Earliest mechanical evidence of cross-bridge activity after stimulation of single skeletal muscle fibers

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ABSTRACT The stiffness of single fibers from frog skeletal muscle was measured by the application of small 2-kHz sinusoidal length oscillations during twitch and tetanic contractions at a range of initial sarcomere lengths. The earliest mechanical signs of activation were a fall in tension (latency relaxation) and a rise in stiffness. The earliest stiffness increase and the earliest tension fall occurred simultaneously at all sarcomere lengths. This suggests a cross-bridge origin for the latency relaxation. The lead of stiffness over tension seen during the rise of tension was substantially established during the latent period. Reducing the size of the twitch by reducing calcium release with D-600 (methoxyverapamil) reduced the latency relaxation and the stiffness development during latency much less than it reduced the twitch tension. For very small twitches the peak of the stiffness response occurred during the latent period and the times of onset of both latency relaxation and stiffness rise were delayed, but remained coincident. This suggests a strong connection between the latency relaxation and the rise of stiffness during the latent period, whereas the connection between these events and positive tension generation appears to be less strong.

INTRODUCTION

Stimulation of a skeletal muscle causes the tension to fall before it begins to rise (Rauh, 1922). This phenomenon has become known as latency relaxation (Sandow, 1944). The relaxation begins at a time after stimulation that is essentially independent of sarcomere length, but has a maximum value and a time course that vary with sarcomere length. As sarcomere length is increased from slack length to ~3.0 μm, the peak value and the time delay to its attainment both increase. Beyond this point the behavior is rather variable, with a decline in amplitude at long lengths being the common feature. In single fibers, Mulieri (1972) reported a plateau from 2.8 to 3.2 μm, Haugen (1982*a*) found a plateau from ~3.15 to 3.35 μm, whereas Haugen and Sten-Knudsen (1976) showed a precipitous decline beyond 3.0 μm. Similar plots for whole muscles were shown by Sandow (1944).

The stiffness of muscle during latency relaxation has been measured by a number of investigators using different methods and reporting somewhat different results. All agree, however, that the stiffness does not decrease with the fall in tension. Herbst and Piontek (1974), using 4.0-kHz sinusoidal length oscillations on whole frog muscles, found the increase in stiffness to commence early in the latency relaxation, though not clearly coincident with the beginning of the relaxation. Wells (1976), using short stretches on fiber bundles, reported that the stiffness rise was "coincident with latency relaxation." Haugen (1982*b*), using short stretches on single frog fibers, reported that the stiffness increase began after the beginning of the tension fall, but well before the subsequent rise. Ford et al. (1986), using very small rapid stretches and releases, reported that the stiffness began to rise during the latent period, but they did not monitor the latency relaxation.

There have been a number of reports that stiffness leads tension throughout the rise of tension when a muscle fiber is tetanized (Ford et al., 1986, and references therein). However, none have clearly shown the relationship between the stiffness rise during the latent period and the lead of stiffness over tension during the rise of tension. Most of the latency relaxation measurements have been made at sarcomere lengths near 3.0 μm, whereas the stiffness measurements are mainly from near maximum overlap of thick and thin filaments (~2.2 μm). The experiments reported here were designed to investigate the relationship between these phenomena by measuring tension and stiffness throughout both the latent period and the rise of tension over a wide range of sarcomere lengths and under conditions of reduced calcium release.

Decreasing the level of activation by decreasing calcium release is a way of further exploring the mechanisms of latency relaxation and stiffness rise. It is important that the agents used for this have no effect on the

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cross-bridges themselves. A valuable agent for such experiments is D-600 (methoxyverapamil or gallopamil). Eisenberg et al. (1983) showed that D-600 could be added to the solution surrounding a fiber and have no effect on the twitch response. In addition, one normal potassium contracture could be elicited in the presence of D-600. However, after one contracture, the fiber was paralyzed. Eisenberg et al. found that 30-μM D-600 solutions produced complete paralysis, but that 2 μM caused ~90% paralysis. Furthermore, the paralysis can be substantially and conveniently reversed by warming the fiber. We found that in 1-μM solutions the degree of paralysis could be graded by varying the number of contractures. Alternatively the paralysis could be graded by completely paralyzing in a higher concentration of D-600 and then partially reversing the paralysis with controlled warming.

A preliminary report of some of these results was presented at a meeting of the Biophysical Society (Claflin et al., 1989).

