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EFFECTS ON SHORTENING VELOCITY OF RABBIT SKELETAL MUSCLE DUE TO VARIATIONS IN THE LEVEL OF THIN-FILAMENT ACTIVATION

BY RICHARD L. MOSS

From the Department of Physiology, School of Medicine, University of Wisconsin, Madison, WI 53706, U.S.A.

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SUMMARY

1. The effect of Ca^{2+} upon maximum shortening velocity (V_{max}) has been investigated in skinned single fibres from rabbit psoas muscles. V_{max} was obtained at 15 °C by measuring the amounts of time (Δt) required to take up various amounts of slack (Δl) imposed at one end of the fibre.

2. During maximal activation with Ca^{2+}, plots of Δl *vs.* Δt were well fitted by a single straight line. Calculation of V_{max} from the slopes of the fitted lines yielded a V_{max} of $4{\cdot}44 \pm 0{\cdot}15$ (S.E. of mean) muscle lengths per second (m.l./s). However, at lower levels of Ca^{2+} activation, plots of Δl *vs.* Δt were biphasic, containing an initial phase of steady high-velocity shortening and a subsequent phase of steady low-velocity shortening. The transition between these two phases occurred following active shortening equivalent to 60–80 nm/half-sarcomere.

3. V_{max} during the high-velocity phase was relatively insensitive to Ca^{2+} concentration between pCas (i.e. $-\log [Ca^{2+}]$) of 4·5 and 6·0; however, V_{max} fell to $3{\cdot}58 \pm 0{\cdot}40$ m.l./s at pCa 6·1 and further to $1{\cdot}02 \pm 0{\cdot}30$ m.l./s at pCa 6·2.

4. V_{max} during the low-velocity phase decreased as Ca^{2+} was lowered within the entire range of pCas studied to a minimum value of $0{\cdot}35 \pm 0{\cdot}09$ m.l./s at pCa 6·2.

5. The degree of thin-filament activation at a particular pCa was varied by partial extraction of troponin-C, which resulted in a permanent though reversible inactivation of parts of the thin filaments. Partial extraction of troponin-C altered the plots of Δl *vs.* Δt obtained at pCa 4·5 to a biphasic form. In addition, V_{max} during the high- and low-velocity phases of shortening was reduced at each pCa > 4·5. V_{max} values obtained in control fibres at low Ca^{2+} concentrations and extracted fibres were in good agreement when generated isometric tensions were equivalent. This was the case for both the high- and low-velocity phases of shortening.

6. Fibres were also activated in the absence of Ca^{2+} by partial removal of total troponin complexes. These fibres developed steady tensions less than 30% of maximum and underwent biphasic shortening, indicating that this phenomenon cannot be the result of shortening-induced dissociation of Ca^{2+} from troponin-C.

7. These results are considered in terms of a proposed population of cross-bridges which at low levels of activation have a low rate constant for detachment. To account for the biphasic nature of shortening, such cross-bridges would present an internal

load to the muscle only after total shortening equivalent to 60–80 nm/half-sarcomere. Reductions in V_{max} could come about if the proportion of slowly dissociating cross-bridges progressively increased as Ca^{2+} was lowered.

INTRODUCTION

The switch-like function of Ca^{2+} to activate contraction of striated muscle is widely recognized (Ebashi & Endo, 1968; Chalovich & Eisenberg, 1982; Laevis & Gergely, 1984). Less certain is a possible role for Ca^{2+} in modulating the kinetics of interaction of myosin with actin once the thin filament is dis-inhibited. *In vitro* biochemical experiments involving the isolated contractile proteins have produced little evidence for such a mechanism, although Lehman (1978) has presented data that suggests a possible Ca^{2+} sensitivity of the actin-activated adenosine triphosphatase (ATPase) activity of skeletal muscle myosin in the absence of the thin-filament-linked regulatory proteins. Measurements of shortening velocity of single skinned fibres from frog skeletal muscles have produced disparate results with regard to possible effects of Ca^{2+}. Teichholz & Podolsky (1970) reported no effect of Ca^{2+} upon maximum shortening velocity (V_{max}) which was obtained by measuring velocities at loads greater than about 0·2 of steady isometric force (P) and extrapolating to zero load. Julian (1971), on the other hand, demonstrated a clear effect of Ca^{2+} on V_{max} using similar methodologies though with substantially more points at less than 0·2 P. Podolin & Ford (1983) recently reviewed this controversy in detail and were unable to resolve the disparity based on the relatively small differences in the experimental protocols used in the two studies.

Published reports in which V_{max} of skinned fibres from rabbit skeletal muscle was measured at various levels of Ca^{2+} activation have also come to differing conclusions. Previous results from this laboratory (Moss, 1982), and by others (Wise, Rondinone & Briggs, 1971), indicated that V_{max} decreased as Ca^{2+} concentrations were lowered. This was found to be the case both when V_{max} was measured using the classic extrapolation method described above and when the slack-test method (Edman, 1979; Julian & Moss, 1981) was used. On the other hand, Brenner (1980) found that the time course of shortening under load was curvilinear, with velocity steadily decreasing with time. Thus, while shortening under a given relative load was generally slower at low concentrations of Ca^{2+}, a unique shortening velocity could not be measured at any Ca^{2+} concentration. This behaviour suggested that the load on the cross-bridges progressively increased during shortening due to the presence of an activation-independent internal load. In our study (Moss, 1982) velocities were measured only during linear phases of shortening, which at high relative loads occurred at long times following the load step.

In the present study, the influence of Ca^{2+} upon V_{max} in mammalian skeletal muscle has been investigated further. The slack-test procedure was used to measure V_{max} since shortening is measured directly under zero applied load. Also, this procedure avoids possible distortion of the shortening records due to uneven tension-bearing capabilities of sarcomeres in series having different initial lengths (Julian & Morgan, 1979). V_{max} was found to vary with Ca^{2+} concentration, and this effect was greatest when total fibre shortening exceeded approximately 80 nm/half-sarcomere. Secondly,

the effect of lowered Ca^{2+} to reduce V_{max} could be reproduced by varying thin-filament activation independent of Ca^{2+} concentration by partially extracting troponin-C from the fibre segments. This latter finding suggests that the effect of Ca^{2+} upon V_{max} is not due to changes in Ca^{2+} concentration *per se* but rather is dependent upon the proportion of functional groups along the thin filament (i.e. seven actin monomers and associated troponin and tropomyosin) that are activated.

A brief report of these findings was made at the Annual Meeting of the (U.S.) Biophysical Society (Moss, Allen & Greaser, 1986*a*).

METHODS

Psoas muscles were obtained from adult male New Zealand rabbits. Bundles of approximately fifty fibres were stripped free while in relaxing solution (Moss, 1979) and were then tied with surgical silk to glass capillary tubes. The bundles were stored at −22 °C in relaxing solution containing 50 % (v/v) glycerol for 3–14 days before use. The procedures for dissecting single fibres and subsequent mounting in the experimental apparatus, as well as the details of the apparatus, have been described previously (Moss, Giulian & Greaser, 1983). Sarcomere length (s.l.) in the relaxed fibre segments was adjusted to 2·5–2·7 μm by changing over-all segment length. Mean s.l. in the fibre segments during maximal Ca^{2+} activation, and prior to any mechanical perturbation, varied between 2·42 and 2·57 μm as determined by photomicroscopy (Moss, 1979). Relaxing and activating solutions contained 7 mM-EGTA, 1 mM-free Mg^{2+}, 20 mM-imidazole, 6·28 mM-total adenosine 5′-triphosphate (ATP), 14·5 mM-creatine phosphate, and sufficient KCl to yield an ionic strength of 180 mM. The concentration of free Ca^{2+} was varied between 10^{-9} M (relaxing solution) and $10^{-4.5}$ M (maximally activating solution). 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 15 °C and for the effects of ionic strength, as described by Fabiato & Fabiato (1979). Free-Ca^{2+} concentrations are expressed as pCas (i.e. $-\log [Ca^{2+}]$, where $[Ca^{2+}]$ is Ca^{2+} concentration) in this report. At each pCa a steady tension was allowed to develop, at which time the segment was rapidly (within 1 ms) slackened to obtain a force base line or a velocity point (described below) and was subsequently relaxed. Active tension was calculated as the difference between total tension in activating solution and the resting tension measured in the same segment while in relaxing solution.

