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ACTIVATION DELAYS IN FROG TWITCH MUSCLE FIBRES

BY R. I. CLOSE

From the Department of Physiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia

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SUMMARY

1. The length dependence of the mechanical latent period and early tension development, uncomplicated by latency relaxation, was determined from responses of single muscle fibres to restimulation at the peak of the isometric twitch at 15 °C.

2. The onset of the reactivation response showed little ($< 1{\cdot}5 \times 10^{-7}$ N) or no latency relaxation.

3. Reactivation latency was minimal (2·8 msec) and constant at 1·9–2·1 μm sarcomere length and it increased by about 3 msec with sarcomere extension to 3·2 μm.

4. Reactivation responses showed two stages of early tension development, an initial phase in which tension acceleration increased, and a phase of maximum responsiveness in which tension acceleration was constant; the transition between the two phases occurred about 4·5 msec after the start of the stimulus at 2·2 μm sarcomere length and was delayed about 4 msec with increase in sarcomere length to 3·2 μm.

5. The square root of the maximum tension acceleration was directly proportional to the degree of overlap of thick and thin filaments in the sarcomere length range 2·3–3·2 μm.

6. It is proposed that the onset of the phase of constant tension acceleration marks the end of the period during which the activator, calcium, is distributed throughout the sarcomere.

7. Analysis of early tension transients in relation to myofibril structure showed that length-dependent changes in reactivation latency and time of onset of constant tension acceleration were probably brought about mainly by alteration of the kinetics of distribution of activator within the myofibril and by changes in the diffusion distance between activator-release sites near the end of the sarcomere and the tension-generating sites.

8. There was a 2 msec myofibril priming delay in the rise of tension in twitch responses that was not seen in reactivation responses; the possible origin of that delay is discussed in relation to structural changes accompanying activation and to competition between calcium-binding structures.

9. The onset of twitch latency relaxation occurred within about 250 μsec after the time corresponding to latency of the earliest reactivation responses and appeared to signal the start of a process that took place after the arrival of calcium among the myofilaments. The origin of latency relaxation is discussed.

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INTRODUCTION

Delay of the early stages of contraction in frog muscle fibres with increase in sarcomere length has been seen as supporting the view that activation calcium is released near the ends of the sarcomere (Guld & Sten-Knudsen, 1960; Sandow,1966; Mulieri, 1972; Haugen & Sten-Knudsen, 1976). A major difficulty that has prevented further analysis along those lines is the presence of latency relaxation (for references see: Haugen & Sten-Knudsen, 1976; Gilai & Kirsch, 1978), a small length-dependent transitory decrease in tension of uncertain origin, that merges with positive tension development and obscures the moment at which actin–myosin crossbridges begin to develop tension.

Latency relaxation is much reduced, however, following a second stimulus during a twitch contraction (Matsumura, 1969) and in the present work it was absent or very small when the second stimulus was given just before the peak of an isometric twitch. In those conditions it has been possible to determine the length dependence of the mechanical latent period.

Analysis of the early tension transients in responses of isolated single muscle fibres has revealed two activation delays that represent different stages in excitation–contraction coupling. One delay is length-dependent and probably involves alterations in both the rate of release of activator and diffusion distance. The other delay is associated with latency relaxation.

METHODS

Frogs (*Litoria aurea*) were collected in Tasmania during summer months and kept for periods up to 2 yr at about 22 °C; the animals fed voluntarily on flies emerging from the pupal stage and continued growing in captivity. Fly pupae were supplied by the C.S.I.R.O.

Preparation

Single muscle fibres about 8 mm in length were dissected from the peroneus muscle of the frog; a piece of tendon about 0·1 mm wide was left attached to each end of the fibre. The fibre was set up in a 5 ml. glass bath constructed from microscope slides. One tendon was held in a stainless-steel clamp, the other was lashed to the stainless-steel wire extension of a tension transducer by a silk thread. The total length of free tendon between the ends of the fibre and points of attachment to the equipment was about 300 μm.

The composition of the bathing fluid was 115 mM-NaCl, 2·5 mM-KCl, 0·85 mM-NaH_2PO_4, 2·15 mM-Na_2HPO_4, 1·8 mM-$CaCl_2$; 10 mg tubocurarine chloride/l. was used in some experiments. Fluid in the bath was replaced at about 0·2–0·5 ml./min.

The temperature of the bath was set within the range of 14·5–15·5 °C and did not vary by more than 0·5 °C during an experiment.

Eighteen of the fibres that were used showed little or no change in contractile responses during the 12 to 24 hr periods of recording. Some information was obtained from thirteen other fibres in experiments that were not completed through failure of either the preparation (usually parting at the myotendon junction) or the equipment.

Fibre dimensions

The sarcomere length of the resting fibre was estimated during experiments by optical diffraction using a 5 mW He/Ne laser. The estimated sarcomere length above 2·2 μm was linearly related to both the fibre length and the micrometer reading on the micromanipulator that was used to adjust fibre length; the line fitted to those data was used to set the fibre for sarcomere lengths below 2·2 μm.

Fibre cross-sectional area (A) was estimated at different places along the fibre from the major (a) and minor (b) widths, measured microscopically, assuming an elliptical shape, i.e. $A = \frac{1}{4}\pi ab$.

Stimulation

The fibre was stimulated transversely along its whole length by square electrical pulses applied between bright platinum plate electrodes that covered the sides of the bath. The pulse was 0·5 msec duration and 1·2 × the threshold voltage; field strengths at 1·2 × threshold ranged from 9 to 11 V/cm. The fibres were rested for 1 min between twitch or double responses and for 3 or 5 min between tetanus responses.

Tension recording

The transducer was a piezoelectric ceramic (PXE 5 Multimorph, Philips, Holland) cantilever with output 129·5 V/N, compliance (including the stainless-steel extension) 5×10^{-4} m/N and natural frequency about 5 kHz in air and 3·6 kHz with fibre attached. The transducer signal was fed to an electrometer amplifier with about 10 × gain and 10 kHz band width. The time constant of the transducer and the electrometer amplifier was 45 sec. The output of the electrometer amplifier was fed to an intermediate amplifier (gain 0·5 to 5 ×) that was used to adjust signals of whole twitch or tetanus responses within the range ±1 V before digitizing. The output of the intermediate amplifier was brought also to a track-and-hold amplifier that was used to obtain greater amplitude resolution (gain 1 to 200 ×) of any selected part of the twitch or tetanus responses.

Piezomotor effects of the stimulus on the transducer caused small artifacts in most records: (1) gradients, averaging about -6×10^{-8} N/msec, in records of latent period and (2) 3·6 kHz oscillations, about 150 nN peak-to-peak at 2·5 msec after the stimulus; those two artifacts had negligible effect on estimates of latency in reactivation responses but both were characterized and cancelled in the records of Fig. 6*A*.

Data processing

Signals from intermediate and track-and-hold amplifiers were digitized and processed by a Hewlett Packard system including 5610A analogue-to-digital converter with 10 bit resolution, 2116B computer, 7970B magnetic tape unit, 7906 disk, 7210 plotter and 1331C oscilloscope. A system of assembler language programmes, written and developed in the laboratory, was used to control all aspects of data collection, and the processing, storage and display of records. Digitized records of whole twitch responses and latent periods were obtained by independent sampling on two input channels at low and high rates under programme control; all timing of the sampling patterns was by 'software clock' based on the computer instruction times with accuracy determined by the 10 MHz timing generator. The sampling programme provided independent control of sampling parameters on two channels; the maximum time resolution for each of two simultaneous inputs was one digital sample every 50 μsec.