METHODS

Dissection, mounting, and apparatus

Twitch fibers were isolated from the tibialis anterior muscle of the frog (*Rana temporaria*). Dissections were performed under dark field illumination at room temperature in a Ringer solution with the following composition (in millimolar): NaCl 115; KCl 2.5; $CaCl_2$ 1.8; Na_2HPO_4 2.15; NaH_2PO_4 0.85; pH 7.2. Experiments were also conducted in this solution. Contractures were induced by brief (~10 s) exposures to a solution of the following composition (in millimolar): potassium aspartate 195; $CaCl_2$ 1.8; Na_2HPO_4 2.15; NaH_2PO_4 0.85; pH 7.2.

Fibers were mounted in a chamber by inserting the wires protruding from a tension transducer and an arm attached to a servomotor through holes cut in the tendons remaining at each end of the fiber. The tendons were secured to each wire using loops of 9-0 monofilament nylon suture. After attachment of the tendons, fibers were stretched until taut. Sarcomere length was then determined from photographs of at least four different areas along the length of the fiber. The fiber length was divided by the average sarcomere length to determine the number of sarcomeres in series. Subsequent sarcomere lengths were set by stretching the fiber to lengths determined by multiplying the desired sarcomere length by the number of sarcomeres in series. The temperature of the Ringer solution was maintained at 2.5 ± 0.2°C for all experiments. The experimental chamber, stimulation circuitry, tension transducer, servomotor, and recording system have been described previously (Julian et al., 1986*a*; Julian and Morgan, 1979).

Measurement of stiffness

Stiffness was measured by adding a small 2-kHz sinusoidal oscillation to the length command signal. The amplitude was calculated to be 0.5 nm peak to peak per half sarcomere. Fiber tension did not affect the motor response at this frequency. 8–16 tension responses were averaged and recorded on a digital oscilloscope (model 4094; Nicolet Instrument Corp., Madison, WI) and stored on floppy disk. The records were then processed by computer according to the flow diagram shown in Fig. 1 using data analysis software (Vu-point; Maxwell Laboratories, Inc., La Jolla, CA). To obtain stiffness, a tension record was first passed through a digital band-pass filter centered at 2.0 kHz, with transition frequencies of 1.0 and 3.0 kHz, and a transition width of 1.0 kHz. The frequency response is shown in Fig. 1. The resulting tension signal was a 2-kHz sine wave of amplitude proportional to the stiffness of the fiber. The second processing step was a full-wave rectification. In the third step the tension signal was passed through a digital low-pass filter with a transition frequency of 1.0 kHz and a transition width of 1.0 kHz. The frequency response of this filter is also shown in Fig. 1. Finally, the tension signal was divided by the mean rectified sinusoidal component of the movement signal to give stiffness.

The tension and stiffness records have all been scaled by the tension and stiffness during an isometric tetanus at a sarcomere length of 2.2 μm (P_o and S_o, respectively) and consequently have units of percent

