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Tension and stiffness of frog muscle fibres at full filament overlap

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Summary

Stiffness measurements in activated skeletal muscle fibres are often used as one means of estimating the number of attached crossbridges on the assumption that myofilament compliances do not contribute significantly to the fibre compliance. This assumption was tested by studying the effects of sarcomere length on fibre stiffness in the plateau region of the length–tension diagram (from 1.96 to 2.16 μm sarcomere length in the tibialis anterior muscle of the frog). Lengthening of the sarcomere across this region in fact, produces only an increase in the proportion of actin filament free from cross-bridges without altering the amount of effective overlap; no change in fibre stiffness is therefore expected if actin filaments are perfectly rigid. The results show that while tetanic tension remained constant within 1.5%, as the sarcomere length was increased from 1.96 to 2.16 μm fibre stiffness decreased by about 4%, indicating that a significant proportion of sarcomere compliance is localized in the actin filaments. A simple model based on the sliding filament theory was used in order to calculate the relative contribution of actin filaments to fibre compliance. In the model it was assumed that fibre compliance resulted from the combination of crossbridge compliance (distributed over the overlap zone) in series with thin filament and tendon compliances. The calculations show that the experimental data could be adequately predicted only assuming that about 19% of sarcomere compliance is due to actin filament compliance.

Introduction

In their experiments on single muscle fibre of the frog, using the spot follower device to eliminate the effects of tendon compliance, Ford and co-workers (1981) showed that fibre compliance was almost exclusively localized in the crossbridges. According to their measurements, at full filament overlap crossbridge compliance was at least 80% and probably well over 90% of the total fibre compliance. The compliance arising from other sarcomere structures (contractile filaments and Z-line) would therefore make a negligible contribution to the sarcomere compliance. On this basis Ford and co-workers (1981) suggested that, at least in the sarcomere length region of full overlap, fibre stiffness can be used as a measure of the number of attached crossbridges. Since then, stiffness measurements have been used by many investigators to measure the number of attached crossbridges in various experimental conditions in intact and skinned fibres (Brenner & Eisenberg, 1986; Brozovich *et al.*, 1988; Cecchi *et al.*, 1982; Cecchi *et al.*, 1987; Goldman & Simmons, 1986; Julian & Morgan, 1981). However, the assumption that fibre stiffness is exclusively localized in the crossbridges has been called into question by the observation, reported by Julian & Morgan (1981), that actin filament compliance alone would contribute some 30% to the total fibre compliance. The experiments reported here were aimed at clarifying this point.

As suggested by Ford and co-workers. (1981) stiffness measurements in the plateau region of the length–tension relation offer one means of estimating the contribution of actin filament to fibre compliance. In fact, lengthening of the sarcomeres from the lower to the upper limit of the plateau region causes only an increase in the length of the segment of actin filament free from crossbridges in series with the overlap zone. If thin filaments are compliant, the fibre stiffness will therefore be lower at the upper limit than at the lower one. To test this expectation we have measured stiffness and tension in single muscle fibres during tetanic contraction along the plateau region of the length–tension diagram.

Materials and methods

Single fibres dissected from tibialis anterior muscles of the frog (*Rana esculenta*) were mounted by means of aluminium foil clips (Ford *et al.*, 1977) between the lever arms of a force transducer and a loudspeaker motor in a chamber provided with a glass floor for ordinary and laser light illumination of the fibres. Experiments were performed at 12° C (± 0.1° C).

