducer was held by a small aluminium clip, which was positioned on the transducer hook in such a way that any lateral, vertical or twisting movements of the fibre during stimulation were minimized (Edman and Reggiani, 1984). For attaching the fibre to motor No.1 (the puller producing sinusoidal length changes), the tendon slip at this end was firmly attached to the puller arm by a strip of Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA). This was wound on the outside of the tendon around the puller arm, leaving a minimum of free tendon outside the end of the puller arm. The Parafilm strip was held in place by attaching its ends to a small hook on the puller arm. Details of the muscle dissection, instrumentation and methods used for measuring fibre length, fibre cross-sectional area and sarcomere length (laser diffraction) are given in Edman and Reggiani (Edman and Reggiani, 1984).

The bathing solution had the following composition (mmol l^{-1}): NaCl, 115.5; KCl, 2.0; $CaCl_2$, 1.8; Na_2HPO_4+NaH_2PO_4 (total concentration), 2.0; pH 7.0. The temperature of the bathing solution was constant to within 0.2°C in a given experiment but ranged from 2.0 to 2.5°C for experiments on different fibres. During the experiment, a glass coverslip (0.1 mm thickness) was placed on top of the muscle chamber in contact with the bathing fluid. In addition to providing a plane upper surface for the laser diffraction measurements, the presence of the coverslip ensured that the bath temperature remained constant along the length of the preparation (to within ±0.1°C) during the experiment. The latter point was tested using a thermistor probe that was moved underneath the cover slip by means of a micromanipulator.

Force transducer

Tension was recorded by means of a semiconductor strain-gauge element (AE 801, Aksjeselskapet Mikroelektronikk, Horten, Norway). The transducer element had been modified to increase the frequency response of the transducer as described by Edman and Lou (Edman and Lou, 1990). The resonant frequency of the force transducer was approximately 19 kHz when the transducer was submerged in the bathing solution.

Stimulation

The fibre was stimulated by passing 0.2 ms current pulses between two platinum plate electrodes placed symmetrically on either side of the preparation approximately 2 mm from it. The stimulus strength was about 15% above threshold. A train of pulses of appropriate frequency (16–22 Hz) was used to produce a fused tetanus of 1 s duration; the tetanic bursts of stimuli being separated by 2 min intervals. A 20–30 min period of regularly paced, tetanic stimulation preceded data collection in each experiment.

Measurement of fibre stiffness

A detailed description of the methods and apparatus used to measure fibre stiffness is given by Edman and Lou (Edman and Lou, 1990). In the present experiments, the fibres were mounted between two electromagnetic pullers (motors Nos 1 and 2) as described above. Motor No. 2, which had the force transducer mounted on its shaft, was used to clamp force to a pre-set level. For stiffness measurements, motor No. 1 produced a sinusoidal length oscillation of constant amplitude throughout the tetanus period. The frequency of the oscillation employed in these experiments was 2–4 kHz, and the peak-to-peak amplitude was 10–11 nm corresponding to approximately 1.7 nm per half-sarcomere with the fibre lengths used. This means that the fibre underwent alternating stretches and releases of <1 nm per half-sarcomere in amplitude about the unperturbed length of the fibre. The stiffness measurement was thus performed within the straight portion of stress–strain relationship of the sarcomere elasticity located above and below the isometric force (e.g. Ford et al., 1977; Månsson, 1989). The stretch and release movements produced during the stiffness measurement were completed in 0.125–0.250 ms and may thus be considered fast enough to provide a useful index of the instantaneous stiffness of active muscle (Huxley and Simmons, 1971; Ford et al., 1977).

The oscillation was initiated just before the onset of stimulation and continued throughout the plateau phase of the tetanus. A stiffness signal was formed by passing the signal from the force transducer through a narrow band-pass filter (Q value 5.5), the optimum frequency of which was set to the actual frequency of the length oscillation used. By rectifying the filtered signal, a direct read-out of the fibre stiffness could be obtained during the course of contraction [for further details, see fig. 1 of Edman and Lou (Edman and Lou, 1990)]. The bandwidth of the rectified signal was DC-1.3 kHz. The force signal was recorded without the superimposed force oscillation by using a notch filter, which produced maximum attenuation at the frequency used for the length oscillation.

The stiffness level recorded during the load-clamp phase was measured by an analysis program written in Labview (National Instruments, Austin, TX, USA). The measurement was made within a time interval of 50 ms after the stiffness trace had passed the initial transient after the onset of force clamp.