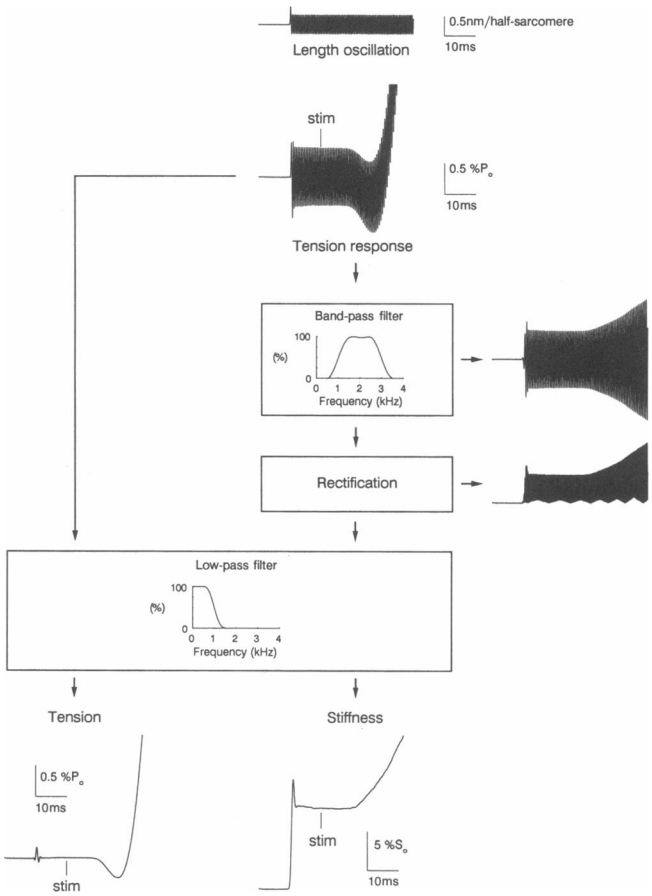

FIGURE 1 Signal processing procedure. To obtain stiffness, the tension response was first passed through a digital band-pass filter. The result was then rectified (full wave), passed through a digital low-pass filter, and finally scaled by the oscillation amplitude. To obtain tension, the 2-kHz oscillation component of the original tension signal was removed by the same low-pass filter used for stiffness processing. An oscillation free tension signal obtained in this way was indistinguishable from an unprocessed tension signal recorded in the absence of length oscillation. The amplitude responses of the filters are shown as percent transmission vs. frequency.

maximum. A tension signal without the component due to oscillation was produced by filtering the tension signal with the same low-pass filter described above. Absolute values for P_o and S_o were 5.97 ± 0.37 mN and 0.157 ± 0.012 mN/μm, respectively (mean ± SEM, n = 11). The mean calculated number of sarcomeres per fiber was 3,258 ± 73 (n = 11).

Reduced calcium release

The records for twitches with reduced calcium release were obtained in the Ringer solution described above, with the addition of 1 μM D-600, (Sigma Chemical Co., St. Louis, MO). After recording control twitches and tetanic contractions in the absence of D-600, the D-600 solution was introduced and further twitches and tetanic contractions recorded. These were always indistinguishable from the control contractions. A series of contractures were then induced with the high potassium solution described earlier (see Methods: Dissection, mounting, and apparatus). For each appropriate level of twitch tension depression, a series of 8–16 twitches were averaged at a sarcomere length of 3.0 μm. After recording twitches at several levels of depression, the normal Ringer solution was reintroduced, and the fiber warmed and maintained at 20–22°C until the twitch no longer increased (~10 min). The solution was then cooled and a final control recorded. Although tetanic tension suffered no irreversible effects from D-600, the twitch tension typically recovered to only about half of the initial value under this procedure. In two experiments, the effect of D-600 on speed of shortening was assessed by applying constant speed shortening movements to a tetanized fiber and recording the tension response. Shortening movements, having speeds near the unloaded shortening speed of the fiber, were applied both before and 15 min after the addition of 8 μM D-600, but before contracture. No changes were observed in the tension responses in the presence of D-600.

In an alternate procedure, the fiber was paralyzed by a single contracture in 8 μM D-600, and the partial paralysis states were reached by carefully controlled warming until a desired twitch size was reached, and then cooling to record the series of twitches. This gave near complete recovery of twitch tension, suggesting that the permanent effects seen in the first procedure were due to the cumulative effects of potassium contractures rather than the D-600.