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Tension was measured by means of a capacitance-gauge transducer (natural frequency between 40 and 60 kHz) similar to that described by Huxley & Lombardi (1980) connected to a phase discriminator circuit (Cecchi, 1983). The sensitivity of the transducers used ranged from 100 to 300 mV per mN and the noise from 0.2 to 0.5 mV peak to peak. Sarcomere length during activity was determined from the laser diffraction pattern of the fibre by means of an opto-electronic system previously described (Bagni *et al.*, 1985). The rise time of the system was 5 μs and the noise corresponded to about 0.2 nm per sarcomere peak to peak. The laser beam was positioned on a region of the fibre, close to the force transducer, selected for uniformity of sarcomere spacing. Sinusoidal and step length-changes were imposed on the muscle fibres by means of a servo controlled loudspeaker motor derived from that already described by Cecchi and coworkers (1982). Fixed-end conditions were obtained by using as a feedback signal the output from the photodiode sensor signalling the position of the loudspeaker lever arm. Length-clamp conditions were obtained by using as a feedback signal the output from the laser diffraction system so as to hold constant the length of the sarcomeres in the selected fibre segment. Tension was measured in length-clamp condition.

Fibre length, cross sectional area and sarcomere spacing were measured under ordinary illumination on the moveable stage of a microscope (ACM Zeiss, F.R.G.). A 40 × dry objective (Zeiss, F.R.G., NA 0.6, working distance 4.9 mm) and 10 × or 25 × micrometer eyepieces were used. Striation spacings were determined by averaging the measurements of sequences of twenty sarcomeres at different points along the fibre. The Ringer's solution had the following composition (in mM l^{-1}): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; phosphate buffer, 3; at pH 7.1.

Stimuli of alternating polarity of 0.5 ms duration and 1.5 threshold strength were applied transversely to the muscle fibre by means of a pair of platinum plate electrodes. Tetanic stimulation was applied in brief (0.6–0.8 s duration) volleys at regular 4 min intervals. Force, fibre length, sarcomere length and intensity of the first order diffraction line were measured on a digital oscilloscope (4094 Nicolet, USA) and stored in its floppy disk memory for further analysis.

Determination of stiffness

Stiffness was determined in fixed-end conditions at the plateau of tetanic contractions elicited at different sarcomere lengths between 1.92 and 2.20 μm. It was measured by the ratio of the instantaneous tension change in response to small (about 0.1% l_0) sinusoidal (frequency, 3–4 kHz) or step (rise time 100 μs) length changes imposed at one tendon end over the amplitude of the length change (see Fig. 1). Although it would have eliminated the necessity to correct for tendon contribution to the computed fibre compliance, we did not use the laser diffraction signal to measure the compliance since the sarcomere length changes were affected by small random errors (about ± 0.4 nm per sarcomere), very likely due to Bragg angle effect (Rüdel & Zite-Ferenczi, 1979), which were not negligible in comparison to the length changes of 0.1% l_0 (2 nm per sarcomere) applied to the fibre to measure the stiffness. Passive tension did not contribute to these measurements, since in the range of sarcomere length studied it was negligible.

Modelling

A simple numerical model based on the sliding filament theory was used to simulate force and stiffness changes and to calculate the contribution of actin filament to total fibre compliance. In the model it was assumed that both crossbridges and thin filaments have a finite Hookean compliance. Fibre compliance results from the combination of tendon compliance in series with the compliance of the spring network representing thin filaments and the crossbridges distributed over the overlap region and with the compliance of the thin filaments between the Z-line and the overlap zone.

To simulate the length–tension relation, the following assumptions were made: (1) in the descending limb of the sarcomere length–tension curve, force is proportional to the amount of overlap between thick and thin filaments in the half sarcomere. (2) The length of overlap becomes maximum when the tips of thin filaments reach the bare zone of the thick filaments. (3) Rounding of the right corner of the plateau of the sarcomere length–tension relation is due to a slight variability in the amount of overlap in the sarcomeres within the fibre cross section in the length-clamped region. This variability was simulated by a slight variation in the lengths of the thin filaments within the half sarcomere. (4) On the left side of the plateau, net production of force declines proportionally to the amount of double overlap between actin filaments from the opposite halves of the sarcomere.

