

STEPWISE SHORTENING IN UNSTIMULATED FROG SKELETAL MUSCLE FIBRES

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SUMMARY

1. We investigated the dynamics of sarcomere length change during imposed stretches and releases of unstimulated single fibres of frog skeletal muscle.

2. Three independent methods were used: an on-line method in which sarcomere length is computed from the striation pattern; laser diffraction; and a segment length tracking device.

3. During steady ramp releases and stretches, both sarcomere and segment length changes occurred in stepwise fashion; i.e. periods of pause were interspersed between periods of rapid shortening.

4. The above result indicates that activation of the fibre is not required to elicit stepwise length changes.

5. Increasing the ramp velocity caused the steps to increase in size and the pauses to decrease in duration.

6. Ramp releases and stretches were imposed at each of several initial sarcomere lengths up to 4.0 μm . Stepwise length changes were observed at all lengths, and their size was independent of initial sarcomere length.

7. The observation of stepwise length changes beyond overlap indicates that the underlying mechanism probably does not lie in synchronous action of cross-bridges; an alternative hypothesis is advanced.

INTRODUCTION

Earlier work from this laboratory has indicated that the sarcomere shortening pattern in both cardiac and skeletal muscle resembles a staircase. Periods of pause, during which there is little or no shortening, alternate with periods of rapid shortening; this confers a staircase-like character on the shortening wave form. The phenomenon was first detected using a high-resolution diffractometer (Pollack, Iwazumi, Ter Keurs & Shibata, 1977; Pollack, Vassallo, Jacobson, Iwazumi & Delay, 1979), and has since been confirmed by high-speed cinemicroscopy of the striation pattern (Delay, Ishide, Jacobson, Pollack & Tirosh, 1981) and by real-time sarcomere length computation based on striation pattern analysis (Jacobson, Tirosh, Delay & Pollack, 1983).

The present experiments were motivated by an early report of evidence for actomyosin interaction in resting fibres (Hill, 1968). This observation raised the

question of whether stepwise phenomena could be observed in fibres that were unstimulated. Because of the controversial nature of the stepwise shortening phenomenon, we employed three independent methods to cross-check our results.

Our observations indicate that stepwise sarcomere length changes do, indeed, occur in unstimulated fibres. Moreover, the wave forms are sufficiently clear, abundant, and regular that a number of significant features of the phenomenon have come to light.

METHODS

Preparations

Fibres from lumbricalis digitorum IV taken from the hind foot of *Rana temporaria* were dissected at room temperature. Slack lengths ranged from 1.0 to 1.7 mm, while fibre diameters were approximately 40–50 μm . A hole was punched in each tendon and the fibre was mounted horizontally between a fixed tension transducer (AME 801E) and the moveable lever arm of a servo motor (Cambridge Technology, Model 303). Temperature was maintained at 7 ± 2 °C, by circulating chilled physiological salt solution parallel to the long axis of the fibre. The composition of the bathing solution was as follows (in mM): NaCl, 111; KCl, 1.8; NaHCO_3 , 2.3; NaH_2PO_4 , 0.18; CaCl_2 , 1.08. Final pH was adjusted with HCl to 7.4. In the earlier experiments on a sporadic basis, and in the latter (approximately half) experiments on a systematic basis, fibres were checked for their ability to contract actively. Fibres that could not sustain an initial 5 s test tetanus were not admitted to the study.

Sarcomere length computation from the striated image

The preparation was transilluminated with light from a 150 W Xenon lamp. An infra-red filter was used to minimize heating of the fibre. The striation image was magnified by a $40 \times$ water immersion objective (Zeiss, N.A. = 0.75) and $12.5 \times$ ocular and projected on to a 128 element photodiode array (Reticon, RE 128EC/17). The field projected on to the photodiodes corresponded to an area in the muscle approximately 50 μm long (i.e. approximately twenty striations) by 5 μm wide with a depth of field of approximately 3 μm .

The photodiode array was scanned every 256 μs and a periodic signal corresponding in frequency to the incident striation pattern was obtained during each scan. The average sarcomere length was computed on-line from this signal using the phase-locked loop method described previously (Myers, Tirosch, Jacobson & Pollack, 1982). Briefly, the phase-locked loop uses a voltage-controlled oscillator to track the frequency of the signal from the array. The voltage output is then proportional to the spatial frequency of the striation pattern falling on the photodiode array. Not all of the striations incident on the array are used for the computation. The first several striation cycles are required for the system to come into 'lock' so that the actual sampling is set to begin only after approximately the tenth striation along the array; the remaining ten to fifteen striations incident on the array are the ones included within the computation window.

Sarcomere length computation by laser diffraction

Laser diffraction was used to complement the phase-locked loop method. In instances in which the protocol involved frequent resetting of initial sarcomere length it was more practical to use the diffraction method. In the latter, no recalibration was required even for large changes of initial length, whereas with the phase-locked loop, the limited dynamic capture range made recalibration necessary. Further, some protocols were repeated with both methods to ascertain whether the results were similar.

The diffraction method was implemented in a manner similar to that developed by Iwazumi & Pollack (1979) and used in earlier studies from this laboratory (Pollack *et al.* 1977, 1979). The specimen was illuminated with a He-Ne laser beam compressed to a diameter of approximately 100 μm . The diffracted light was collected with a $40 \times$ water immersion objective (Zeiss, N.A. = 0.75), and projected on to a 128 element linear photodiode array (Reticon, RE 128EC/17). The array was scanned at 256 μs intervals, and for each scan the position of the median of the first order was computed on-line. From this signal the sarcomere length was determined.