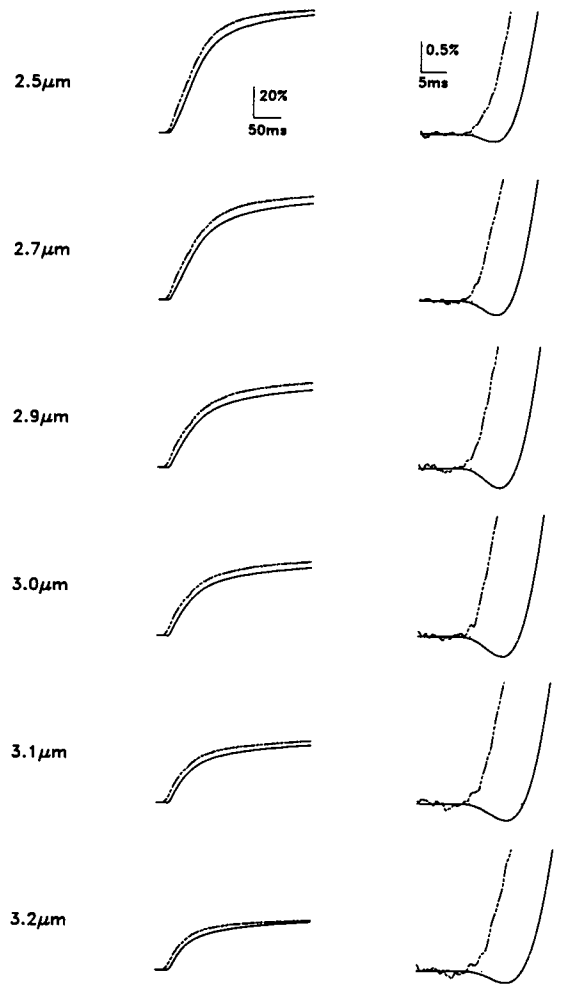

FIGURE 2 The rise of stiffness and tension at a range of sarcomere lengths and on two different scales. Solid traces are tension, broken traces are stiffness. Tension and stiffness are expressed relative to the isometric tetanic tension and stiffness measured at a sarcomere length of 2.2 μm (P_o and S_o, respectively). The vertical calibration bars indicate the scales in percent of P_o and S_o. Each pair of traces in the right-hand column shows the initial portion of the corresponding traces in the left-hand column. For both scales, the beginning of the traces corresponds to the time at which the first stimulus was delivered. Sarcomere lengths are indicated at the left. Note that, in all cases, the onset of the rise of stiffness is simultaneous with the onset of the fall of tension, and that the lead of stiffness over tension is largely established during the latent period.

RESULTS

Variation of sarcomere length

A typical set of stiffness and tension plots is shown in Fig. 2. In all plots the continuous line is the tension record and the broken line is stiffness. For each sarcomere length the records are shown on two scales. The left-hand panels show the whole of the rise of tension and stiffness, while the right-hand panels show the latent period and the first several milliseconds of tension development on expanded time and amplitude scales. Resting tension levels (not shown) were similar to those reported previously for fibers from the tibialis anterior muscle of the frog (Claflin et al., 1989; Fig. 4 *B*).

The expanded records of continuously measured stiffness of single fibers consistently showed that the earliest fall in tension did not precede the earliest rise in stiffness. Below a sarcomere length of 2.5 μm the latency relaxation was very small but remained discernable to lengths as short as 2.1 μm. At 2.5 μm there was a clear latency relaxation and a clear stiffness rise coincident with it. As the sarcomere length was increased, the latency relaxation increased in amplitude and time to peak, as previously reported (Guld and Sten-Knudsen, 1960; Mulieri, 1972; Haugen and Sten-Knudsen, 1976; Bartels et al., 1979). The stiffness developed during the latent period also increased with sarcomere length, at least for sarco-

mere lengths out to 3.0 μm. However, the time of onset of the stiffness rise appeared coincident with the fall in tension at all sarcomere lengths.

The slow records of Fig. 2 support the previous reports that stiffness rises ahead of tension throughout the development of tension. Furthermore, this was so at all sarcomere lengths tested, from the shortest lengths where latency relaxation was visible up to 3.2 μm. The records of Fig. 2 also show that the lead was substantially acquired within the latent period. At a sarcomere length of 3.0 μm, for example, the interval between the onset of stiffness rise and the time required for tension to recross the passive level was 10.5 ± 0.6 ms (mean ± SEM, $n = 4$), whereas the lead of stiffness over tension at the time tension reached 50% of maximum was 11.0 ± 0.4 ms. This point is more clearly shown in Fig. 3, in which stiffness is plotted against tension during the early part of the rise.

Reduced calcium release

The effect of reduced calcium release is shown in Fig. 4. The traces in Fig. 4 *A* show the original twitch at a sarcomere length of 3.0 μm, before the addition of D-600, superimposed on a twitch recorded 15 min after substituting the normal bathing solution with a solution containing 8 μM D-600. The records are virtually indistinguishable and show the typical latency relaxation of ~0.3% of maximum isometric tetanic tension. The traces shown in Fig. 4 *B* were recorded after a potassium contracture, which resulted in total paralysis, followed by warming to 14°C and finally cooling to 2.5°C. The warming resulted in partial recovery of the tension response. Traces in Fig. 4, *C–E*, all recorded at a temperature of 2.5°C, show progressive recovery of twitch tension achieved by warming to 16°, 18°, and 22°C, respectively. During partial paralysis, the amplitude of the latency relaxation was decreased much less than the twitch tension, so that it was possible to reach a situation where the latency relaxation was larger than the subsequent twitch (see, for example, Fig. 5 *B*).