Statistical smoothing of some records was carried out off-line, where indicated below, using a programme developed by Ms E. Elekessy and described elsewhere (Henry, Bishop, Tupper & Dreher, 1973). In principle the method obtained the smoothed value for a sample from neighbouring samples that were weighted according to the normal distribution. The term n/δ, used below, refers to the severity of smoothing, determined by selecting the nth neighbouring sample as having the weight corresponding to the first standard deviation (δ) on the normal probability curve. Attenuation due to smoothing was −0·1 dB at 179, 217 and 270 Hz with n/δ set to 3·0, 2·5 and 2·0 respectively. Consequently, biological signals such as latency relaxation, approximately equivalent to a 200 Hz sine wave, were passed with virtually no attenuation.

Resolution of tension transients

The end of the latent period and the start of linear sections of records were resolved by successive linear regression analysis of increasing numbers of data samples (10, 11, 12, ...) in smoothed records ($n/\delta = 3{\cdot}0$), starting at one end of a linear section and working toward the point where it became non-linear; the standard error of the slope of the line fitted to a section of record was plotted on an arbitrary scale against the time of the last incremental sample. Such analysis yielded a curve that passed through a minimum at the point where a record diverged from a straight line (e.g. Fig. 7*a*, *e*). The difference between estimates of latency by statistical analysis and by subjective means was 0·03 msec (±0·19 msec S.D., $n = 35$, $P > 0{\cdot}2$). Points of deviation from linearity could be resolved within 50 μsec in simulated records having random noise comparable to that in experimental records.

RESULTS

The results described below were obtained from fibres at 15 °C in which the length:twitch tension curve was linear above about 2·3 μm sarcomere length and extrapolated to zero tension at roughly 3·7 μm (cf. Close, 1972).

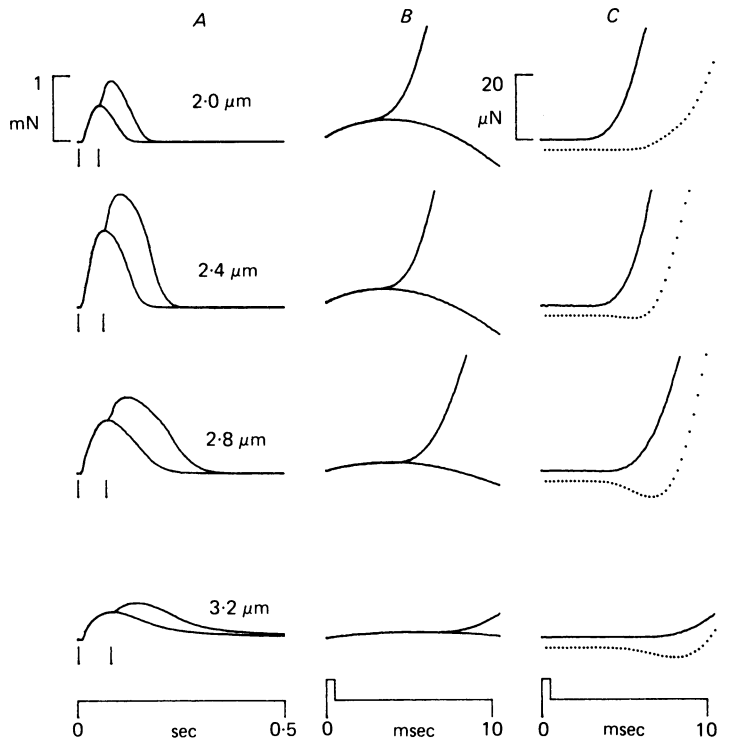

Fig. 1. Isometric responses of a fibre to single and paired stimuli; each record is the average of ten responses. The horizontal rows of records are for responses at sarcomere lengths 2·0–3·2 μm as indicated. Records in column *A* are the twitch response following one stimulus and the superimposed response to two stimuli; the time of stimulation is marked by vertical bars under the records. The superimposed records in *B* are the peak of the twitch (concave down) and the onset of the response to the second stimulus; the second stimulus occurred at the start of the records as indicated by the marker on the time scale. The continuous curves in *C* are the tension difference between the curves for control and restimulated responses in *B*. The dotted curves in *C*, offset vertically for clarity, are the onset of the twitch response after the first stimulus. The 20 μN scale applies to records in *B* and *C*. At 2·2 μm sarcomere length, the fibre cross-sectional area was $6{\cdot}66 \times 10^{-9}$ m^2 and the maximum isometric tetanic tension was $3{\cdot}32 \times 10^5$ N/m^2. Stimulus amplitude: 9 V/cm. Temperature: 15 °C. Fibre reference: 031078.

Initial and reactivation latencies

Some records used for studying latency and early tension development are illustrated in Fig. 1. The left column (Fig. 1 *A*) shows records of isometric twitch responses of a fibre at different sarcomere lengths with superimposed records of double responses in which a second stimulus was given a few milliseconds before the peak

of the control twitch. In the central column (Fig. 1*B*) both the peak of the control twitch and the onset of the response to the second stimulus of the events of Fig. 1*A* have been recorded and plotted at 50-fold greater tension resolution; the records show reactivation latency, the time between the start of the second stimulus and onset of the response. Simple records of reactivation latency, obtained for each length by subtracting the control curve from the double-response curve in Fig. 1*B*, are plotted as difference tension against time in the right column (Fig. 1*C*, continuous curves);

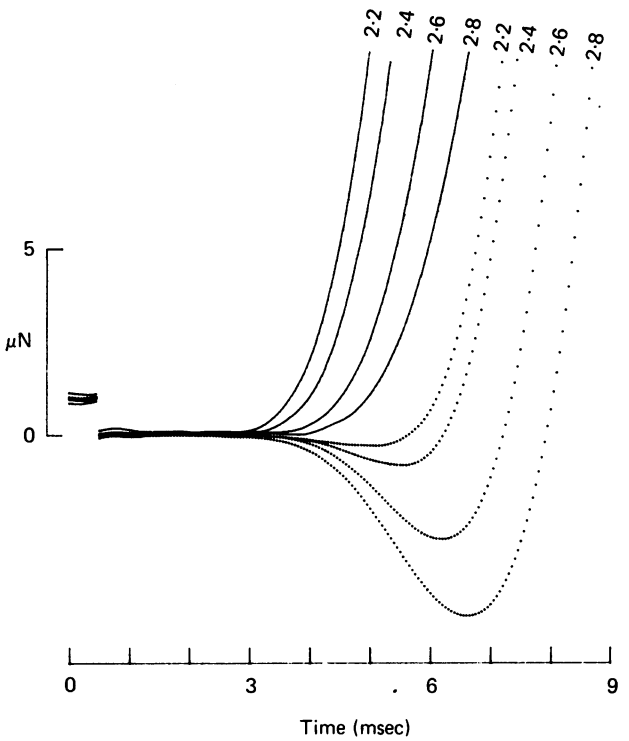

Fig. 2. The onset of tension development in the twitch after a single stimulus (dotted curves) and the response to a second stimulus given a few milliseconds before the peak of an isometric twitch (continuous curves). The stimulus started at zero on the time scale. The recordings were made with the fibre at initial sarcomere lengths from 2·2 to 2·8 μm, as shown at the top of the curves. The records are from the same series as those in Fig. 1 and data were smoothed ($n/\delta = 2{\cdot}5$).

corresponding records of the onset of the control twitch after the first stimulus are shown as dotted curves in the same column.