Tensions (P) at submaximally activating levels of calcium were expressed as a fraction of P_0, the tensions obtained during maximal activation at pCa 4·5. Every third or fourth contraction was done at pCa 4·5 in order to assess any decline in fibre performance (Moss, 1979). Ca^{2+}-activated tensions measured after partial extraction of troponin-C were expressed as a fraction of P_0 obtained under control conditions.

Shortening velocities were measured using the slack-test procedure (Edman, 1979; Moss, Giulian & Greaser, 1982) in which the duration of unloaded shortening (Δt) was recorded as a function of the amount of slack (Δl) introduced at one end of the fibre segments. For pCas at which P/P_0 was greater than 0·5, various amounts of slack were introduced in succeeding contractions. For $P/P_0 < 0{\cdot}5$, the fibre segment was repeatedly slackened and re-extended during continuous Ca^{2+} activation. The latter protocol yielded results which were virtually identical to data obtained during successive activations at low Ca^{2+} concentrations. Plots of Δl *vs.* Δt were fitted with one or more straight lines and V_{max} values, in muscle lengths per second (m.l./s), were calculated as the slopes of the fitted lines (Moss *et al.* 1983). The slack-test plots were determined to be biphasic (i.e. consisting of two linear phases) by initial visual inspection. Straight lines were then fitted by the least-squares error method. Such fits were done only if each phase of the plot contained at least four data points, though in most cases the lines were fitted to seven or more data points.

The partial extraction of troponin-C was done by bathing the fibre segments in a solution containing 20 mM-Tris, 5 mM-EDTA, pH 7·85 (Cox, Comte & Stein, 1981; Moss, Giulian & Greaser, 1985) at 12 °C for 120 min. Troponin-C was recombined into the fibre segments by bathing the segments for 40 min in relaxing solution containing approximately 1 mg purified troponin-C/ml

(Moss *et al.* 1982). The segment was subsequently washed in relaxing solution to remove excess troponin-C.

In all experiments, the extraction of troponin-C was quantified by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Giulian, Moss & Greaser, 1983). Each fibre segment was divided into two pieces. The first of these was dissolved in SDS-containing sample buffer to permit quantitation of the troponin-C content of untreated fibre segments. The second segment was mounted in the chamber between the force transducer and motor and was used for the measurements of tension and shortening velocity both prior to and following partial extraction of troponin-C. This segment was subsequently dissolved in sample buffer for SDS–PAGE. Several experiments were done to investigate the reversibility of the effects of troponin-C removal. In these instances, the fibre segments were divided into three pieces, two of which were tied into the experimental chamber (Moss *et al.* 1982), to allow assessment of troponin-C content at each stage of the experimental protocol. As we have done previously, troponin-C content in the control and extracted fibres was expressed as a fraction of the troponin-I content in these same fibres (Moss *et al.* 1985), which is not affected by the troponin-C extraction protocol. The gels were scanned using a laser-light scanning densitometer (BioMed Instruments, Fullerton, CA, U.S.A.), and the ratio of the areas of the peaks corresponding to troponin-I and troponin-C was calculated.

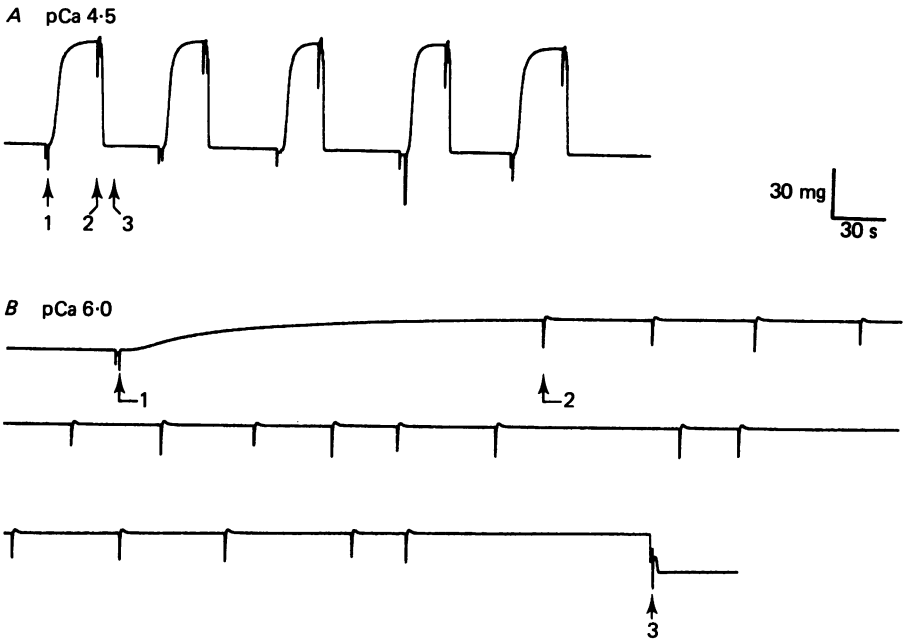

Fig. 1. Original slow time-base recordings of tension obtained during velocity measurements at maximal and submaximal levels of Ca^{2+} activation. In *A*, the fibre was activated at time point '1', slackened and subsequently relaxed at '2', and re-extended to its original length at '3'. This cycle was repeated to obtain slack-test data points for five slack steps of differing amplitudes. In *B*, the fibre was activated at time point '1', slackened and subsequently re-extended at '2', and slackened and relaxed at '3'. Fibre No. 11184.

RESULTS

Slow time-base recordings of tension during the experimental protocol are shown in Fig. 1. At pCa 4·5, and at submaximal Ca^{2+} concentrations corresponding to $P/P_0 > 0{\cdot}5$, multiple data points for the velocity determinations were obtained in successive contractions in order to minimize deterioration of sarcomere length

uniformity that was otherwise observed during prolonged activation. Thus, re-extension of fibres to take up imposed slack was done while the fibres were relaxed. Steady tensions measured in the first and last contractions of a series at pCa 4·5 usually agreed within 2 % of P_0 and never differed by more than 7 %. At high pCa

