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FACTORS INFLUENCING THE ASCENDING LIMB OF THE SARCOMERE LENGTH–TENSION RELATIONSHIP IN RABBIT SKINNED MUSCLE FIBRES

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SUMMARY

1. The length dependence of Ca^{2+}-activated tension within the ascending limb of the length–tension relationship, corresponding to sarcomere lengths below about 2·25 μm, was investigated in skinned fibres from rabbit psoas muscle. At high [Ca^{2+}] a shallow phase and then a steep phase of tension decline were observed as sarcomere length was reduced, while at low [Ca^{2+}] tension decreased monotonically with decreases in sarcomere length. The sarcomere length at which the ascending limb intersected zero tension was greater for lower concentrations of Ca^{2+}.

2. The length–tension relationship from maximally activated fibres changed when filament lattice spacing was reduced by osmotic compression. Relationships obtained in the presence of 5% (w/v) dextran T500 more distinctly demonstrated both the shallow and steep portions of the ascending limb than did relationships from untreated fibres.

3. As striation spacing was decreased a progressive decline in the Ca^{2+} sensitivity of tension development was observed. Tension–pCa relationships from both control and dextran-treated fibres underwent a rightward shift (i.e. to a higher [Ca^{2+}]) by 0·23 pCa units as sarcomere length was reduced between 2·46 and 1·54 μm.

4. Fibre stiffness was studied by applying a 3·3 kHz sinusoidal length change at one end of the fibre and measuring the resultant tension change. At submaximal activation (pCa 5·8), stiffness increased relative to tension as sarcomere length was decreased below $\sim$ 2·4 μm, suggesting that there is an activation-related internal load at low [Ca^{2+}]. At maximal activation, a significant increase in this ratio occurred only at sarcomere lengths less than $\sim$ 1·8 μm, and presumably involved collision of the thick filaments with the Z-lines.

5. Length-dependent changes in the Ca^{2+} sensitivity of tension development do not appear to be the result of shortening-induced dissociation of Ca^{2+} from troponin-C, the Ca^{2+} binding subunit of troponin. Fibres activated in the absence of Ca^{2+}, by the partial removal of whole troponin complexes, produced length–tension relationships similar to those observed in the same fibres before troponin removal at a submaximal [Ca^{2+}] yielding similar active tensions.

INTRODUCTION

The relationship between isometric tension and sarcomere length has been studied extensively in both living and skinned single-fibre preparations of vertebrate skeletal muscle. However, the basis of the ascending limb of this relationship, corresponding to sarcomere lengths shorter than the optimum for tension development, remains a subject of controversy. Gordon, Huxley & Julian (1966*a*, *b*) established the shape of the ascending limb for tetanically stimulated single muscle fibres from the frog and found a shallow portion (corresponding to sarcomere lengths between about 2·05 and 1·70 μm) and a steep portion (between sarcomere lengths of 1·70 and 1·30 μm). In the shallow portion, increasing overlap of the thin filaments from opposite ends of the sarcomere was thought to interfere with cross-bridge attachments as sarcomere length was decreased. The steep portion of the relationship was thought to result from the compression of the ends of the thick filaments against the Z-lines.

Others (Taylor & Rudel, 1970; Rudel & Taylor, 1971; Lopez, Wanek & Taylor, 1981) have proposed that at short sarcomere lengths activation may be length dependent, since tetanic tension at short lengths was enhanced by the application of twitch-potentiating agents such as nitrate. These results suggest that the amount of Ca^{2+} released during tetanic stimulation may vary with changes in sarcomere length. Consistent with this suggestion, Schoenberg & Podolsky (1972) found that the steep portion of the ascending limb was substantially elevated in maximally activated skinned fibres from frog muscle when compared to intact fibres, although the shallow portion had the predicted form. However, Moss (1979) found good agreement between length–tension relationships determined in frog skeletal muscle fibres, first while living and tetanically stimulated and then in the same fibres at high levels of Ca^{2+} activation following skinning. These results, obtained in preparations in which Ca^{2+} was directly controlled, were inconsistent with the idea that a variation in Ca^{2+} release is a major determinant of the form of the ascending limb in living muscle fibres. More recently, Julian & Morgan (1981) have found no effect of twitch-potentiating agents on tetanic tension in the range of sarcomere lengths between 2·00 and 1·65 μm in intact muscle fibres from the frog.

In the present study, skinned single fibres from rabbit psoas muscle were used to investigate the effects of variations in the level of steady Ca^{2+} activation on the sarcomere length–tension relationship. Due to better sarcomere length uniformity and the longer thin filaments in rabbit, we have found this relationship to differ from that previously established in frog skinned fibres (Moss, 1979). Also, the increase in filament lattice separation which occurred upon skinning was found to distort the length–tension relationship. The concentration of activating Ca^{2+} had a pronounced effect on the form of the ascending limb. Measurements of stiffness as a function of sarcomere length suggest that there is an activation-related internal load which arises at low Ca^{2+} concentrations as a result of shortening and which is not apparent at high concentrations of Ca^{2+}.

METHODS

Bundles of approximately fifty fibres were dissected from psoas muscles of adult male New Zealand rabbits. The bundles were tied to glass capillary tubes and stored at −22 °C in relaxing solution containing 50% (v/v) glycerol for 3–21 days before use (Moss, Giulian & Greaser, 1983).

Individual fibres were pulled from each bundle and segments 2–4 mm in length were mounted in the experimental chamber containing relaxing solution. The dissecting and mounting procedures as well as the details of the experimental apparatus have been described previously (Moss *et al.* 1983*a*). Mean sarcomere length in the relaxed fibre segments was initially adjusted within the range 2·45–2·70 μm by changing the over-all length of the segment (L_0). In this paper all mean values are expressed $\pm$S.D., unless otherwise indicated. Fibre width and sarcomere length were measured at rest and during activation directly from photomicrographs at 250 × magnification (25 × objective, 10 × eyepiece). This photographic method was similar to that described earlier (Moss, 1979). Fibre cross-sectional area was calculated by equating fibre width and diameter, assuming a circular cross-section as the fibre rounds up when briefly removed from solution. These measurements were made from photographs of the fibres while in air, but within 5 s of removal from the bathing solution to minimize shrinkage due to evaporation (Ferenczi, 1986).

The experimental solutions contained 7 mM-EGTA, 1 mM-free Mg^{2+}, 20 mM-imidazole, 6·28 mM-total ATP, 14·5 mM-creatine phosphate, 10 mM-caffeine, various free-Ca^{2+} concentrations, and sufficient KCl to yield an ionic strength of 180 mM. The pH of all solutions was adjusted to 7·0 with KOH. The concentration of free Ca^{2+} was varied between 10^{-9} (relaxing solution) and $10^{-4.5}$ M (maximally activating solution) and is expressed as pCa ($-\log [Ca^{2+}]$) in this report. The computer program of Fabiato & Fabiato (1979) was used to calculate the final concentrations of each metal, ligand and metal–ligand complex in solution based on the stability constants listed by Godt & Lindley (1982). The apparent stability constant for Ca–EGTA was corrected to 10 °C and for the effects of ionic strength, as described by Fabiato & Fabiato (1979). In several experiments, 5% (w/v) dextran T500 (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added to both relaxing and activating solutions in order to compress the fibre volume (Maughan & Godt, 1979). Dextran-containing solutions were mixed as described by Maughan & Godt (1981*b*).