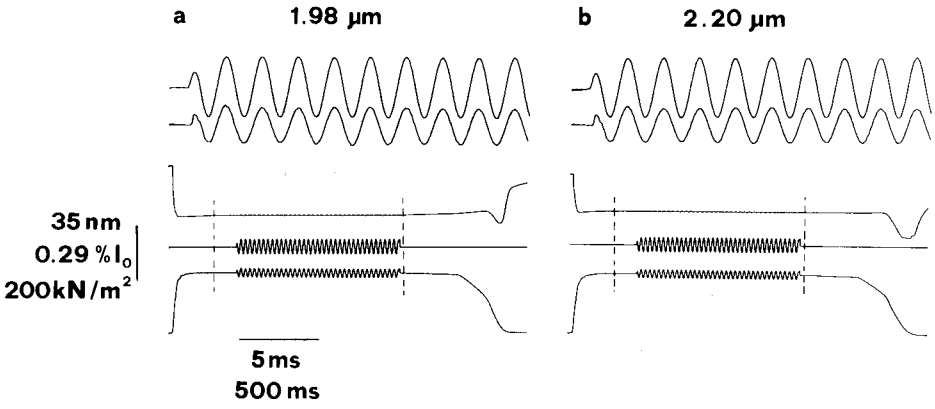

Fig. 1. Sample records illustrating stiffness measurements in a tetanized fibre at 1.98 μm (a) and 2.20 μm (b) sarcomere length. Traces show, from bottom to top, tension, fibre length, sarcomere length, again tension and length both expanded 8 times horizontally and 4 times vertically. Time calibration is 5 ms within the dashed lines and 500 ms outside. Note that no phase shift is present between force and length sinusoids.

In the computations, the lengths of a number of thin filaments (usually 300) were assumed to be normally distributed in the half sarcomere. The lengths of the thick filaments and their bare zone and the mean length of the thin filaments were selected in order to account for the following characteristics of the sarcomere length-tension curve reported by Bagni *et al.* (1988) for single fibres from frog tibialis anterior: (1) the intercept of the descending limb on the sarcomere length axis that corresponds to the sum of thin and thick filament lengths is 3.53 μm. (2) The extrapolated upper limit of the plateau corresponding to the sum of the thin filament length plus the bare zone length on the thick filaments is 2.16 μm. The proportionality coefficient relating force to overlap was assumed to be 1. The numerical parameters to be identified were therefore (a) the mean and the standard deviation of thin filament length and (b) the proportionality coefficient relating force decline on the ascending limb to double overlap. The geometrical parameters that best simulate the sarcomere length–tension relation are the following: actin filament length, 1.98 $\pm$ 0.04 μm; myosin filament length, 1.55 μm; bare zone length, 0.18 μm. The comparison between model computations and experimental data is reported in Fig. 2.

The sarcomere length-overlap relation computed by the model was used to calculate the effects of sarcomere length on fibre stiffness. Since in the present experiments stiffness measurements were made in fixed-end conditions, the contribution from tendon compliance has been considered by adding an exponential elasticity in series with the sarcomere structures. Under our experimental conditions, tendon compliance at full filament overlap has been previously estimated using the striation follower to detect length changes at sarcomere level, to be about 20% of the total fibre compliance (Cecchi *et al.*, 1987). Sarcomere compliance was calculated according to equation A10 reported by Ford and co-workers (1981), with the additional simplification that Z-line and myosin compliance were assumed to be zero. This simplification has no effect on actin filament compliance calculation in the region of full overlap. Several computer runs were performed to find the combination of crossbridge and actin filament compliances that best approximates the experimental stiffness data. For simplicity it was assumed that on the left side of the plateau, sarcomere compliance could still be described by Ford and co-workers' (1981) equation A10, without any additional complication due to the formation of thin filament double overlap. The calculated sarcomere compliance is only slightly affected by this assumption since our stiffness data do not extend appreciably to the left side of the plateau.