Measurements of stiffness with reduced calcium are

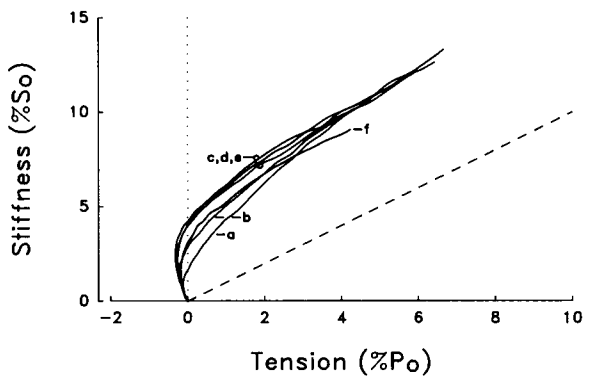

FIGURE 3 Stiffness plotted against tension during the early stages of tension development. The traces are marked as follows: (*a*) 2.5 μm; (*b*) 2.7 μm; (*c*) 2.9 μm; (*d*) 3.0 μm; (*e*) 3.1 μm; and (*f*) 3.2 μm. Plotting the results in this way shows the extent to which the relation between tension and stiffness varies with sarcomere length. Tension and stiffness are both expressed relative to the isometric tetanic tension and stiffness at a sarcomere length of 2.2 μm (P_o and S_o, respectively). The dashed line represents the relationship that would result if stiffness and tension were directly proportional. Note that after the latent period, the relationship is approximately proportional. This plot was made from the results shown in Fig. 2.

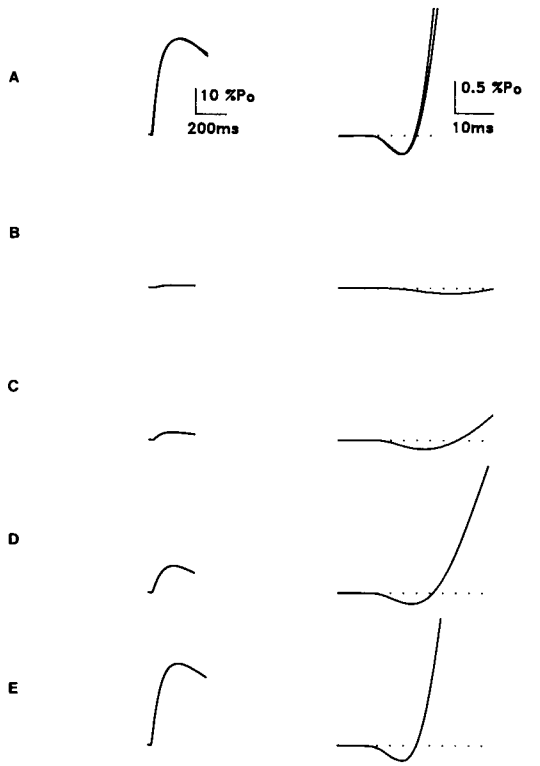

FIGURE 4 The effects of progressive recovery from paralysis with D-600. Each record is the response to a single stimulus pulse. Responses are shown on two different scales. For both scales, the beginning of the trace corresponds to the time at which the stimulus pulse was delivered. The low-gain recordings (left-hand column) were terminated after the peak of the twitch response was reached. (*A*) superimposed traces of responses before and 15 min after introduction of 8 μM D-600. Note that if no contracture has taken place, D-600 has no effect on the twitch tension response. (*B*) after completely paralyzing the fiber (no tension response, not shown) with a potassium contracture, then warming briefly to 14°C and returning to 2.5°C. (*C*) the fiber was warmed further to 16°C then returned to 2.5°C; (*D*) 18°C, then returned to 2.5°C; and finally (*E*) the D-600 was removed and the fiber was warmed to 22°C for 10 min, then returned to 2.5°C. Note the reversibility and the much greater effect on tension development than on latency relaxation. Sarcomere length was 3.0 μm.

shown in Fig. 5. The signal noise was clearly more noticeable when measuring the small sinusoidal variations superimposed on an already small tension. Despite these problems, several clear and consistent observations were made. The initial tension fall and initial stiffness rise remained simultaneous and the lead of stiffness over tension persisted. Furthermore, the stiffness rise during the latent period did not decrease in proportion with the later development of tension. The result of this was that in extreme cases such as that shown in Fig. 5 *B*, the stiffness had peaked and begun to decline before any positive tension developed. Records in which the stiffness began to decline well before the peak of tension were common, providing a counter-example to the suggestion by Bagni et al. (1988) that stiffness depends only on the value of tension and is independent of how the tension came to have that value.

