Variation in myoplasmic Ca^{2+} concentration during contraction and relaxation studied by the indicator fluo-3 in frog muscle fibres

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1. The fluorescent dye fluo-3, in its permeant acetoxymethyl form, was used to monitor calcium transients during twitch and tetanus of single fibres isolated from the anterior tibialis muscle of *Rana temporaria* (2–5 °C).

2. Fluo-3 was loaded into the muscle fibre by diffusion. Under the experimental conditions used, approximately 45% of maximal fluorescence was reached during a 1 s fused isometric tetanus. Fluo-3 had no detectable effect on the mechanical response of the fibre.

3. The free calcium concentration in the myoplasm, $[Ca^{2+}]_i$, and its variation with time, was calculated from the fluorescence signal by accounting for the on- and off-rate constants for the binding of calcium to the dye. The time course of the calcium transient during twitch and tetanus determined in this way agreed well with previous measurements based on fast-reacting calcium-sensitive dyes.

4. $[Ca^{2+}]_i$ declined steeply during the initial phase of force relaxation in both twitch and tetanus, but exhibited a secondary rise that closely coincided with the pseudoexponential fall of tension after the shoulder in the tetanus myogram. The rate of decay of $[Ca^{2+}]_i$ during relaxation and the rate of decline of force both became progressively reduced by repetitive stimulation.

5. Stretch and shortening ramps performed during the plateau of an isometric tetanus had no detectable effect upon the calcium transient during the movement. By contrast, shortening and stretch imposed during the linear phase of relaxation both led to an increase of $[Ca^{2+}]_i$ and to a steepening of the relaxation phase.

6. The results strongly suggest that the non-uniform length changes that are known to occur along a muscle fibre during relaxation enhance the release of calcium from the contractile system. The calcium mobilized in this way probably accounts for the transitory increase of $[Ca^{2+}]_i$ that is observed during the latter part of force relaxation.

The transient release of calcium from the sarcoplasmic reticulum of frog muscle fibres during contractile activation has been studied by several investigators using different calcium indicators. Following the pioneering study by Jöbsis & O'Connor (1966), the first attempts to quantify the temporal relationship between the calcium transient and active force were reported by Ashley & Ridgway (1968), who injected the photoprotein aequorin into intact single muscle fibres. Limitations imposed by the kinetics of the aequorin reaction with calcium and by the non-linearity between aequorin light emission and calcium concentration (Allen, Blinks & Prendergast, 1977) have led researchers to the use of other calcium indicators. Much work has been carried out with metallochromic calcium indicators, agents that undergo absorbance changes in the presence of calcium (Miledi, Parker & Schalow, 1977; Kovács, Ríos & Schneider, 1979; Maylie, Irving, Sizto & Chandler, 1987*b*,*c*). These measurements, however, are influenced by changes in sarcomere length and fibre geometry and therefore require that the contractile response is abolished or at least greatly reduced.

The introduction of the tetracarboxylate dye fura-2 (Grynkiewicz, Poenie & Tsien, 1985), which in the presence of calcium shows a shift in the fluorescence excitation spectrum, has opened the possibility of measuring calcium transients in fibres with residual (Baylor & Hollingworth, 1988) and full contractile capacity (Lee, Westerblad & Allen, 1991). In the present experiments the new

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fluorescent dye fluo-3 (Minta, Kao & Tsien, 1989), in its permeant acetoxymethyl form, has been used to measure simultaneously calcium transients and contractile force in intact single muscle fibres of the frog. The dye has visible excitation and emission wavelengths, and, when complexed with calcium, shows a large increase in fluorescence without shifts in the excitation and emission spectra. Although these features complicate the calibration procedures, they allow measurements to be made without utilizing the ratio between signals. Fluo-3 is now being increasingly used in muscle physiology (Garcia, Compagnon, Vergara & Stefani, 1989; Hollingworth, Harkins & Baylor, 1990) and has been found suitable for applying imaging techniques to muscle fibres (Vergara, DiFranco, Compagnon & Suarez-Isla, 1991). While it is clear that fluo-3 does not meet all the requirements for an ideal intracellular calcium indicator (see Tsien, 1988), the results presented in this work demonstrate that fluo-3 is suitable for gaining information about changes in the myoplasmic calcium ion concentration during contractile activity, especially under conditions when the muscle fibre undergoes a length change.

Fluo-3 has been used in this study to elucidate further the calcium transient that is associated with force relaxation during twitch and tetanus in frog muscle fibres. Particular interest has been focused upon the nature of the transitory increase in myoplasmic free calcium concentration that occurs during the latter part of relaxation, as first observed by Cannell (1986). For this purpose controlled length changes (shortening and elongation) have been imposed on the fibre at different times during the tetanus while the intracellular calcium ion concentration has been monitored by fluo-3. Evidence will be presented to show that the transient increase in myoplasmic free calcium concentration during relaxation is causally related to the non-uniform changes in sarcomere length that normally occur during the pseudoexponential phase of relaxation in a fixed-end tetanus (Cleworth & Edman, 1969, 1972). Some of the results have been presented in a preliminary form (Caputo & Edman, 1991; Edman, Sun, Lou & Caputo, 1992).

METHODS

Preparation and mounting

Single muscle fibres were dissected from the tibialis anterior muscle of *Rana temporaria*. The frogs were killed by decapitation followed by destruction of the spinal cord. After dissection the fibres were mounted horizontally in a thermostatically controlled Perspex chamber between a force transducer (AE801; Aksjeselskapet Mikroelektronikk, Horten, Norway) and a stainless-steel hook fixed to the bottom of the experimental chamber or an arm extending from the moving coil of an electromagnetic puller. Clips of aluminium foil were attached to the tendons (Edman & Reggiani, 1984), and the side parts of the clips were tightly folded around the hooks on the force transducer and at the opposite attachment site. The setting of the clips was carefully adjusted to minimize any lateral, vertical or twisting movements of the fibre during contractile activity, this being an essential procedure in order to avoid movement artifacts in the calcium measurement. Determination of sarcomere length was made in the fibre at rest by direct microscopy at ×400 magnification. For this measurement the spacing taken up by sequences of twenty sarcomeres was determined at two or more places along the fibre, and a mean value of the sarcomere length formed. The overall fibre length was adjusted to provide a resting sarcomere length of 2·2 μm.