Furthermore, the contribution of actin filament to total fibre compliance was also directly estimated from the experimental stiffness data obtained in the sarcomere length region (2.0–2.08 μm) where tension and presumably the effective overlap was strictly constant (see Appendix). This approach eliminated the necessity of making any assumption about: (i) mechanism responsible for the drop of tension on the ascending limb, (ii) amount and shape of tendon, Z-line and thick filament compliances, (iii) amount of overlap variability. In fact, as shown in the Appendix, actin contribution to fibre compliance is a linear function of the stiffness change across the sarcomere length region where effective overlap is constant.

Results

Figure 3 shows typical records of fixed-end and length-clamped tetanic contractions in a single muscle fibre at 1.97 and 2.53 μm sarcomere length. The record at

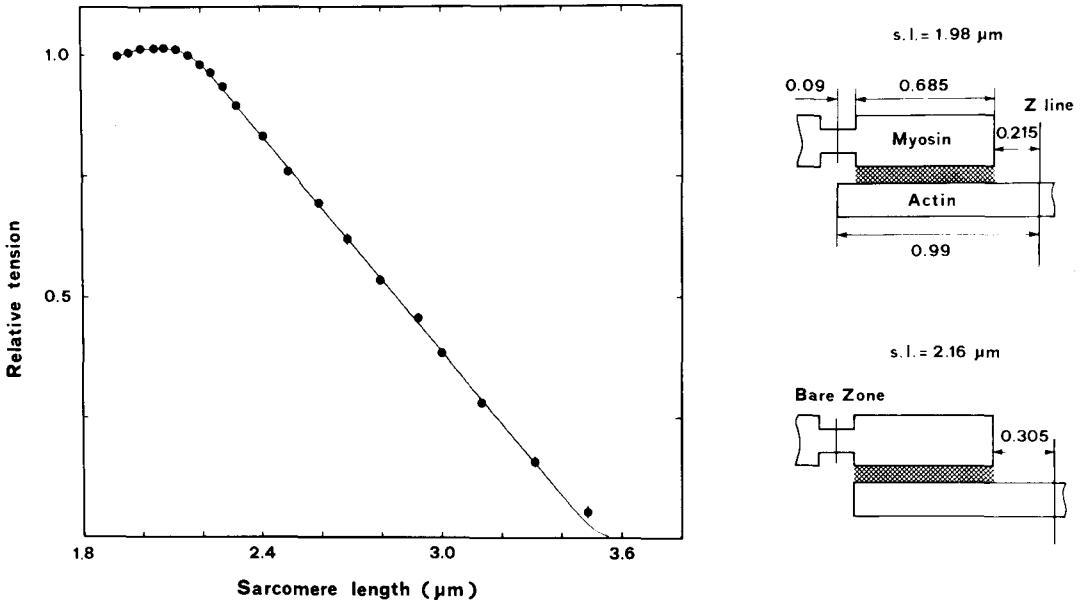

Fig. 2. Left: fitting of the model response to the experimental data of the whole length–tension relation reported by Bagni and co-workers (1988). The consequence of assuming a standard deviation of filament overlap of $\pm$ 0.02 μm per half sarcomere is a small rounding of the plateau corners and a small upward curvature of the descending limb in the proximity of the intercept on the abscissa. Right: schematic diagram illustrating the modifications in the half sarcomere geometry produced by increasing sarcomere length from 1.98 to 2,16 μm. It can be seen that as a consequence of this lengthening there is an increase of the portion of actin filament free from crossbridges from 0.215 to 0.305 μm. If the actin filament is compliant this increases the total compliance of the half sarcomere (lengths are expressed in μm).