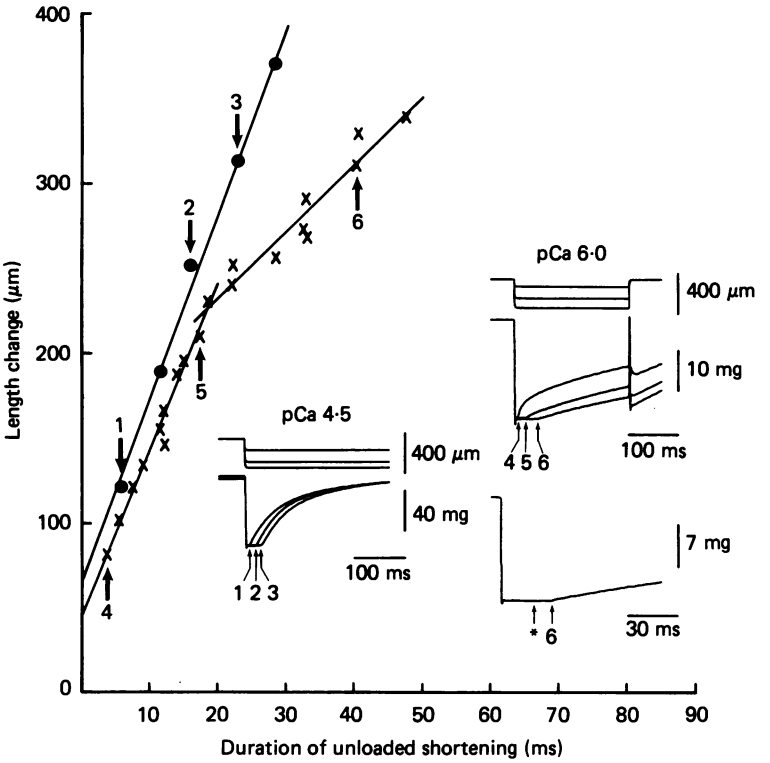

Fig. 2. Plots of slack-test data obtained at pCa values 4·5 (●) and 6·0 (×). The insets show original tracings of length (upper) and tension at each pCa. The arrows indicate the time points at which tension redevelopment commenced, with the numbers indicating the corresponding points on the plots. At pCa 4·5 the fibre was relaxed prior to re-extension, while at pCa 6·0 the fibre was re-extended shortly after the redevelopment of tension. The middle panel shown for pCa 6·0 is a series of superimposed tension traces recorded at four times the sensitivity as those at pCa 4·5. The lower panel contains the tension trace corresponding to data point 6 in the middle panel, but at an increased sensitivity and a faster time base to allow better visualization of the time at which tension began to redevelop. The arrow labelled '*' indicates the time point at which tension would have redeveloped had V_{max} remained high. Note that in the lower panel the expanded sensitivity of the oscilloscope during the print-out to the X–Y recorder was such that steady tension prior to the length step was off scale. Fibre No. 11184; s.l. = 2·42 μm; over-all length of 2·69 mm.

values, yielding tensions of 0·50 P_0 or less, activation was maintained continuously during the collection of data points. The fibres were re-extended within 10–50 ms of the onset of tension recovery following the slack step. A brief tension transient after re-extension was followed by recovery to the level of steady tension that had been developed prior to the slack step. Steady tensions before and after each cycle of release and re-extension did not differ significantly.

Records of length and tension obtained during slack-test measurements of V_{max} at two different Ca^{2+} concentrations are shown for one fibre segment in Fig. 2. At pCa 4·5, the rise of tension from zero following the uptake of imposed slack was abrupt and easily discernible. Subsequent tension redevelopment at the shorter lengths reached levels that were at or near the value just prior to the slack step. A plot of the amount of slack imposed *vs.* the duration of unloaded shortening was well fitted

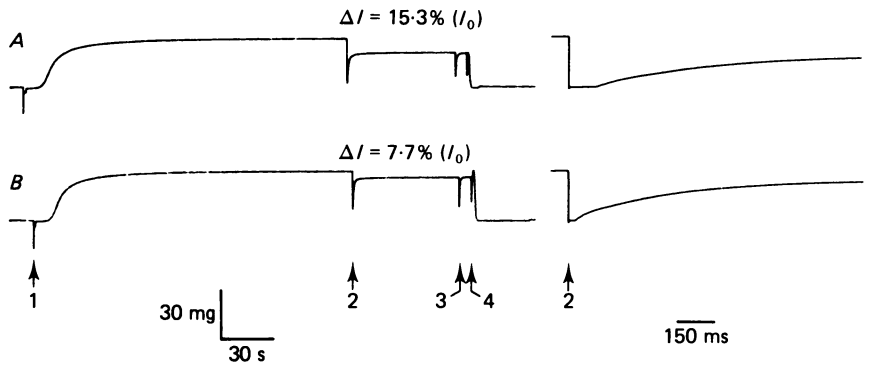

Fig. 3. Slow and fast time-base recordings of tension demonstrating reduced levels of isometric tension following slack-test measurement. In both *A* and *B*, the muscle fibre was activated in solution of pCa 5·9 at time point '1'. The fibre was slackened at '2', which is also shown at the right in each panel on a much faster time base to demonstrate that zero tension was reached following the slack step (length traces not shown). At '3', the fibre was again briefly slackened and subsequently re-extended to the post-slack step length to obtain a measure of the steady isometric tension developed after the slack step. The fibre was relaxed at '4' and was then re-extended to its original length. Steady tensions following the slack steps, as a percentage of the original pre-step value, were 72% in *A* and 88% in *B*. Fibre No. 42485; s.l. = 2·45 μm; over-all length (l_0) of 2·69 mm.

by a single straight line, from which a V_{max} of 4·03 m.l./s was calculated. At lower levels of Ca^{2+} activation (pCa 6·0 in Fig. 2), the rates-of-rise of tension following the uptake of imposed slack were slow relative to that observed during maximal activation. None the less, the time points at which tension began to redevelop were clearly evident. The plot of slack-test data in this instance appeared to be biphasic, and was well fitted by two straight lines intersecting at a point (referred to as the 'break point') corresponding to shortening of 81 nm/half-sarcomere (nm/h.s.). V_{max} for length changes less than 81 nm/h.s. was 3·65 m.l./s and for greater extents of shortening was 1·46 m.l./s. To simplify the presentation of data, the phase of shortening at lengths below the break point will be called 'high velocity' and the phase at lengths beyond the break point will be called 'low velocity'.

In several activations at low Ca^{2+} in which recovery was allowed to proceed to completion, tension typically failed to redevelop to pre-step levels (Fig. 3). This reduction in isometric-tension-generating capability most likely does not represent shortening inactivation described previously by Edman (1975) and Ekelund & Edman (1982), since the lowered tension can be maintained indefinitely. Rather, variations in steady tensions following the length change might reflect sarcomere-length-dependent changes in the Ca^{2+} sensitivity of tension development.