Solutions

The Ringer solution had the following composition (mM): NaCl, 115·5; KCl, 2·0; $CaCl_2$, 1·8; $Na_2HPO_4 + NaH_2PO_4$, 2·0; pH, 7·0. The temperature of the bathing fluid ranged between 2 and 5 °C in the different experiments, but was maintained to within $\pm$0·2 °C in any given experiment.

Fluo-3 AM (25 μg, the acetoxymethyl form of fluo-3; Molecular Probes, Eugene, OR, USA) was dissolved in 5 μl dimethyl sulphoxide (DMSO) and 1–2 μl Pluronic F-127 solution containing 75% (w/w) DMSO, and then mixed with about 1·5 ml normal Ringer solution to provide the final solution for loading the dye. According to information given by Molecular Probes, Inc., the purity of fluo-3 AM varied slightly from lot to lot but was stated to be 95–96% as determined by high-performance liquid chromatography.

Stimulation

The fibres were stimulated by passing rectangular current pulses (0·2 ms duration) between two platinum plate electrodes placed symmetrically on either side of the fibre approximately 4 mm apart. The stimulus strength was 15–20% above threshold.

Loading of fluo-3 and measurement of fluorescence

The muscle chamber, of which the bottom was a glass slide, was mounted on the stage of a Zeiss Axiovert 35 microscope equipped with an epifluorescence attachment. The light source was a 100 W mercury lamp driven by a stabilized power supply. The filter combination used for fluo-3 was 450–490/510/520 (excitation/dichroic/barrier). A manual shutter was used to illuminate the fibre only during recording of the light signals. The light signals were collected from an area with a diameter of about 1 mm, which was kept constant during the experiment.

After several isometric tetanic contractions the fibre was loaded with the dye by immersing the fibre at room temperature (20–22 °C) in Ringer solution containing 10–20 μM fluo-3 AM for about 60 min. The fluo-3 AM diffusing into the fibre was hydrolysed by cellular esterases and trapped inside the fibre in the form of fluo-3 (Kao, Harootunian & Tsien, 1989). After the fibre had been loaded with fluo-3, the dye was removed from the bath by perfusing the chamber with ordinary Ringer solution at a rate of about 5 ml min^{-1}. This rate of perfusion was maintained constant throughout the experiment. The fibre was thereafter stimulated to produce a 1 s isometric tetanus at regular 2 min intervals for at least 20 min before the actual experiment was started.

In addition to the light emitted by the calcium–fluo-3 complex, two other sources contributed to the measured light signal: (1) a dye-independent component including autofluorescence from the fibre and light originating from the mercury lamp, i.e. light escaping through the filters; (2) light produced by dye that is not complexed by calcium. Some of the

dye taken up by the fibre may be bound to cellular constituents (i.e. myoplasmic and membranous proteins) and/or taken up by internal organelles. It has been reported that about 70% of fluo-3 was bound to myoplasmic constituents when the dye was pressure injected into the fibre (Hollingworth *et al.* 1990). In the present study, fluo-3 was loaded into the fibre by diffusion from the extracellular fluid, and therefore there might be an additional quantity of dye bound to membrane structures on the fibre surface.

The image of the muscle fibre formed by a ×10 objective lens was collected by an ocular piece (×10) on a phototransistor (bandwidth 3 kHz) whose output was fed into a current-to-voltage converter. The optical signals were fed, together with the output of the force transducer bridge circuit after proper scaling, into a data acquisition and analysis system (Asystant+; Asyst Software Technologies, Inc., Rochester, NY, USA). The data were stored on diskettes for later analysis.

Calibration of fluo-3 signal

The reaction between calcium and fluo-3 is given by:

$$\text{Calcium} + \text{fluo-3} \rightleftharpoons \text{calcium–fluo-3}. \quad (1)$$

At equilibrium the intracellular free calcium concentration, $[Ca^{2+}]_i$, can be estimated from the fluorescence signal (F) by the equation:

$$[Ca^{2+}]_i = ((F - F_{min})k_-)/((F_{max} - F)k_+), \quad (2)$$

in which F_{max} is the maximum fluorescence signal, F_{min} is the fluorescence of the dye itself, and k_- and k_+ denote the off-rate and on-rate constants, respectively, for calcium binding to the dye.

Under dynamic conditions, i.e. during contractile activity, the rate of change of the fluorescence signal, dF/dt, should also be considered:

$$dF/dt = [Ca^{2+}]_i(F_{max} - F)k_+ - (F - F_{min})k_-. \quad (3)$$

In the fibre the value of F contained the resting fluorescence (F_{rest}) and ΔF, the change of fluorescence over and above F_{rest}. Equation (3) can therefore be rewritten as:

$$[Ca^{2+}]_i = \frac{(\Delta F + F_{rest} - F_{min})k_- + dF/dt}{(F_{max} - F_{rest} - \Delta F)k_+}. \quad (4)$$

The free calcium concentration at rest, $[Ca^{2+}]_{iR}$, is given by:

$$[Ca^{2+}]_{iR} = \frac{(F_{rest} - F_{min})k_-}{(F_{max} - F_{rest})k_+}. \quad (5)$$

Substituting $(F_{rest} - F_{min})$ in eqn (4) according to eqn (5) provides the following relation:

$$[Ca^{2+}]_i = \frac{[Ca^{2+}]_{iR}(F_{max} - F_{rest})k_+ + \Delta F k_- + dF/dt}{(F_{max} - F_{rest} - \Delta F)k_+}. \quad (6)$$

The numerical value of k_- for fluo-3 in the myoplasm has been estimated to be 55 s^{-1} at 16 °C (Hollingworth *et al.* 1990). K_d, the dissociation constant of the calcium–fluo-3 complex ($= k_-/k_+$), has been determined to be approximately 0·40 μM in a water solution *in vitro* at 22 °C (range 0·40–0·46 μM; Eberhard & Erne, 1989; Minta *et al.* 1989; Lattanzio & Bartschat, 1991). However, K_d is likely to have a 10 times higher value in the myoplasm inside the fibre (Harkins, Kurebayashi, Hollingworth & Baylor, 1991). Using this assumption, and taking account of the temperature dependence of the on- and off-rate constants (Lattanzio & Bartschat, 1991), the following values of k_- and k_+ were derived and used in the present study (2–5 °C): $k_- = 35\ s^{-1}$, $k_+ = 7\ \mu M^{-1}\ s^{-1}$.