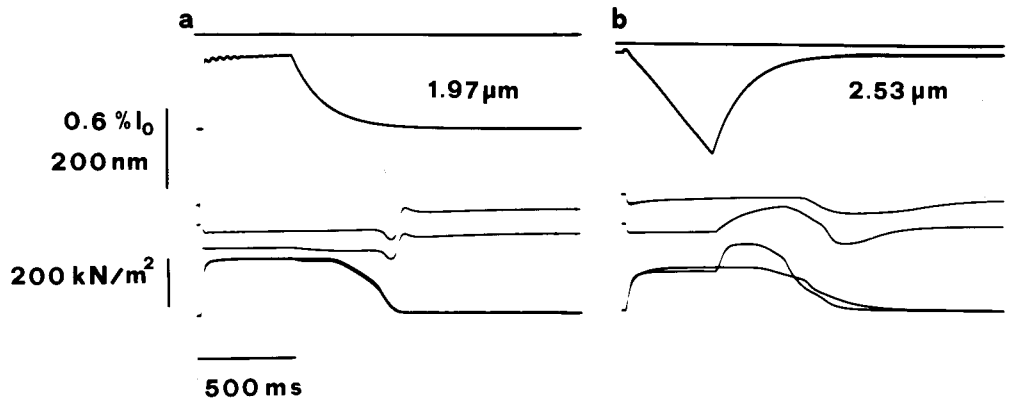

Fig. 3. Fixed-end and length-clamp tetanic contractions in single muscle fibres at two different sarcomere lengths: 1.97 μm (a) and 2.53 μm (b). Traces from top to bottom: fibre length (fixed-end and length-clamp); sarcomere length (fixed-end and length-clamp); tension (fixed-end and length-clamp superimposed). The length-clamp is applied during the tetanus rise when the tension is about 0.5 P_0. In (a) to maintain s.l. constant, the fibre is quickly stretched to a longer length while in (b) the fibre is released at almost constant speed. When the length-clamp is ended, during the tetanus plateau, length traces return slowly to their resting level. Downward deflections indicate sarcomere shortening. Note that the application of length-clamp abolishes the slow creep of tension present in normal isometric contraction at 2.53 μm.

2.53 μm shows the effectiveness of the length-clamp system in eliminating the slow creep of tension that follows the rapid development of tension in fixed-end contractions. No difference is present between fixed-end and length-clamped tetani at 1.97 μm since after an initial shortening sarcomere length remains constant. Figure 1 shows experimental records used for stiffness measurements. The sinusoidal length oscillations applied to one fibre end produce sinusoidal tension changes at the other end. Note that both at 1.98 and 2.2 μm, length and force are perfectly in phase. The absolute stiffness value measured in this fibre at 2.20 s.l. corresponded to a Young modulus of 39.7 MN m^{-2}. The mean value of y_0 (the amount of instantaneous shortening necessary to discharge completely the tetanic tension) measured in 9 fibres was 0.61 ± 0.024% l_0.

Figure 4 shows the length-tension and the length-stiffness relationships at sarcomere lengths near the optimum for tension development, obtained from 10 length-clamped segments from as many fibres. Individual data points were obtained by 0.04 μm class averaging of tension, stiffness and sarcomere length. Tension and stiffness were expressed relative to the values at 2.16 μm. It can be seen that, in agreement with data reported by Bagni *et al.*, (1988), tension varied less than 1.5% between 1.92 and 2.16 μm and it was constant between 2.00 and 2.08 μm. On the contrary, stiffness was not constant across the plateau region but increased linearly with decreasing sarcomere length and at 1.96 μm was about 4% greater than at 2.16 μm. Stiffness change in the region where tension remained strictly constant (2.00–2.08 μm) was about 16% per μm, a value smaller than that (21% per μm) reported by Julian & Morgan (1981). Beyond 2.16 μm stiffness fell approximately in proportion to tension.

As stated in the Introduction, the observed lack of proportionality between stiffness and tension can be due to the contribution of thin filament to the compliance of the whole fibre. This was tested using the sliding filament model described in detail under Materials and methods.