All experiments in this report were conducted at 10·0 $\pm$ 0·2 °C to reduce the likelihood that striation non-uniformities would develop within the fibres. Tensions (P) at submaximally activating levels of Ca^{2+} were expressed as a fraction of P_0, the tension obtained during maximal activation at pCa 4·5 in the s.l. range 2·4–2·6 μm. At any given pCa, steady tension was allowed to develop, at which time the segment was rapidly (within 1 ms) slackened to obtain a force base line. For Ca^{2+} concentrations at which P/P_0 was less than 0·50 the fibres were re-extended from slack during maintained activation, while at higher Ca^{2+} concentrations, the fibres were relaxed prior to re-extension. Active tension was calculated as the difference between total tension in activating solution and the resting tension measured at the same segment length while in relaxing solution. Every third or fourth contraction was performed at pCa 4·5 in order to assess any decline in fibre performance (Moss, 1979). Over the course of an experiment, P_0 declined by less than 10% in most fibres.

At Ca^{2+} concentrations for which P/P_0 was greater than 0·50, a single data point was collected during a given activation, each time changing over-all length while the fibre was relaxed (Fig. 1*A*). This protocol minimized the deterioration of sarcomere length uniformity. At tensions of 0·50 P_0 or less, the fibres were successively shortened while activated (Fig. 1*B*). After a steady tension had developed at a given sarcomere length the fibre was photographed, tension was measured, and then the over-all length was adjusted in order to decrease sarcomere length. Steady tension was again achieved before this procedure was repeated at the new sarcomere length.

The relationship between relative isometric tension (i.e. P/P_0) and pCa was also measured in several fibre segments at one or more sarcomere lengths (Fig. 5). A maximum of six tension–pCa relationships was obtained from a particular single fibre. At a given sarcomere length two tension–pCa relationships were determined: one using control solutions and another with solutions containing 5% dextran. Following measurements in the presence of dextran, fibres were thoroughly rinsed in relaxing solution prior to further measurements.

To determine fibre stiffness at each sarcomere length, small amplitude (0·03–0·06% L_0) sinusoidal length changes, at a frequency of 3·3 kHz, were continuously applied via a torque motor (Model 300s, Cambridge Technology, Inc., Cambridge, MA, U.S.A.) to one end of the fibre segment. The peak-to-peak amplitudes of the length (ΔL) and resulting tension oscillations (ΔP) were measured, ten sequential measurements being averaged for each stiffness determination (i.e. $\Delta P/\Delta L$). There was a phase shift of approximately 58 deg, at pCa values of 4·5 and 5·8 with force lagging ΔL. This was due almost entirely to a 4-pole filter in the force-detection circuit, each pole contributing an approximate 15 deg lag at the frequency used in this study, and with negligible effects on the amplitude of the tension response (personal communication, B. Rohr, Cambridge Technology,

Inc.). Consistent with this interpretation, fibres in rigor were found to demonstrate similar phase lags, at both high and low levels of maintained tension achieved by small changes in over-all length. A capacitance gauge tension transducer (Model 407, Cambridge Technology, Inc.), having a sensitivity of 2 mV/mg and resonant frequency of 6 kHz, was used. Tension and displacement were recorded on an Explorer III B digital oscilloscope (Nicolet Instruments Corp., Madison, WI, U.S.A.), using a sampling interval of 20 μs, and were stored on floppy disks for later analysis.

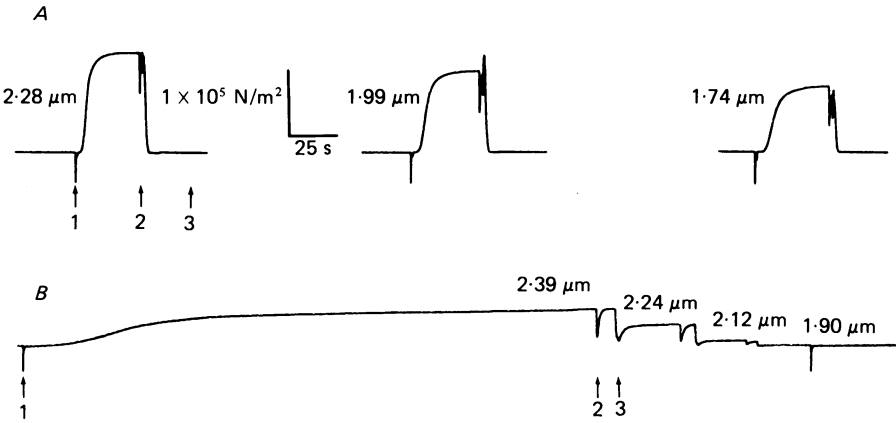

Fig. 1. Original slow time-base recordings of tension obtained at pCa 4·5 (*A*) and 5·9 (*B*) for various sarcomere lengths (indicated in μm). In both *A* and *B*, the fibre was activated at time point '1' and steady tension was measured at '2'. At time point '3' the over-all segment length was shortened, either while the fibre was relaxed (*A*) or during activation (*B*). Sarcomere lengths and corresponding tensions were measured at pCa 4·5 as follows (sarcomere length, tension): 2·28 μm, $1{\cdot}20 \times 10^5$ N/m^2; 1·99 μm, $9{\cdot}74 \times 10^4$ N/m^2; 1·74 μm, $7{\cdot}89 \times 10^4$ N/m^2. At pCa 5·9, the following were obtained: 2·39 μm, $4{\cdot}37 \times 10^4$ N/m^2; 2·24 μm, $2{\cdot}65 \times 10^4$ N/m^2; 2·12 μm, $5{\cdot}91 \times 10^3$ N/m^2; 1·90 μm, $9{\cdot}10 \times 10^2$ N/m^2. Fibre No. 10885; resting sarcomere length = 2·57 μm at an over-all length of 2·99 mm.

In another series of experiments, whole troponin complex was partially removed from psoas fibres by utilizing contaminant protease activity found in preparations of myosin light chain-2 from bovine masseter muscle, as has been described previously (Moss, Allen & Greaser, 1986). Fibres were bathed at room temperature (22–24 °C) in a solution containing 50 mM-KCl, 10 mM-EDTA, 5 mM-PO_4, pH 7·00 and 1 mg/ml of the myosin light chain-2 preparation to remove troponin. Treatment times were varied between 4 and 8 h depending upon the amount of Ca^{2+}-insensitive (i.e. in relaxing solution) tension that we wished to achieve. In these experiments a control length–tension relationship was determined at a pCa yielding tension between 0·30 and 0·50 P_0 at a sarcomere length of 2·6–2·7 μm. To obtain a Ca^{2+}-insensitive tension equal to this control tension, the fibre was then soaked in the solution to remove troponin. After the treatment fibres were first placed in a relaxing solution containing 4·1 mM-ATPγS, instead of ATP, to suppress the tension generated in normal relaxing solution and thereby reduce the structural deterioration of the fibre which might otherwise have occurred. When Ca^{2+}-insensitive tension was approximately equal to the control value obtained at low Ca^{2+}, a second length–tension relationship was determined at pCa 9·0.

