The intracellular Ca^{2+} transient and tension in frog skeletal muscle fibres measured with high temporal resolution

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1. The purpose of this study was to determine, with high temporal resolution, the relationship between the intracellular Ca^{2+} transient (ICT) and the mechanical responses of intact, single skeletal muscle fibres of frogs following stimulation by a single, brief depolarization.

2. The time course of the ICT was monitored using the Ca^{2+}-sensitive fluorescent dyes mag-fura-2 (furaptra) and mag-fura-5. The mag-fura dyes have a low affinity for Ca^{2+} and have been shown to track the ICT with no appreciable kinetic delay. Continuous records of mag-fura fluorescence, tension and stiffness responses were obtained simultaneously at high time resolution at a sarcomere length of 2·9 μm. Experimental temperature was 3 °C.

3. When a delay of 0·4 ms due to the low-pass filter associated with the photodetector was included, the onset of the fluorescence response preceded the onset of latency relaxation (the small fall in tension that precedes positive tension generation) by $3{\cdot}1 \pm 0{\cdot}2$ ms (mean ± S.E.M., $n = 8$). After its onset, the mag-fura fluorescence signal continued to change rapidly (indicating increasing intracellular [Ca^{2+}]) to an extreme level that occurred $1{\cdot}5 \pm 0{\cdot}5$ ms (mean ± S.E.M., $n = 7$) before tension had recovered to its resting level following latency relaxation. The time delay from the extreme of the fluorescence signal to the peak of the tension signal was 239 ± 27 ms (mean ± S.E.M., $n = 6$).

4. It is concluded that the intracellular concentration of Ca^{2+} begins to rise before any detectable mechanical changes occur during a twitch contraction and begins to decrease before the onset of positive tension development.

Electrical stimulation of frog twitch muscle fibres produces three phenomena that precede the onset of positive tension generation. Intracellular [Ca^{2+}] increases transiently (Blinks, Rüdel & Taylor, 1978; Baylor, Chandler & Marshall, 1982), tension undergoes a small transient decrease known as latency relaxation (Sandow, 1944) and longitudinal stiffness begins to increase (Hill, 1950). Elucidation of the mechanism of excitation–contraction coupling requires that the relative timing of these events be accurately known. We have reported (Claflin, Morgan & Julian, 1990; Morgan, Claflin & Julian, 1990) that the onsets of latency relaxation (LR) and the increase in muscle fibre stiffness occur simultaneously. However, the precise time relations between the intracellular Ca^{2+} transient (ICT) and the mechanical responses following stimulation have not been determined.

If the onsets of LR and the increase in stiffness are the earliest detectable signs of a Ca^{2+}-mediated influence on cross-bridges in response to electrical stimulation, then it follows that the onset of the ICT must precede these first mechanical events. Our first aim was to test this hypothesis by obtaining high time-resolution records of the onset of the ICT. This was achieved by working at a low temperature (3 °C) and using rapid Ca^{2+} reporters (mag-fura-2 and mag-fura-5). Close & Lännergren (1984) have reported that the onsets of the ICT and LR are virtually simultaneous, but the temporal resolution of their results was limited by the use of a relatively high temperature (15 °C) and relatively slow Ca^{2+} reporter (arsenazo III).

Our second aim was to relate, with improved temporal accuracy, the time of the peak of the ICT to the time course of the tension generated during a twitch. Previous reports of the time course of the ICT during twitch contractions have been from experiments in which either the muscle fibres were stretched to extreme sarcomere lengths to avoid motion artifacts (e.g. Baylor *et al.* 1982), or relatively slow Ca^{2+} indicators such as aequorin were used (e.g. Ashley & Ridgway, 1970; Blinks *et al.* 1978). A unique contribution of the experiments reported here is that substantial filament

overlap has been maintained in the muscle fibres (sarcomere length, 2·9 μm), thus preserving a more physiological state while recording fluorescence signals generated by the ICT with little or no kinetic delay. A brief report of these results has been presented to the American Biophysical Society (Claflin, Morgan, Stephenson & Julian, 1992).