Model calculations show that the experimental tension points can be closely approximated (continuous line in fig. 4) by assuming a standard deviation of filament overlap of ± 0.023 μm per half sarcomere, and a proportionality coefficient relating force decline on the ascending limb to double overlap, equal to 0.2. Calculated stiffness could simulate experimental data only when actin filaments were assumed to have a significant compliance. The closest approximation to experimental stiffness data was obtained when actin filaments were assumed to contribute by 15% to the compliance of the whole fibre calculated at 2.16 μm sarcomere length (dashed line in fig. 4). The dotted line shows the computed stiffness resulting from assuming that all the sarcomere compliance resides in the crossbridges. In all the computations tendon compliance was assumed to contribute by 23% to the total fibre compliance at 2.16 μm.

As shown in the Appendix, about the same amount (15.4% at 2.08 μm sarcomere length) of actin contribution to fibre compliance was calculated directly from the experimental stiffness data obtained in the sarcomere length region (2.00–2.08 μm) where tension was strictly constant.

Discussion

Sarcomere length–tension relation

As previously reported (Bagni *et al.*, 1988), the plateau region of the sarcomere length–tension diagram in tibialis

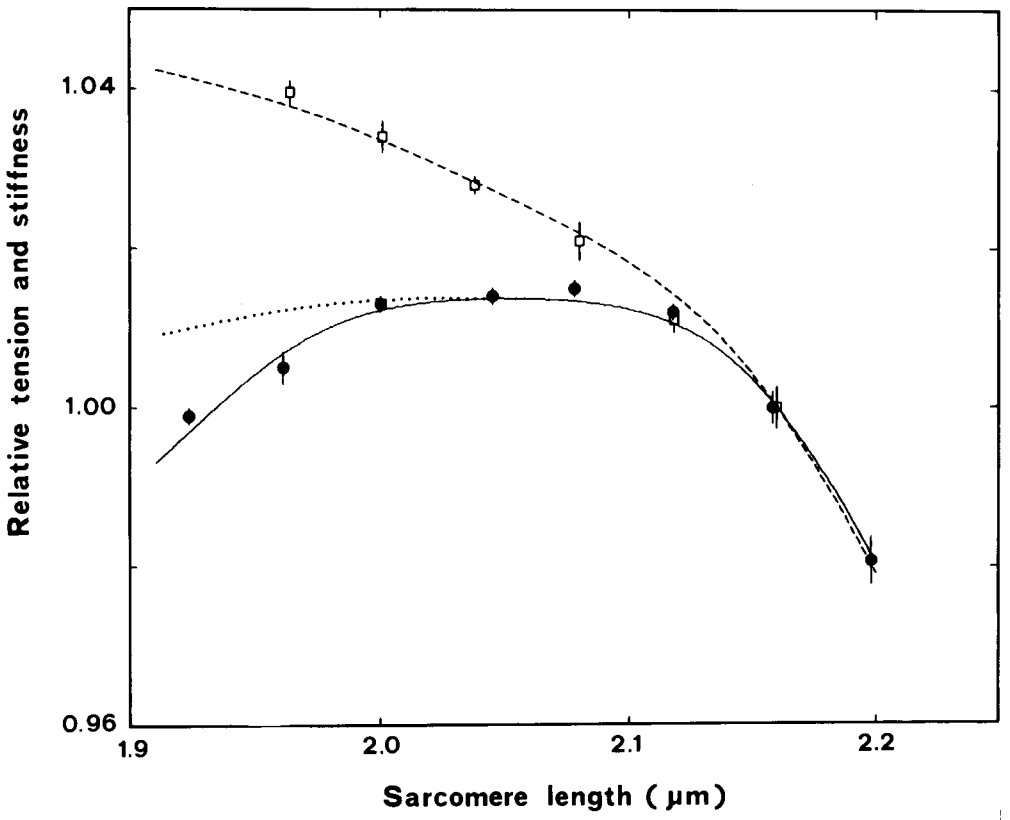

Fig. 4. Sarcomere length–tension and stiffness relations over the plateau region of the length–tension diagram. Individual data points were obtained by 0.04 μm class averaging of tension, stiffness and sarcomere length data from ten fibres. Continuous and dashed lines show the fitting of the model to experimental force and stiffness data, respectively. Stiffness (open squares) can be adequately fitted only by assuming that a significant proportion (15%) of fibre compliance is localized in the actin filaments. Dotted line shows the model response obtained assuming that actin filaments are not compliant and no change in stiffness is produced by the formation of double overlap. The small downward curvature is due to the nonlinearity of the tendon compliance which was assumed to be of exponential shape and contributing 23% to the total compliance.