Segment length computation

As a supplement to the two methods described above, we repeated several of the experiments on both toe and tibialis anterior single fibres using a new segment length detection system (Granzier, Brozovich, Rowinski, Myers & Pollack, 1984). The method is, in principle, similar to that developed by Edman & Hoglund (1981) and used subsequently by Edman, Elzinga & Noble (1982), except for several refinements. Thin black cat hairs, nominally 5–15 μm wide and 50–100 μm long, were placed along the fibre on its upper surface, oriented normal to the fibre axis and spaced 300–800 μm apart. The image of a section of the fibre containing two hairs was split such that one hair was projected on to one photodiode array, while the other was projected on to a second similar array (Reticon, RL256C/17). Cylindrical lenses were used to compress the rod-like image of each hair to a dot, thereby increasing the intensity of the image on the array. A processing circuit computed the location of the median of each marker profile during each scan. The spacing between the two medians, computed every 256 μs , then gave the segment length.

The method achieves its high spatial resolution by virtue of the method of locating the median of the image of each hair. The image signal, encompassing some ten to twenty photodiode elements, is first integrated, giving the area signal. Total area is stored as a reference. The stored value is then compared on a continuous basis with the area signal derived from the subsequent scan, and when the latter equals half the former, the time is noted. This time corresponds to the median location along the array for the latter scan. Because it is determined on a continuous basis (using a ramp signal), the median is resolved more finely than the spacing of individual photodiode elements. Depending on various factors we have been able to achieve a resolution on the order of 0.1 % of segment length.

Controls for artifacts

To test for possible effects of axial translational movement of fibres across the optical axis (Altringham, Bottinelli & Laktis, 1984; Pollack, 1984*a*), calibrated gratings were moved across the optical field at a constant velocity using the moveable lever arm of the servo motor. Because of the finite window width in the phase-locked loop method, translation is expected to introduce small oscillations in the output wave form as the striations pass through the window. We found that the number of oscillations corresponded to the number of dark lines on the grating that crossed the optical axis. The peak-to-peak amplitude of the observed oscillation was approximately 1 nm (though it grew considerably when illumination levels were made subnormal). This is within the noise level of our sarcomere length records. The same experiment repeated with the diffraction method gave no detectable oscillation.

As an additional test for translation-induced steps with the phase-locked loop method, in twelve regions from three fibres, we compared the number of steps on sarcomere length records with the number of fibre striations crossing the optical axis. The latter was measured with the aid of natural markers, or, in cases in which translation velocity was low, striation movements could be followed by eye. Steps on the sarcomere length records were treated as 'oscillations' superimposed on an otherwise smooth wave form. The number of such oscillations showed no correlation with the number of striations crossing the axis.

The possibility that translation-induced oscillations may be mistaken for steps is circumvented in the segment length method. However, other potential artifacts may be inherent in this method. In a separate communication (H. L. M. Granzier, J. A. Myers & G. H. Pollack, in preparation) careful consideration has been given to the following possible sources of artifact: discreteness of the photodiode array; longitudinal and lateral fibre translation; variations of intensity across the field; and spurious fluctuations in the marker signal. All have been ruled out quantitatively as potential causes of the pauses and steps.

Experimental protocol

Before each experiment the optical system was calibrated using a series of etched gratings (American Holographic). Five gratings with line spacings between 1.97 and 3.67 μm were permanently mounted in the chamber at the same level as the fibre so that the optical system could be calibrated in the same plane of focus in which experimental observations were made.

Unstimulated fibres were shortened or stretched at constant velocities ranging between 0.2 and 2.0 fibre lengths/s by feeding a trapezoidal signal to the servo motor. Fibres had been

intentionally mounted through long tendinous connexions, so that by virtue of the large end-compliance, the sarcomeres were not constrained to follow exactly the movements of the lever arm; i.e. imposition of a smooth ramp change of muscle length did not preclude stepwise changes of sarcomere length. Position of the lever arm was controlled accurately within $0.5 \mu\text{m}$, and there was no detectable oscillation in its movement. Except for studies with stretched fibres, initial sarcomere lengths were set nominally at $2.5 \mu\text{m}$ (range $2.3\text{--}2.7 \mu\text{m}$).

To be certain that the fibre was not going slack during release, we monitored tension at the fixed end of the fibre. Resting tension was typically 5% of maximum tetanic tension and decreased steadily toward zero during the release. Only after substantial shortening, i.e. late in the release, did the tension occasionally reach zero; thus, the fibres were not buckled.

Sarcomere length signals during stretch and release usually became noisier as the region under observation was shifted toward the end of the fibre nearer to the lever arm. The noise increase is presumably caused by increased axial and lateral translation nearer to the moving end. However, it was usually possible to obtain clean records from regions extending from the fixed end to a point 30–50% of the way along the fibre with the phase-locked loop method and slightly further with the laser diffraction method.

A total of fifty-six fibres were studied with the optical methods, and twenty-one with the segment length method.

Data analysis

With the phase-locked loop and laser diffraction methods, the sarcomere length signal was attenuated, offset, and sent to a DEC PDP-15 computer by means of a 10-bit analog to digital converter. Digitized signals were stored on disk cartridges. For analysis, sarcomere length change records were displayed on the video screen of the computer and break points were identified with a cursor. The computer then drew line segments between break points using a least-squares fit method. From the line segment record, step size and duration as well as pause duration were computed automatically (Fig. 1).