RESULTS

The light photomicrographs shown in Pl. 1 demonstrate a representative range of fibre widths and striation spacings utilized in this study. For the fibre segment shown, sarcomere lengths of 2·31, 2·00 and 1·47 μm, during activation, corresponded to widths of 65, 68 and 79 μm, respectively. Including all fibres used in this study,

sarcomere length during activation ranged from 2·65 to 1·35 μm and fibre widths increased about 20% between the longest and shortest lengths in this range. Slow time-base recordings of tension at pCa 4·5 and 5·9 are shown in Fig. 1*A* and *B*. In each case, as sarcomere length was reduced by changing over-all fibre length, steady isometric tension declined.

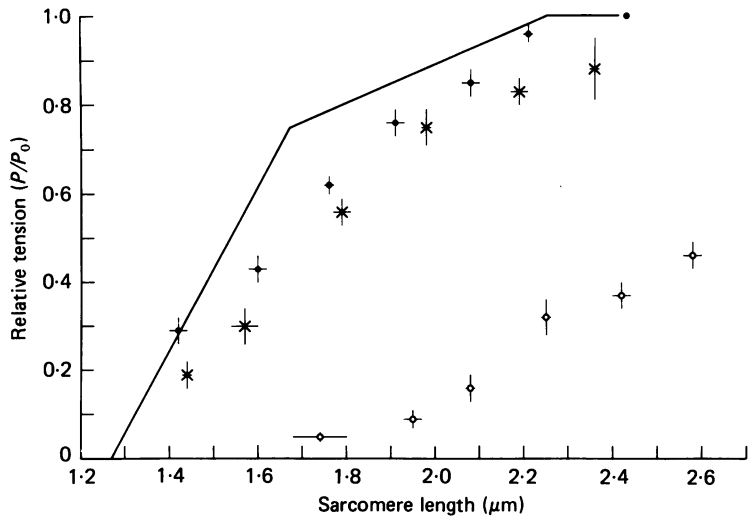

Fig. 2. Plot of relative tension (P/P_0) *vs.* sarcomere length obtained at pCa values 4·5 (●), 5·6 (×) and 5·8 (○). Error bars represent ±S.E. of mean. All data points represent the means of three to twenty tension determinations within a particular sarcomere length range. The continuous line illustrates the predicted length–tension relationship for tetanically stimulated rabbit skeletal muscle (see text for further explanation).

The relationships between relative isometric tension and sarcomere length for various levels of activation are presented in Fig. 2. At pCa 5·8, where P/P_0 was 0·46±0·03 at a sarcomere length of 2·58±0·06 μm, a decrease in striation spacing to 1·60–1·70 μm resulted in a decrease of Ca^{2+}-activated tension to zero. In relationships determined at lower [Ca^{2+}] (i.e. higher pCa) reductions to zero tension occurred at a still longer sarcomere length (~ 1·8 μm). At higher [Ca^{2+}], yielding tensions greater than 0·50 P_0, zero tension was not observed since we were unable to resolve sarcomere lengths less than ~ 1·4 μm. Linear extrapolations of these plots indicate that at pCa 5·6 zero tension would be attained at a sarcomere length of ~ 1·30 μm, while at pCa 4·5 zero tension would be expected at 1·20 μm.

The shapes of the relationships presented in Fig. 2 also varied with [Ca^{2+}]. At maximal activation, tension did not change significantly until sarcomere length was decreased below ~ 2·2 μm, at which point tension slowly declined and then, at ~ 1·9 μm, fell off more rapidly as sarcomere length was decreased further. The plot for pCa 5·6 was similar, except that at sarcomere lengths less than ~ 1·95 μm the plot was somewhat steeper. At pCa 4·5 the slope of the steep portion of the ascending limb was 0·98 P_0/μm, while at pCa 5·6 it was 1·06 P_0/μm. At a still lower [Ca^{2+}] (pCa 5·8) the length–tension relationship appeared to be monotonic with a steady decrease in tension associated with decreasing sarcomere length. Neither a plateau nor a shallow

phase was apparent at this low [Ca^{2+}], and the slope of the relation was 0·52 P_0/μm.

The length–tension relationship at pCa 4·5 does not fit particularly well to the relation that would be predicted for rabbit skeletal muscle (continuous line, Fig. 2), based on the results from tetanically stimulated frog muscle (Gordon *et al.* 1966*b*) and taking into account the longer thin filament lengths in mammalian skeletal muscles (2·24 μm for hydrated I segments from rabbit psoas muscle, Huxley, 1963; see also Woledge, Curtin & Homsher, 1985). As a test of whether these differences were due to previously observed changes in filament lattice volume following the skinning procedure, fibres were bathed with a non-penetrating high molecular weight polymer in order to laterally compress the filament lattice (Godt & Maughan, 1977).

Fibres bathed in maximally activating solutions containing 5% (w/v) dextran were noticeably compressed (~ 15%) compared to untreated fibres (Pl. 2) without significantly affecting striation spacing. This concentration of dextran has been shown to compress skinned frog fibres back to their diameters measured prior to skinning or when the fibre was in oil (Maughan & Godt, 1981*a*). Osmotically compressed fibres developed more tension at a particular sarcomere length and pCa than did the swollen, dextran-free fibres. In Fig. 3, during maximal activation at a sarcomere length of 2·44 μm, the dextran-treated fibre in *B* developed 22% more tension than the same fibre, in *A*, before treatment. When sarcomere length was decreased to 2·01 μm the dextran-treated fibre at pCa 4·5 (*F*) developed 28% more tension than the untreated fibre (*E*). At pCa 6·1 these differences were even greater. At the longer sarcomere length the dextran-treated fibre (*D*) developed more than twice as much tension as the untreated fibre (*C*) and, when the sarcomere length was decreased to 2·01 μm, the fibre developed twenty times more tension in the presence of dextran (*H*) than in its absence (*G*). Consequently, the two conditions generated different length–tension relationships (Fig. 4). The ascending limb in the presence of dextran more nearly resembled the predicted relationship in mammalian muscle. In untreated fibres the steep and shallow portions of the ascending limb were less distinct and fell beneath the predicted relationship.

As a further investigation of the effect of filament lattice separation on the length–tension relationship, tension–pCa relationships were determined at various sarcomere lengths (Fig. 5). Control curves at each sarcomere length demonstrated a steeper relationship as compared to curves obtained in the presence of dextran. As sarcomere length was decreased in control fibres, the steepness of each curve increased and the mid-point of each curve shifted to a lower pCa. After shortening from a sarcomere length of $2{\cdot}46 \pm 0{\cdot}05$ to $1{\cdot}54 \pm 0{\cdot}06$ μm, both control and dextran tension–pCa relations underwent a rightward shift by approximately 0·23 pCa units, that is, became less sensitive to Ca^{2+} as assessed at P/P_0 equal to 0·50 (Table 1). As might be expected on the basis of Fig. 3, dextran treatment resulted in a leftward shift of the tension–pCa relation relative to control, indicating an increase in the Ca^{2+} sensitivity of tension development at each sarcomere length.