METHODS

Dissection, mounting, apparatus and stiffness measurements

The experiments were performed on intact single twitch fibres isolated from the tibialis anterior muscle of the frog (*Rana temporaria*). Frogs were killed by decapitation followed by double-pithing after being immersed in ice water for no less than 10 min. Dissections were performed under dark-field illumination at room temperature in a Ringer solution with the following composition (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85; pH 7·2. Fibres were mounted horizontally in a chamber filled with the same solution and maintained at a temperature of 3 °C. One end of each fibre was attached to a servomotor and the other was attached to a tension transducer. Unless otherwise noted, records are from fibres stretched to a mean sarcomere length of 2·9 μm. At this sarcomere length, substantial filament overlap remains (almost 50 %) and the amplitude of the LR is nearly maximal (Morgan *et al.* 1990). The length of the fibres used in this study was 6·95 ± 0·29 mm (mean ± S.E.M., $n = 8$) when average sarcomere length was 2·1 μm. Details of the dissection, mounting, solutions and apparatus have been described previously (Claflin *et al.* 1990).

Stiffness was monitored by applying a small (0·5 nm per half-sarcomere, peak-to-peak) sinusoidal oscillation to one end of the fibre and recording the resultant tension oscillations at the other end. Continuous stiffness and tension signals were extracted from the oscillating tension response using digital signal processing techniques. Using this protocol, simultaneous records of tension and stiffness could be obtained from a single oscillated tension record. In practice, each oscillated tension record stored for analysis was the average of sixteen to thirty-six successive twitch contractions, separated in time by 10–15 s. The responses were averaged, so it was necessary to phase-lock the sinusoidal oscillations to the stimuli and to verify that the oscillation frequency was stable over the period of time that the averaging took place. Details of the technique for continuously and simultaneously monitoring tension and stiffness have been described previously (Claflin *et al.* 1990).

Monitoring the intracellular Ca^{2+} transient

The ICT was monitored using the Ca^{2+}-sensitive fluorescent dyes mag-fura-2 (furaptra) and mag-fura-5 (Molecular Probes, Inc., Eugene, OR, USA). The fluorescent dyes were loaded by incubating intact fibres for 30 min at 20 °C in a Ringer solution containing 5 μmol l^{-1} of the membrane-permeant acetoxymethyl (AM) ester of the dye. The AM form passes through the sarcolemma and is hydrolysed by cytoplasmic esterases to a membrane-impermeant free acid, resulting in loading of the entire fibre. After the loading period, the bathing solution was replaced with dye-free Ringer solution and bath temperature was reduced to 3 °C, a process that required approximately 10 min to complete. The amount of dye loaded using this protocol was sufficient to produce a fluorescence response that was easily measured, but not great enough to cause a change in the contractile response of the fibre as a result of dye loading.

To measure fluorescence, the stage of an upright light microscope was replaced by the chamber containing the loaded fibre. Fluorescence was excited by epi-illumination from a 75 W xenon source. Light was passed through a bandpass 'excitation' filter, reflected through an angle of 90 deg by a dichroic mirror, and passed through a microscope objective before reaching the fibre. Fluorescence was collected by the objective and passed through the dichroic mirror and a bandpass 'barrier' filter to a photomultiplier tube for detection. For the mag-fura dyes, the excitation filter was centred at 380 nm, the dichroic mirror reflected wavelengths shorter than 400 nm and passed those longer than 400 nm, and the barrier filter was centred at 510 nm. When exciting the mag-fura dyes using a wavelength of 380 nm, an increase in $[Ca^{2+}]$ causes a reduction in dye fluorescence. The current output from the photomultiplier was converted to voltage and then passed through an active low-pass filter (8-pole, Butterworth) with a cut-off frequency of 2 kHz before being displayed, along with the simultaneously acquired tension response, on the screen of a digital oscilloscope (model 4094; Nicolet Instruments Corp., Madison, WI, USA). Due to its phase characteristics, the low-pass filter introduced a time delay that depended slightly on frequency: 0·39 ms at 50 Hz and 0·40 ms at 500 Hz (as determined using pure sinusoidal waveforms). The digital low-pass filter used for the tension and stiffness signals did not introduce delay. All records were stored for later analysis. Each fluorescence record stored for analysis was the average of thirty-six successive fluorescence responses obtained during twitch contractions separated in time by 10–15 s. This time period was sufficient for the fluorescence to return to baseline levels when using the mag-fura dyes.