Acceptance of data into the pool was based on a systematic set of criteria (Jacobson *et al.* 1983). The aim was to eliminate in an objective way noisy or questionable signals, and to choose steps in a consistent manner. The following criteria were applied uniformly to all records by the computer program (cf. Fig. 1). For pauses: minimum duration 1 ms; maximum allowable 'tilt', 3 nm; maximum allowable difference of velocity before and after the pause, 30%. For steps: no step was included in the data pool unless it was surrounded by two pauses that met the above criteria. These criteria tended to ensure that pauses and steps were distinct entities, and that noise was not likely to be mistaken for signal.

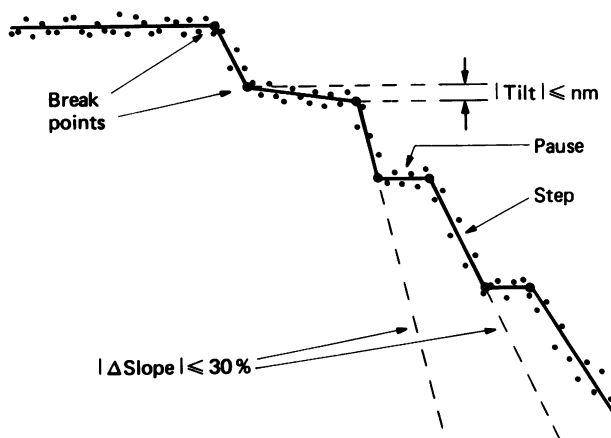


Fig. 1. Schematic drawing of method used for computer analysis of stepwise shortening patterns. Break points are first selected manually on the computer display. The computer then performs a least-squares fit between points. Step sizes are computed automatically from the piecewise linear fit. Only those steps and pauses satisfying the criteria (see text) are accepted into the data pool.

RESULTS

Characteristics of steps and pauses

Fig. 2*A* shows a representative record of the response to an imposed trapezoidal muscle length change obtained with the phase-locked loop method. In general, the records showed cascades of steps during both the shortening and lengthening phase. Steps were sometimes more distinct during shortening than during lengthening.

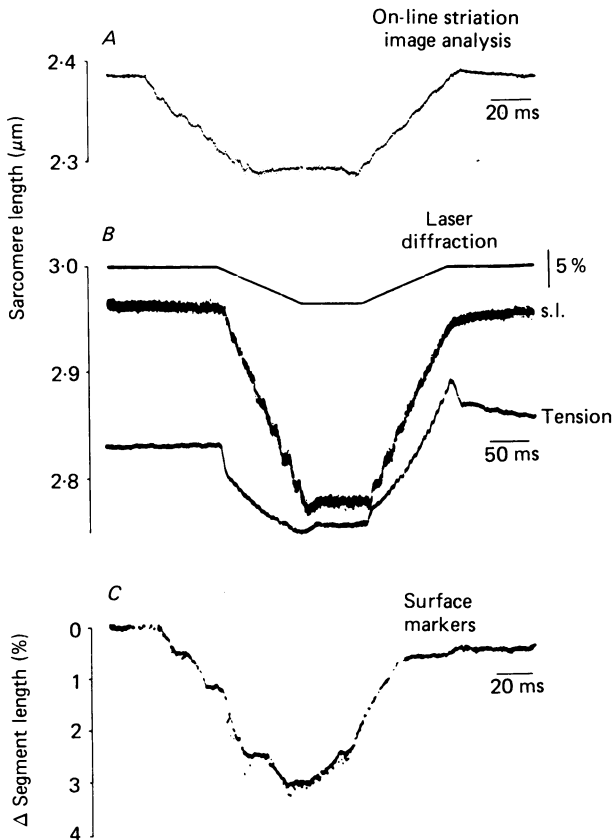


Fig. 2. Response to trapezoidal length change imposed on single, unstimulated, skeletal muscle fibres. *A*, phase-locked loop method, based on analysis of the striated image. *B*, laser diffraction method; muscle length (top) and tension (bottom) wave forms are included. *C*, segment length method; initial segment length, $800 \mu\text{m}$.

Occasionally, signals broke into large oscillations (cf. Fig. 7*A*); this occurred if the striated image falling on the photodiode array deteriorated for any of a number of reasons and the detector was unable to sustain its 'lock' on to the signal. In specimens with clear striation patterns, relatively noise-free records could be obtained in an abundantly wide variety of regions. Of these, some 95% showed regular cascades of steps; only in rare instances were the traces smooth.

Fig. 2*B* shows a record obtained under comparable conditions using the laser diffraction method. Such records were generally similar to those obtained with the

phase-locked loop method, except that the noise level was somewhat higher and the frequency of observation of step cascades was lower. We found such cascades in approximately three-fourths of fibres.

Fig. 2C shows a representative record obtained under similar conditions with the segment length method. Pauses and steps are evident. In twenty-one such preparations, five produced traces that could be regarded as smooth. In the other sixteen fibres, wave forms ranged from those in which only a few short hesitations could be discerned to those in which the regular cascade of steps was qualitatively indistinguishable from those obtained with the other two methods. Although we did not subject these records to computer analysis, we estimate that records from at least twelve of the twenty-one fibres contained steps that would have satisfied the acceptance criteria.