$[Ca^{2+}]_{iR}$ was assumed to be 0·07 μM in accordance with previous intracellular measurements in frog muscle fibres using calcium-selective microelectrodes (Coray, Fay, Hess, McGuigan & Weingart, 1980; López, Alamo, Caputo, DiPolo & Vergara, 1983; Kubota, Hagiwara & Fujimoto, 1990).

$(F_{max} - F_{rest})$ was determined at the end of the experiment by exposing the fibre to a solution containing 0·1 mg ml^{-1} saponine and 95 mM $CaCl_2$. The fluorescence signal rose to a maximum (F_{max}) within approximately 10 s in response to this treatment, after which the fibre began to show signs of deterioration with various degrees of contracture along the length of the preparation. $(F_{max} - F_{rest})$ was determined in eight experiments that were ended by saponine treatment. The peak amplitude of the fluo-3 signal recorded during the isometric twitch in the same experiments, expressed as a fraction of $(F_{max} - F_{rest})$, was determined in each case, and a mean value of this ratio was formed from the eight experiments. The latter value was used as a reference for calibrating the $(F_{max} - F_{rest})$ value in all experiments presented in this study.

Student's t test was used for determinations of statistical significance. All statistics are given as means ± S.E.M.

RESULTS

Fluo-3 signals associated with contractile responses

The loading of fluo-3 into the fibre had no discernible effect on the fibre's capacity to produce force. This is illustrated in Fig. 1, in which two tetani are compared before and after loading of the dye. The two tetani can be seen to be nearly identical, indicating that the accumulation of dye in the

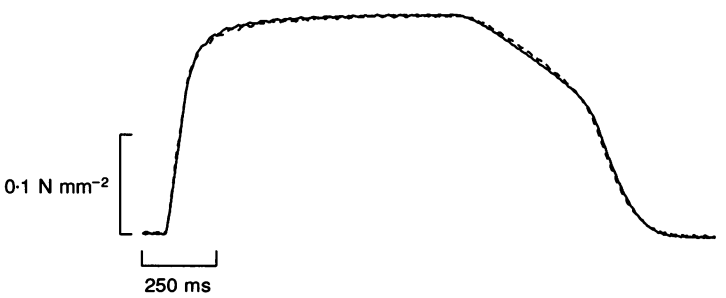

Figure 1. Lack of influence of fluo-3 on contractile response
Superimposed records of isometric tetani of a single muscle fibre before exposure to the dye (continuous line) and after the fibre had been loaded with fluo-3 (dashed line). Sarcomere length, 2·20 μm; temperature, 2·3 °C.

fibre does not add to the calcium buffer capacity to an extent that contractility is affected.

Figure 2 illustrates typical records of tension and fluo-3 fluorescence signals in a fibre stimulated at different frequencies. The records in *A* represent isometric tension while those in *B* show the corresponding fluorescence signals. With increasing stimulation rate there is a stepwise rise of the fluorescence signal. This is not due to a progressive increase of fluorescence with each stimulus, but rather to a steady accumulation of the calcium–dye complex between the stimuli throughout the tetanus period. Following a tetanus or even a single twitch, after the fibre has relaxed completely, the fluorescence signal is maintained at a level higher than the resting level for tens of seconds, indicating that, in agreement with the findings of Cannell (1986) and Hollingworth *et al.* (1990), the basal $[Ca^{2+}]_i$ remains high for a prolonged time. Taking into account the increase in basal $[Ca^{2+}]_i$ during repetitive stimulation, it appears that the peak amplitude of the individual fluorescence signals tends to remain constant or to decrease slightly during a fused or partially fused tetanus.

The complete return of the fluorescence signal to the pre-stimulation level after a resting period is demonstrated in Fig. 2*B*. Here the original resting fluorescence level is indicated by a dashed horizontal line in a series of recordings (*a*–*e*) separated by 2 min resting periods. The peak amplitude of the fluorescence signal evoked by the first stimulus in each record can be seen to be nearly identical in traces *a*–*e*, suggesting that the state of fluo-3, and the distribution of calcium, inside the fibre were the same at the onset of stimulation in all cases. This consistency of the fluo-3 measurement was typically maintained for at least 2 h after the initial run-in period that followed the loading of the dye (see Methods). This clearly indicates that there was no substantial bleaching of the dye over this period. The brief exposure of the fibre to light during the experiment (a few seconds exposure every 2 min in connection with stimulation; see Methods) is likely to account for the constancy of the fluorescence signal.

The records of Fig. 2 also confirm that during repetitive stimulation both the decay phase of the fluorescence signal and the decline of force become progressively slower, as has also been reported for aequorin (Blinks, Rüdel & Taylor, 1978). The slowing of the decay phase was discernible after only one or two twitches and became more pronounced as the stimulation frequency was raised. Finally, a secondary increase of the fluo-3 signal appeared during the decay phase, similar to that observed with aequorin (Cannell, 1986) and fura-2 (Lee *et al.* 1991). The time course of this secondary increase of fluorescence was the same all along the fibre. However, the amplitude of the transient varied slightly from one region to another. This phenomenon will be further described in the following section.