RESULTS

Fig. 1 illustrates force-clamp records from a single muscle fibre that was stimulated to produce a fused tetanus at 2.20 μm sarcomere length and released to shorten against a constant load during the tetanus plateau. The stiffness record (middle trace) represents the force response to a 2 kHz length oscillation that was applied to one end of the fibre starting just before the stimulation and continuing throughout the tetanus plateau as described under Materials and methods. As can be seen, there was an abrupt drop in stiffness at the start of the force-clamp manoeuvre and the stiffness remained quite stable during the shortening phase.

Eight experiments were performed in which force and stiffness were recorded as shown in Fig. 1 with measurements at 2.20 and 2.60 μm sarcomere length. This range of sarcomere lengths was selected to avoid interference in the measurements from passive elasticity in the fibres (see Edman, 1979). Two or three different force-clamp levels were studied in the same fibre at the two lengths. By performing the stiffness measurements at the same force level at the two sarcomere lengths, it was ensured that elastic elements acting in series with the myofilaments (such as tendons and fibre attachments) were equally extended. Furthermore, by using identical load-clamp levels at the two sarcomere lengths, an equal number of myosin motors can be presumed to be involved in force production at the two lengths.

Fig. 2 illustrates example records of force and stiffness from two fibres at 2.20 μm and 2.60 μm sarcomere lengths. The rising phase and plateau of the tetanus, including the load-clamp phase, are shown superimposed at the two lengths. Determinants of the force rise time

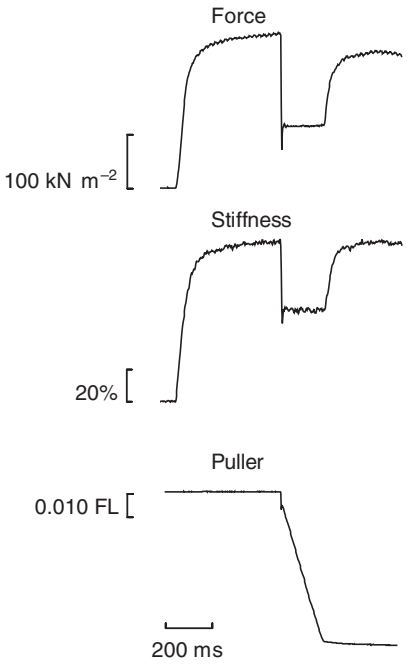

Fig. 1. Simultaneous records of force, stiffness and puller movement during load-clamp recording performed during fused tetanus of a single muscle fibre. Sarcomere length: 2.20 μm. FL=fibre length. Temperature, 2.1°C.

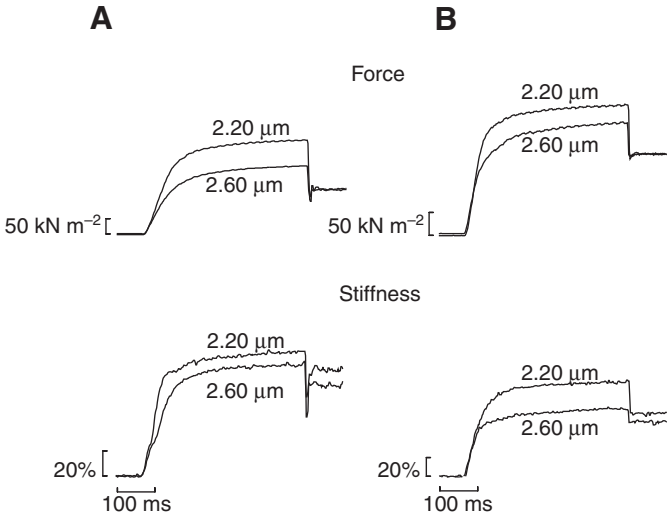

Fig. 2. Superimposed records of force (upper) and stiffness (lower) recorded at 2.20 μm and 2.60 μm sarcomere lengths. Two different load-clamp levels are illustrated in two separate muscle fibres A and B. Load-clamp levels (fraction of tetanic force at 2.20 μm sarcomere length): A, 0.46; B, 0.59. Note that whereas the load-clamp level is the same at the two sarcomere lengths in A and B, the fibre stiffness recorded during the load-clamp phase is markedly different at the two lengths. Temperature: A, 2.0°C; B, 2.1°C.