Early phases of tension development in twitch and second response are shown in more detail in Fig. 2. The records for the twitch response (dotted curves, Fig. 2) show latency relaxation, which began about 2·8 msec (from high resolution plots) after the start of the stimulus at all lengths and deepened from about 50–100 nN at 1·9 μm sarcomere length to 5·9 μN at 3·0 μm. In contrast, the records of reactivation latency (continuous curves, Fig. 2) showed no latency relaxation and earlier onset of positive tension.

In earlier work the inflexion in the descending phase of latency relaxation, i.e. the moment of maximum rate of tension decrease, was taken as the end of the twitch latent period and time for onset of positive tension (Sandow, 1944, 1966); that inflexion is at 5·5 msec in the 2·8 μm record in Fig. 2, about 1·5 msec after the onset of the reactivation response at the same length. In fact, neither the records of latency relaxation in Fig. 2, nor their time derivatives, provide any clue that could be used to identify unequivocally the time at which actin–myosin crossbridges began to develop tension in twitch responses. What has now become clear is that activation can occur earlier than was thought previously. Indeed, true twitch latency for actin–myosin interaction might even be the same as reactivation latency and be as short as 2 msec at room temperature (Q_{10} about 2·2); that possibility is important in connexion with identification of sites of other physical changes thought to occur during the latent period.

Although the twitch latent period has not been identified, it is clear from Fig. 2 that the rise of tension in the twitch occurs at least 2 msec later than in the reactivation response at the same length. That delay in twitch contraction, referred to below as the myofibril priming delay, is evident in the 2·2 μm record even though latency relaxation is small at that length. As a result of earlier tension rise, the reactivation response at 2·2 μm exceeded the twitch response by more than 20 μN, i.e. 3·0 mN . mm^{-2}, within 5 msec of stimulation. That difference is much greater than the initial tension, cf. about 0·46 mN . mm^{-2} at 2·3 μm (Lannergren & Noth, 1973), and the 2 msec delay in rise of twitch tension cannot be attributed simply to a discharge in passive tension during latency relaxation.

Reactivation latency at different tensions and times

At sarcomere lengths about 2·2 μm, the reactivation latency at the peak of contraction was the same for twitches (twitch:tetanus ratio $\geqslant$ 0·5) and larger twitch-like responses to several stimuli (e.g. 4 pulses at 67 Hz) even though tension differences at the time of restimulation ranged up to 1·9-fold. Furthermore, at any sarcomere length (1·9–3·0 μm), there was no consistent difference in reactivation latency following restimulation at either twitch peak or twitch half-relaxation time. As the transverse tubule conduction time is about 1 msec at 15 °C (Gonzalez-Serratos, 1971) and diffusion of calcium in the myofibril may take several milliseconds, it seems likely that the method always measured latency for onset of contraction in the peripheral region of the sarcomere. The interpretation used here is that tension-generating structures at the surface of peripheral myofibrils are the first ones to be activated during a twitch and the first ones to become available for reactivation.

Resolution of the contraction onset

On dimensional grounds (Huxley, 1972; Mobley & Eisenberg, 1975) it was estimated that there were about $3{\cdot}45 \times 10^6$ thick filaments and $6{\cdot}72 \times 10^8$ crossbridges in every sarcomere in the fibre that is represented in Figs. 1–3. As the maximum isometric tetanic tension for that fibre was 2·21 mN, each active actin–myosin crossbridge must have developed at least $3{\cdot}3 \times 10^{-12}$ N. The resolving power of the equipment for those recordings was $5{\cdot}1 \times 10^{-8}$ N. The noise in the records in Figs. 1, 2 and 3 was equivalent to about $1{\cdot}5 \times 10^{-7}$ N, or the tension output of about $4{\cdot}6 \times 10^4$

crossbridges. The peripheral thick myosin filaments, those at the surface of peripheral myofibrils, would have numbered about $2{\cdot}38 \times 10^4$; consequently, the equipment was quite sensitive enough to resolve the combined tension development of one pair of crossbridges in each half length of all of the peripheral myosin filaments (about $3{\cdot}0 \times 10^{-7}$ N per sarcomere).

Similarly, the fibre represented in Figs. 4, 7 and 9*A* developed about 4×10^{-12} N per crossbridge and the noise in those records was equivalent to the output of about three crossbridges, or fewer, per half sarcomere length of all peripheral thick filaments.

In the discussion below, it is assumed that onset of tension at the end of a latent period signals the arrival of a threshold amount of activator at the actin–myosin juxtaposition nearest the calcium-release sites.

Reactivation latency at different sarcomere lengths

Fig. 3*A*, *B* show some typical records of onset of contraction at short and long sarcomere lengths following restimulation just before the peak of an isometric twitch. The differentiated records show quite clearly the time at which the record of the response to the second stimulus diverges from that of the control twitch: Reactivation latency was about 2·8 msec at 2·2 μm sarcomere length and about 6·25 msec at 3·2 μm sarcomere length. Records of the tension difference between control and reactivation responses of Fig. 3*A*, *B* provide a more convenient means of depicting the onset of reactivation responses, particularly at the higher magnification used to search for reactivation latency relaxation, and such curves are shown in Fig. 3*C*. The 3·2 μm record in Fig. 3*C* shows (1) no sign of mechanical change during the extended latent period and (2) abrupt onset of tension development. The records in Fig. 3, not complicated by latency relaxation, provide the first clear evidence for length-dependent delays in the onset of contraction.

A series of difference tension curves obtained from another fibre, is shown in Fig. 4 to illustrate the effect of sarcomere length changes on reactivation latency. The latent period was constant at short sarcomere lengths and increased non-linearly with extension from about 3·25 msec at 2·1 μm to about 5·75 msec at 3·0 μm sarcomere length. Similar results obtained for several fibres are plotted in Fig. 5*A*.

Reactivation latency is proportional, approximately, to the square of the increase in sarcomere length beyond a particular length. An estimate of that length has been made for each fibre by subracting the mean reactivation latency at short sarcomere lengths ($\leqslant 2{\cdot}1$ μm) from the latency at longer lengths and plotting the square root of the time difference against length; results for four fibres are shown in Fig. 5*B*. The regression line (pooled data) in Fig. 5*B* meets the length scale at 2·05 μm and the mean intercept obtained from analysis of data for each fibre was 2·07 μm, $\pm$ 0·03 μm (S.D.); a more accurate estimate of this critical length should be made by measuring sarcomere length during contraction.