V_{max} data from seventeen fibre segments are shown in Fig. 4, in which two V_{max} values were plotted at each pCa for which biphasic shortening was observed. The mean V_{max} during high-velocity shortening at pCa 4·5 was 4·44 ± 0·15 m.l./s (± 1 S.E. of mean). The phase of high-velocity shortening was relatively insensitive to Ca^{2+} concentrations between pCa values 4·5 and 6·0; however, V_{max} fell to 3·58 ± 0·40 m.l./s

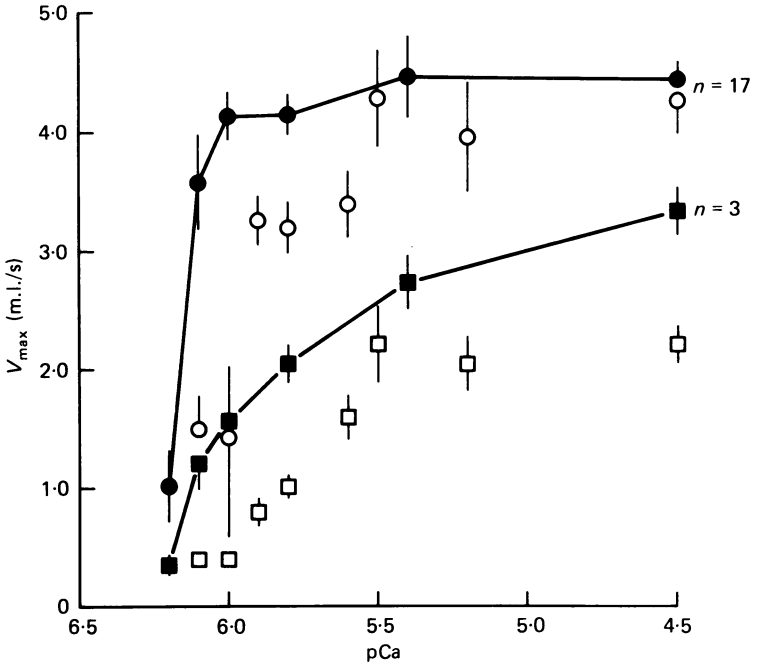

Fig. 4. Plot of mean V_{max} *vs.* pCa, obtained from control fibres (● and ■) and fibres following partial extraction of troponin-C (○ and □). The circles represent V_{max} values measured during the high-velocity phase of shortening, and the squares are values during the low-velocity phase. Error bars represent ± 1 S.E. of mean. All data points represent the mean of twelve to seventeen V_{max} determinations except the low-velocity point at pCa 4·5 which is the mean of three values.

at pCa 6·1 and further to 1·02 ± 0·30 m.l./s at pCa 6·2. The V_{max} values obtained during the low-velocity phase of shortening declined within the entire range of pCas studied, from a mean of 3·34 ± 0·20 m.l./s at pCa 4·5 to 0·35 ± 0·09 m.l./s at pCa 6·2. For every case in which a biphasic plot of slack-test data was observed, the amount of active shortening to the length corresponding to the break in the plot was measured (Fig. 5). It should be noted that the occurrence of biphasic plots of slack-test data was relatively rare at pCa 4·5 (three of forty-two fibres studied). For the remaining pCa values, the extent of shortening at high velocity varied from 85·5 ± 2·5 nm/h.s. at pCa 5·6 to 66·3 ± 8·6 nm/h.s. at pCa 6·1.

Control experiments were done to investigate whether the breaks in the slack-test plots were due to the length change *per se* or were instead the result of attaining a particular sarcomere length during the shortening time course. If the latter were the case, it could be argued that the reduction of V_{max} beyond the break point was

due to structural interference, such as thin-filament overlap, that would retard shortening. While the range of sarcomere lengths employed in this study would argue against this mechanism, a direct test of this possibility was performed by measuring

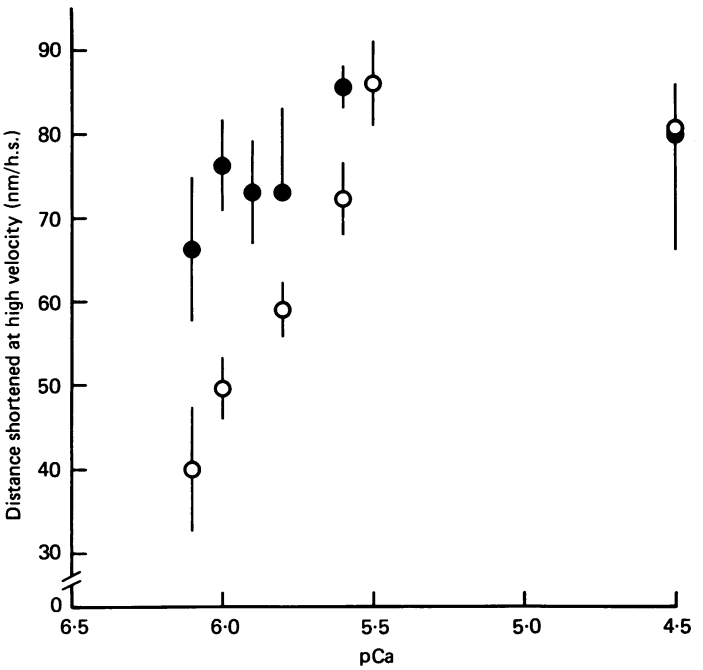

Fig. 5. Plot of the distance shortened during the high-velocity phase of shortening as a function of pCa. Each point is the mean (±1 S.E. of mean) of twelve to seventeen determinations except for the control data point at pCa 4·5 ($n = 3$). ●, data from untreated control fibre segments; ○, data from fibres from which troponin-C had been partially extracted.

V_{max} at a given pCa employing two different initial sarcomere lengths. In the example shown in Fig. 6, slack-test data was obtained at pCa 6·10 employing initial average sarcomere lengths of 2·46 and 2·32 μm. V_{max} during each of the phases of shortening at 2·32 μm was slower than the comparable values at 2·46 μm. This presumably resulted from the lower level of active tension developed at the shorter sarcomere length (0·28 P_0 *vs.* 0·36 P_0 at 2·46 μm), as discussed below in connexion with Fig. 9. Of more immediate interest in this experiment, the amount of shortening to the break point was similar in the two cases: 79 nm/h.s. at 2·32 μm and 83 nm/h.s. at 2·46 μm. These results indicate that biphasic shortening is related to the extent of shortening and is not a result of structural interference encountered at a particular sarcomere length, at least within the range of sarcomere lengths that were employed. If such interferences were the mechanism of biphasic shortening, decreasing the initial sarcomere length by 0·14 μm (Fig. 6) would be expected to reduce the amount of over-all shortening to reach the break point by 175 μm (i.e. 0·14 × the number of sarcomeres in series) which clearly was not observed.

As a test of whether the effects of Ca^{2+} on V_{max} observed above (Figs. 4 and 5) were due to changes in Ca^{2+} concentration *per se*, measurements of V_{max} and the extent of high-velocity shortening were made using fibres from which troponin-C was

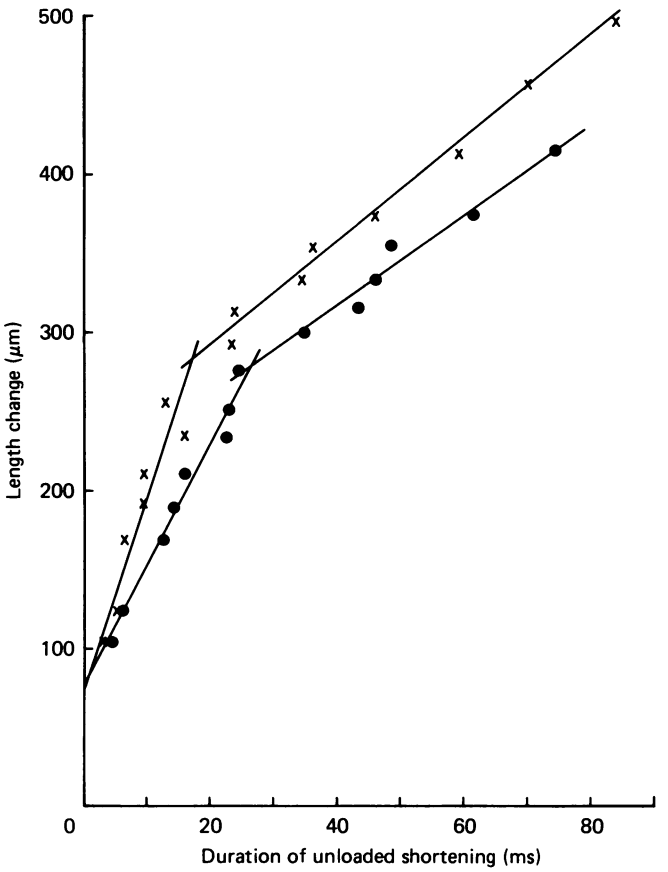

Fig. 6. Plots of slack-test data obtained from the same fibre segment at sarcomere lengths of 2·46 μm (×) and 2·32 μm (●). At 2·46 μm, V_{max} was 4·03 m.l./s during the high-velocity phase and 1·06 m.l./s during the low. At 2·32 μm, the corresponding V_{max} values were 2·53 m.l./s and 0·95 m.l./s, respectively. Fibre No. 121684; over-all length was 3·10 mm at a sarcomere length of 2·46 μm.