and tetanus amplitude have been described in detail previously (Edman and Josephson, 2007; Edman and Reggiani, 1987) and are not considered in the present study. The clamp tension differs in A and B but can be seen to be the same at the two sarcomere lengths in the respective fibre. By contrast, in both fibres, the stiffness recorded during the load-clamp phase is clearly lower at 2.60 μm than at 2.20 μm sarcomere length. This change in stiffness is most likely to be attributable to the fact that the free (non-overlapping) portion of the thick and thin filaments is greater at the longer sarcomere length. Assuming that the thick and thin filaments have a length of 1.55 and 1.94 μm, respectively, and the width of the *Z* disk is 0.05 μm (see Edman and Reggiani, 1987), the portion of the thick and thin filaments outside the overlap region can be estimated to be 0.81 μm at 2.20 μm sarcomere length and 1.61 μm at 2.60 μm sarcomere length.

For the subsequent analysis of the filament compliance, it was of relevance to establish how the fibre stiffness recorded at 2.60 μm sarcomere length ($S_{2.60}$) related to that measured at 2.20 μm ($S_{2.20}$) as the force during the load-clamp manoeuvre was varied. Fig. 3 summarizes the results from eight individual muscle fibres in which the $S_{2.60}/S_{2.20}$ ratio was measured at different loads ranging between 40 and 70% of the tetanic force at optimum length. The results show that within the range of loads investigated, the $S_{2.60}/S_{2.20}$ ratio was quite constant, the individual data points varying between 0.83 and 0.97. The regression of the $S_{2.60}/S_{2.20}$ ratio upon force results in a nearly horizontal line with the $S_{2.60}/S_{2.20}$ ratio ranging from 0.89 to 0.91.

The following equation was used to calculate the myofilament compliance (C) at the different force levels displayed in Fig. 3:

$$\frac{S_{2.60}}{S_{2.20}} = \frac{F_{2.60} \times 0.81/1.61C\left(F_{2.20} + 1/C\right)}{\left(F_{2.60} + 0.81/1.61C\right)F_{2.20} \times 1/C} . \quad (1)$$

This equation is based on stiffness measurements ($S_{2.20}$ and $S_{2.60}$) carried out on the fibre as a whole at two different degrees of filament overlap, 2.20 and 2.60 μm sarcomere lengths, respectively. With the experimental design used (stiffness measurements performed during load-clamp shortening at constant force), $S_{2.20}$ and $S_{2.60}$ provide a measure of the compound stiffness formed by the active cross-bridges and the free portions of the myofilaments at the two sarcomere lengths. The cross-bridge stiffness ($F_{2.20}$ and $F_{2.60}$) is assumed to be proportional to the active force during the load-clamp phase normalized to the maximum tetanic force at full overlap. As stated earlier, $F_{2.20}$ and $F_{2.60}$ were very nearly the same in each set of load-clamp recordings at the two sarcomere lengths. The myofilament compliance (the reciprocal of myofilament stiffness) is assumed to be proportional to the free portions of the filaments and is denoted by C at 2.20 μm sarcomere length and $1.61C/0.81$ at 2.60 μm sarcomere length (cf. above). The compound stiffness (A_T) of two elastic elements (A_1 and A_2) acting in series is given by $A_T=(A_1\times A_2)/(A_1+A_2)$. This relationship is formulated in Eqn 1 using the expressions for fibre stiffness, cross-bridge stiffness and myofilament stiffness given above.

The use of active force as an index of cross-bridge stiffness presupposes that there is a fairly constant ratio of 'weak' to 'strong' cross-bridges at the different clamp levels, shortening velocities and sarcomere lengths investigated (see Discussion). The validity of this assumption cannot be fully established at the present time. However, the findings by Ford and colleagues (Ford et al., 1981; Ford et al., 1985) that the instantaneous sarcomere stiffness varies almost in proportion to the measured force, at different sarcomere lengths and at different speeds of shortening, support the view that the ratio of 'weak' to 'strong' cross-bridges is indeed maintained quite constant under the test situations studied in the present investigation.