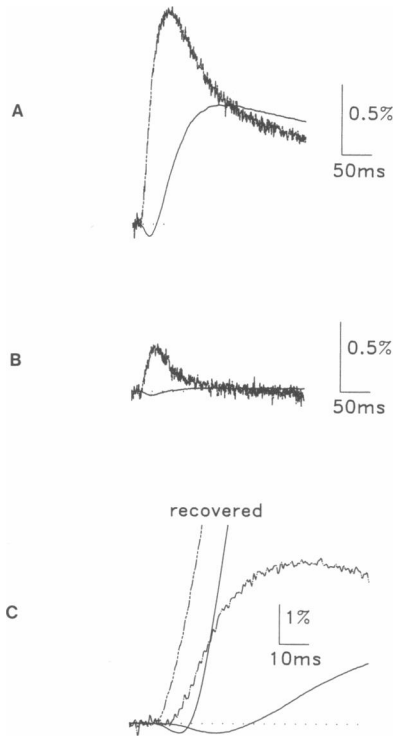

FIGURE 5 Stiffness in a fiber that is almost paralyzed. In all cases the solid traces are tension, the broken traces are stiffness, and the beginning of each trace corresponds to the time at which the stimulus was delivered. The vertical calibration bars indicate the scales in percent of P_o and S_o, the maximum isometric tension and stiffness, respectively, measured at a sarcomere length of 2.2 μm. (*A*) In this example the peak twitch tension was 2.8% of that of the nonparalyzed fiber whereas the amplitude of the latency relaxation was 29% of control. (*B*) The same fiber as that shown in *A* with further depression. Peak twitch tension was 0.082% of control whereas latency relaxation was still 10% of control. (*C*) The records shown in *A* have been expanded and superimposed upon traces from the same fiber after recovery. The vertical calibration bar in *C* corresponds to the recovery records. The traces from the partially paralyzed fiber in *C* have been scaled by a factor of 2.7 with respect to the vertical calibration bar. This scaling was applied so that the latency relaxation of both tension records would appear equal, facilitating comparisons of the times of onset of latency relaxation and stiffness. All records in C share the same time base. Sarcomere length was 3.0 μm in all cases.

Fig. 5 *C* shows stiffness and tension traces on expanded scales for a typical very small twitch, superimposed upon responses from the same fiber after recovery. The vertical scales for both tension and stiffness have been adjusted to make the amplitude of the latency relaxation appear the same in the control (recovered) and depressed cases, to facilitate judgement of timing. The very small tensions involved in the depressed case resulted in rather noisy traces, even with signal averaging. However, it is clear that at these extreme levels of depression, the times of onset of latency relaxation and stiffness increase are delayed without separation in time of the two events.

The relation between the different parameters of the twitch are shown on logarithmic scales in Fig. 6. Tension generation is represented by the peak of the twitch, latency relaxation by the amplitude of tension fall during the latent period, and the early stiffness by the magnitude of the stiffness at the time of the peak latency relaxation. D-600 is seen to affect tension generation much more severely than latency relaxation or early stiffness. A reduction of the twitch by 1,000-fold is accompanied by about a 10-fold reduction in latency relaxation. The points for early stiffness development closely follow the

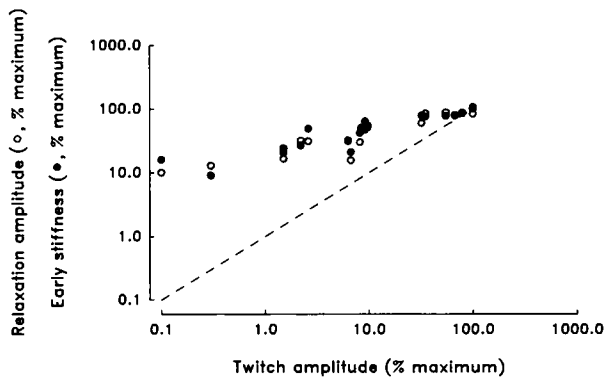

FIGURE 6 Latency relaxation and stiffness plotted as functions of twitch tension for various degrees of paralysis. Early stiffness is defined here as the stiffness at the peak of latency relaxation. All parameters have been expressed as a percent of their initial (i.e., preparalysis) values and all measurements were made at a sarcomere length of 3.0 μm. Points after recovery are included. The dashed line represents the relationship that would result if relaxation amplitude and early stiffness were porportional to peak twitch tension at all levels of paralysis. Note the close correlation between the latency relaxation amplitude and the early stiffness rise and the substantially greater depression of peak twitch tension. Collected results from five fibers.

latency relaxation, again showing the close link between the early stiffness rise and the latency relaxation.