The Hill plot transformation of the tension–pCa data in Fig. 5 (Moss, Swinford & Greaser, 1983) yielded quantitative descriptors of the tension–pCa relations. The Hill coefficients (n_1 for $P/P_0 > 0{\cdot}50$, n_2 for $P/P_0 < 0{\cdot}5$), the pCa at which P/P_0 was 0·50

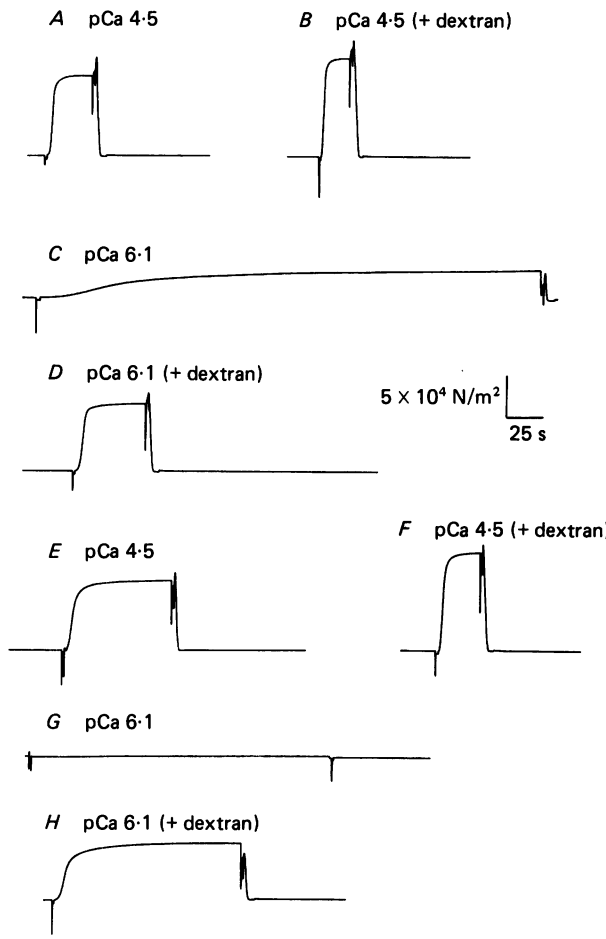

Fig. 3. Slow time-base tension records demonstrating the increased tension development in osmotically compressed fibres when compared to dextran-free fibres. The steady tensions developed by this fibre at pCa 4·5 and pCa 6·1 at a sarcomere length of 2·44 μm (*A–D*) and 2·01 μm (*E–H*), with and without dextran, were as follows (in N/m^2): *A*, $9{\cdot}03 \times 10^4$; *B*, $1{\cdot}16 \times 10^5$; *C*, $3{\cdot}37 \times 10^4$; *D*, $7{\cdot}84 \times 10^4$; *E*, $8{\cdot}23 \times 10^4$; *F*, $1{\cdot}14 \times 10^5$; *G*, $2{\cdot}78 \times 10^3$; *H*, $6{\cdot}55 \times 10^4$. Fibre No. 8885; resting sarcomere length = 2·69 μm at an overall length of 3·32 mm.

(pCa_{50}), and the absolute and relative widths of maximally activated fibres at each of four mean sarcomere lengths are summarized in Table 1. In both control and dextran solutions n_1 showed little change until sarcomere length was reduced to $1{\cdot}54 \pm 0{\cdot}06$ μm, at which a notable increase occurred. At all intervals, n_1 in the control solutions was less than n_1 in the presence of dextran, while n_2 values were greater in the absence of dextran at each sarcomere length. Unlike n_1, n_2 values showed no consistent changes with decreasing sarcomere length. As expected, the relative widths of both control and dextran-treated fibres increased as the fibres were shortened. The compression of the fibres in the presence of dextran remained at a constant $\sim 0{\cdot}85$ of control width at each sarcomere length.

The possibility existed that at least some of the rapid decline in tension at short sarcomere lengths during low-level Ca^{2+} activation represented the accumulation of internal load, possibly due to long-lived cross-bridge attachments, as the fibre was progressively shortened during maintained activation. To test this possibility experiments were done in which each tension measurement at low Ca^{2+} was preceded by

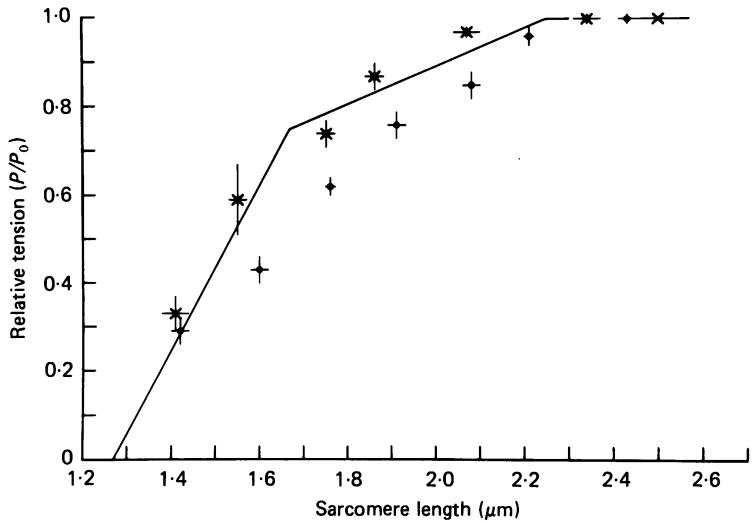

Fig. 4. Plot of relative tension (P/P_0) *vs.* sarcomere length obtained at pCa 4·5 in untreated (●) and dextran-treated (×) fibres. Control and dextran tensions were scaled to P_0 values obtained in dextran-free and dextran-treated solutions, respectively. Error bars represent ± S.E. of mean. All data points represent means of four to twenty tension determinations within a particular sarcomere length range. The continuous line illustrates the predicted length–tension relationship for tetanically stimulated intact rabbit skeletal muscle.

a maximal activation at the same sarcomere length (Fig. 6). Two length–tension relationships were first determined for a given fibre (Fig. 6*B* and *C*), one at maximal [Ca^{2+}] and another at a submaximal [Ca^{2+}], either pCa 5·8 or 5·9 (data not shown), at which P/P_0 was less than 0·50. Additional length–tension data, referred to as 'experimental', were then obtained as follows. The fibre was allowed to develop maximum tension at pCa 4·5 and sarcomere length and tension were measured (indicated by 1 in Fig. 6*A*); (2) the fibre was shortened; (3) this new sarcomere length and steady tension were measured; (4) the fibre was placed into the higher pCa solution, and (5) sarcomere length and steady tension were again measured. The fibre was then relaxed (6) before activating and shortening it again. Two experimental length–tension plots were generated this way (connected by the lines in Fig. 6*B* and *C*). In each fibre similar control and experimental relationships were observed at pCa 4·5; however, the experimental data points obtained at the higher pCa, which were each immediately preceded by a maximal activation, lay above the control points over most of the sarcomere length range that was investigated. Data from two fibres are presented in Fig. 6, since we were concerned that some of this extra tension was due to an extension of the fibre, as a result of elastic recoil at the points of attachment, when the fibre was placed into the lower Ca^{2+} solution and developed tension declined