Care was taken to attach each fibre to the motor and tension transducer in such a way that fibre motion was minimized during a twitch. Two loops of 0·3 metric monofilament nylon surgical suture were used at each end of the fibre to tie the remaining tendon to wires protruding from the motor arm and the tension transducer. One tie at each end was placed within 50 μm of the fibre end. The response of the fibre to twitch stimulation was then monitored using a microscope. If fibre motion (either lateral or with respect to the plane of focus) was observed, the attachment of the fibre was adjusted to minimize the motion. This process was repeated until the fibre remained essentially motionless during contraction. The intrinsic fluorescence of the fibres was then exploited to further reduce the possibility of signal contamination by motion artifact. This was done by exciting the loaded fibre at a wavelength of 480 nm and monitoring the resulting fluorescence at a wavelength of 535 nm during twitch contractions. The resting fluorescence response of the loaded fibres to excitation at 480 nm was, on average, 25 % of the resting response to excitation at 380 nm (± 10 % S.E.M., $n = 6$). Since the fluorescence from the mag-fura dyes is not sensitive to Ca^{2+} when excited by 480 nm light, any changes in the light signal observed during contraction under these conditions are most likely to be due to fibre motion. A search was performed along the length of the fibre to find an area from which no light fluctuations were observed during contraction while exciting at 480 nm, and subsequent mag-fura fluorescence signals were obtained from such an area. The diameter of the 'circle' of excitation illumination used in these experiments varied from 0·1 to 0·8 mm.

RESULTS

Fluorescence and LR onsets

The time interval between the onset of the fluorescence signal and the onset of the LR signal was determined for each fibre as follows. The signals were first scaled such that the difference between the resting level and the minimum level reached following stimulation was the same for both. The fluorescence signal was then truncated at the point at which it had dropped by 20 % of the maximum decline. The 20 % level was chosen arbitrarily after calculations using 10, 20 and 30 % resulted in only small (< 5 %) variations in time interval estimates. The root-mean-square (r.m.s.) difference between the fluorescence signal and the tension signal was then computed over the time interval occupied by the truncated fluorescence signal. This was calculated by finding the difference for each point of the sampled waveforms (sample rate, 50 or 100 kHz), squaring it and summing over all the points. The result was divided by the number of points and then its square-root was computed and stored. The truncated fluorescence signal was then shifted with respect to the tension signal and the r.m.s. difference between the two signals was again computed. This process of shifting and computing the r.m.s. difference was repeated, using a simple search technique, until the time shift that resulted in the minimum difference between the truncated fluorescence signal and the tension signal was determined. This time shift was taken as the interval between the onsets of the fluorescence and LR signals. The result of this process, applied to typical fluorescence and tension records from one fibre, is illustrated in Fig. 1, where the unshifted records are shown in *A* and the same two records with the fluorescence signal shifted ahead in time by 2·25 ms (the shift that minimized the r.m.s. difference between the two responses) are shown in *B*.