Earliest sign of mechanical responses. Comparison of the onset of twitch and reactivation reaponses was complicated by differences in corresponding latencies of different fibres (Fig. 5) and by small artifacts (see Methods). Measurement difficulties were partly overcome by (1) plotting the deviations of data from a line fitted to the latent period (legend, Fig. 6) against time referenced to the onset of the earliest

reactivation responses for the fibre and (2) characterizing the transducer vibrations in the latent period and cancelling that signal throughout the record. Results from three fibres in Fig. 6*A* show that twitch latency relaxation and the earliest reactivation responses begin at about the same time after stimulation; there was no instance of twitch latency relaxation beginning earlier than the time corresponding to the minimum reactivation latency. On the contrary, analysis of the first detectable

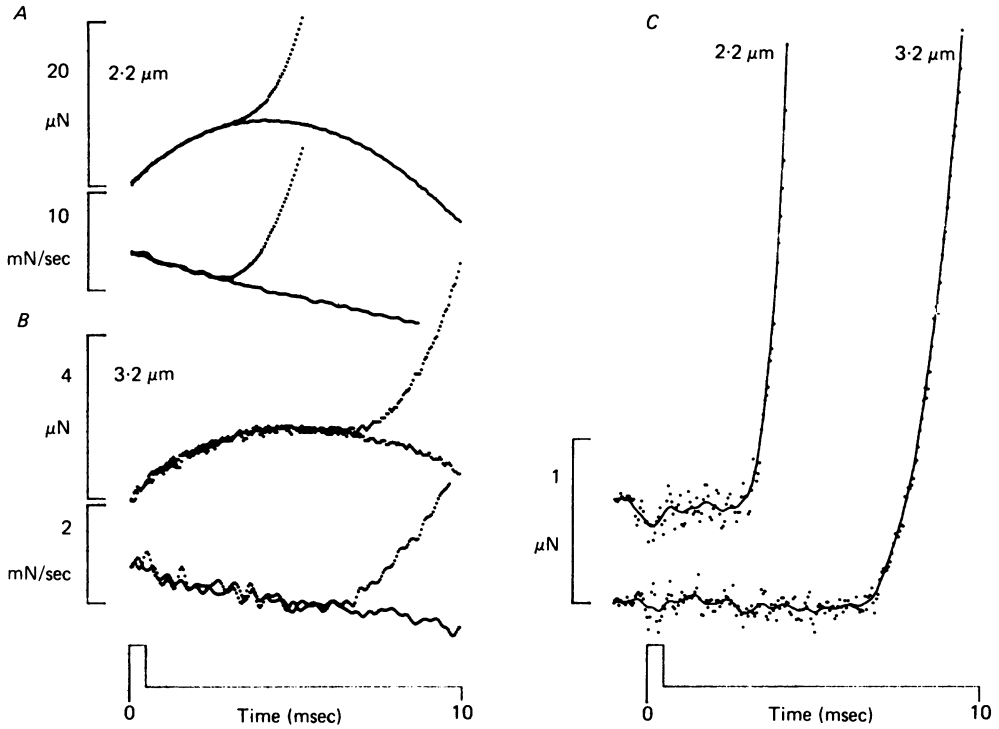

Fig. 3. Reactivation latency in a fibre following restimulation near the peak of an isometric twitch. In *A*, the upper pair of superimposed records are the peak of the control twitch and the onset of the response after restimulation; the lower pair of records are the derivatives of the records of tension. The records in *A* were obtained at initial sarcomere length 2·2 μm and similar records at 3·2 μm are shown in *B*. The reactivation stimulus occurred at the start of the records as shown on the time scale. The lower end of each rate scale is zero. The records are from the same series as those in Figs. 1 and 2. The derivatives were smoothed ($n/\delta = 2{\cdot}5$).

The records in *C* are the tension difference between the curves for control and restimulation responses in both *A* and *B*; in each record the original data are plotted as points about the continuous smoothed curve ($n/\delta = 3{\cdot}0$).

mechanical change in high resolution plots of averages of 267 records (five fibres) gave 2·80 msec (±0·28 msec, S.D.) for minimum reactivation latency (1·9–2·1 μm sarcomere length) and 3·05 msec (±0·22 msec, S.D.) for the delay to onset of twitch latency relaxation; the difference between those apparent latencies, 0·25 msec (±0·15 msec, S.D., $n = 5$), was significant ($P < 0{\cdot}02$, paired t test) and variations in the two times were correlated directly ($r = 0{\cdot}85$). As latency relaxation starts after the same delay

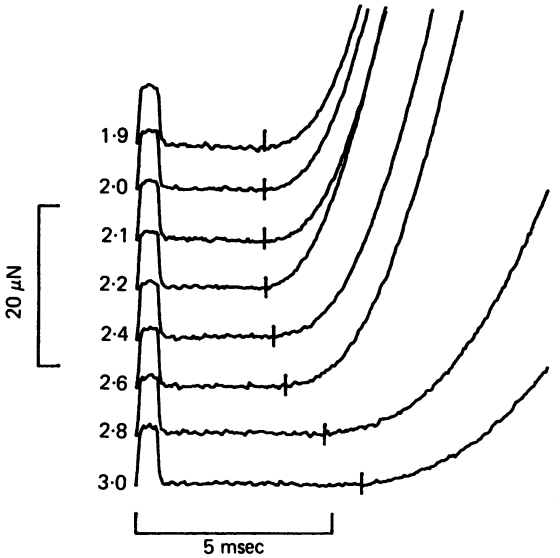

Fig. 4. Reactivation latency at different sarcomere lengths. The difference tension curves are for responses to restimulation by a single stimulus given a few milliseconds before the peak of an isometric twitch. Each record is from one control twitch and one restimulated response. The stimulus artifact is at the start of each record and a vertical bar marks the end of the latent period. At 2·2 μm sarcomere length the cross-sectional area of the fibre was $5{\cdot}5 \times 10^{-9}$ m^2, the peak isometric twitch tension was $3{\cdot}2 \times 10^5$ N/m^2 and the maximum isometric tetanic tension was $4{\cdot}04 \times 10^5$ N/m^2. Stimulus amplitude: 9 V/cm. Temperature: 15 °C. Fibre reference: 160878.

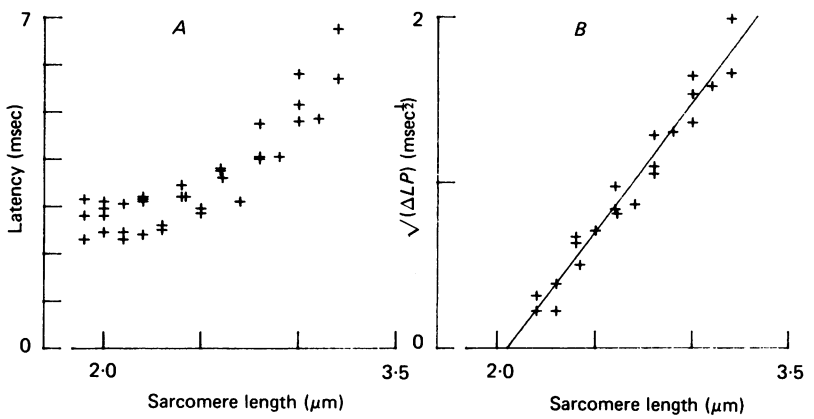

Fig. 5. Graph *A* shows the influence of sarcomere length (*SL*) on latency (*LP*) of the response to a stimulus given a few milliseconds before the peak of the isometric twitch. Results from four fibres; each point is from the average curve of five or ten difference tension records. The mean reactivation latency in the sarcomere length range 1·9–2·1 μm was $2{\cdot}8 \pm 0{\cdot}3$ msec S.D. Graph *B* shows the square-law relation between the change in latent period (ΔLP) and sarcomere length; the change in latency, obtained separately for each fibre from the results in *A*, is the difference between the mean latency of the fibre at sarcomere lengths 1·9–2·1 μm and the latency at other lengths. Two inexplicable divergent results at 2·2 μm, 0·15 msec and 0·25 msec higher than expected, were excluded. The regression line in *B* is $\sqrt{(\Delta LP)} = 1{\cdot}56SL\text{-}3{\cdot}21$.

at all lengths its onset probably signals the arrival of activation calcium among the myofilaments and not some event preceding calcium release as suggested by Gilai & Kirsch (1978).