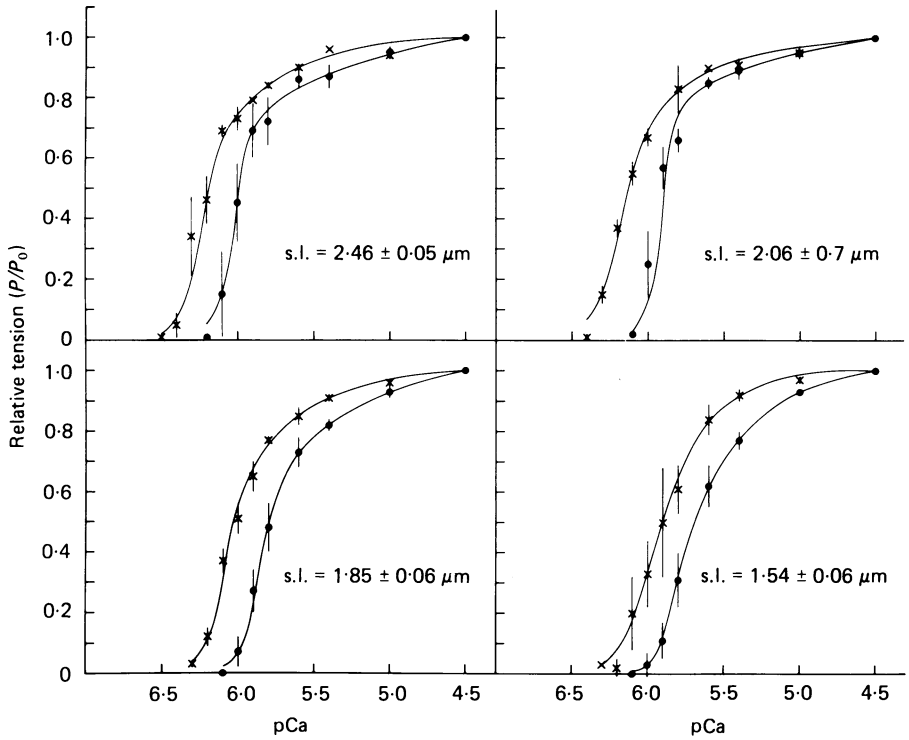

Fig. 5. Plots of relative tension *vs.* pCa comparing control (●) and dextran-treated (×) fibres at four mean sarcomere lengths (s.l.). Curves were fitted to this data using the equation:

$$P/P_0 = \frac{[Ca^{2+}]^n}{Q^n + [Ca^{2+}]^n},$$

where n is the Hill coefficient corresponding to a particular $[Ca^{2+}]$ and Q is the $[Ca^{2+}]$ at which the Hill plot intersects the x axis. Separate curves were fitted to data above and below 0·5 P_0 (see Table 1). Error bars represent ±S.D. Data points represent means of three to five tension determinations at each pCa.

TABLE 1. Quantitative descriptions of the tension–pCa relationships shown in Fig. 5. Hill coefficients (n_1 for $P/P_0 > 0{\cdot}50$ and n_2 for $P/P_0 < 0{\cdot}50$), the pCa at which P/P_0 was 0·50 (pCa_{50}), and the absolute (W) and relative (W/W_0) widths of maximally activated fibres in both control and dextran-treated fibres at each of four mean sarcomere lengths are summarized

	Control	Dextran	Control	Dextran
Sarcomere length ± S.E. of mean	2·46 ± 0·02		2·06 ± 0·03	
n_1	1·03	1·30	0·88	1·17
n_2	5·50	5·11	6·54	4·20
pCa_{50}	5·97	6·18	5·88	6·13
W ± S.E. of mean	65·8 ± 6·7	56·1 ± 5·0	67·9 ± 7·5	57·7 ± 6·2
W/W_0	1·00	0·85	1·03	0·88
	Control	Dextran	Control	Dextran
Sarcomere length ± S.E. of mean	1·85 ± 0·02		1·54 ± 0·02	
n_1	1·15	1·39	1·51	2·18
n_2	5·45	5·23	5·80	4·08
pCa_{50}	5·80	6·03	5·74	5·94
W ± S.E. of mean	67·6 ± 5·6	57·2 ± 4·5	83·2 ± 4·2	70·5 ± 3·3
W/W_0	1·03	0·87	1·27	1·07

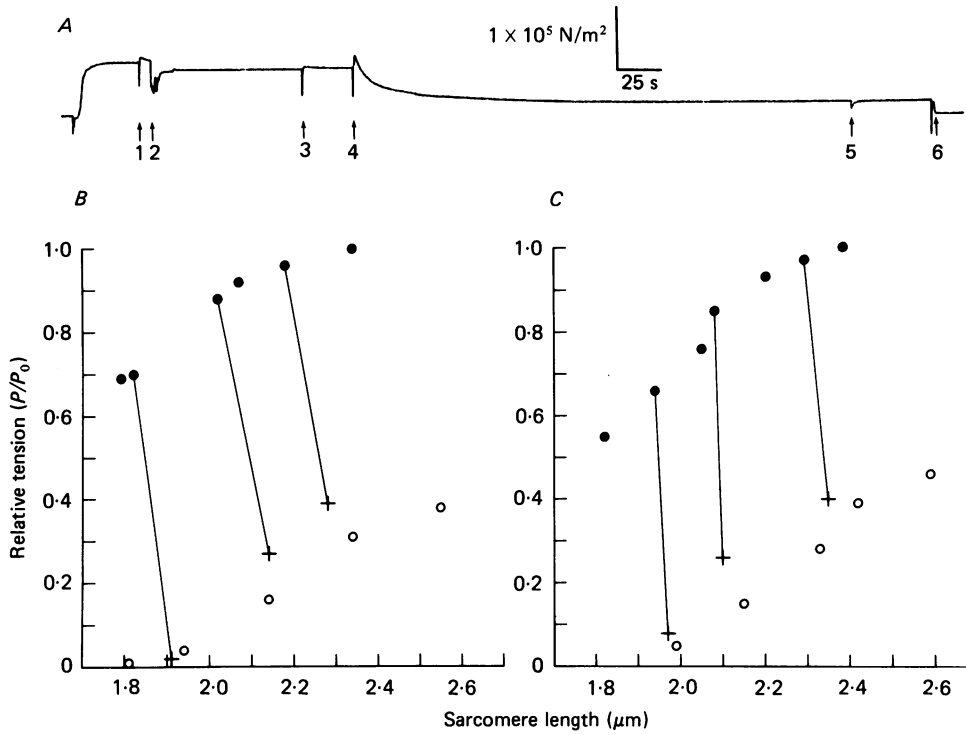

Fig. 6. In *A*, original slow time-base recordings of tension demonstrate a portion of the experiment done to minimize a suspected active internal load. At time point '1' sarcomere length and tension were measured at maximal activation, at '2' the fibre was shortened, and this new sarcomere length and tension were measured at '3'. The fibre was placed into pCa 5·8 at time point '4', where sarcomere length and steady tension were measured at '5' before relaxing the fibre at '6'. This procedure was repeated several times to generate data points for the experimental length–tension plots (connected by the lines) shown in *B* and *C*. ●, pCa 4·5; + and ○, pCa 5·8. These plots are compared to control plots of steady developed tension as a function of sarcomere length and demonstrate that a component of the decline in tension with decreasing sarcomere length during continuous low-level activation can be eliminated by intervening activations at pCa 4·5. Similar results were obtained from three additional fibres. Fibre No. 10785 (*B*); resting sarcomere length = 2·60 μm at an over-all length of 2·44 mm. Fibre No. 10985 (*C*); resting sarcomere length = 2·70 μm at an over-all length of 3·54 mm.

(see Fig. 6*B*). However, this possibility seems unlikely since in Fig. 6*C* sarcomere lengths before and after placement into pCa 5·8 were virtually the same and experimental points still lay well above the control. These results demonstrate that a component of the decline in tension with decreasing sarcomere length during continuous low-level activation can be eliminated by intervening activations at pCa 4·5.