The delay between the onsets of the fluorescence and LR responses was 2·7 ± 0·2 ms (mean ± S.E.M., $n = 8$) when mag-fura-5 was used as the Ca^{2+} reporter. When mag-fura-2 was used ($n = 2$), the delay times were 2·3 and 3·2 ms. Figure 2 shows a typical response obtained using mag-fura-5 to report Ca^{2+} while, at the same time, monitoring both the tension and stiffness responses of the fibre. The first response of the fibre is a decrease in fluorescence, indicating an increase in intracellular [Ca^{2+}], followed 3·2 ms later by a simultaneous increase in stiffness and decrease in tension. It should be noted that these reported delay times are underestimated by approximately 0·40 ms due to the phase characteristics of the low-pass filter through which the photomultiplier signal passed (see Methods).

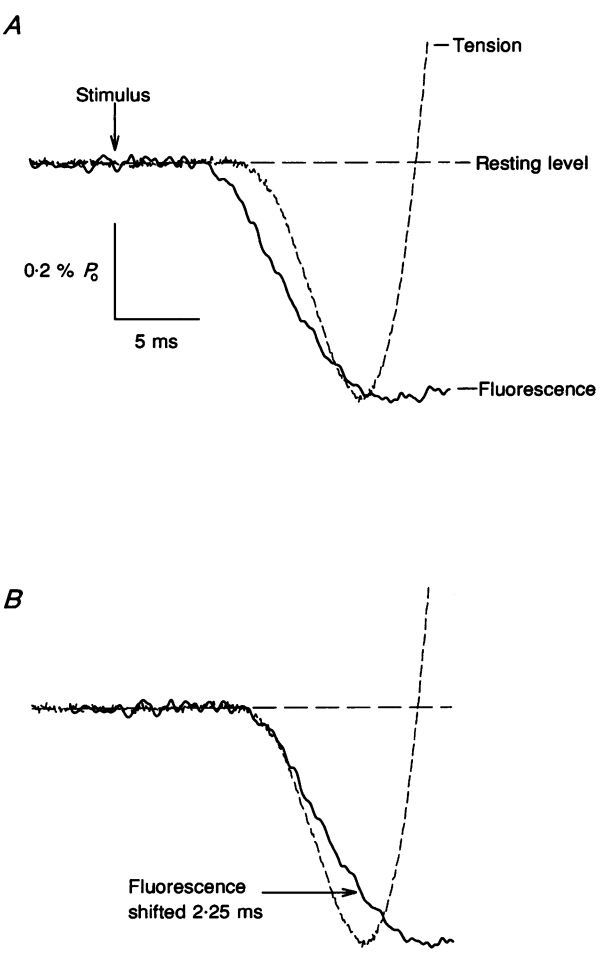

Figure 1. The interval between the onsets of fluorescence and latency relaxation (LR)
A, typical tension (dashed line) and mag-fura-2 fluorescence (continuous line) recorded simultaneously from one fibre in response to single electrical stimuli (36 sweeps averaged; sample rate, 100 kHz). P_0 is the isometric tension measured during the plateau of a contraction at a sarcomere length of 2·1 μm. Fluorescence records are scaled such that the minimum level reached matches the minimum tension level reached during LR. Note that, for mag-fura-2 excited at a wavelength of 380 nm, an increase in [Ca^{2+}] causes a decrease in fluorescence. The time interval between the onsets of the fluorescence signal and the tension relaxation was estimated by the procedure described in the text. For the pair of records shown in *A*, this procedure produced a minimum r.m.s. difference when the fluorescence response was shifted ahead in time by 2·25 ms. *B*, the records shown in *A* are duplicated here with the fluorescence record shifted ahead by 2·25 ms, the shift required to minimize the r.m.s. difference between the two records as described above. Mean sarcomere length was 2·9 μm and temperature was 3 °C.