partially extracted. In these fibres, the level of activation was dependent both upon the extent of troponin-C extraction and on the pCa of the bathing medium. Troponin-C content of the extracted fibres averaged 65 % of control values measured in segments from the same fibres (Pl. 1). Following extraction, isometric tension at pCa 4·5 declined to 48·0 ± 4·1 % of P_0 obtained in the same segments prior to treatment. V_{max} during the high-velocity phase of shortening was not significantly changed (4·26 ± 0·27 m.l./s *vs.* 4·44 ± 0·15 m.l./s in the untreated segments) following the extraction procedure. However, in all but two cases the plot of slack-test data at pCa 4·5 became biphasic following the extraction (Fig. 7), with a mean V_{max} of 2·21 ± 0·16 m.l./s for the slow phase of shortening. This effect was reversed by

recombination of troponin-C into the fibre segments (Fig. 8). At submaximal levels of Ca^{2+} (pCa 6·10 in Fig. 7), V_{max} values for both the high- and low-velocity phases of shortening were found to be lower after partial extraction of troponin-C.

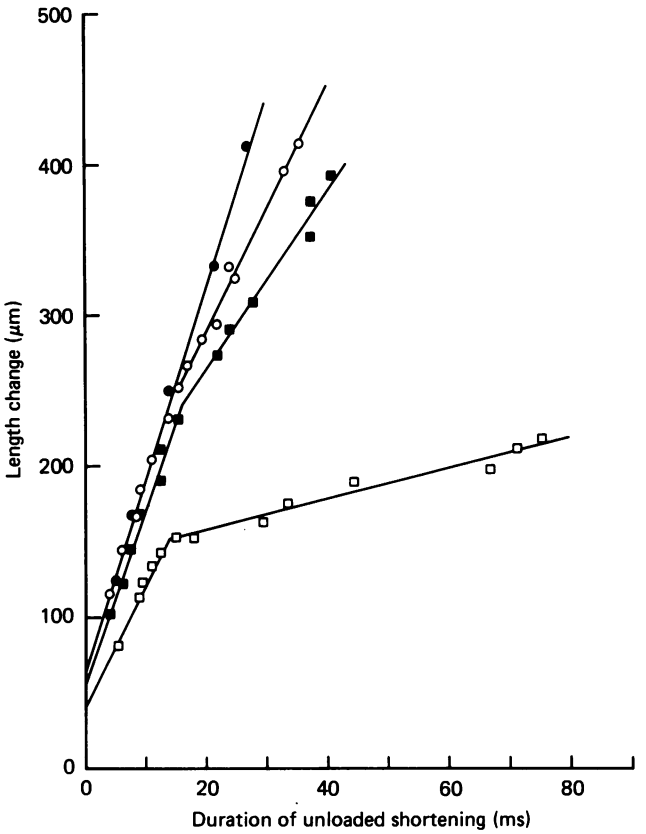

Fig. 7. Plots of slack-test data obtained from a control fibre segment (● and ■) and the same segment following partial extraction of troponin-C (○ and □). Circles, pCa 4·5; squares, pCa 6·1. V_{max} values were measured as follows:

		V_{max} (m.l./s)	
pCa	Velocity phase	Control	Post-troponin-C extraction
4·5	High	3·83	3·67 (line not shown)
	Low	—	2·42
6·1	High	3·40	2·37
	Low	1·75	0·30

Fibre No. 112684; s.l. = 2·42 μm; over-all length of 3·37 mm.

Mean V_{max} values obtained at several pCas following the extraction procedure are shown in Fig. 4. However, an unexpressed variable in plotting such data *vs.* pCa is the lowered troponin-C content of the partially extracted fibres. Reductions in troponin-C content have previously been shown to reduce the Ca^{2+} sensitivity of

tension development in rabbit psoas fibres (Moss *et al.* 1985). Thus, V_{max} values for both the control and extracted fibres were plotted against the steady isometric tension, as percentage control P_0, developed just prior to the slack step (Fig. 9). When plotted in this way, control V_{max} values measured during the high-velocity

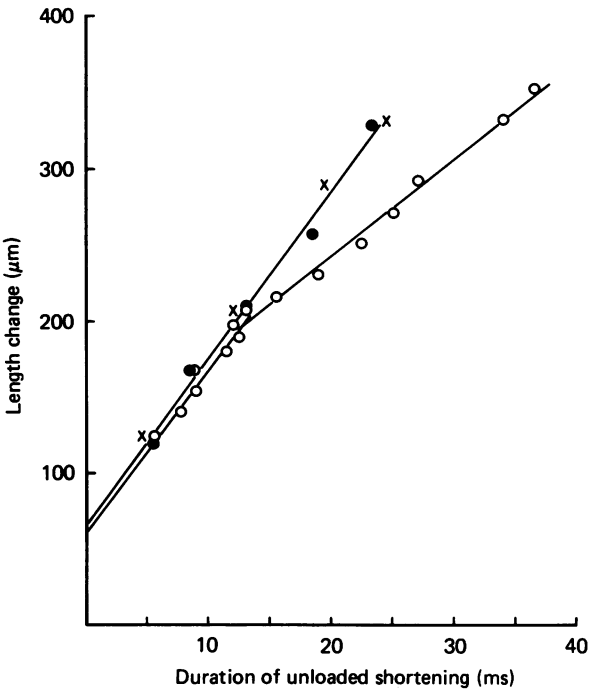

Fig. 8. Plots of slack-test data from one fibre segment demonstrating the reversibility of the effects of troponin-C extraction on V_{max}. The following V_{max} values and tensions were obtained:

	V_{max} (m.l./s)		Steady tension (fraction of control P_0)
	High velocity phase	Low velocity phase	
Control (●)	4·89	—	1·00
Troponin-C-extracted (○)	4·81	2·83	0·47
Troponin-C-recombined (×)	4·68	—	0·99 (line not shown)

All measurements were done at pCa 4·5. Fibre No. 112884; s.l. = 2·55; over-all length of 2·28 mm.

phase of shortening remained approximately constant at pCa values for which the tension-generating capability was greater than about 30 % P_0, and decreased at still lower tension values. The mean high-velocity data points from partially troponin-C-extracted fibres agreed remarkably well with V_{max} values measured in control fibres at submaximal levels of Ca^{2+} when the tension-generating capabilities of the fibre segments were similar. Likewise, the low-velocity data obtained following partial extraction of troponin-C varied with the isometric-tension-generating capability of the fibres in much the same was way as the control segments.