Stiffness measurements in the absence of dextran were undertaken to determine whether the length dependence of tension development at low [Ca^{2+}] could be explained on the basis of an internal load opposing contraction. At a mean sarcomere length of 2·58 $\pm$ 0·05 μm, the mean elastic modulus at pCa 4·50 was $2{\cdot}14 \times 10^7$ N/m^2 ($n = 4$). At low levels of activation, the measured stiffness significantly increased

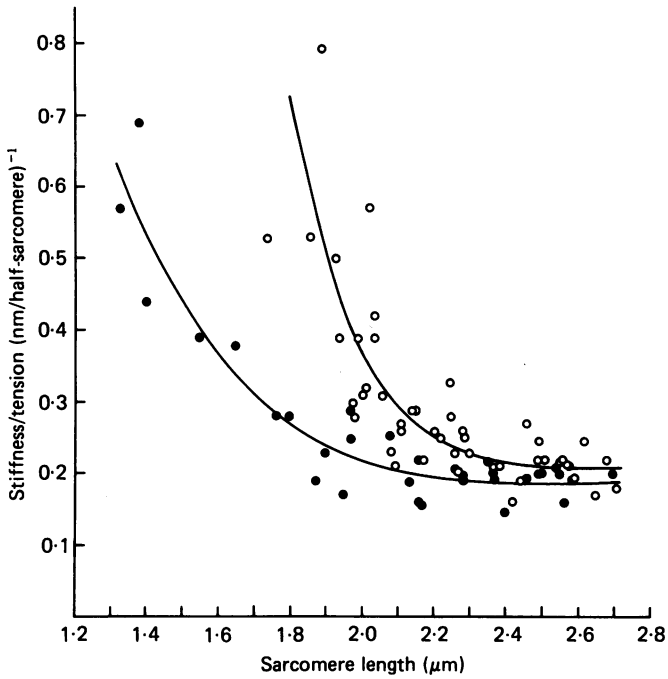

Fig. 7. Variation of stiffness relative to tension as a function of sarcomere length. Measurements were made in twelve fibres at pCa 4·5 (●) and 5·8 (○). Length changes were expressed in terms of nm/half-sarcomere.

relative to tension as sarcomere length was reduced below ~ 2·4 μm (Fig. 7). With full overlap of the thick and thin filaments (sarcomere length = ~ 2·6 μm), the mean absolute stiffness at pCa 4·5 ($n = 4$) was 22·87 N/m and at pCa 5·8 ($n = 7$) it was 9·45 N/m. At a sarcomere length of ~ 1·9 μm the mean absolute stiffness decreased to 14·35 and 3·85 N/m respectively, while the stiffness/tension ratio was little changed at pCa 4·5 and increased at pCa 5·8 from 0·22 to 0·50 (nm/half-sarcomere)$^{-1}$. An equivalent increase in stiffness relative to tension was observed at pCa 4·5 only when sarcomere length was reduced to ~ 1·4 μm. At low [Ca^{2+}] a steady rate of tension decline, which was faster than the decrease in absolute stiffness, occurred as sarcomere length was decreased, while at high [Ca^{2+}] the stiffness/tension ratio remained approximately constant until sarcomere length was reduced below ~ 1·8 μm where it is likely that the ends of the thick filament collide with the Z-lines giving rise to a substantial passive internal load.

A series of experiments was done to investigate whether sarcomere-length-dependent changes in the Ca^{2+} sensitivity of tension development result from a shortening-induced dissociation of Ca^{2+} from the troponin-C subunit of troponin. This is a factor to be considered since length–tension relationships were obtained by changing length during continuous activations at low [Ca^{2+}] or by introducing slack while the fibre was relaxed preceding higher Ca^{2+} activations. Whole troponin was partially removed from the skinned fibres yielding active tensions of 0·30–0·50 P_0 when the fibres were placed in relaxing solution. In other words, after partial removal

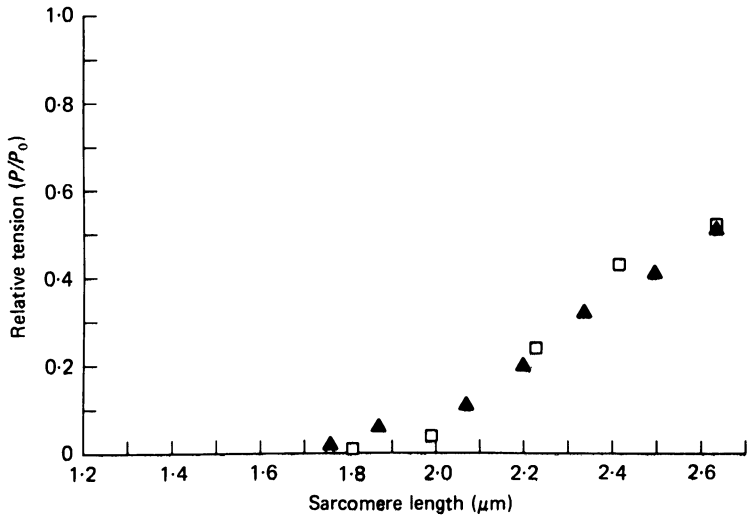

Fig. 8. Plot of relative tension (P/P_0) *vs.* sarcomere length from one fibre segment. Control measurements, obtained at pCa 5·9 (□), are compared to measurements made after the partial removal of whole troponin, where a low level of activation occurred in the absence of Ca^{2+} at pCa 9·0 (▲). Note that Ca^{2+}-insensitive tension at the initial sarcomere length (2·64 μm) was approximately equal to tension measured before the removal of troponin at pCa 5·9. This procedure was performed on three additional fibres with similar results. Fibre No. 101485; resting sarcomere length = 2·76 μm at an over-all length of 4·48 mm.

of whole troponin, a low level of activation of these fibres occurred in the absence of Ca^{2+}. When this Ca^{2+}-insensitive tension was approximately equal to the tension measured before the extraction procedure at submaximal Ca^{2+} concentrations (pCa 5·8 or 5·9), a length–tension relationship at pCa 9·0 (relaxing solution) was determined and compared to the control relation (Fig. 8). Clearly, the two relationships are very similar making it unlikely that the changes observed in the length–tension relationship at submaximal Ca^{2+} concentrations are caused by dissociation of activator Ca^{2+} from troponin-C as the fibre shortens.

DISCUSSION

Effects of osmotic compression on Ca^{2+}-activated tension

The main results of this investigation indicate that the Ca^{2+} sensitivity of tension development in mammalian skinned single muscle fibres depends upon sarcomere length and this is reflected in alterations of the ascending limb of the length–tension relationship as $[Ca^{2+}]$ is reduced. During maximal activation at pCa 4·5 a relationship different from that established by Gordon *et al.* (1966*a*, *b*) in intact single frog muscle fibres was observed here (Fig. 2). Most likely these differences are due to the types of fibres studied. Rabbit muscle has longer thin filaments than frog muscle and for this reason each would be expected to have unique length–tension relationships (Woledge *et al.* 1985). In addition, during the skinning process these fibre preparations swell (Matsubara & Elliott, 1972; Godt & Maughan, 1977; Matsubara, Umazume & Yagi, 1985) causing an increase in the filament lattice separation above

that seen in intact fibres. The length–tension relationship in maximally Ca^{2+}-activated dextran-treated fibres, where this separation and fibre volume was decreased, closely resembled that expected for tetanically stimulated, intact mammalian fibres (Fig. 4).