Fluorescence and tension time courses

The relationship between the peak of the ICT and the time course of the tension response during a twitch was determined by using mag-fura-5 to monitor changes in intracellular Ca^{2+} while simultaneously recording the tension response. Typical records showing the relationship between the mag-fura-5 fluorescence (inverted to facilitate comparisons) and tension responses are shown in Fig. 3 on two different time scales. After its onset, the mag-fura-5 fluorescence signal continued to rise rapidly to a maximum level that occurred 1·1 ± 0·5 ms (mean ± S.E.M., $n = 7$) before the tension signal had recovered to its resting level following LR. The difference between these two time points was statistically significant (Student's paired t test, $P = 0·044$). When the 0·4 ms delay introduced by the photomultiplier low-pass filter (see Methods) is added, our best estimate for this difference is 1·5 ms. That is, the fluorescence signal had reached a peak and begun to return to its resting level before net positive tension generation began. After reaching its maximum level, the fluorescence signal decreased rapidly and returned nearly to resting level by the time the tension peak occurred. The time delay from the peak of the fluorescence signal to the peak of the tension signal was 239 ± 27 ms (mean ± S.E.M., $n = 6$).

It should be noted that, because of their relatively large dissociation constant (K_d) values, the mag-fura dyes are not capable of detecting small changes in $[Ca^{2+}]$ near the resting level. Consequently, the small elevations above baseline that persist in the fluorescence level during tension relaxation, observed when more sensitive Ca^{2+} indicators are used (Baylor & Hollingworth, 1988; Klein, Kovacs, Simon & Schneider, 1991), were not expected and not detected here. It should also be noted that, although the mag-fura dyes are sensitive to Mg^{2+} as well as Ca^{2+}, any changes in $[Mg^{2+}]$ are thought to be slow compared to the time course of the ICT and should not contribute significantly to the fluorescence changes recorded during a twitch contraction (Konishi, Hollingworth, Harkins & Baylor, 1991). No attempts were made to calibrate the fluorescence signals in terms of absolute Ca^{2+} concentrations.

Fluorescence records were also obtained from some of the fibres after stretching to an average sarcomere length of 4·0 μm to determine whether artifact due to fibre motion was contaminating the responses during contraction and relaxation at 2·9 μm. At an average sarcomere length of 4·0 μm tension generation and the concomitant potential for motion artifact are virtually eliminated. Figure 4 shows records from such an experiment on two different time scales. In Fig. 4*A* it can be seen that the time course of the