During a single twitch the time to peak fluorescence and the duration of the fluorescence signal at 50% of the peak amplitude (t_{50}) were found to be 25·9 ± 0·7 and 69·6 ± 2·0 ms (means ± S.E.M., $n = 7$), respectively (Table 1). These times

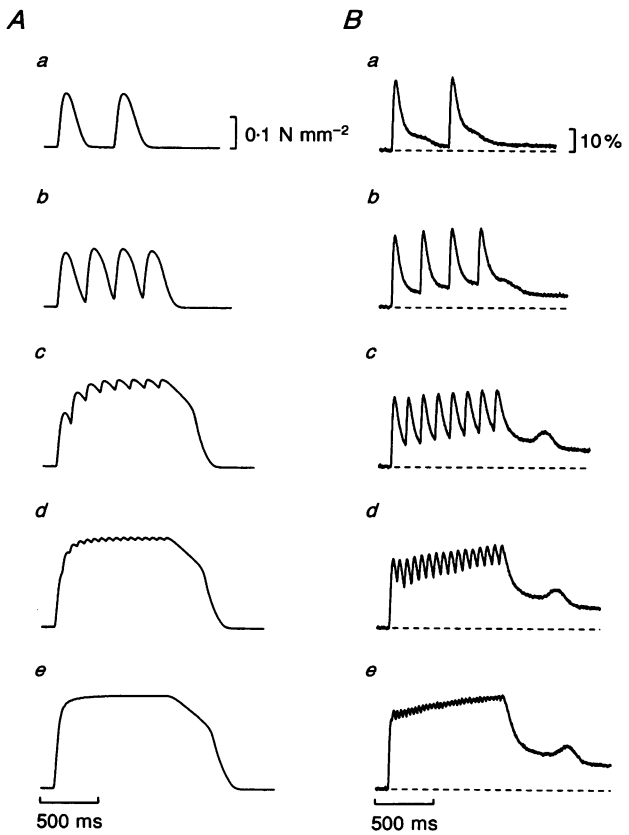

Figure 2. Example records of force and fluo-3 signals at different degrees of mechanical fusion
A, force; *B*, fluo-3 signal. The stimulation frequency was raised to provide a steady increase in mechanical fusion in records *a*–*e*. Dashed lines in *B* indicate resting fluorescence level. Note that (i) the fluo-3 signal is still above the resting level at a time when the isometric force has relaxed completely; and (ii) the fluo-3 signal exhibits a secondary rise during the latter part of relaxation, forming a distinct hump in the fluorescence record as the mechanical fusion is increased and the relaxation phase is prolonged. Sarcomere length, 2·20 μm; temperature, 3·5 °C.

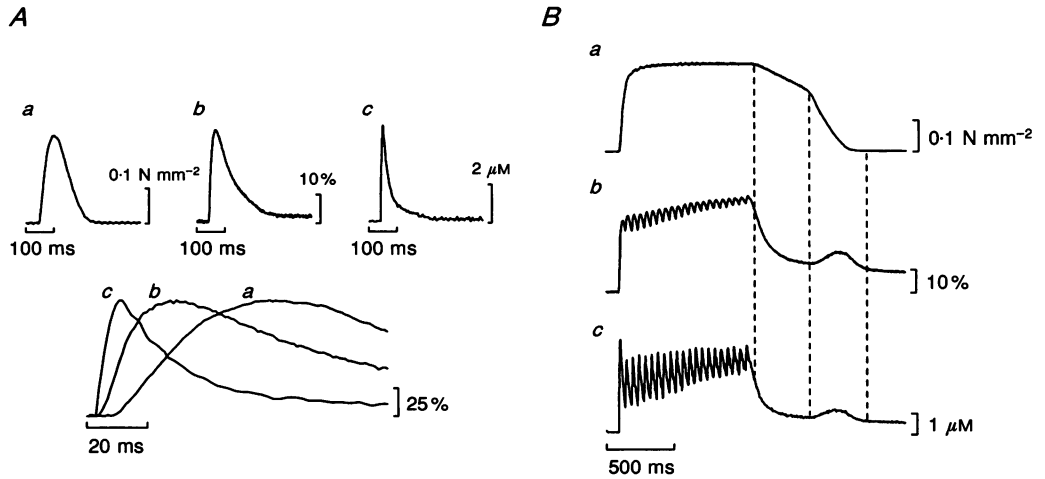

Figure 3. Time relations of isometric force, fluo-3 signal and calculated calcium transient
A, single twitch; *B*, 1 s tetanus. Traces *a*, isometric force; traces *b*, fluo-3 signal; traces *c*, calculated calcium transient. The lower part of *A* shows the three records of the upper portion of *A* superimposed on a faster time base. The vertical dashed lines in *B* indicate the onset of force decline (left), the occurrence of the tension shoulder (middle) and the time of complete relaxation (right). Note that the secondary rise of the fluo-3 signal and of the calculated calcium transient starts at the tension shoulder. Sarcomere length, 2·20 μm; temperature, 3·5 °C (*A*) and 2·7 °C (*B*).

are similar to those recorded with fura-2 (Baylor & Hollingworth, 1988) and aequorin (Blinks *et al.* 1978) but substantially longer than those derived with fast-reacting dyes such as metallochromic (Maylie *et al.* 1987*c*; Baylor & Hollingworth, 1988) and purpurate calcium indicators (Hirota, Chandler, Southwick & Waggoner, 1989; Konishi & Baylor, 1991) and furaptra (Konishi, Hollingworth, Harkins & Baylor, 1991).

Transformation of the fluo-3 signals

Given the relatively low value of approximately 0·40 μM for the K_d of the calcium–fluo-3 complex obtained *in vitro* (see Methods), one might expect that the dye would become saturated with calcium during a twitch or a tetanus. In the present work the peak fluorescence signals obtained during single twitches or tetani were expressed as a percentage of the value of $(F_{max} - F_{rest})$ (see Methods). The fluorescence signal during a twitch was found to be merely 32·2 ± 2·4% ($n = 8$) of $(F_{max} - F_{rest})$, while in the case of a 1 s fused tetanus the corresponding value was 45·2 ± 2·2% ($n = 8$). These results clearly indicate that fluo-3 does not become saturated with calcium during twitch or tetanus. This agrees with the conclusion reached by Harkins *et al.* (1991), who reported that the value of K_d inside the fibre is approximately 10 times the K_d value derived *in vitro*.