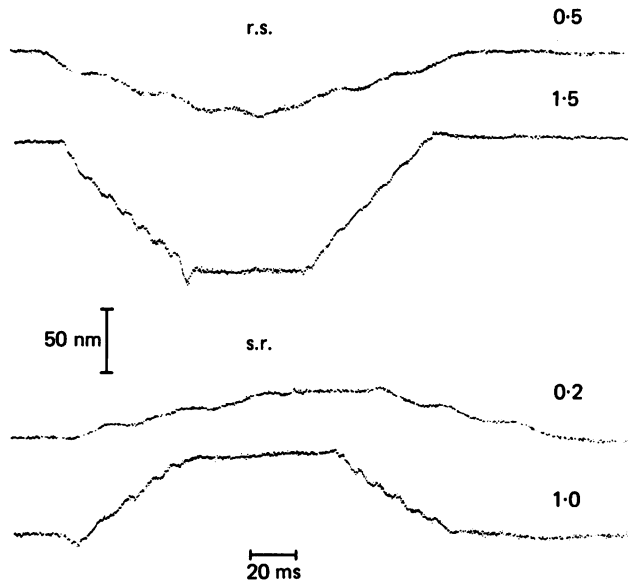


Fig. 3. Effect of variation of speed of stretch/release. Records are taken from the same region of the same fibre using the phase-locked loop method. r.s., release followed by stretch; s.r., stretch followed by release. Figures to the right of each trace refer to speed of release/stretch in fibre lengths/s. Initial sarcomere length, $2.4 \mu\text{m}$.

The variation in ease of detectability of steps among the three methods is understandable if synchrony of shortening steps does not extend infinitely. The sampled volume varies with the method. At the one extreme, the phase-locked loop method samples a roughly cylindrical region some $30 \mu\text{m}$ long and $3\text{--}5 \mu\text{m}$ in diameter, while at the other extreme, the segment length method samples a cylindrical region roughly 0.5 mm long and at least some fraction of the full fibre width. The frequency of detection of cascades of steps thus varied inversely with the sampled volume. Presumably, modest asynchrony makes detection of cascades of steps less likely as the sampled volume increases.

Fig. 3 shows a series of wave forms measured with the phase-locked loop method.

Each was obtained at a different imposed ramp velocity. The wave forms of the Figure appear qualitatively similar, except that when the imposed velocities were higher, the pause durations were shorter and shortening during the step occurred more rapidly. At the lowest of the imposed velocities, the transition between pause and step occurred somewhat less abruptly.

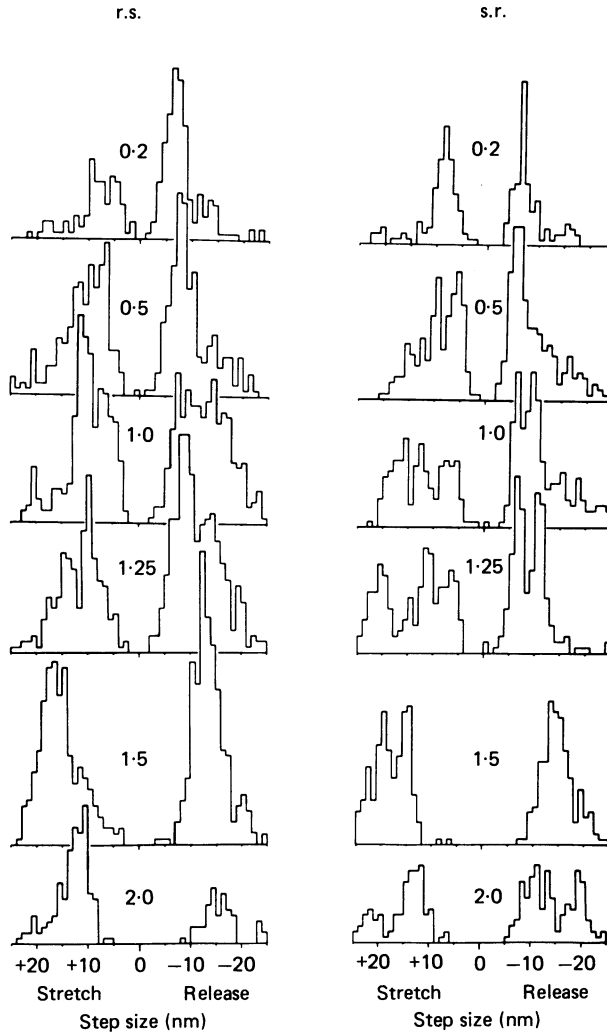


Fig. 4. Distributions of step size at a series of imposed ramp velocities, measured with the phase-locked loop method. r.s., release followed by stretch; s.r., stretch followed by release. Numbers at the centre of each histogram refer to the speed of release/stretch in fibre lengths/s.

The pauses shown in Fig. 3 are mostly 'flat'. However, some pauses on these and other records showed noticeable slope, either upward or downward. These slopes are likely to arise from axial translation of the fibre across the optical axis, a factor difficult to avoid in experiments such as these. Translation causes a progressive

renewing of the region sampled by the sensor. The effects of any inhomogeneity of sarcomere length will thus be superimposed upon the local sarcomere shortening signal. Suppose, for example, between the beginning and the end of a pause, translation induces a shift of sampled sarcomere length. If the difference in length between the new and old populations were as little as 1 nm (0.04%), an otherwise flat pause would take on a noticeable slope. We have not yet checked this explanation systematically, but in one experiment in which we measured both the distribution