Osmotically compressed fibres developed more tension at any given pCa than untreated fibres indicating that the lateral separation of thick and thin filaments is an important factor in force generation (see Figs. 3 and 5), as previously suggested by Maughan & Godt (1981*b*) and confirmed by Kawai & Schulman (1985). Recent findings by Gulati & Babu (1985) suggested that force was invariant over a similar range of fibre diameters, although the basis for this apparent discrepancy is unclear. An increase in tension with decreased filament lattice spacing cannot be explained on the basis of changes in the component vectors of cross-bridge force, since previous calculations indicated that the force acting parallel to the long axis of the thick filament remains virtually constant despite changes in lateral filament separation within the physiological range (Julian, Moss & Sollins, 1978; Schoenberg, 1980). Instead, reducing the thick to thin filament separation may affect the rate constant of cross-bridge attachment by increasing the probability of contact between the myosin head and the actin filament.

Dextran-treated fibres were also shown to have an increased sensitivity to Ca^{2+} when compared to untreated fibres at the same sarcomere length (see Fig. 5, also Maughan & Godt, 1981*b*). The Ca^{2+} sensitivity of a functional group (defined structurally as seven actin monomers, one troponin, one tropomyosin) within the thin filament may vary depending upon the state of activation of immediately adjacent functional groups (Murray & Weber, 1980; Grabarek, Grabarek, Leavis & Gergely, 1983; Moss, Giulian & Greaser, 1985; Moss *et al.* 1986). In control fibres at low levels of free Ca^{2+} (pCa 6·1), very few functional groups (and possibly only portions of these) will be activated, making this kind of co-operativity less likely. Activation of functional groups could also be enhanced by increased cross-bridge binding within a functional group causing an increase in the affinity of the binding of Ca^{2+} to the low-affinity sites on troponin-C (see Bremel & Weber, 1972). In a compressed fibre at the same $[Ca^{2+}]$, such co-operativity may occur to a greater degree than in a control fibre due to facilitated contact between thick and thin filaments. This enhancement of the binding of cross-bridges and Ca^{2+} within a functional group could thus increase the likelihood that adjacent groups would be activated.

A similar argument may help to explain the apparent decrease in Ca^{2+} sensitivity as sarcomere length was decreased (Table 1), that is, co-operative activation of the thin filament at low levels of Ca^{2+} decreased as lateral filament separation increased. Consistent with this notion, n_1, the slope of the Hill plot at high $[Ca^{2+}]$, was greater at each sarcomere length when tension was measured in the presence of dextran. However, n_2, calculated for low concentrations of Ca^{2+}, *decreased* when dextran was added, a result that we are presently unable to explain. The value of n_1, in both control and dextran-treated fibres, decreased when sarcomere length was adjusted from a mean of 2·46 to 2·06 μm, but n_1 actually increased with further reductions in sarcomere length. This initial decrease is consistent with the idea that increases in filament separation would reduce co-operative activation of the thin filament. The finding that n_1 increases at still lower sarcomere lengths may reflect a steepening of

the *relative* tension–pCa relationship due to the presence of an internal load, either active or passive, which would offset some of the tension generated by the fibre. This would have the effect of truncating the tension–pCa relationship so that zero tension expressed at the ends of the fibre would actually represent a positive Ca^{2+}-activated tension that is nulled by an opposing force due to the internal load. Thus, the steepened relationships at short sarcomere lengths might not accurately reflect the Ca^{2+} regulation of cross-bridge attachments.

The role of internal loads in modulating tension development

The decline in tension with decreasing sarcomere length at maximal activation can be explained by geometrical factors, as suggested by Gordon *et al.* (1966*b*). In this study, stiffness/tension ratios during maximal activation remained virtually constant until sarcomere length was decreased below about 1·8 μm, where this ratio increased significantly (Fig. 7). Until the sarcomere length became quite short, both stiffness and tension decreased nearly proportionately, an observation that is consistent with the idea that the shallow ascending limb is the result of thin filament overlap interfering with cross-bridge attachments. In the steep portion of the ascending limb, corresponding to sarcomere lengths below about 1·7 μm, stiffness increased relative to tension, presumably because of the collision of the ends of the thick filaments with the Z-lines. These results are similar to those obtained in tetanically stimulated frog skeletal muscle by Bressler & Clinch (1975) and Halpern & Moss (1976). More recently, Julian & Morgan (1981) found that stiffness in intact single fibres from frog muscle increased over the plateau region of the length–tension relationship and did not fall in proportion to tension over the sarcomere length range of 1·7–2·0 μm. In their report absolute stiffness rose to a peak at a sarcomere length of between 1·8 and 1·9 μm before slowly declining with further decreases in sarcomere length. The basis for this difference from our results is unclear, but may involve species differences, temperature, and the vibration frequency used to make the stiffness measurements.

Several lines of evidence suggest that the shape of the ascending limb at low Ca^{2+} is in large part determined by the presence of a shortening-dependent internal load which is not evident during maximal activation. When a maximally activated fibre was shortened and then placed into a submaximal Ca^{2+} solution the resulting point on the length–tension curve lay above a control point, which was obtained at a similar sarcomere length by simply shortening during submaximal activation (see Fig. 6). By shortening a maximally activated fibre, we assured that the maximum number of cross-bridges appropriate to that length would be attached to the thin filament (i.e. full activation) prior to placement in the low-Ca^{2+} solution. Conversely, when the fibre was shortened during submaximal activation, only a portion of the thin filament was disinhibited. This development of tension above the control with intervening activations at pCa 4·5 suggests that there is an internal load associated with shortening at low $[Ca^{2+}]$. A possible basis for this load, suggested by Moss (1986), involves a population of cross-bridges at low levels of activation which has a low rate constant for detachment. As sarcomere length is reduced, these slowly detaching cross-bridges may be compressed giving rise to a tension that opposes contraction. It should be noted that the effect reported in Fig. 6 is probably not related to the

hysteresis in the tension–pCa relationship reported previously in barnacle fibres (Ridgway, Gordon & Martyn, 1983), since there was no consistent difference in the tension values obtained by the two methods at the initial length.

Stiffness at submaximal [Ca^{2+}] increased relative to tension over the entire ascending limb (Fig. 7) indicating that in addition to filament geometry, other factors contribute to the reduction in tension with decreasing sarcomere length. Slowly detaching cross-bridges at low Ca^{2+} would result in an increased stiffness/tension ratio with shortening; however, it is also possible that a passive load is involved. If a small passive load were present at a sarcomere length $\leqslant 2{\cdot}4$, one could expect an increase in the stiffness/tension ratio with shortening at low [Ca^{2+}], which might not be apparent at high [Ca^{2+}] due to the greater development of tension. The structural basis for a passive internal load is at present uncertain but could, for example, result from the collision of thin filaments in the centre of the sarcomere. To determine whether long-lived cross-bridges contribute to the observed increase in the stiffness/tension ratios at short lengths during submaximal activation, stiffness and tension were measured in several fibres at various sarcomere lengths during continuous activation with a low level of Ca^{2+}. Then these measurements were again performed, but first the fibres were maximally activated and shortened before transfer to the low-Ca^{2+} solution. By doing this we hoped to prevent the formation of long-lived, negative-tension-generating cross-bridges. Without these cross-bridges, tensions *at a particular sarcomere length* would be expected to increase and the absolute stiffness/tension ratio to decrease relative to control values at the same pCa. Our results were mixed, with some fibres demonstrating little change in the stiffness/tension ratio relative to control while others showed a definite decrease in this ratio when the fibre was maximally activated before being placed in a submaximal Ca^{2+} solution.