There was usually no detectable latency relaxation in reactivation responses (see Fig. 3). However, tension decreases of about 0·1–0·2 μN following reactivation at the twitch peak were evident in some responses of some fibres at 3·0 μm sarcomere length

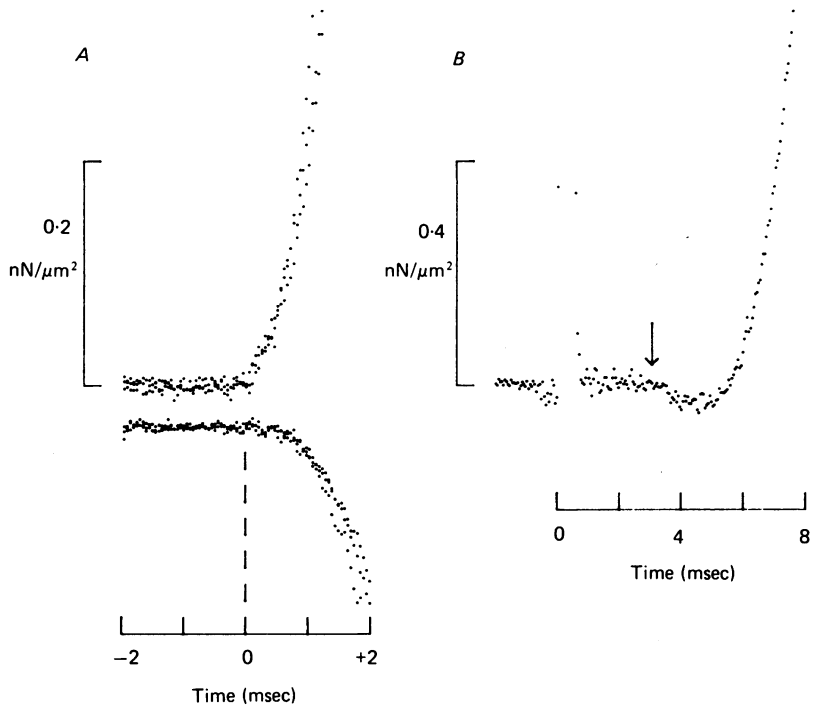

Fig. 6. *A*, earliest mechanical changes in twitch latency relaxation (lower record) at 2·8–3·2 μm sarcomere lengths and reactivation responses (upper record) at 1·9–2·1 μm sarcomere lengths. Results from three fibres. The average records for each fibre plotted with the time of onset of the reactivation response as the zero time reference; zero time was on average 2·9 msec after the start of stimulus. Data for each fibre are deviations from the regression line fitted to forty samples in the latent period from −2 to 0 on the time scale. Small transducer oscillations (see Methods) were cancelled digitally. Fibre cross-sectional areas: 6650, 5500, 6660 μm². Fibre references: 310778, 160878, 031078. *B*, average of eight selected difference tension records of reactivation responses at twitch peak showing latency relaxation at long sarcomere lengths (2·8–3·0 μm); stimulus started at zero time. The arrow indicates the minimum reactivation latency (3·05 msec) in other average responses at 2·1 μm sarcomere length. Fibre described in Fig. 4 legend.

and those decreases were identified tentatively as latency relaxation. An average of some selected records showing reactivation latency relaxation is shown in Fig. 6*B*; minimum reactivation latency for that fibre was about 3·05 msec (arrow). The particular value of that record is that it shows reactivation latency relaxation starting after about the same delay as twitch latency relaxation (Figs. 1, 2; legend, Fig. 6) and, therefore, that the time course of events in excitation–contraction coupling up to that stage is the same in twitch and reactivation responses.

Arsenazo III calcium transients recorded by Suarez-Kurtz & Parker (1977) at 10 °C and by Miledi, Parker & Schalow (1977) at 5 °C suggest those signals would begin about 2·4 msec after stimulation at 15 °C, and the indodicarbocyanine fluorescence signal started about 2·8 msec after the stimulus at 15 °C (Oetliker, Baylor & Chandler, 1975); both estimates are about the same as the minimum reactivation latencies (Fig. 5*A*) and there could be only a brief delay between onset of calcium release and earliest tension change at short lengths. Furthermore, the correspondence between starting times of those optical signals, in fibres that were stretched to minimize movement, and the earliest mechanical change at short sarcomere length indicates that the time of onset of release of activation calcium has little or no length dependence; the same seems to hold for other published records of optical signals that have been attributed to changes in either sarcoplasmic calcium ion concentration or potential across sarcoplasmic reticulum membranes associated with calcium release. This observation is supported by the fact that the time of onset of latency relaxation is not altered by length changes (e.g. Haugen & Sten-Knudsen, 1976). Further work should be carried out to record calcium transients during reactivation responses to determine directly whether calcium release begins at the same time at all lengths.

Early development of tension

The difference tension recorded from one fibre at 2·6 μm sarcomere length and its rate of change with respect to time are shown in records *b* and *c* respectively of Fig. 7. There are two phases of tension development: (1) an initial phase of increasing tension acceleration and (2) a phase of constant acceleration of tension; resolution of the transition times is described below. During constant acceleration of tension,

$$\frac{d^2P}{dt^2} = nk \tag{1}$$

and

$$P = kt^n \tag{2}$$

where P is tension, t is time and n and k are constants. The power of time in eqn. (2) can be estimated from the slope $(n-1)$ of log-log plots of the rate of change of tension against time. Such analysis of time derivatives of the records in Fig. 4 showed the slope was very near unity at all sarcomere lengths from 1·9 to 3·0 μm (mean slope: $1{\cdot}03 \pm 0{\cdot}03$, S.D.) and that n was virtually 2. In other words, tension developed by a fibre following restimulation increases approximately as the square of time after onset. This is shown in Fig. 7 where difference tension has been plotted on both linear (record *b*) and square root (record *d*) scales of tension.

The square-law relation between tension and time in reactivation responses is consistent with the interpretation that the early phase of the mechanical response involves two relatively slow reactions (Ashley & Moisescu, 1972; Ashley, Moisescu & Rose, 1974).

The two phases of tension development are much clearer in square-root records (Fig. 7*d*) than in first derivative records (Fig. 7*c*). Both the end of the latent period (3·80 msec) and the time of onset of constant tension acceleration (5·75 msec) could be resolved quite accurately from the minimum in the standard error of slope curves (Fig. 7*a*, *e*) that are described under Methods and in the legend of Fig. 7. The two phases of tension development are shown most clearly in the record of deviations (Fig. 7*f*) of observed data from the regression line fitted to the square-root record (Fig. 7*d*) between 5·75 and 8·0 msec.

Small differences between control twitch and restimulated response during the latent period can cause distortion of the square root plot. This was minimized where

necessary by averaging data samples of the last millisecond or so of the latent period and using that figure as a zero tension reference.