Attempts were made to quantify the transient changes in $[Ca^{2+}]_i$ during activity, following the procedure of estimating the free calcium concentration from the fluo-3 signal described in the Methods section. Figure 3 illustrates results from such an analysis based on measurements during twitch and tetanus. Figure 3*A* shows traces of twitch force, fluo-3 signal and calculated free calcium

Table 1. Comparison of fluo-3 signal and calculated calcium transient during isometric twitches in seven isolated muscle fibres

	Fluo-3 signal		Calculated $[Ca^{2+}]_i$ transient		
Experiment	Time to peak (ms)	t_{50}* (ms)	Concentration (μM)	Time to peak (ms)	t_{50}* (ms)
910522	28	73	4·7	9·0	29
911021	24	69	5·9	8·0	17
920304–1	27	70	4·9	9·0	25
920304–2	27	75	4·7	9·0	28
920305	24	63	5·0	9·0	22
920306	24	62	5·3	7·5	22
920312	27	75	5·1	8·5	25
Mean ± S.E.M.	25·9 ± 0·7	69·6 ± 2·0	5·1 ± 0·2	8·6 ± 0·2	24·0 ± 1·5

*t_{50} denotes the duration of the fluo-3 signal and of the calcium transient at 50% of peak amplitude.

concentration. The same traces are shown superimposed (lower traces) on an expanded time base to illustrate further the difference that the transformation procedure introduces in the time course of the calcium signal. The three signals can be seen to reach their peak values 61, 28 and 11 ms after the stimulus. It is notable that the onset of the calcium transient is about 1 ms ahead of the fluo-3 signal. The transformation procedure used to calculate the calcium signal from the fluo-3 signal takes into account the on- and off-rate constants of the reaction between fluo-3 and calcium (see Methods).

Figure 3*B* shows the time relation between force, fluo-3 signal and calculated free calcium concentration, $[Ca^{2+}]_i$, during a 1 s tetanus. In this case, although mechanical fusion was complete, individual transients could still be detected in the fluo-3 signals; after the transformation procedure the individual transients in the calcium signals during the fused tetanus become more distinct.

Table 1 summarizes results obtained in seven fibres. It can be seen that the time to peak fluorescence and the t_{50} value of the fluorescence signal were 26 and 70 ms, respectively, during a single twitch. After transformation of the fluo-3 signal these times were reduced to 8·6 and 24 ms. The mean peak calcium concentration obtained during a twitch was 5·1 ± 0·2 μM ($n = 7$).

Prolonged tetanic stimulation, like repetitive fatiguing stimulation (Edman & Mattiazzi, 1981) is known to increase the relaxation time by slowing the initial linear phase of relaxation and delaying the occurrence of the 'shoulder' in the force myogram (e.g. Hou, Johnson & Rall, 1991). The results shown in Fig. 4 confirm this observation and, furthermore, demonstrate that slowing of relaxation is associated with a slower decay of the myoplasmic free calcium concentration and a later appearance of the secondary rise of $[Ca^{2+}]_i$. As can be seen in Fig. 4, the onset of the secondary rise of $[Ca^{2+}]_i$ coincided with the shoulder of the mechanogram in the three different situations studied. The close relationship between the two events is further illustrated in Fig. 5, which summarizes results from three experiments similar to that shown in Fig. 4. Here the time of onset of the secondary rise of myoplasmic free calcium concentration has been plotted against the time of occurrence of the tension shoulder, both times being measured from the last stimulus. The pooled data in Fig. 5 show that the two measurements correlated well over a 3-fold change of the time elapsing from the last stimulus to the attainment of the tension shoulder. The *peak* of the secondary rise of myoplasmic free calcium concentration occurred when the tetanic force had declined to 12·0 ± 2·5% of the maximal value (measurements performed during 1 s tetani in 10 fibres). Taken together, these results would seem to make clear that the secondary $[Ca^{2+}]_i$ transient is a phenomenon associated with the pseudoexponential phase of relaxation, i.e. the phase starting at the tension shoulder.

Effect of imposed length changes on the calcium signals

Previous experiments have demonstrated that the pseudoexponential phase of relaxation is associated with

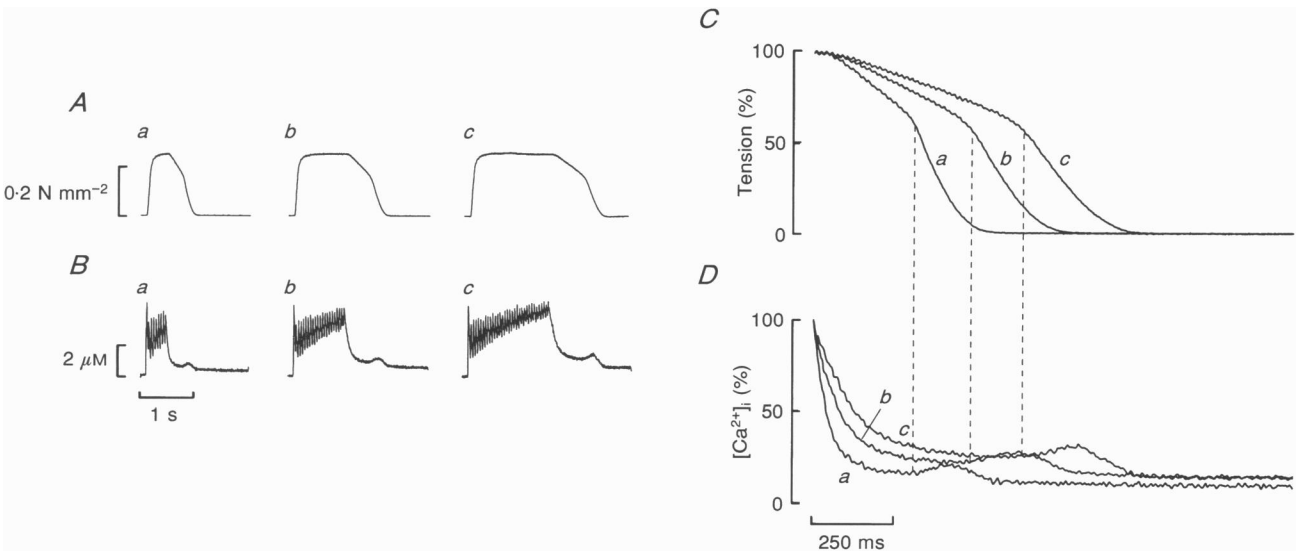

Figure 4. Isometric force and computed $[Ca^{2+}]_i$ during isometric tetani of varied duration
A, force; *B*, $[Ca^{2+}]_i$. Duration of tetanic stimulation: *a*, 0·4 s; *b*, 1·0 s; *c*, 1·5 s. In *C* and *D*, the relaxation phases of the mechanograms, and the corresponding calcium transients, shown in *A* and *B* are superimposed on a faster time base starting from the last peak of the calcium transient in each case. Note the progressive slowing of relaxation as the duration of the tetanus is increased. Also note that the secondary rise of the calcium transient maintains the same relation to the tension shoulder in all three cases, as indicated by the dashed vertical lines. Sarcomere length, 2·20 μm; temperature, 2·9 °C.