The myofilament stiffness was calculated from Eqn 1 for various values of force according to the regression line in Fig. 3. The results of this calculation are shown in Fig. 4 in which the filament stiffness is expressed as a multiple of the maximum cross-bridge stiffness (see above). Stiffness values, referring to both 2.20 μm (open symbols) and 2.60 μm (closed symbols) sarcomere lengths, are

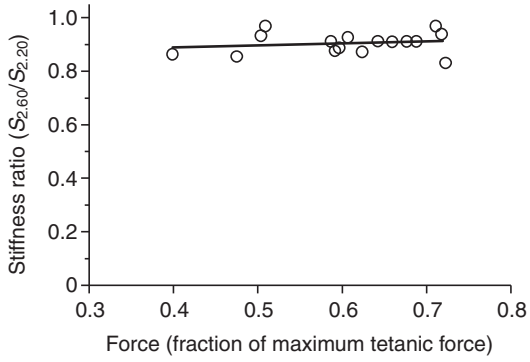

Fig. 3. The ratio of fibre stiffness recorded at 2.60 μm sarcomere length ($S_{2.60}$) to that recorded at 2.20 μm sarcomere length ($S_{2.20}$) plotted against the force held by the fibre during the load-clamp phase. Force is expressed as a fraction of the tetanic force measured at 2.20 μm sarcomere length. Data collected from eight individual muscle fibres with 1–3 different load-clamp levels tested in each fibre. Line: linear regression of stiffness ratio upon force. The slope of the line does not deviate from zero, $P>0.5$. Each data point is based on 1–3 recordings at 2.20 and 2.60 μm sarcomere length.

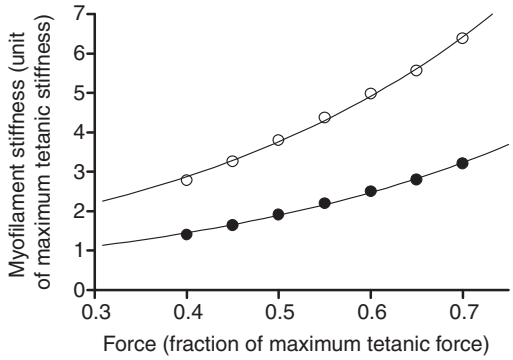

Fig. 4. Myofilament stiffness calculated for different force levels based on data presented in Fig. 3. Open symbols: myofilament stiffness at 2.20 μm sarcomere length. Closed symbols: myofilament stiffness at 2.60 μm sarcomere length. Lines: least-squares fitting of exponential growth $y=0.99\exp(2.67\times)$ (upper curve) and $y=0.50\exp(2.67\times)$ (lower curve). $R^2=0.99$. Force is expressed as a fraction of the tetanic force measured at 2.20 μm sarcomere length.

plotted for comparison in Fig. 4. The myofilament stiffness can be seen to increase progressively as the load on the fibre was raised from 40 to 70% of the maximum tetanic force. At full overlap, i.e. at 2.20 μm sarcomere length, and at 40% of maximum load on the fibre, the calculated myofilament stiffness was approximately 2.5 times the maximum cross-bridge stiffness. The myofilament stiffness more than doubled as the load was increased to 70% of maximum tetanic force. The myofilament elasticity thus exhibits the characteristics of a non-Hookean spring.

DISCUSSION

Evidence was produced in two independent X-ray diffraction studies, both performed on frog striated muscle, suggesting that the thick and thin filaments have a finite stiffness that is comparable with that residing in the cross-bridges during tetanic activity (Huxley et al., 1994; Wakabayashi et al., 1994). The existence of filament compliance has later been corroborated in several studies on various isolated muscle preparations (see earlier), even by measurements on isolated actin filaments (Kojima et al., 1994). Subsequent studies on skinned skeletal muscle fibres have further elucidated the existence of myofilament compliance and its contribution to the kinetic properties of the muscle fibre during activity (Martyn et al., 2002).

In the present study, an approach has been used to evaluate the spring-like properties of the myofilaments in an intact muscle fibre. The stiffness measurements (for details, see Materials and methods) were performed during load-clamp recordings at two selected sarcomere lengths, 2.20 and 2.60 μm, i.e. over a range where the resting tension is negligible (Edman, 1979). An important point in these measurements is the fact that the same load was held by the fibre during the load-clamp phase at the two lengths. This ensures that passive elastic elements acting in series with the myofilament system, such as tendons and attachments to the transducer arm, remained constant during the stiffness measurements at the two sarcomere lengths. Furthermore, by using the same clamp-force at the two sarcomere lengths, the number of myosin motors involved in force production can be presumed to be the same in the two situations. The load held by the fibre during the stiffness measurement was limited to be within the range 40–70% of maximum tetanic force for the following reasons: (1) the force employed during the load-clamp test was not allowed to exceed the isometric tetanic force at 2.60 μm sarcomere length in order to avoid stretching the muscle fibre; (2) previous measurements (performed in the same experimental setup as that used in the present study) have demonstrated that the error in the fibre stiffness measurement due to tendon compliance is negligible at force levels greater than 40% of maximum tetanic force (Edman et al., 1997).