Phase of constant acceleration of tension. The time of onset of constant tension acceleration (5·75 msec in Fig. 7) increased with sarcomere length from about 4·6 msec

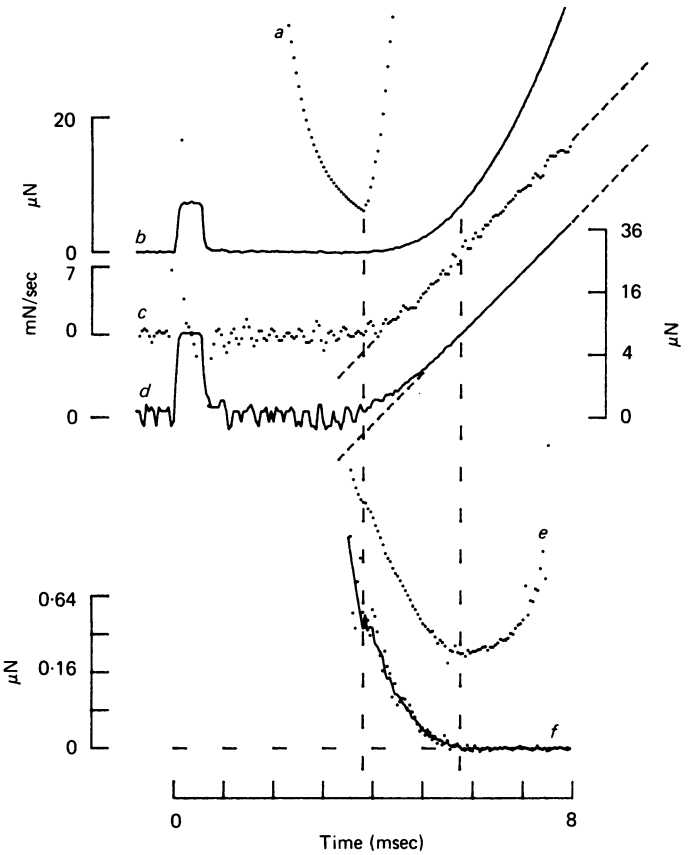

Fig. 7. Tension transients following restimulation at the peak of an isometric twitch. *b*, difference tension (P) at 2·6 μm sarcomere length (unsmoothed average of five records). *c*, rate of change in tension in *b*; regression line fitted between 5·75 and 8·0 msec is, $dP/dt = 5{\cdot}23t - 2{\cdot}16 \times 10^{-2}$ (P in Newtons, t in seconds). *d*, unsmoothed square-root transform of *b*; regression line fitted between 5·75 and 8·0 msec is $\sqrt{P} = 1{\cdot}616t - 6{\cdot}66 \times 10^{-3}$ (P in Newtons, t in seconds). *a*, standard error of slope curve (see Methods) from regression analysis of *b* starting at 1·0 msec; minimum signals onset of tension in *b*. *e*, standard error of slope curve from analysis of *d* starting at 8·0 msec; minimum signals onset of constant acceleration of tension. Curves *a* and *e* on arbitrary scales. *f*, deviation of unsmoothed record *d* (dots) and smoothed ($n/\delta = 3{\cdot}0$) record *d* (curve) from the regression line fitted to record *d*. The fibre is described in Fig. 4 legend.

at 2·1 μm to about 8·5 msec at 3·2 μm, as illustrated in Fig. 8 (upper curve); the lower curve in the same figure represents the corresponding reactivation latencies; second degree polynomials provided the best descriptions of the data. There was direct correlation between the relative deviations of data for corresponding delays about

their respective polynomial curves ($r = 0{\cdot}64$, $n = 27$, $P < 0{\cdot}001$). Onset of constant tension acceleration in a reactivation response occurred at about the same time after stimulation as minimum tension in the control twitch latency relaxation, suggesting the two events signal the same stage in activation; differences between those times were not significant ($P > 0{\cdot}8$). Relations between reactivation latency, time of onset of constant tension acceleration and the relative positions of myofilaments and sarcoplasmic reticulum are described under Discussion.

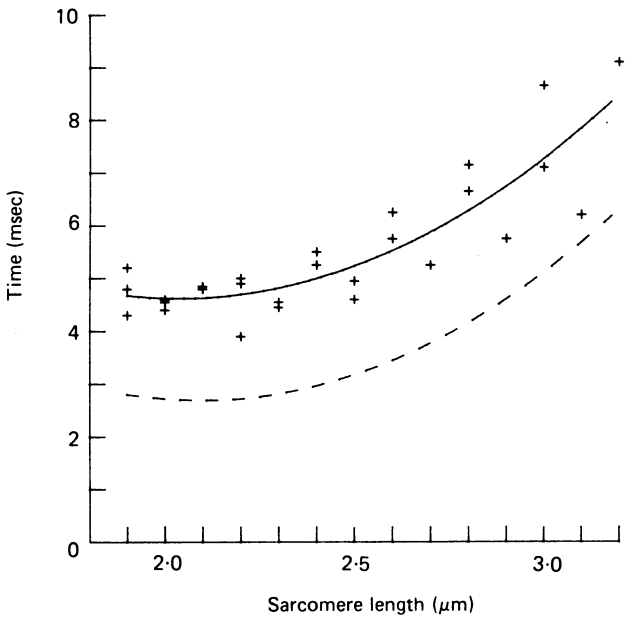

Fig. 8. Time between start of stimulus and onset of the phase of constant acceleration of tension (crosses) at different sarcomere lengths; the curve fitted by polynomial regression is $y = 2{\cdot}84x^2 - 11{\cdot}55x + 16{\cdot}37$ where y is ordinate and x is abscissa. The lower curve represents the corresponding reactivation latencies from the same records and is described by $y = 2{\cdot}96x^2 - 12{\cdot}41x + 15{\cdot}68$. Results from the three fibres described in legend of Fig. 6*A*.

During constant acceleration of tension, both the derivative of the square root of tension and the square root of tension acceleration were proportional to the length of the zone of overlap of thin and thick myofilaments (L_f); that is,

$$\frac{d\sqrt{P}}{dt} = k^{\frac{1}{2}} \propto L_f, \quad \frac{\sqrt{d^2P}}{dt^2} = (nk)^{\frac{1}{2}} \propto L_f$$

This is illustrated in a simple way in Fig. 9*A* for square root transforms of tension development at 2·2 μm and 3·0 μm sarcomere lengths. When the record at longer length was scaled ($\times$ 2·33) for full filament overlap it gave the dotted curve. The slope of the 2·2 μm record after 4·5 msec is virtually the same as that of the scaled 3·0 μm record after 8·5 msec.

The slopes of square-root records in the constant-acceleration phase, obtained by

linear regression analysis, are plotted against sarcomere length in Fig. 9*B*. The maximum slope, unity on the arbitrary scale, occurred at 2·0–2·3 μm sarcomere length. The slopes decreased linearly with sarcomere extension beyond about 2·3 μm and regression analysis of data for individual fibres gave maximum rate (1·0 on