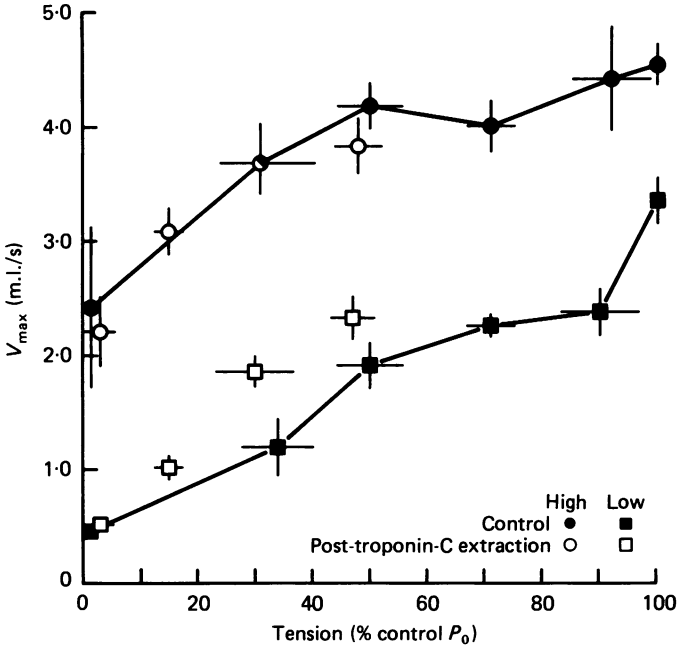

Fig. 9. Plot of V_{max} as a function of steady isometric tension developed prior to the slack step. Tension was varied by adjusting the pCa of the activating solution and is expressed in each case as a percentage of the maximum isometric tension at pCa 4·5 developed by the same fibre segment prior to extraction. Each point represents the mean (±1 S.E. of mean) of four or more individual determinations. The horizontal bars represent ±1 S.D. The symbol key is the same as that described in the legend of Fig. 4.

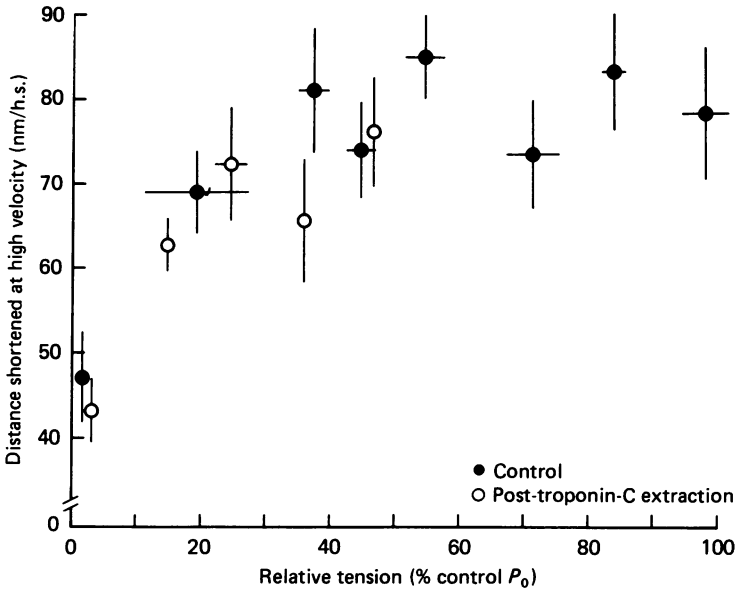

Fig. 10. Plots of the distance shortened during the high-velocity phase of shortening as a function of the steady isometric tension developed prior to the slack step. The descriptions of symbols, error bars and the determination of relative tensions are similar to those in the legend of Fig. 9.

The distance shortened during the high-velocity phase was also plotted as a function of the tension-generating capabilities of the fibre segments (Fig. 10). In the control segments, the extent of high-velocity shortening was invariant with tension-generating capability down to about 30 % P_0, decreasing to 69·0 ± 4·9 nm/h.s. (± S.E. of mean, $n = 5$) at a mean tension-generating capability of 19·3 % P_0 and to

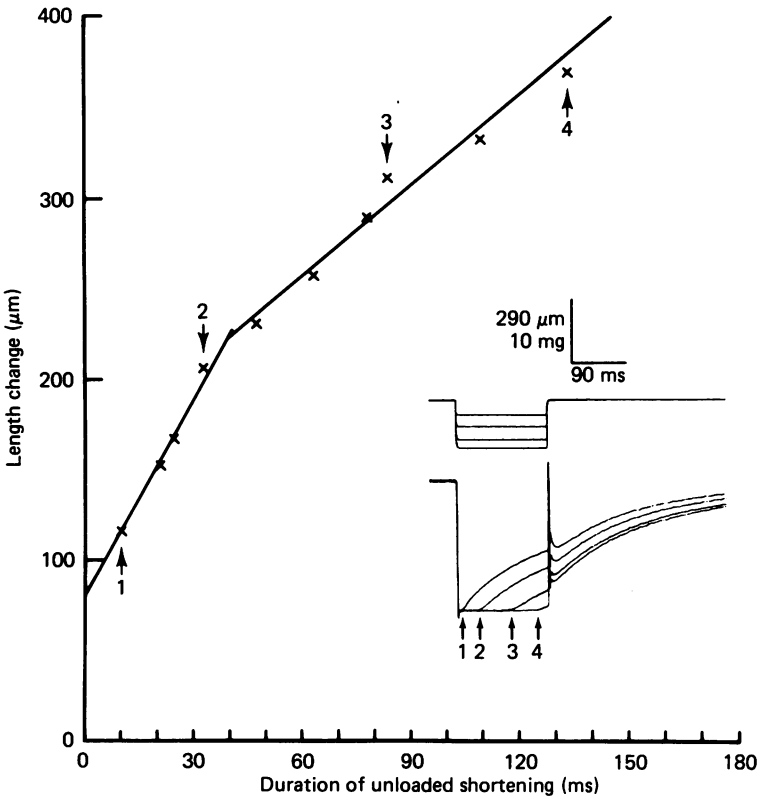

Fig. 11. Plot of slack-test data from a fibre segment activated in the absence of Ca^{2+} by partial extraction of whole troponin. The inset contains original records of length (upper) and tension (lower) used in constructing this plot, with the numbered points on the plot indicating the corresponding tension take-up points on the tension traces. The slack-test data was recorded in a manner similar to that described in Fig. 1 *B*. Steady tension at pCa 9·0 following partial extraction of troponin was 21 % of that developed at pCa 4·5 prior to extraction. V_{max} during the high-velocity phase of shortening was 1·10 m.l./s, and during the low-velocity phase was 0·51 m.l./s. Fibre No. 73184; s.l. = 2·52 m.l./s; over-all length of 3·47 mm.

47·0 ± 5·3 nm/h.s. (± S.E. of mean, $n = 6$) at 1·8 % P_0. There was good agreement between the mean value of this parameter in similar ranges of tension-generating capability obtained before and after troponin-C extraction.