The form of the ascending limb at low levels of activation does not depend on [Ca^{2+}] *per se*

From the results of experiments involving the partial removal of whole troponin complexes from fibres (Fig. 8), we conclude that sarcomere-length-dependent changes in the Ca^{2+} sensitivity of tension development are not due to a shortening-induced dissociation of Ca^{2+} from troponin-C. Ridgway & Gordon (1984) examined the effects of post-stimulus length changes on the levels of Ca^{2+} in the myoplasm, and, using the bioluminescent protein aequorin as a Ca^{2+} indicator in barnacle muscle, observed extra light (extra Ca^{2+}) when a fibre was shortened during the declining phase of the Ca^{2+} transient. They inferred that this additional Ca^{2+} was released from myofibrillar proteins. However, in this investigation we observed a shortening-induced 'deactivation' in the absence of Ca^{2+} by activating the fibre via the partial removal of troponin. The similar decrease in tension with shortening seen here both in the presence and absence of Ca^{2+} is consistent with the idea that shortening-induced detachment of cross-bridges decreases the co-operative role played by attached cross-bridges, which allows more cross-bridge attachments regardless of the level of Ca^{2+} activation (Murray & Weber, 1980). Our finding in skinned fibres does not contradict those of Ridgway & Gordon (1984) in living fibres since in their preparations activation, as determined by the aequorin light signal, was transient whereas in our preparations activation was maintained at a steady level. Thus, it may be that

in living fibres at low levels of Ca^{2+} activation, shortening-induced inactivation of tension would result from both a reduction in the co-operative activation of the thin filaments by cross-bridges and an accumulation of internal load as well as the dissociation of Ca^{2+} from troponin-C causing inactivation of functional groups (i.e. the effect of shortening on tension in living fibres should be greater than that which we have seen in skinned fibres).

In conclusion, the ascending limb of the sarcomere length–tension relationship in skinned fibres appears to be influenced by a number of factors. At high levels of Ca^{2+}, the shape of this portion of the relationship is most likely determined by filament overlap, with the possibility that some of the fall-off in tension with decreasing sarcomere length results from a concomitant increase in filament lattice spacing. However, length–tension relationships at low $[Ca^{2+}]$ are probably depressed due to the effects of increased filament spacing to reduce co-operative activation of the thin filament. In addition, variations in the stiffness/tension ratio as a function of sarcomere length are consistent with the presence of an activation-dependent internal load which would be expected to progressively reduce total tension as the fibre is shortened.

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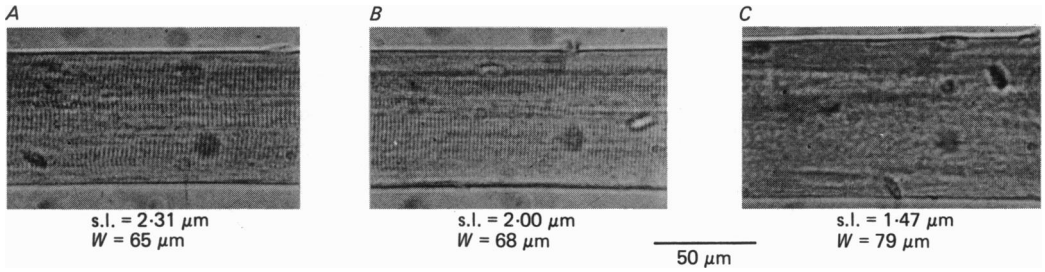
EXPLANATION OF PLATES

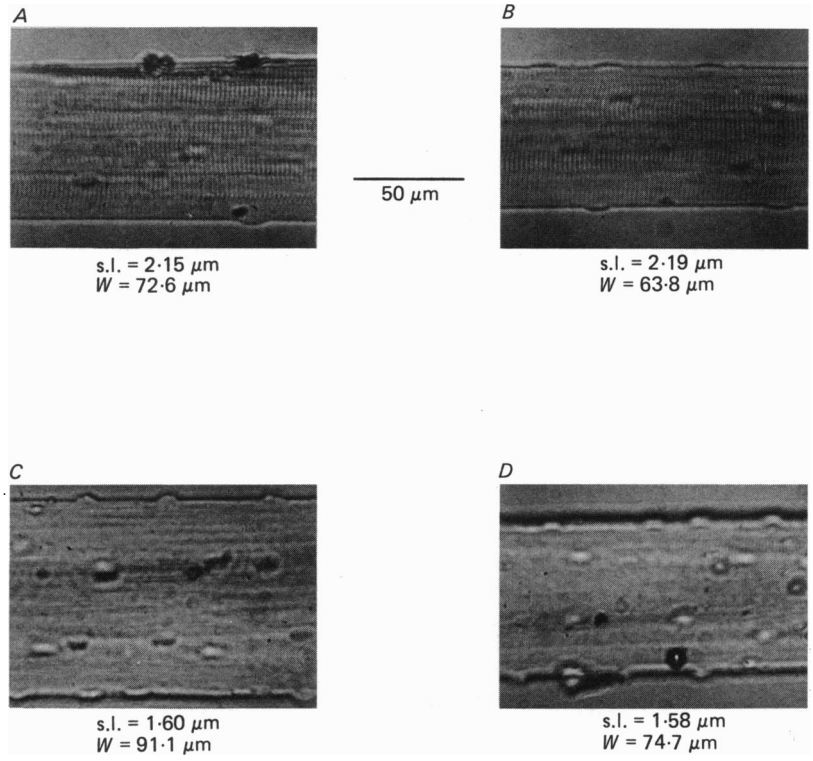
PLATE 1

High-power (250 ×) light photomicrographs of a portion of a fibre illustrating a representative range of sarcomere lengths (s.l.) and fibre widths (W) observed during maximal activation at pCa 4·5. Fibre No. 121285; resting sarcomere length = 2·52 μm at an overall length of 3·07 mm.

PLATE 2

Light photomicrographs of fibre No. 82085 demonstrating the effects of dextran treatment on width (W) and sarcomere length (s.l.) in a maximally activated fibre. Only a slight change in sarcomere length was observed between control (*A* and *C*) and dextran-treated (*B* and *D*) fibres. When over-all length was reduced from 3·09 mm (*A* and *B*) to 2·17 mm (*C* and *D*) there was an approximately proportionate decrease in sarcomere length and an increase in width that was greatest in the untreated (control) fibre.


A
B
C
s.l. = 2·31 μm
W = 65 μm
s.l. = 2·00 μm
W = 68 μm
50 μm
s.l. = 1·47 μm
W = 79 μm


A
B
50 μm
s.l. = 2·15 μm
W = 72·6 μm
s.l. = 2·19 μm
W = 63·8 μm
C
D
s.l. = 1·60 μm
W = 91·1 μm
s.l. = 1·58 μm
W = 74·7 μm