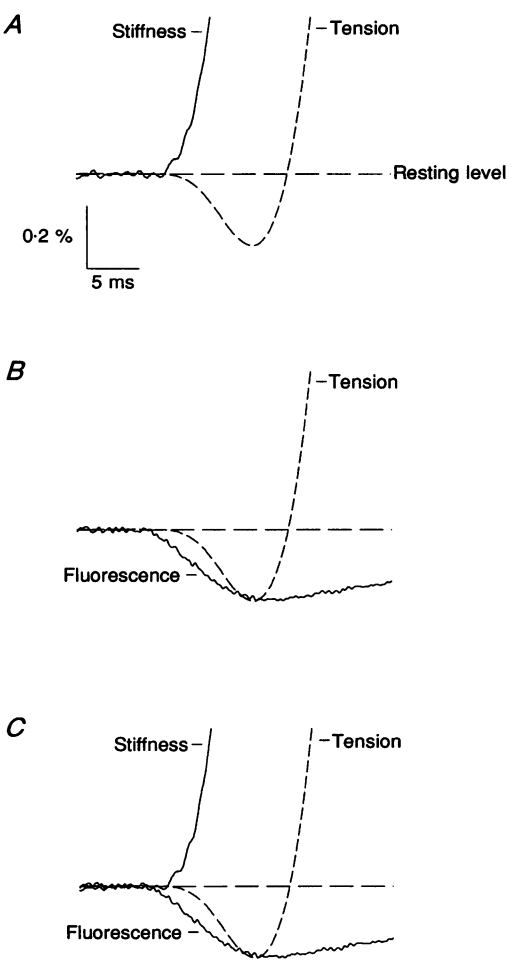

Figure 2. The onsets of latency relaxation (LR), stiffness and fluorescence
Simultaneous records of the onsets of LR, stiffness and mag-fura-5 fluorescence signals obtained in response to single electrical stimuli (36 sweeps averaged; sample rate, 50 kHz). To facilitate comparisons, stiffness and tension records have been paired in *A*, fluorescence and tension in *B*, and all three records are shown in *C*. The vertical scale shown in *A* is calibrated in per cent of maximum tension or stiffness obtained at a sarcomere length of 2·1 μm during the plateau of a tetanic contraction. The scaling of the fluorescence signal was arbitrarily chosen such that the minimum fluorescence level matched the minimum tension level during LR as in Fig. 1. The time scale shown in *A* applies to all records. The delay between the onset of the fluorescence signal and the onset of the LR for this fibre was 3·2 μs. The beginning of the trace corresponds to the time at which the stimulus was delivered. Mean sarcomere length was 2·9 μm and temperature was 3 °C.

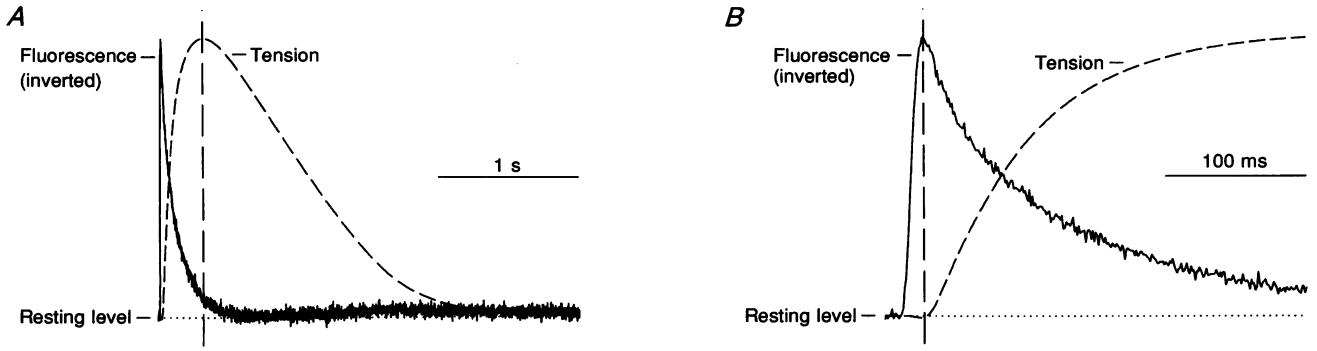

Figure 3. Simultaneously recorded twitch tension and mag-fura-5 fluorescence
Typical twitch tension (dashed line) and mag-fura-5 fluorescence (continuous line) recorded simultaneously in response to single electrical stimuli (36 sweeps averaged; sample rate, 1 kHz; fluorescence records inverted to facilitate comparisons with the tension records). *A*, records shown on a slow time base and scaled such that the peaks of the tension and fluorescence responses match. Note that the mag-fura-5 signal has declined to near its resting level by the time tension reached its peak (dashed vertical line). *B*, the records shown in *A* are duplicated on a time base that has been expanded by a factor of 10. Note that, for this fibre, the peak of the fluorescence signal occurs 2·6 ms before tension recrosses its resting level following latency relaxation (dashed vertical line). Mean sarcomere length was 2·9 μm and temperature was 3 °C.

(inverted) fluorescence response at an average sarcomere length of 2·9 μm is very similar to the response at 4·0 μm, indicating that the record obtained at 2·9 μm contained no significant distortions due to motion artifact. Upon close inspection of the records during the late stages of tension relaxation, slight fluctuations can be seen in the fluorescence record obtained at 2·9 μm that are not present at 4·0 μm. Such fluctuations were sometimes, but not always, apparent (for example, the records shown in Fig. 3*A* have virtually none) and are most likely to be due to residual fibre motion at the shorter sarcomere length during relaxation. It should be noted that, because the fluorescence signal has reached a peak and begun the return to resting level before any significant tension is generated, it is extremely unlikely that its onset and rise are contaminated by motion artifact.