anterior fibres, extends from about 1.96 to 2.16 μm, with an absolute flat region from 2.0 to 2.08 μm. The rounding of the left and right corners of the plateau is probably due to some non uniformity in the amount of overlap, arising from variability of sarcomere length, filament length and misalignment of the filaments in the sarcomeres (Gordon *et al.*, 1966; Edman & Reggiani 1987). In our model we assumed that overlap non uniformity is entirely due to variability in actin filament length; however, the conclusion would not have been substantially different if the variability were localized in different sarcomere structures. A close approximation to the experimental data was obtained assuming a variability of the thin filament length normally distributed with a standard deviation of ± 0.023 μm per half sarcomere. This figure is very close to the standard deviation of ± 0.0175 μm per half-sarcomere computed by Gordon *et al.* (1966) on the assumption that the rounding of the plateau corners is attributable to non uniformity of sarcomere lengths within the length-clamped segment. On the other hand, a much higher standard deviation of overlap length (± 0.21 μm per half sarcomere) has been computed by Edman & Reggiani (1987) using a model similar to ours. The discrepancy is probably due to the different shape of their length-tension relation which lacks a true flat region.

Sarcomere length–stiffness relation

As clearly shown in Fig. 4, stiffness does not remain constant across the plateau region but it increases by about 4% as the sarcomere length decreases from 2.16 to 1.96 μm. Since the only consequence of decreasing the sarcomere length from 2.16 to 1.96 μm is the reduction of the portion of actin filament free from crossbridges, without any change in effective overlap, this increase in stiffness indicates that a significant portion of fibre compliance is localized in the actin filament itself.

The comparison of the model response with the experimental data (Fig. 4) shows that in order to fit our stiffness measurements it is necessary to assume that at an s.l. of 2.16 μm about 15% of the total fibre compliance (including tendon contribution) is localized in the actin filament. The value estimated with the model is fundamentally independent from the assumptions about the

consequences of double overlap formation. In fact, as shown in the Appendix, a value very close to that produced by the model is that directly calculated from the experimental data using an approach that does not require any assumption, not only about the mechanism responsible for the ascending limb of the length-tension relationship, but also about tendon, Z-lines and myosin compliances.

As stated in Materials and methods, our stiffness measurements were made in fixed- end conditions, they therefore included a contribution from tendon compliance. Since at tetanus plateau (at sarcomere lengths close to the optimum) the tendon compliance represents about 20% of the total, this means that the contribution of the actin filaments to the sarcomere compliance rises to about 19%. Ford and co-workers., (1981) gave a maximum possible value for the compliance of the elastic structure outside the crossbridges of about 20% of total compliance. This value includes contributions from actin, myosin and Z-line, while our value of 19% refers only to actin contribution. If we assume that compliance per unit length is the same for all actin filaments in parallel and for all myosin fillaments in parallel, the overall filament compliance would be about 32%, a value sensibly higher than that reported by Ford and co-workers, (1981). However, this discrepancy might be due to our experiments being done at higher temperature.

Our stiffness measurements were made using small sinusoidal oscillations, they therefore involved small stretches and releases while Ford and co-workers, (1981) used only step releases. Because of the non-linearity of the elastic behaviour of the fibre (Ford *et al.*, 1977) stiffness measured with oscillations (involving stretches) is slightly higher than that measured only with releases. However, this complication would not alter our conclusion since our stiffness measurements are expressed relatively to the value measured at 2.16 μm and are independent of the absolute stiffness value. In addition, in a few experiments in which stiffness was measured with step releases we found the same results.