A series of experiments was done to investigate whether biphasic shortening at low levels of thin-filament activation results from a shortening-induced dissociation of Ca^{2+} from troponin-C. Single skinned fibres were bathed in a solution containing

20 mM-EDTA, 50 mM-KCl and 5 mM-phosphate buffer (pH 7·0) and a slow muscle myosin-light-chain preparation (1 mg/ml) previously demonstrated to contain a protease activity that cleaves whole troponin complexes from skinned psoas fibres (Moss, Allen & Greaser, 1986*b*). Incubation for a period of 2 h at 20 °C resulted in the loss on average of 12 % of total troponin and yielded a tension in relaxing solution that was 0·25 P_0. The sum of this Ca^{2+}-insensitive tension and the additional tension

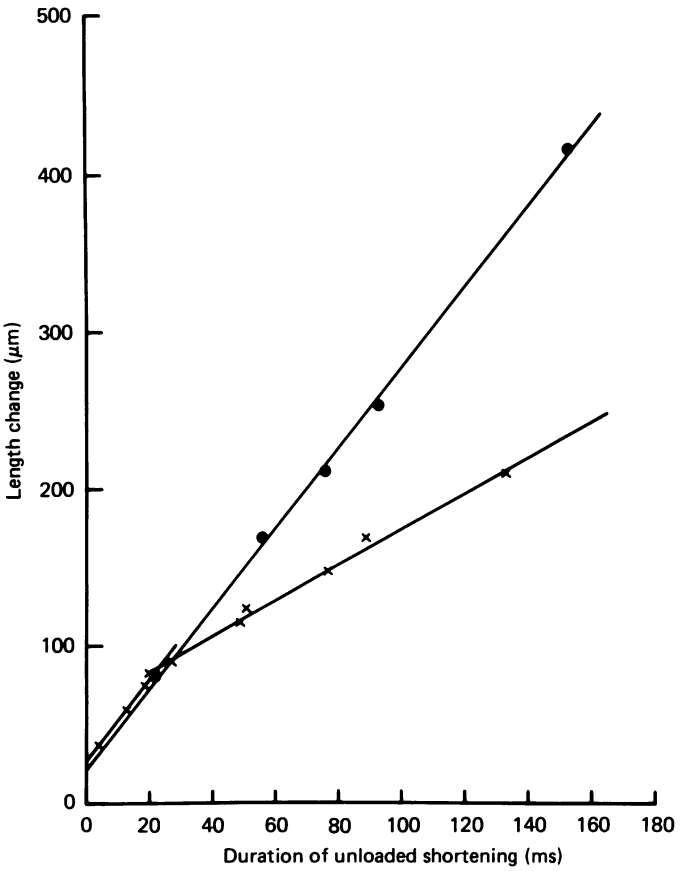

Fig. 12. Plot of slack-test data from a single skinned fibre from a rabbit soleus muscle. Fibre No. 9485; s.l. = 2·49 μm; over-all length of 3·30 mm. ●, pCa 4·5; ×, pCa 6·4.

developed during a subsequent activation at pCa 4·5 was approximately equal to P_0 measured in the same fibres prior to incubation. Thus, a low-level activation of these fibres was achieved in the absence of Ca^{2+}. Slack-test data obtained from one of these fibres while in relaxing solution is shown in Fig. 11. Clearly, the plot of slack-test data is biphasic, with a break corresponding to a length change of 52 nm/h.s. The V_{max} value calculated for the fast phase was 1·10 m.l./s and for the slow phase 0·51 m.l./s. Based on these results obtained at pCa 9·0, it is highly unlikely that the transition from the fast phase to the slow phase of unloaded shortening involved the dissociation of activator Ca^{2+} from troponin-C.

To see whether biphasic shortening is a characteristic of only fast-twitch muscle, and thus perhaps related to the presence of fast-type regulatory proteins (particularly troponin), slack-test data of an exploratory nature was obtained from several skinned fibres from rabbit soleus muscle at various levels of Ca^{2+} activation (Fig. 12). This muscle contains predominantly fibres of the slow type in which V_{max} has previously been shown to be sensitive to Ca^{2+} (Moss, 1982). In the particular case presented in Fig. 12, slack-test data at pCa 4·5 was well fitted by a single straight line yielding a V_{max} of 0·79 m.l./s. However, the data at pCa 6·4 ($P/P_0 = 0·18$) was biphasic with a break point corresponding to active shortening of 29 nm/h.s. V_{max} was 0·82 m.l./s during the high-velocity phase of shortening and 0·32 m.l./s during the low-velocity phase. Thus, biphasic shortening at low levels of Ca^{2+} activation appears to be a property common to both fast- and slow-twitch mammalian muscles.

DISCUSSION

The results of this study show that there is a pronounced effect of variations in the levels of free Ca^{2+} upon V_{max} in mammalian fast-twitch skeletal muscle. The magnitude of this effect appears to differ depending upon the extent of shortening of the muscle prior to the measurement. V_{max} values measured during extents of shortening less than 60–80 nm/h.s. are relatively invariant at Ca^{2+} concentrations corresponding to active tensions of 30–100 % P_0, but fall off sharply as Ca^{2+} is reduced to achieve tensions below this range. For greater extents of shortening, a phase of slower shortening is observed. V_{max} values measured during this phase were found to progressively decline when the tension-generating capabilities of the fibres were reduced from about 70 % P_0 by varying free Ca^{2+}. Consideration of our earlier results (Moss, 1982) in light of the present findings indicates that the effects we reported then were based largely on comparisons of V_{max} values obtained during the high-velocity phase of shortening at maximal activation and during the low-velocity phase of shortening at partial levels of activation.

It should be emphasized that the biphasic time course of unloaded shortening reported in the present paper would not be evident in many cases unless a relatively large number of data points were obtained for extents of shortening less than 60–80 nm/h.s. A small number of data points in this range would yield V_{max} values between those for high- and low-velocity ranges, since the effect of these points would be to increase the slope of a straight line that otherwise would be fitted to only the low-velocity points. In addition, failure to identify the high-velocity phase of shortening would give rise to an over-estimate of the extension of series elements during tension development at low levels of activating Ca^{2+}. Since the ordinal intercept of the straight line fitted to slack-test data is a measure of the extension of series elements (see Julian, Moss & Waller, 1981), lines fitted only to data in the low-velocity phase of shortening will yield an apparent and paradoxically greater extension of series elements (such as connexions to the apparatus) at low Ca^{2+} concentrations than at high (see Moss, 1982).

Identification of two distinct phases of unloaded shortening may provide a basis for partially resolving the differing conclusions of the reports from Brenner (1980) and Moss (1982) regarding a possible effect of Ca^{2+} upon V_{max} in rabbit skeletal muscle.

Both investigators found that the velocity of shortening under load decreased with elapsed time following the imposition of the load, although there was disagreement as to the Ca^{2+} dependence and time course of curvilinear shortening. Brenner (1980) found continual slowing of velocity at all loads and Ca^{2+} concentrations, while Moss (1982) observed such slowing only at high relative loads. In the latter report, curvilinear shortening was greatest at low Ca^{2+} concentrations and even then contained at least one interval of linear shortening. These differences aside, the back-extrapolation of velocity–length relationships done by Brenner (1980) to obtain estimates of V_{max} during the initial phase of shortening would yield velocities corresponding to the high-velocity phase of shortening identified in the present study. Unless measurements were made at very low Ca^{2+} concentrations (yielding tensions much less than 30 % P_0), an effect of Ca^{2+} on shortening velocity would not be evident. In contrast, Moss's measurements were done during constant-velocity shortening at long times after the imposition of load, corresponding to the low-velocity phase of shortening. A necessary caution in this comparison is that the velocity data from the earlier studies were obtained under load, so that shortening of the fibre preparations could be distorted by sarcomere-length non-uniformities (Julian & Morgan, 1979). None the less, re-evaluation of some of the data obtained previously (e.g. Fig. 3 of Moss, 1982) indicates that in order to achieve a steady shortening velocity under moderate loads, the fibres shortened by approximately 50 nm/h.s. exclusive of elastic recoil. This value is in good agreement with the value of 60–80 nm/h.s. obtained in the present study for the transition between the fast and slow phases of shortening.