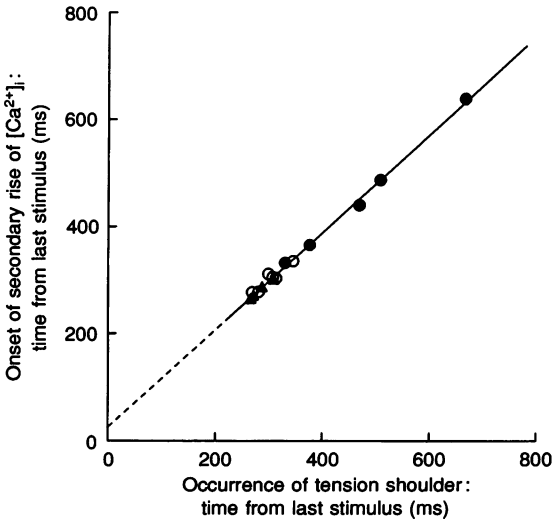

Figure 5. Relationship between the time of onset of the secondary rise of the calcium transient (t_{Ca}) and the time of occurrence of the tension shoulder ($t_{shoulder}$) during isometric relaxation
The data refer to three muscle fibres (identified by different symbols) that were stimulated to produce isometric tetani of different durations. Values denoted by filled circles are from the fibre used in Fig. 4. The straight line is the least-squares regression of t_{Ca} upon $t_{shoulder}$. The slope of this line is 0·91 (correlation coefficient, 0·992; $n = 15$).

non-uniform length changes along the muscle fibre, in that some segments undergo a considerable amount of shortening during this phase while other ('weaker') segments are being stretched (Cleworth & Edman, 1969, 1972; Huxley & Simmons, 1970; Edman & Flitney, 1982). The time course of the secondary calcium transient, described in the preceding section, has a striking resemblance to the time course of the segmental length changes, suggesting that the two phenomena are related. The possibility was considered that the secondary calcium transient might reflect enhanced release of calcium from intracellular binding sites caused by the non-uniform sarcomere movements. In order to test this point experiments were carried out in which slow ramp movements, shortening or stretch, were imposed on the fibre at various times during tetanic activity. Tension and calcium transients were recorded both during the tetanus in which the movement was carried out and during

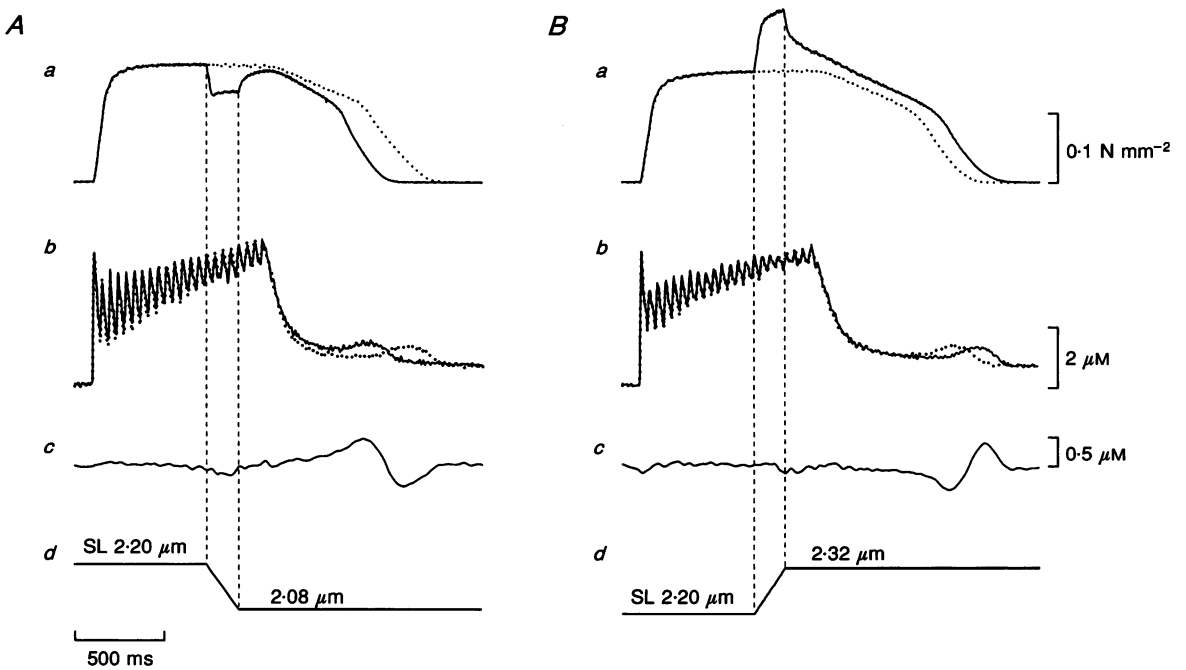

Figure 6. Records of force and calcium transient in a muscle fibre subjected to length changes during the plateau of a fused (1 s) tetanus
A, shortening; *B*, stretch. Traces *a*, force; traces *b*, calcium transient. The superimposed dotted traces in *a* and *b* are isometric control runs performed at the initial sarcomere length (SL). Traces *c* show the computed difference in $[Ca^{2+}]_i$ between test and control runs (see text for further details). Traces *d*, position of puller arm indicating the sarcomere length before and after the movement. Note that the two length changes had no significant effect on $[Ca^{2+}]_i$ during the tetanus plateau. Temperature, 2·9 °C.