Our value for the actin filament contribution to total compliance, is considerably smaller than the value of 30% reported by Julian & Morgan (1981). However it should be noted that Julian & Morgan (1981) did not consider the rounding of the corners of the length–tension diagram and that the theoretical length–tension diagram they used was the one derived from experiments on semitendinosus fibres (Gordon *et al.*, 1966), while their stiffness measurements were made on tibialis anterior muscle. This procedure may have influenced the fitting since tibialis anterior and semitendinosus fibres have a slightly different length–tension relationship (Bagni *et al.*, 1988).

If the actin filament is relatively compliant it must elongate as the muscle fibre goes from rest to maximum tetanic tension. Since the actin filaments contribute to about 20% of sarcomere compliance and the total elongation of the elastic structure of the half sarcomere at the tetanus plateau (y_0) is about 4 nm (Ford *et al.*, 1977), this means that the actin filaments elongate by 0.8 nm per half sarcomere. In principle it should be possible using X-ray diffraction techniques to observe if a real elongation is taking place during tetanic contraction. Actually, data reported in the literature (Huxley & Brown, 1967; Kress *et al.*, 1986) have shown that no significant change in the spacing of the second actin layer line reflections is produced during contraction. However, it must be considered that the relative length change of the actin filament is very small; assuming for simplicity that the elongation is uniformly distributed along the thin filament, the change would be only 0.8/900, i.e. less than 0.1% This is certainly below the resolution of X-ray diffraction experiments.

The principal conclusion from the results presented in this paper is that a significant portion of the sarcomere compliance of a fibre tetanically stimulated at sarcomere lengths near the optimum is localized in the actin filaments. This implies that in order to precisely correlate fibre stiffness measurements with the number of attached crossbridge it is necessary to consider a contribution from the actin filament compliance.

Appendix

As stated in Materials and methods, present experimental data can give a direct estimate of the contribution of actin filaments to fibre compliance if the analysis of the sarcomere length–stiffness relation is limited to that range of sarcomere lengths where tension is maximal and strictly constant (present data show that from 2.00 to 2.08 μm tension changes are less than 0.2%). In fact, since in this region the tension is constant, it is reasonable to assume that, in spite of slight variabilities in filament length, the effective overlap between thin and thick filaments is maximal and remains constant, while no double overlap is formed. Under these circustances, the contribution to fibre compliance by all the elastic structures except actin is constant and equation A10 from Ford and co-workers, (1981) can be simplified to:

$$C(s) = C_A(l_A(s) - V) + K \qquad 2.00 \leq s \leq 2.08\ \mu m \quad (1)$$

where $C(s)$ is the compliance of the fibre as a function of the sarcomere lengths s, C_A is thin filament compliance per unit length; $l_A(s)$ is the actin filament length from Z-line to the end of the effective overlap with myosin; V is a constant equal to 2/3 the maximal effective overlap and K is a constant given by the sum of crossbridges, myosin and tendon contribution to fibre compliance in the region where effective overlap is maximum, plus the constant contribution from the Z-line. From equ. 1 actin compliance per unit length can be expressed in terms of the difference between the compliances of the fibre at the upper (C_{upper}) and lower (C_{lower}) limits of the flat region of the sarcomere length–tension diagram:

$$C_A = \frac{C_{upper} - C_{lower}}{l_{A\,upper} - l_{A\,lower}} \quad (2)$$

where $l_{A\,upper} - l_{A\,lower}$ corresponds to the length change of the half-sarcomere between the upper and lower limits of the flat region.

In present experiments, the fibre compliance measured at 2.00 μm sarcomere length was on average 1.25% lower than that measured at 2.08 μm. From these data, using equ. 1 and the geometrical parameters reported in Fig. 2, it can be calculated easily that the relative contribution of actin to fibre compliance at 2.08 μm sarcomere length is 15.4%. This estimate is very close to that obtained from the model analysis of all the experimental data points.

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