DISCUSSION

Reliability and accuracy of the results

Measurement of stiffness by sinusoidal length changes applied to a fiber is subject to errors arising from several sources. The existence of mounting compliance in the absence of a segment length clamp will give artifactually low results, particularly at low levels of tension. However, we are not primarily concerned with the absolute level of the stiffness, but with the timing of the stiffness changes. Mounting compliance could only serve to make stiffness changes at low tensions less apparent, further strengthening our conclusion that tension fall does not precede stiffness rise.

Length oscillation may significantly disturb the processes that are being measured. While it is difficult to exclude this possibility completely, we have minimized it by using small length changes. Tests made by comparing contractions with and without oscillation confirmed that the generated tension was not affected.

The stiffness of a cross-bridge may depend on its orientation, so that two dynamic conditions with the same number of attached cross-bridges may not give the same stiffness. In these experiments however, only the changes in stiffness at the onset of an isometric contraction are critical to the conclusions.

Comparison with previous results

These experiments provide further confirmation of a difference in the time course of development of tension and stiffness during a normal twitch or tetanus. Our results clearly support the suggestion of Ford et al. (1986), that most of this lead of stiffness over tension is established during the latent period. On the interpretation that the stiffness lead is brought about by a "stiff but not tension generating" cross-bridge state (see interpretation), this finding suggests that the population of such bridges approaches or even reaches a maximum before the tension recrosses the passive level.

The first stiffness change appears somewhat earlier in our records than in those of Haugen (1982*b*) and of Herbst and Piontek (1974). Herbst and Piontek were using whole muscle, so that the greater end compliance may have masked the very early rise of stiffness. Haugen measured the response to ramp stretches at various times, giving a discrete time measurement rather than the continuous measurement used here. Consequently, his conclusions were very dependent on a few particular measurements between the beginning of the tension fall and the attainment of maximum rate of fall, where he found no measurable change, but we found a small increase. If the major portion of the stiffness trace during latency relaxation in our Fig. 2 was back extrapolated, it would produce a plot very similar to that of Haugen (1982*b*, Fig. 2). However, our results show a small but definite rounding of the stiffness rise that is not apparent in Haugen's results. It is, however, much too large to be an artifact of the filtering. The vertical stiffness scaling chosen in our Fig. 2 is relatively much greater than that shown by Haugen, so that our increase would be barely visible on a scale comparable to his. The disagreement is, then, slight in terms of the differences between the traces at any point, but significant in the time of the first stiffness increase.

Previous experiments involving the reduction of calcium release have also shown a greater effect on tension than on latency relaxation. For example, Gilai and Kirsch (1978) showed reductions of the twitch to as low as 10% of control without change in latency relaxation amplitude, using deuterium dioxide and dantrolene. Use of D-600, however, allows investigation of much greater reductions in calcium release. Although direct effects of D-600 on the contractile apparatus cannot be completely ruled out, the present results indicate that the compound has no apparent effect on speed of shortening or the isometric twitch or tetanic responses, including latency relaxation. Eisenberg et al. (1983) reported similar findings but did not monitor shortening speed or latency relaxation. Berwe et al. (1987) proposed that D-600 binds to sites on the T-tubular membrane, from the intracellular side, with a high affinity in the depolarized state, interfering with normal activation.

Interpretation

Ford et al. (1986) have detailed the reasons for assigning the difference in the time courses of tension and stiffness rise to cross-bridge dynamics, rather than to internal shortening or tendon compliance. They and others have consequently postulated an intermediate cross-bridge state, preceding the normal tension-generating state in the cross-bridge cycle. Cross-bridges in this state are postulated to be attached, to have significant stiffness, but to generate little or no tension.