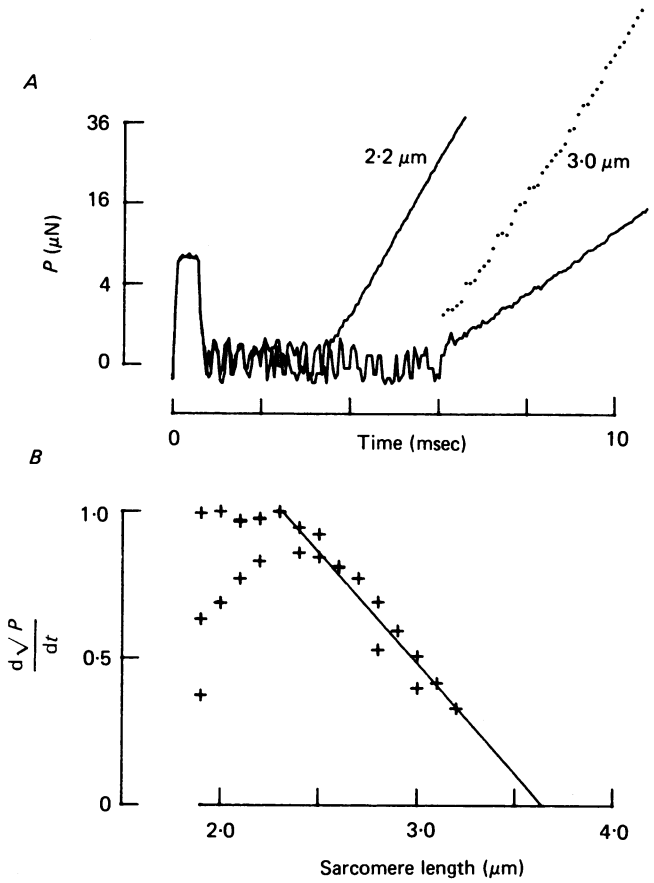

Fig. 9. Influence of sarcomere length (μm) on early tension development following restimulation at the peak of an isometric twitch. *A*, records from the series in Fig. 4 with difference tension plotted on a square-root scale. The dotted curve was obtained by multiplying the 3·0 μm record by 2·33 and plotting every second data sample in the range 6–10·5 msec after start of stimulus. Stimulus artifact is at start of records. *B*, $d\sqrt{P}/dt$ during constant acceleration of difference tension (P) plotted on arbitrary scale against sarcomere length (SL). The regression line fitted to pooled data for $SL \geqslant 2{\cdot}3$ μm has slope $-0{\cdot}754$ and ordinate intercept 2·745. Unity on ordinate corresponds to rates $8{\cdot}8–11{\cdot}6 \times 10^{-2}$ $N^{\frac{1}{2}}$/sec. Results from the three fibres described in legend of Fig. 6*A*.

ordinate) at 2·29 μm ($\pm 0{\cdot}07$ μm, S.D.) and abscissa intercept at 3·65 μm ($\pm 0{\cdot}08$ μm, S.D.); the difference in sarcomere length over that range is virtually the same as the length of the section of a thick filament that bears myosin projections, and the intercept at 3·65 μm is nearly the same as the combined length of thin and thick filaments (Page & Huxley, 1963). Consequently, the slope of a square-root record is proportional to length of filament overlap.

The result in Fig. 9*B* shows that $d\sqrt{P}/dt$ and not dP/dt is proportional to the length of the filament overlap zone. This means that at $L_f = 0{\cdot}5$, for instance, both the number of crossbridges that can form and the mean rate of tension change per crossbridge are one half their value at full filament overlap. More work should be carried out to determine whether the rate effect is a result of some kind of cooperative action among crossbridges or whether specific factors such as interfilament distance and amount of activation calcium affect either movement of myosin projections toward actin or subsequent structural changes leading to tension.

The results in Fig. 9 show that during constant tension acceleration fibres had reached a steady state of maximum responsiveness that was length dependent. It is proposed that onset of the phase of constant acceleration of tension at any length signals the time when activation calcium has become evenly distributed in the myofibrils throughout the sarcomere. That proposal is consistent with results of Ashley & Moisescu (1972) showing that the rate of rise of tension in frog skinned fibres was linearly related to time in experimental conditions where myofibrillar calcium concentration was held constant.

DISCUSSION

Correspondence between times of onset of the earliest reactivation response, latency relaxation and optically recorded calcium transients (above) provides good evidence that the arrival of activator in the myofilament array occurs at the same time in twitch and reactivation responses and that the time is not altered significantly by length changes. That result is consistent with observations that length changes have little influence on membrane events such as action potential (Häkansson, 1957; Edman & Kiessling, 1971), charge movement and mechanical threshold (Hui & Gilly, 1979). The remaining steps in excitation–contraction coupling that possibly contribute to length-dependent changes in reactivation latency are as follows.

(*a*) Rate of release of calcium from the sarcoplasmic reticulum.

(*b*) Diffusion of calcium through the myofibril.

(*c*) The activation process itself, i.e. binding of calcium to troponin and subsequent structural changes in the troponin–tropomyosin complex.

(*d*) Movement of a myosin projection toward a thin filament and force development.

At present, consideration of length-dependent delays in activation pivots on identification of the site of calcium release and possible diffusion delays.

Site of calcium release. It is unclear from available physiological evidence whether activation calcium ions are released throughout the sarcoplasmic reticulum or from a specialized region.

If calcium were released uniformly and synchronously from all regions of the sarcoplasmic reticulum, the diffusion distance between the longitudinal reticulum and the binding sites of troponin in the filament overlap zone would be quite small, about the same ($< 0{\cdot}04$ μm) as the interfilament distance (Huxley & Brown, 1967) at all sarcomere lengths, and variation in activator diffusion time (*b*, above) would be far less than the 3 msec differences in reactivation latencies (Fig. 5). It has been shown above that the probable time for onset of calcium release from the sarcoplasmic reticulum is independent of sarcomere length and that the delayed onset of

reactivation responses is abrupt (Figs. 3*C*, 9*A*). It is therefore not clear how relatively large variations in reactivation latency could be accounted for in terms of a model in which there is always negligible diffusion distance between calcium-release sites and tension-generating sites.

On the other hand, the length–dependence of the latent period can be understood if it is assumed that calcium is distributed to the contractile filaments in a wave that moves longitudinally through the myofibril in each half length of sarcomere. In view of earlier work on half-sarcomere activation (Huxley & Taylor, 1958) the direction of movement of such a wave in each half sarcomere would be probably toward the centre (M line) of the sarcomere. Assuming a length of 1·6 μm for thick myosin filaments (Page & Huxley, 1963), the results in Fig. 5 show that reactivation latency was unchanged with increase in sarcomere extension up to the length where the ends of thick filaments were about 0·25 μm away from the ends of the sarcomere. There is good agreement between this finding and results of electron microscopy showing that terminal cisternae are located at the ends of the sarcomere in frog fibres (Huxley, 1959) and that their longitudinal dimension is about 0·2–0·25 μm (Huxley, 1964; Page, 1965; Peachey, 1965; Franzini-Armstrong, 1970; A. R. Luff, personal communication); a similar finding was reported by Mulieri (1972). In terms of the sliding filament model of muscle the results mean that the time for movement of activator is minimal and constant when parts of thick filaments are adjacent to terminal cisternae, and that it increases approximately as the square of the distance between the ends of thick filaments and the near edge of terminal cisternae as the sarcomere is extended beyond 2·1 μm. That result is consistent with the hypothesis (e.g. Huxley, 1957) that activation calcium ions are released initially near the ends of the sarcomere, probably from terminal cisternae, and that they move to the myofilaments by diffusion.

Length-dependent delays in activation. Fig. 10 shows that both the onset of tension at the end of the reactivation latent period and the onset of the phase of constant tension acceleration (Fig. 7) probably signal major events in the distribution of activation calcium ions in the half sarcomere. In comparing times (t_1, t_2) and distances (d_1, d_2), defined in Fig. 10*A*, *B*, allowance has been made (ordinate Fig. 10*C*) for the estimated 0·7 msec delay in activation of central myofibrils due to the time required for conduction along transverse tubules (cf. Gonzalez-Serratos, 1971). The equality of the ratios of those times and distances (Fig. 10*C*), over a wide range of sarcomere lengths, is consistent with the view that activation calcium ions are distributed longitudinally at fairly constant speed at any particular length, that the onset of tension signals the arrival of the activation wave at the ends of the thick filaments and that the onset of constant tension acceleration signals the time when it reaches the ends of thin filaments at the other end of the filament overlap zone; the same result has been obtained with a numerical diffusion model of the myofibril in which activator is released from a circumferential compartment near the end of the sarcomere and allowed to diffuse longitudinally, with binding, toward the centre of the sarcomere (unpublished observations).