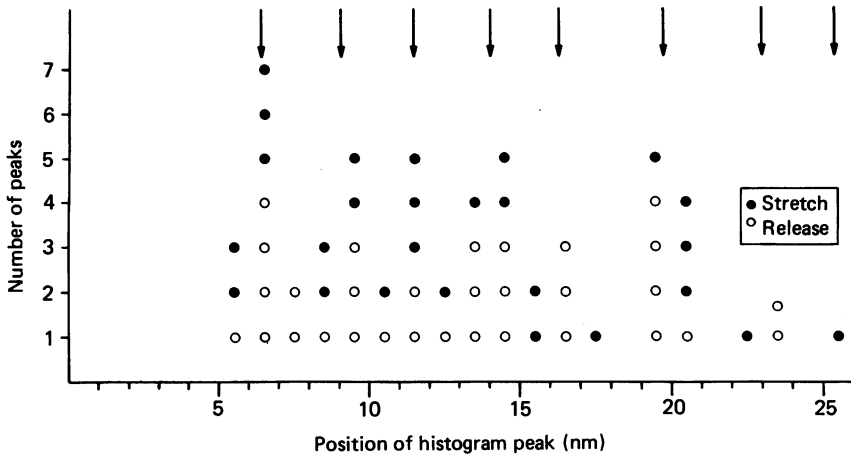


Fig. 5. Distribution of major peaks of the histograms of Fig. 4. See text for criteria used for peak selection. Centre of gravity of each peak is shown by arrow above.

of sarcomere lengths along the fibre and the amount of axial translation, we were able to compute the 'error' due to the fact that sarcomeres of different length had entered the optical field. When this error was subtracted, the 'slope' of the pauses diminished significantly and the pauses became almost flat.

The histograms of Fig. 4 show the distributions of step size at each of the imposed velocities studied with the phase-locked loop method. Each histogram is divided into a stretch portion (left) and a release portion (right). The two sets of histograms correspond to the two conditions: when the release preceded the stretch (r.s.), and when the stretch preceded the release (s.r.). Each histogram shows a relatively broad distribution, and there is a progressive shift of the mean step size toward larger values at higher velocities.

Distinct peaks appear in some of the histograms. To test whether these occurred at preferred values of step size, we plotted the location of these peaks on Fig. 5. Peaks were included only if they satisfied the following criteria for distinctness: height $\geq \Sigma$ surrounding valley heights and $\geq 20\%$ of the highest histogram peak; width ≤ 3 nm at 80% of peak height. The Figure shows a cluster of peaks at 20 nm, and another near 6 nm. The intervening values (arrows) are less distinct. Variations of criteria affected details of the histogram but not its general features.

Fig. 6A shows the relationship between imposed ramp velocity and mean step size. These results were obtained from the data of Fig. 4. The Figure shows that mean step size increased with imposed ramp velocity. We could find no consistent difference