With the approach used, the stiffness measurement was specifically focused on the free, non-overlapping portions of the thick and thin filaments without distinction between the two filaments. Excluded from the measurements are the anchoring sites of the filaments at the *M* line and *Z* disk, as the measurements would only include those portions of the filaments that become free of overlap when the sarcomeres are extended from 2.20 to 2.60 μm.

It is worth pointing out that the stiffness measurement presented in the current study refers to the myofilaments *in situ* in the fibre, i.e. in an environment where the actin and myosin filaments are surrounded by, and interwoven with, a number of auxiliary filaments, such as titin and desmin that make up the cytoskeleton (e.g. Maruyama et al., 1977; Wang and Ramirez-Mitchell, 1983; Wang et al., 2001; Erickson, 1997; Granzier et al., 1997; Linke et al., 1998; Granzier et al., 2002; Kreplak et al., 2008). These cytoskeletal structures may be regarded as an integral part of the 'myofilament elasticity' measured in intact whole muscle or isolated muscle fibres irrespective of the measuring technique used. The contribution to the measured filament stiffness coming from the cytoskeletal structures is still difficult to assess and requires further studies to be settled. Interaction between the titin and actin filaments may occur (see Granzier et al., 1997; Yamasaki et al., 2001) and this could affect the tensile properties of the actin filament. It is of interest to mention in this connection that the intact muscle fibre behaves as a constant volume (see Edman, 1999). The fibre diameter thus varies as an inverse square root function of the sarcomere length. This means that elastic structures that are oriented transversely, like desmin, may in fact also respond to longitudinal length changes of the fibre.

It is now generally believed (see Gordon et al., 2000) that the myosin cross-bridge cycle is initiated in a weakly bound state that

translates into a strongly bound state that is associated with force production. Bridges in the weakly bound state are thought to contribute to fibre stiffness but not to active force. In the above calculations, it was implicitly assumed that the ratio of 'weak' to 'strong' cross-bridges remains the same during loaded shortening at the sarcomere lengths and clamp levels tested. This assumption is based on the findings by Ford and colleagues (Ford et al., 1981; Ford et al., 1985), who demonstrated that the measured sarcomere stiffness varies almost in proportion to the developed force, both during active shortening at various loads and velocities of shortening and during isometric contraction at various degrees of filament overlap. These observations support the view that the ratio of 'weak' to 'strong' cross-bridges is maintained fairly constant at the different force levels and sarcomere lengths studied.

Earlier studies suggest that the stiffness of the thick and thin filaments is similar in magnitude to that residing in the myosin cross-bridges during maximal activity (Huxley et al., 1994; Wakabayashi et al., 1994). The present results indicate that the myofilament stiffness at full overlap (2.20 μm sarcomere length) markedly exceeds the cross-bridge stiffness and, furthermore, that it increases in magnitude with increasing tension on the muscle fibre. The calculated myofilament stiffness was thus found to more than double as the load on the fibre was raised from 40 to 70% of maximum isometric force. This result is in fair agreement with previous stiffness measurements reported by Higuchi and colleagues (Higuchi et al., 1995), who estimated the thin filament compliance by imposing small cyclical length changes on skinned muscle fibres in rigor at sarcomere lengths where the overlap between the thick and thin filaments is complete. Their results suggest, in accordance with the present data, that the thin filament stiffness is nearly doubled as the load on the fibre is increased from 50 to 150 $kN\,m^{-2}$, i.e. over a force range similar to that covered in the present experiments. Non-linearity of myosin compliance has similarly been suggested in two recent X-ray diffraction studies, in which muscle force was varied by the muscle relaxant BDM (Griffith et al., 2006) and by recording the myosin reflections at various speeds of active shortening (Huxley et al., 2006).

In conclusion, with the approach used in the present study, it has been possible to evaluate the stiffness of the free portions of the thick and thin filaments, relative to the cross-bridge stiffness, in intact single muscle fibres. The data indicate that the filament stiffness calculated at optimum filament overlap (2.20 μm sarcomere length) exceeds that residing in the cross-bridges at full activation. The myofilament elasticity has the characteristics of a non-Hookean spring; the stiffness of the filaments increases 2.5-fold as the tension held by the fibre is raised from 40 to 70% of maximum tetanic force.

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