If it is the first rise of stiffness rather than the development of tension that signals the first cross-bridge binding, albeit in an intermediate state, then our results show that the fall of tension that constitutes latency relaxation begins at the same time as the attachment of

cross-bridges. This was true at all sarcomere lengths investigated, and at all degrees of reduced calcium release due to D-600, even when delays in the onset were produced. The observation that both latency relaxation and the rise of stiffness during the latent period remained substantial and closely correlated to each other when tension development was almost abolished by D-600 reinforces the connection between latency relaxation and binding in the intermediate state. These connections are most naturally explained by assuming that cross-bridge binding is the cause of the latency relaxation. In such a scheme, cross-bridges in the intermediate state could actually exert an extremely small pushing force to account for the tension fall (Matsumara, 1969). Alternatively, the state of attachment that has been suggested to exist in relaxed muscle may produce a positive tension, and the stiff intermediate state produce no tension. This, then, would be a return to the explanation of latency relaxation proposed by D. K. Hill (1968), with the modification of the intermediate state.

In contrast to the apparent tight coupling between latency relaxation and the early stiffness rise, the very different effects of D-600 on latency relaxation and tension generation indicate a substantial degree of uncoupling between them. If latency relaxation was due to cross-bridge binding, and calcium was only involved as a switch allowing the transition from the relaxed to the intermediate state, and subsequent transition to the tension-generating state was not under the direct control of calcium, then a proportionate decrease in latency relaxation and tension development would be expected. The opposite observation (i.e., very different changes in latency relaxation and tension development as shown in Fig. 6) could be taken as an indication that latency relaxation is not a cross-bridge phenomenon. This would require the conclusion that the same times of onset of latency relaxation and stiffness increase, and similar variations in these parameters with the amount of calcium released, are merely coincidental. An alternative explanation would be to propose that the two cross-bridge transitions (relaxed to intermediate and intermediate to tension generating) are both independently controlled by calcium, but that the second requires a higher concentration of calcium, that is, is less sensitive. Reducing the calcium would then affect the second transition more than the first, thus affecting tension generation more than the stiffness increase and tension fall during latency.

Such a dual activation process is quite compatible with the current thinking about muscle activation. According to the x-ray diffraction work of H. E. Huxley and colleagues (Kress et al., 1986), structural changes linked to troponin-tropomyosin occur soon after muscle stimulation, before tension development. According to this scheme, the structural changes lead to attachment of myosin cross-bridges and could account for the early increase in stiffness reported here. The results from solution biochemistry studies (Chalovich et al., 1981; Chalovich and Eisenberg, 1982), however, led to the proposal that a step in the actomyosin-ATPase reaction cycle subsequent to cross-bridge attachment is sensitive to calcium. This could account for the finding that positive tension generation follows the increase in stiffness. Reports that, in skinned fibers, the rate constant for the formation of tension producing cross-bridges is sensitive to the concentration of calcium (Brenner, 1988; Metzger et al., 1989) are also consistent with these views. The precise way in which such a dual activation scheme regulates both cross-bridge attachment and a step in the actomyosin-ATPase cycle in living muscle remains to be elucidated.

A dual activation proposal could also overcome a long-standing difficulty with suggestions that latency relaxation is a cross-bridge phenomenon. The time of the onset of latency relaxation is independent of sarcomere length but the time of the peak of latency relaxation increases with sarcomere length over much of the range (Fig. 2). The time from the release of calcium to the attainment of sufficient concentration to cause the first cross-bridge transition (to attached, stiff, but not tension generating) could be so small that its variation with sarcomere length could not be seen, but the higher concentration needed for tension generation would be reached only after a greater diffusion delay. Increases in this greater delay at longer sarcomere lengths would be large enough to measure (see Close, 1981). At extreme levels of depression, such as that shown in Fig. 5 *C*, the amount of calcium released is presumably very small. Under such conditions, the time required for the calcium concentration to become sufficient to cause the first cross-bridge transition could become observable and account for the increase in the time between stimulus and the onsets of stiffness increase and latency relaxation in fibers nearly paralyzed by D-600.

The finding that stiffness increase, and by inference cross-bridge binding, is intimately connected with latency relaxation and the lead of stiffness over tension imposes new constraints on cross-bridge models of muscle. Similarly, the evidence for two control points in the cross-bridge cycle has wide ranging implications in a number of areas, such as the possible variation with calcium concentration of the unloaded shortening speed of skinned fibers (Julian et al., 1986*b*).

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