The underlying cause for two distinct phases of unloaded shortening is not apparent from the data of this study. In order to explain curvilinear shortening under load, Brenner (1980) previously suggested the presence of an internal load that was independent of the free Ca^{2+} and that increased as a function of the distance shortened. Such an explanation is inconsistent with our results since shortening in the high- and low-velocity phases is linear. Also, in most cases there was no evidence of a slow phase of shortening at pCa 4·5. In those few instances of slow-phase shortening at pCa 4·5 the control fibre was most likely partially deficient in troponin-C content, as we have previously observed in some fibres following storage (Moss *et al.* 1985).

A mechanism that would account for two phases of shortening is one in which the average rate constant for detachment (g) of the cross-bridges decreases substantially following shortening equivalent to 60–80 nm/h.s., since in cross-bridge kinetic models (Huxley, 1957) mechanical V_{max} is highly sensitive to g. In terms of Huxley's model, a reduction in g would lead to an increase in the proportion of total cross-bridges bearing negative force, thereby increasing the resistance to further shortening of the muscle. The observation that the fibres undergo a linear phase of high-velocity shortening before reaching the slow phase suggests that cross-bridges at negative strain are not simply under compression since this would result in a gradual increase in internal load with shortening. Also, 60–80 nm/h.s. is at least an order of magnitude greater than current estimates of the range of displacements that an attached cross-bridge can accommodate (e.g. Ford, Huxley & Simmons, 1977). Of potential interest in this regard is the recent report by Yanagida, Arata & Oosawa (1985) in which it was concluded on the basis of light scattering and fluorescent microscopic

techniques, that the working distance of an attached cross-bridge is 60 nm or greater. A necessary concern is that these results were obtained from myofibrils from crab leg muscles and may not be directly applicable to vertebrate striated muscles. Alternatively, in order to explain the range of high-velocity shortening in the present study, an internal loading of the muscle could arise due to buckling of the S2 link of myosin back upon itself before a substantial force opposing contraction could be borne. Ultimately, such bridges would have to dissociate in order for V_{max} to remain constant during the low-velocity phase of shortening.

An alternate and initially attractive mechanism with which to explain the biphasic time course of shortening was a possible inactivation of the fibre due to a shortening-dependent release of Ca^{2+} from low-affinity sites on troponin-C, thereby decreasing the number of actin sites available for cross-bridge binding. This mechanism was proposed by Ridgway, Gordon & Martyn (1983) to account for their results obtained from aequorin-injected barnacle muscle in which an emission of extra light was correlated with extent of shortening and inactivation of developed tension. However, such a mechanism is unlikely to be the cause of biphasic shortening in the present case, since fibres that were activated in the absence of Ca^{2+} by partial removal of troponin complexes clearly demonstrated both high- and low-velocity phases of shortening (Fig. 11), and also showed reduced steady-tension levels (similar to those in Fig. 3) following the slack-test measurements.

Thus, at present, the slow phase of shortening in mammalian skeletal muscle appears to reflect the presence of a population of slowly cycling cross-bridges during partial Ca^{2+} activation. The proportion of cross-bridges that would be expected to be of this type would increase as Ca^{2+} concentration was lowered in order to explain the relationship between V_{max} during low-velocity shortening and pCa. The mechanism for the observed decrease in V_{max} during high-velocity shortening when Ca^{2+} concentration is lowered (Figs. 4 and 9) is unknown. However, this decrease is probably not due to changes in Ca^{2+} concentration *per se* since similar decreases in V_{max} can be achieved at much higher Ca^{2+} concentrations in partially troponin-C-extracted fibres (Fig. 9). Also, since V_{max} can be altered by varying thin-filament activation independent of Ca^{2+} concentration (Figs. 9 and 11), it is likely that Ca^{2+} has little or no effect via the thick filament to modulate the kinetics of interaction of myosin with actin, a statement that applies equally to the effect of Ca^{2+} on the low-velocity phase. Instead, the mechanisms may, for example, involve changes in the co-operativity of cross-bridge binding to the thin filaments (see Bremel & Weber, 1972) such that at low levels of Ca^{2+} activation the rate constant for detachment of some or all of the attached cross-bridges is reduced.

Finally, the applicability of these results to the long-standing controversy regarding an effect of Ca^{2+} on V_{max} in frog skeletal muscles is uncertain. While shortening records obtained under load are curvilinear in studies by Podolsky and his colleagues (Teichholz & Podolsky, 1970; Thames, Teichholz & Podolsky, 1974), this is not evident in the records obtained by Julian (1971) and Julian & Moss (1981). This difference may be a result of the brief periods of shortening used in the latter studies, although in this case the pronounced effects of Ca^{2+} upon V_{max} would not be expected since the extents of shortening employed should correspond to the high-velocity phase of shortening in rabbit muscle. Alternatively, if a high-velocity phase of shortening

does exist in frog muscle, there is at present no compelling reason to expect that the Ca^{2+} dependence of V_{max} measured in this phase should necessarily be the same as that seen in rabbit skeletal muscle.

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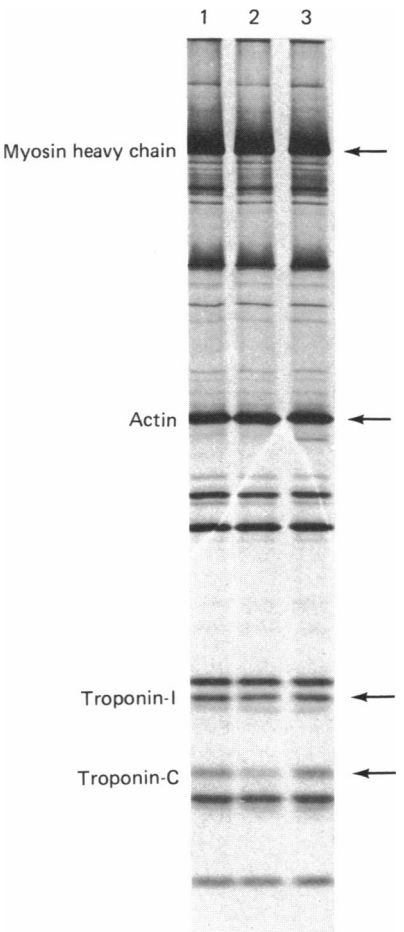
1
2
3
Myosin heavy chain
Actin
Troponin-I
Troponin-C

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EXPLANATION OF PLATE

Silver-stained, SDS-polyacrylamide gel of segments of the same single fibre prior to the extraction procedure (lane 1), following partial extraction of troponin-C (lane 2) and following recombination of troponin-C (lane 3). Segment length applied to the gel in each case was approximately 0·3 mm. The raw densitometric scans of this gel yielded the following amounts for each of the relevant proteins, in arbitrary area units.

	Lane 1	Lane 2	Lane 3
Myosin light $chain_1$	722	740	718
Troponin-I	511	491	531
Troponin-C	376	213	365
Myosin light $chain_2$	884	931	932
Myosin light $chain_3$	292	323	311

In this case, the content of troponin-C following the extraction procedure (lane 2) was 59% of that in the control fibre (lane 1), when troponin-C content is expressed as a fraction of the troponin-I content of the same segment.