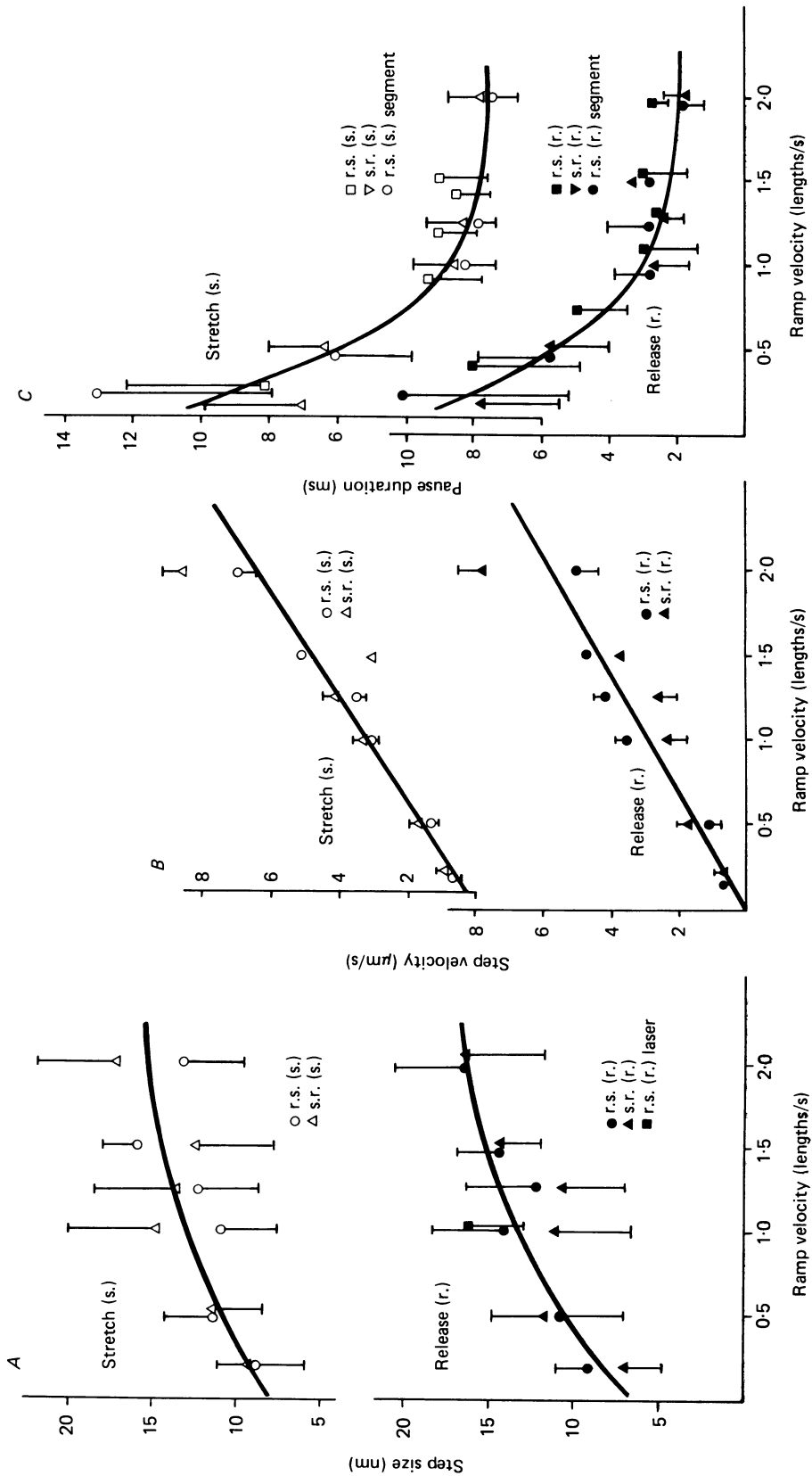


Fig. 6. Effects of variation of imposed ramp velocity on features of the stepwise shortening pattern. Based on data obtained with the phase-locked loop method except where noted. Key: r.s.(s.) for example, indicates that the imposed trapezoidal wave form consisted of a release followed by a stretch, and that it was the stretch (s.) portion that is under consideration.

in step size between release and stretch at a given velocity. The result was also independent of whether the initial length change was negative or positive. Test results obtained at a velocity of 1 length/s with the laser diffraction method (filled square) gave a value of step size indistinguishable from that obtained with the phase-locked loop method.

TABLE 1. Step size as function of initial sarcomere length

Initial sarcomere length (μm)	Method	Step size (nm)	s.d. size (nm)	<i>n</i>
2.53	L.	16.6	3.6	29
2.54	P.I.I.	17.5	3.5	39
2.64	L.	15.0	4.5	26
2.70	L.	18.1	3.1	15
2.76	L.	16.8	3.5	16
2.81	L.	16.6	4.4	11
2.88	L.	13.7	3.5	20
2.96	L.	17.6	5.0	10
3.03	L.	17.1	3.3	12
3.08	L.	15.8	3.6	33
3.17	L.	15.0	4.4	8
3.60	P.I.I.	14.1	1.8	4
3.68	L.	15.6	3.5	23
3.69	L.	15.0	2.7	12
3.71	L.	16.5	2.1	18
3.75	L.	15.2	2.8	20
3.80	P.I.I.	16.8	8.8	8
4.02	P.I.I.	16.9	5.2	5

Mean step size (laser) = 16.0 nm.

Mean step size (P.I.I.) = 16.3 nm.

Mean step size for s.l. $\leq 3.60 \mu\text{m}$ = 16.2 nm.

Mean step size for s.l. $\geq 3.60 \mu\text{m}$ = 16.0 nm.

All data obtained during shortening ramp at 1 length/s. L., laser; P.I.I., phase-locked loop; s.l., sarcomere length.

Fig. 6*B* shows that as the release velocity increased by a factor of ten, the sarcomere step velocity also increased by a factor of ten; from approximately $0.7 \mu\text{m/s}$ to approximately $7 \mu\text{m/s}$. Thus, step velocity increased linearly with fibre velocity and was, of course, sufficiently higher than muscle velocity to allow time for pauses. Again, there was no difference between stretch and release. The graph shows generally small scatter of results at a given velocity, indicating a tight coupling between step velocity and imposed ramp velocity.

Pause duration decreased sharply with increasing ramp velocity from 11 ms at 0.2 lengths/s to 1.8 ms at 2.0 lengths/s (Fig. 6*C*). This feature was also similar for both stretch and release. The tendency for pause duration to reach a minimum plateau at higher ramp velocities may be real or may reflect the fact that pauses of less than 1 ms duration were excluded from the analysis. Although the time resolution of $256 \mu\text{s}$ allowed for detection of shorter pauses, we felt that resolution of such fine features of the wave form encroached on the limitations imposed by the

noise of the method. Fig. 6C also shows results obtained with the segment length method. The pause durations followed the same functional dependence on ramp velocity as the result obtained with the phase-locked loop method.

Shortening behaviour in stretched fibres

We also imposed trapezoidal length changes on fibres set at each of a series of extended lengths. For technical reasons (see Methods), sarcomere length changes were measured primarily using laser diffraction in these experiments. Table 1 shows that the mean step sizes did not depend on initial sarcomere length. At the velocity used

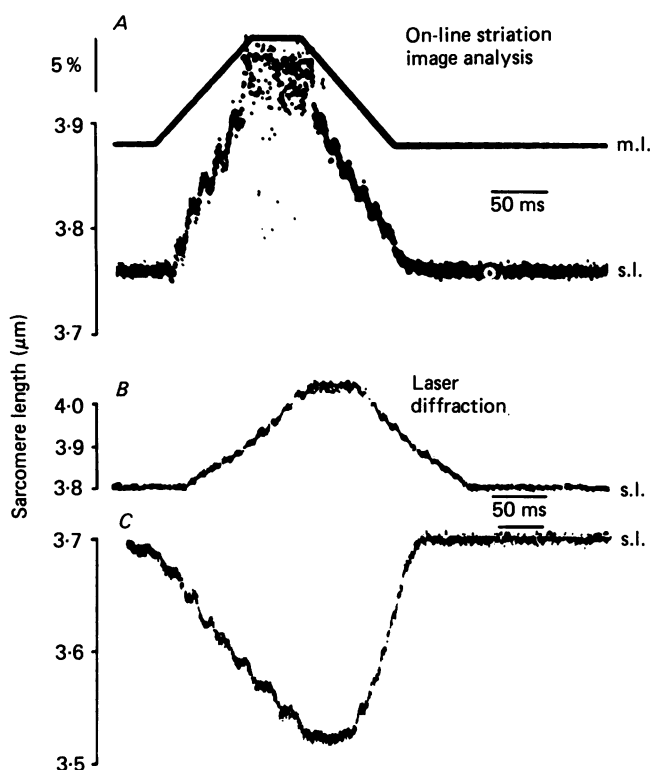


Fig. 7. Sarcomere length changes in highly stretched fibres. *A*, phase-locked loop method. *B* and *C*, laser diffraction method. In panel *C* the sarcomere length passes through 3.6 μm ; the anticipated boundary between non-overlap and overlap of thick and thin filaments. m.l., muscle length; s.l., sarcomere length.