The marked length dependence of the delay to onset of constant tension acceleration (Fig. 8, upper curve) is probably unaffected by changes in lateral dimensions of the filament lattice; even at long sarcomere lengths the interfilament distance is about

50 times greater than the diameter of a hydrated calcium ion. Variation in that delay could conceivably reflect changes in release of calcium from the sarcoplasmic reticulum, activation and tension generation (*a*, *c* and *d* above); all could be consistent with the results in Fig. 10*C*. However, analysis of fourteen sets of data from Fig. 8 (2·4–3·2 μm) showed that at any length the quantity d_1/t_1, defined in Fig. 10, was on average 0·95 ($\pm$0·25, S.D.) times the quantity $(d_2-d_1)/(t_2-t_1-0{\cdot}7)$ and the difference between those speeds was not quite significant ($P > 0{\cdot}1$). There was

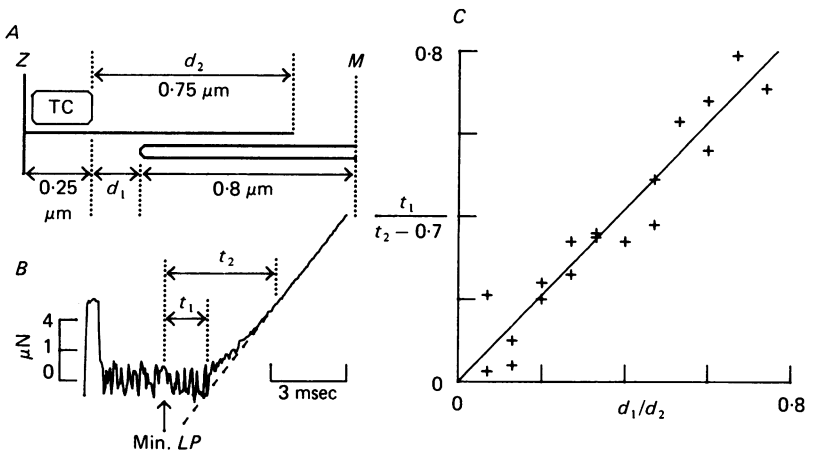

Fig. 10. Relations between myofibril structure and delays in the early tension transients of reactivation responses at different lengths. *A*, diagram of a half-sarcomere with thin filament (1 μm), thick filament (0·8 μm) and terminal cisterna (TC). *B*, square root transform of a single difference tension record at 2·6 μm sarcomere length. Min *LP* is the reactivation latency at short sarcomere length; end of t_1 phase marks the onset of tension and end of t_2 phase marks the onset of constant acceleration of tension; t_1 and t_2 (msec). *C*, relation between delay times and diffusion distances, defined in *A* and *B*, at different sarcomere lengths (2·2–3·2 μm). Data from Fig. 8; one point with Fig. 10*C* co-ordinates 0·07, 1·0 was rejected. Regression analysis gave slope 1·05, ordinate intercept $-0{\cdot}003$ and correlation coefficient 0·96.

no evidence, therefore, of a major difference in apparent speed of longitudinal spread of activation in the myofibrillar I and A zones. It follows that possible length dependent changes in the molecular events of activation and tension generation (*c* and *d* above), that would presumably alter t_1 and t_2 but not t_2-t_1, could not have been large enough to account for delay changes of several milliseconds; indeed, the quantity $t_1 . d_2/d_1 - t_2 + 0{\cdot}7$, an approximate measure of the duration of those molecular events, was small (0·1 $\pm$ 0·35 msec, S.D.) and not correlated apparently with length.

The simplest interpretation of the length dependence of changes in reactivation latencies is that they are brought about mainly by changes in the kinetics of distribution of activator within the myofibril, most likely through an effect on calcium release from the sarcoplasmic reticulum, and by changes in diffusion distance between calcium-release sites and the tension-generating sites.

Delay associated with latency relaxation. In twitch responses there was a surprisingly long delay in activation, hitherto unrecognized, that was not seen in reactivation

responses. The delay of about 2 msec in the rise of twitch tension at short lengths (2·2 μm, Fig. 2), compared with that in the reactivation response, was associated with latency relaxation but it is not clear whether the two were causally connected. If activator were released from the whole surface of the terminal cisterna, the thick filaments at the surface of the neighbouring myofibril would have been very near the release sites at short sarcomere lengths (2·0–2·2 μm) and the delay in twitch tension development could not have been due to diffusion. Furthermore, the delay was probably not due to slower release of calcium from the sarcoplasmic reticulum in the twitch, compared with that in reactivation responses, because calcium transients diminish during repetitive stimulation (Blinks, Rüdel & Taylor, 1978). It seems as though the delay was brought about by a priming process that took place within the myofibril.

Myofibril priming delay could be due to a change which, once completed at the onset of a twitch, persists throughout the greater part of that response and is not repeated fully on restimulation at the twitch peak. Structural changes in myofilaments and competition among various structures for calcium ions could possibly contribute to the delay.

Structural changes in the contractile filaments may be relatively slow and delay the twitch onset. For example, calcium binding to troponin occurs simultaneously at calcium–magnesium sites and calcium-specific sites at physiological levels of magnesium (Levine, Thornton, Fernandes, Kelly & Mercola, 1978), but a relatively slow conformational rearrangement following displacement of magnesium by calcium may contribute to the myofibril priming delay if the resulting state of the molecule were an essential forerunner of activation and contraction. Alternatively, if latency relaxation were involved in structural alterations, the myofibril priming delay may result from transitory interference with normal crossbridge action.

Competition for activation calcium between the tension-controlling, calcium-specific sites on troponin and other sites could slow the movement of activator through the myofibril in the early phase of a twitch response. A possible interpretation is that some tension-controlling sites on troponin are available for reactivation at twitch peak whereas other sites, that initially bound some activation calcium, are still occupied because they have either relatively high affinity for calcium or, as for the calcium–magnesium sites on troponin (Johnson, Charlton & Potter, 1979), relatively low exchange rate for removal of calcium.

Origin of latency relaxation. The suggestion that activation calcium is released near the ends of the sarcomere is not necessarily inconsistent with proposals that latency relaxation results from changes in thick and thin filaments in their overlap zone (Huxley & Brown, 1967; Hill, 1968; Huxley, 1974), even though latency relaxation starts after the same delay at all extensions. It is conceivable that binding of calcium to the thin filament near the end of the sarcomere may have long range effects that could alter the force between neighbouring thick and thin filaments in the overlap zone. Indeed, the fact that latency relaxation is maximal when one half of the crossbridge-bearing part of the thick filaments is overlapped by thin filaments (i.e. sarcomere length about 2·9 μm), and is virtually zero at both maximum and zero overlap, strongly suggests that latency relaxation involves interaction between whole filaments. The puzzling length-dependent increase and decrease of latency relaxation

could be explained by a class of model in which (1) total resting force between adjacent, overlapping thick and thin filaments is directly related to length of overlap and inversely related to the lateral spacing of the filaments and (2) the proportion of resting interfilamentary force that is discharged during latency relaxation increases with amount of calcium bound to the thin filament.

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