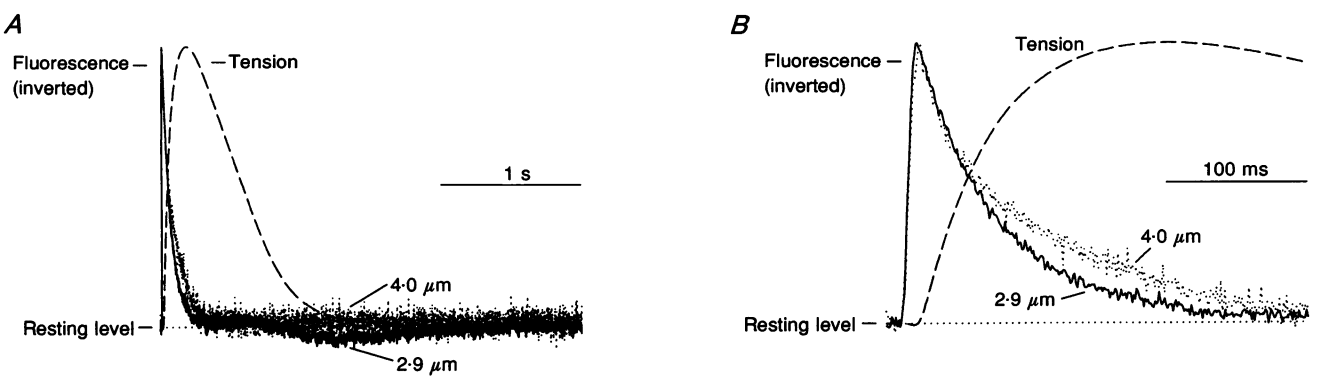

Figure 4. The contribution of motion artifact to the fluorescence signals
Twitch tension (dashed line) and mag-fura-5 fluorescence (continuous line) recorded simultaneously at a mean sarcomere length of 2·9 μm, and mag-fura-5 fluorescence (dotted line) recorded at a mean sarcomere length of 4·0 μm. For all records, 36 sweeps were averaged and sample rate was 1 kHz. The fluorescence records have been inverted and scaled to facilitate comparisons with the tension records. The amplitude of the inverted fluorescence record at 4·0 μm was about 50 % of that at 2·9 μm when each was normalized by its corresponding resting level. *A*, records shown on a slow time base and scaled such that the peaks of the tension and fluorescence responses match. Note the small fluctuations in the fluorescence signal obtained at a sarcomere length of 2·9 μm following its return to baseline and the absence of such fluctuations in the signal obtained at 4·0 μm. These fluctuations are most likely to be artifacts due to movement of the fibre during tension relaxation. *B*, the records shown in *A* are duplicated on a time base that has been expanded by a factor of 10. Temperature was 3 °C.

DISCUSSION

The mag-fura dyes were chosen for these experiments because of their potential for reporting rapid changes in Ca^{2+} levels. Mag-fura-2 and mag-fura-5 have K_d values for Ca^{2+} of 53 μM (Raju, Murphy, Levy, Hall & London, 1989) and 25 μM (Molecular Probes, Inc.), respectively. Based on these relatively large K_d values, the kinetics of the reactions of these dyes with Ca^{2+} should be rapid. Consequently, the mag-fura dyes would be expected to track rapid changes in intracellular Ca^{2+} with high fidelity. Konishi *et al.* (1991) have reported that the kinetics of the reaction between mag-fura-2 and Ca^{2+} are more rapid than those of the Ca^{2+}–antipyrylazo III reaction which are, in turn, much more rapid than those of the Ca^{2+}–arsenazo III reaction (Palade & Vergara, 1982; Baylor, Quinta-Ferreira & Hui, 1983). Indeed, mag-fura-2 has been shown by Konishi *et al.* (1991) to track the ICT in frog skeletal muscle fibres with 'little or no kinetic delay'. An additional advantage of the low affinity Ca^{2+} reporters is that, at concentrations sufficient to generate easily measured fluorescence responses, they did not affect the contractile response of the fibres. This is, presumably, because they do not significantly buffer the ICT at such concentrations. Consequently, it was possible to make fluorescence, tension and stiffness measurements simultaneously during the same contraction, thus removing uncertainties inherent in making the measurements during separate contractions as was required in other studies.