(1 length/s), step size remained approximately 16 nm at each initial sarcomere length, not significantly different from the results obtained at the same imposed velocity with the phase-locked loop method (Fig. 6A).

To determine whether stepwise behaviour in unstimulated fibres requires actomyosin interaction, we examined records of sarcomere length changes in fibres stretched to sarcomere lengths greater than 3.6 μm , i.e. beyond overlap. Fig. 7 shows several representative records. Though the quality of the records is inferior to those

obtained at shorter sarcomere lengths, stepwise behaviour is apparent. Similar results were obtained using both optical imaging (*A*) and laser diffraction (*B* and *C*). Panel *C* shows that there is no obvious change of the quality of the pattern as the sarcomere length passes through the boundary between non-overlap and overlap. The step sizes obtained at lengths beyond overlap are indeed similar to those measured at the shorter sarcomere lengths (Table 1). It appears that there is a continuum that is not broken at the boundary between overlap and no overlap.

DISCUSSION

The results indicate that stepwise phenomena may indeed be observed in unstimulated fibres, both during stretch and release. All three methods show comparable features and these, in turn, are at least qualitatively similar to those observed earlier with activated specimens (Pollack *et al.* 1977, 1979; Delay *et al.* 1981; Jacobson *et al.* 1983; Pollack, Tirosh, Brozovich, Lactis, Jacobson & Tameyasu, 1984).

Are steps real?

Since the time stepwise sarcomere shortening was first identified using optical diffraction (Pollack *et al.* 1977), the phenomenon has remained controversial. Because sarcomere length measurements necessarily reflect the collective behaviour of large numbers of myofibrillar sarcomeres, the very fact that discrete behaviour is observed indicates that the elements contained within the sampled volume must be changing their length synchronously and pausing synchronously. This feature has provided ample reason for questioning the validity of these observations (Rüdel & Zite-Ferency, 1979; Altringham *et al.* 1984; but see Pollack, 1984*a*).

On the other hand, the stepwise phenomenon has now been confirmed using a number of independent methods. We have observed steps by analysing successive frames of high-speed cinemicrographic records (4000 frames/s) of the striation pattern taken during contraction (Delay *et al.* 1981), and this result has recently been confirmed in another laboratory (H. Sugi & M. Toride, unpublished observations). The present phase-locked loop method also makes use of the striation image, and shows steps with higher resolution than the cine method. The present segment length method is independent of the optical properties of the striations, and also shows clear steps, as do records published by Edman *et al.* (1982, Fig. 8) using an essentially similar method. Two additional segment length methods, one using glass micro-electrode tips to delineate segments (Housmans, 1984), and another using natural markers to delineate segments (T. Tameyasu, T. Toyoki & H. Sugi, unpublished observations), also show steps.

In spite of confirmation by several optical and non-optical methods, we carried out a number of control experiments to test for possible artifact. We were particularly concerned with the potential effects of fibre translation with the optical methods (in the segment length method the sampled population remains invariant). Moving a grating across the optical field at right angles to the rulings produced corresponding oscillations which were barely discernible above the noise floor in the phase-locked loop signal and indiscernible in the diffraction signal. In experiments on fibres, we could find no correlation between the amount of translation and the number of steps,

and in fact, were able to find clear stepwise shortening in regions approaching the fixed tendon, where translation was minimal. This supplements earlier tests in which we demonstrated that relative translation of fibre and sensor produces oscillations of 2 nm or less (Jacobson *et al.*, 1983).

On the basis of these controls, and on the similarity of results obtained with various independent methods (Fig. 6; Table 1), we conclude that the steps appear to be a genuine feature of muscle dynamics.

Properties of steps and pauses in unstimulated fibres

The observation of stepwise shortening in unstimulated fibres was at first unexpected. We had envisioned the stepwise process as related to the mechanism of active shortening and wondered whether the reported low level of activation in unstimulated fibres (Hill, 1968) could be sufficient to elicit the phenomenon. We found the stepwise patterns in unstimulated fibres to be every bit as apparent as in stimulated fibres. Evidently, whatever process gives rise to the steps in activated fibres may also mediate their appearance in unstimulated fibres where the level of activation is markedly lower.

An interesting aspect of the results was that the patterns of steps during a release were quite similar to the patterns of steps during a comparable stretch (Fig. 3). The similarity extended quantitatively to step sizes (Fig. 6A), step velocities (Fig. 6B) and pause durations (Fig. 6C). This implies that whatever process gives rise to the steps is reversible.

The pattern of step sizes followed an interesting distribution (Fig. 5). Step sizes tended to favour certain discrete values separated from one another by 2–3 nm. Although the distinctness of some of the histogram peaks is questionable, a similar pattern of discrete sizes with 2–3 nm intervals has been noted in activated fibres (Jacobson *et al.* 1983). Some differences in the exact positions of the peaks in the two sets of data are apparent, but the limited resolution of the histograms, taken together with the breadth of each peak, may limit the value of a more detailed comparison at this stage.