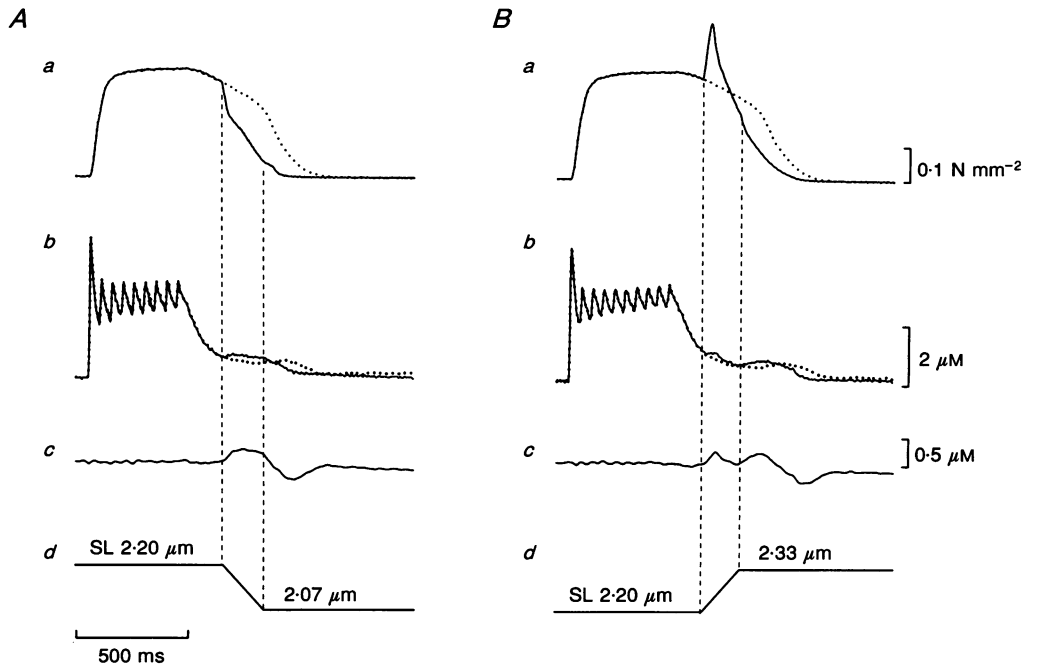

Figure 7. Records of force and calcium transient in a muscle fibre subjected to length changes during the linear phase of relaxation of a fused (0·5 s) tetanus
A, shortening; *B*, stretch. For an explanation of records *a–d*, see legend to Fig. 6. Note that both shortening and stretch performed during this phase of the tetanus led to a momentary rise of $[Ca^{2+}]_i$ during the movement. Temperature, 2·7 °C.

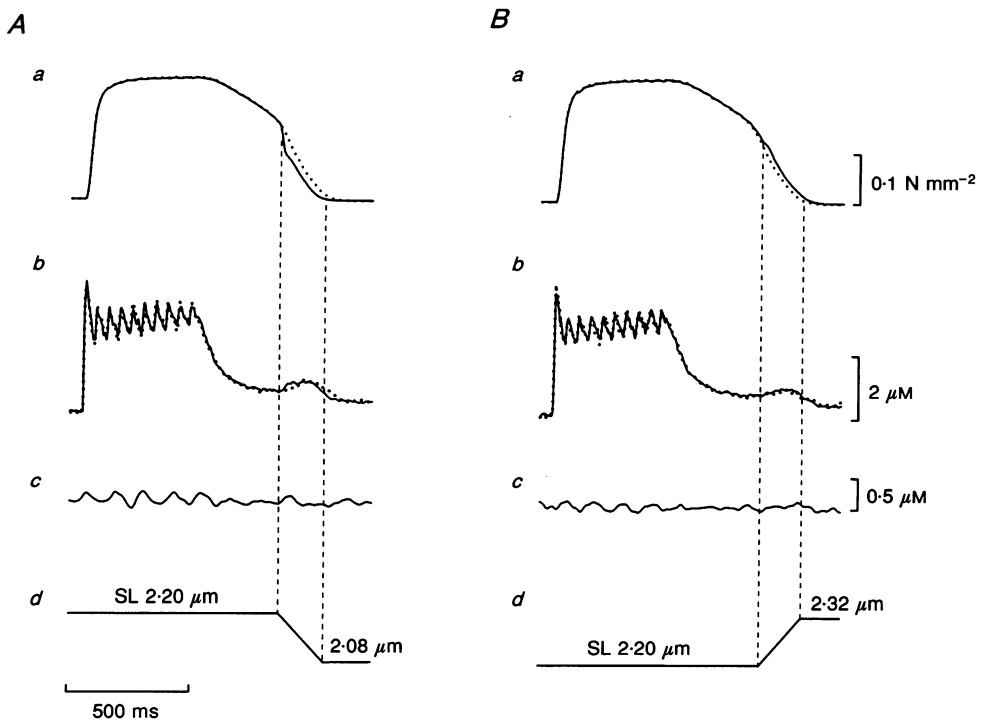

Figure 8. Records of force and calcium transient in a muscle fibre subjected to length changes after the tension shoulder during relaxation of a fused (0·5 s) tetanus
A, shortening; *B*, stretch. For an explanation of records *a–d*, see legend to Fig. 6. Note that the length perturbations, which coincided with the 'hump' of the calcium transient, did not cause any marked additional rise of $[Ca^{2+}]_i$. Temperature, 2·0 °C.

isometric control tetani at the initial and final sarcomere lengths, respectively. The calcium transients derived during the two control runs were, in general, nearly identical. This would seem to exclude the possibility that the observed effects of stretch and shortening upon the calcium transient reflect a spacial variation of the fluorescence signal within the region of fibre investigated. The experiments were performed within a range of sarcomere lengths between 2·05 and 2·35 μm, the amplitude of the length change being 0·05–0·15 μm per sarcomere to correspond to the length changes encountered during the relaxation phase (Edman & Flitney, 1982).