Fluorescence, LR and stiffness onsets

It is generally acknowledged that an increase in $[Ca^{2+}]$ in the myoplasm of skeletal muscle is required for the initiation of cross-bridge attachment and state transitions that, in turn, result in force generation (for review, see Ashley, Mulligan & Lea, 1991). Ford, Huxley & Simmons (1981, 1986) have presented evidence that cross-bridge attachment is responsible for the increase in muscle stiffness that occurs during tension development. We have shown previously that the earliest detectable mechanical changes following electrical stimulation of an intact single skeletal muscle fibre are a simultaneous decrease in tension and increase in stiffness, and postulated that both are due to cross-bridge attachment (Claflin *et al.* 1990; Morgan *et al.* 1990). If these mechanical events are due to cross-bridges that are activated by Ca^{2+}, then they must be preceded by an increase in myoplasmic $[Ca^{2+}]$. The present results show that the onset of the ICT does, indeed, precede the earliest mechanical changes during the activation of contraction in skeletal muscle. This finding is important because it provides accurate knowledge of the temporal relationship between two key events that occur during muscle activation; the release of Ca^{2+} and the attachment of cross-bridges in a 'non-resting' state.

Fluorescence and tension time courses

Previous reports of the time course of the ICT fall into one of two categories. Most commonly, the corresponding tension response is ignored because the fibre has been stretched such that no filament overlap exists in most of the fibre and very little tension is generated (e.g. Baylor *et al.* 1982). This is done to avoid the artifacts often introduced by fibre motion during contraction. We found that we were nearly able to eliminate the motion artifact problem by carefully attaching the fibres to the apparatus and then scanning the length of the fibre to choose a motion-free segment. It is evident from Fig. 4, in which fluorescence records from one fibre at average sarcomere lengths of 2·9 μm (~43 % of maximum filament overlap) and 4·0 μm (where almost no filament overlap remains) are superimposed, that motion artifact is contributing very little to the records obtained at 2·9 μm. The largest discrepancy between the two records occurred during the fall in fluorescence, when the highly stretched fibre declines at a somewhat slower rate. This fall in the rate of decline with increasing sarcomere length has also been reported by Konishi *et al.* (1991).

In reports that do include corresponding tension records, the ICT was monitored with a Ca^{2+} sensor that introduced considerable kinetic delay. Using aequorin as the Ca^{2+} indicator, Blinks *et al.* (1978) reported that the peak of the ICT occurs approximately at the time that the rate of rise of tension is maximal, well after positive tension generation begins. Similarly, Close & Lännergren (1984), using arsenazo III, showed the peak of the ICT occurring after tension has begun to rise above the resting level. The present report is unique in that it combines rapidly responding dyes with a relatively motion-free intact single fibre preparation, thus allowing the recording of the time course of the ICT with high fidelity while simultaneously recording the tension response under more physiological filament overlap conditions. The results show that the rates at which $[Ca^{2+}]$ increases in the myoplasm following the onset of the ICT and then decreases during positive tension generation are very rapid. That is, compared to the time course of the tension response, the ICT is very brief. The peak of the ICT occurs before positive tension generation begins following LR, much earlier than previously thought. Since the Ca^{2+}-specific sites on troponin-C are thought to bind calcium very rapidly (Ashley *et al.* 1991), the present results emphasize the marked difference between the time courses of this binding and active tension generation.

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