The effect of variation of ramp velocity on step size was modest. Though the speed of the ramp was increased over a range of 10:1, the mean step size increased by only about a factor of two (Fig. 6A). Since the higher ramp velocities give rise to higher rates of change of tension we assumed that the latter might constitute the critical variable determining the size of the step. However, this is apparently not the case: large steps also occurred late in the release, when the rate of change of tension was considerably lower than at the beginning. Furthermore, releases (at 1 length/s) from extended lengths, where resting tension was considerably elevated, gave similar step sizes of 16 nm despite substantially higher rates of change of tension. An alternate possibility is that the imposed ramp velocity, itself, is the factor that determines step size; i.e. that once the sarcomere begins shortening from the previous pause, the distance it will shorten is a function of the amount of 'slack' imposed on the fibre by the motor. This possibility remains to be explored.

The effect of variation of ramp velocity on pause duration was more dramatic (Fig. 6C). Our working hypothesis has been that during the pause state the contractile apparatus is locked in a state of quasi-equilibrium; and that when this equilibrium

is disturbed by increasing or decreasing the load by a sufficient amount, the fibre will begin its step. In such a case, the onset of the sarcomere length change would be precipitated most rapidly when the load is changed most rapidly. The shortest pauses did occur when the ramp velocity was highest. This was equally true for shortening ramps and lengthening ramps.

The velocity of sarcomere length change during the step varied similarly to the velocity of the imposed ramp (Fig. 6*B*). This is hardly surprising since sarcomere shortening would be expected to be limited by the velocity imposed on the ends of the fibre. In other words, while the step may have some intrinsic maximum velocity, presumably the one seen when the end of the fibre is forced to move fast enough to present no load, any lower velocity of movement restricts the velocity with which the sarcomere may step.

Steps in stretched fibres

We also observed steps when the sarcomeres were stretched to the point where there should have been no overlap of thick and thin filaments. Steps were seen during shortening and lengthening. Both the imaging method and the laser diffraction method gave steps of comparable size. Further, these steps were similar in size to those found at lesser degrees of stretch (Table 1); i.e. there was no discontinuity at the boundary between overlap and no overlap. Thus, the steps seen at these long and shorter sarcomere lengths appear to be caused by the same type of mechanism. The result is evidently unexpected, and we cannot rule out the possibility that minimal areas of overlap in some regions over the cross-section of the fibre might have given rise to the steps. However, we regard such a possibility as unlikely, considering the fact that our measurements included sarcomere lengths to beyond $4.0\ \mu\text{m}$.

From these results it appears that the process giving rise to the stepwise phenomenon does not *require* actomyosin interaction. We emphasize that this tentative conclusion does not discount the possibility that actomyosin interaction is normally integrated into the mechanism of step generation; only that it may not be essential.

Toward an hypothesis

Although we originally envisioned the steps to arise out of thick/thin filament interaction (Jacobson *et al.* 1983), the present results indicate that steps can be observed in the absence of such interaction. How then might the steps arise?

Beyond overlap, Z-Z continuity is not lost; it is retained through a set of filaments that interconnect the end of each thick filament with the nearest Z line. Although such 'connecting' filaments or 'gap' filaments are not broadly acknowledged, they have been repeatedly documented (Huxley & Peachey, 1961; Locker & Leet, 1975; Magid, Ting-Beall, Carvell, Kontis & Lucaveche, 1984), and are now characterized biochemically (Wang, 1984; Wang, Ramirez-Mitchell & Palter, 1984). These connecting filaments apparently support much of the resting tension, particularly beyond overlap (Magid *et al.* 1984).

An inescapable consequence of this architecture is that a change of sarcomere length is necessarily associated with a change in either the connecting filament length or the thick filament length. Thus, the steps observed at extended lengths must arise out

of step length changes of either the connecting filament or the thick filament. It is generally accepted that the thick filament length remains constant at these extended lengths; if so, the source of the steps would appear to lie in the connecting filaments.

In fibres at shorter length, the situation is more complex. A stepwise length change of the connecting filament might still generate sarcomere length steps in fibres that have substantial resting tension. However, steps are observable (in activated fibres) at lengths well below those at which there is substantial resting tension (Pollack *et al.* 1977, 1979), lengths where the connecting filaments would be expected to be slack. It thus seems unlikely that they could still be responsible for the generation of the sarcomere shortening steps.

At these shorter lengths two *a priori* hypotheses seem possible. The steps could arise out of some kind of synchronized cross-bridge action; stepwise motion could then be transmitted to the Z line through the thin filament. Alternatively, the step-like motion could arise out of the stepwise length changes of the thick filaments, and be similarly transmitted. A large number of studies since the mid-fifties indicate that substantial thick filament shortening may accompany sarcomere shortening at these lengths (for review cf. Pollack, 1983). If filament shortening occurred locally instead of uniformly along the entire filament, this possibility would not necessarily conflict with X-ray diffraction observations (Pollack, 1984 *b*).

Either way, the simplest hypothesis invokes two separate sources, a situation that tends to blunt Occam's razor. We have either stepwise length changes of the connecting filament plus synchronized cross-bridge action, or stepwise length changes of both the connecting filament and the thick filament.

At this stage we cannot rule out either of the two combinations. On the other hand, thick filaments and connecting filaments bear at least some structural similarity. Both, for example, are aggregates of long slender rods with 14.3 nm axial repeat spacing (Koretz & Wang, 1984). It may therefore not be implausible to propose that shortening or lengthening might occur in stepwise fashion in either one. This proposal, however, remains to be tested.

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