Figure 6*A* shows results from an experiment in which the fibre was shortened (0·36 lengths s^{-1}) from an initial sarcomere length of 2·20 to one of 2·08 μm during the plateau of a tetanus. Superimposed records of tension (*a*) and calcium transients (*b*) obtained during the shortening run and during the isometric control performed at the initial sarcomere length are illustrated. Trace *c* shows the calculated difference in myoplasmic free calcium concentration between test and control runs. For this calculation a mean was formed from the two control runs at the initial and final sarcomere lengths, respectively. Figure 6*B* shows a similar experiment in which the fibre was stretched from a sarcomere length of 2·20 μm to one of 2·32 μm during the tetanus plateau. As is evident from Fig. 6*A* and *B*, neither shortening nor stretch during the tetanus plateau produced any significant change in $[Ca^{2+}]_i$ during the movement. The length perturbations did, however, affect the subsequent relaxation phase, as is illustrated in Fig. 6. Shortening during the tetanus plateau leads to an earlier attainment of the tension shoulder, whereas stretch has the opposite effect. A length change imposed on the fibre during the plateau of tetanus also affects the pattern of segmental length changes that start at the tension shoulder (illustrated in Fig. 2 of Edman, Caputo & Lou, 1993). Since these effects are outside the scope of the present study, they are not further elaborated here.

Figure 7 shows the effects of shortening and stretch when the movements were applied during the linear phase of relaxation before the tension shoulder. It can be seen that both shortening and stretch led to an increase of $[Ca^{2+}]_i$, and to an increased rate of relaxation during the movement, indicating that the release of bound calcium was speeded up. However, there was a clear difference between the effects of the two movements, as can be seen by comparing *A* and *B* in Fig. 7. Thus, whereas shortening caused an increase in $[Ca^{2+}]_i$ that was maintained throughout the movement, stretch induced an initial increase of $[Ca^{2+}]_i$ that was followed by a return towards the pre-stretch level. The results shown in Fig. 7*A* and *B* were confirmed in five experiments altogether, in which the amplitudes of shortening and stretch varied between 0·05 and 0·15 μm per sarcomere. In these experiments the mean increase in $[Ca^{2+}]_i$ during the movement was computed relative to the two control runs performed at the initial and final lengths. This calculation was performed from computerized recordings (see Methods) at a time resolution of 1 ms per point. In the five experiments $[Ca^{2+}]_i$ was found to increase by 0·157 $\pm$ 0·005 μM during shortening and by 0·087 $\pm$ 0·004 μM during stretch, both values being significantly different from zero at the 0·1% level.

Finally, Fig. 8 demonstrates that when shortening and stretch were imposed after the time of the shoulder no clear effects upon $[Ca^{2+}]_i$ were detectable. Furthermore, the two movements can be seen to cause only slight changes of the mechanogram. It is reasonable to presume that the dissociation of calcium from its binding sites starts to increase at the time of the tension shoulder, i.e. when the large sarcomere movements normally begin during relaxation (Cleworth & Edman, 1969, 1972; Huxley & Simmons, 1970). Any additional movement imposed at this time apparently contributes little to the mobilization of bound calcium.

DISCUSSION

Fluo-3 has recently been used for monitoring calcium signals in muscle fibres (Garcia *et al.* 1989; Hollingworth *et al.* 1990). The advantages of this dye for the application of imaging techniques have recently been highlighted by Vergara *et al.* (1991). In this work we show that fluo-3 can be successfully used for monitoring calcium transients during contractile activity. The large fluorescence changes that occur during contractile activation, compared with the low fluorescence at rest, makes the fluo-3 signal relatively insensitive to fibre movements and also makes the signal relatively free of noise. Furthermore, the fact that binding of calcium to the dye does not involve a spectral shift makes it feasible to obtain information on the calcium transient from a single response. However, the lack of spectral shift of the calcium–fluo-3 complex precludes a calibration method that is based on the ratio of the signals obtained at two wavelengths. This disadvantage may be circumvented by using other, somewhat less convenient calibration procedures. Finally the long wavelength excitation and emission characteristics of this dye allows the use of conventional optics.

The dissociation constant (K_d) of the calcium–fluo-3 complex has been stated to be 0·4 μM when measured *in vitro* (for references, see Methods). However, a 10-fold higher value of K_d is likely to exist in the myoplasm, as estimated by Harkins *et al.* (1991). The latter value of K_d is in accord with the present observation that the calcium–fluo-3 signal is highly sensitive to changes of intracellular Ca^{2+} concentration within the range of concentrations (1–10 μM) dealt with during contractile activity. In line with this view, the fluorescence signal recorded during fused tetani was found to be

approximately 45% of the maximal fluorescence obtained after saponine treatment in the presence of high calcium concentrations.

An apparent disadvantage of fluo-3, when compared with other, fast-reacting dyes (see earlier), is the relative slowness of the fluorescence signal. However, this can be adequately corrected by taking account of the on- and off-binding rates of the calcium–fluo-3 complex (for details, see Methods). After correction, the time to peak amplitude and the t_{50} value of the calcium signal were 8·6 and 24 ms, respectively, at 2–5 °C. These numbers accord reasonably well with the values of the same parameters obtained with the fast-reacting dyes antipyrylazo III (Maylie *et al.* 1987*c*; Baylor & Hollingworth, 1988), purpurate-3,3′ diacetic acid (PDAA) (Hirota *et al.* 1989; Konishi & Baylor, 1991), tetramethylmurexide (TMX) (Maylie, Irving, Sizto, Boyarsky & Chandler, 1987*a*; Konishi & Baylor, 1991) and the fluorescent dye furaptra (Konishi *et al.* 1991). With the latter dyes, the time to peak amplitude and the t_{50} value of the calcium–dye signal were reported to range from 5 to 7 ms and from 6 to 15 ms, respectively, at 16–18 °C.

Using other indicators, such as antipyrylazo III or furaptra, several previous authors have reported that during tetanic stimulation, peak fluorescence decays after the first transient, after which the individual peaks reach a constant level (Maylie *et al.* 1987*c*; Konishi *et al.* 1991). The present results confirm that during mechanical fusion in an isometric tetanus the individual transients remain clearly distinguishable. With continued tetanic stimulation, however, there is a clear tendency for increased fusion of the calcium transients and a steady rise of the individual peaks.

Using aequorin with improved resolution techniques, Cannell (1986) demonstrated that after the end of a tetanus the calcium concentration stays above the resting level for a relatively long (several seconds) time. A similar observation has been reported by Lee *et al.* (1991) using fura-2. Our own results, based on measurements with fluo-3, confirm the slow return of the free calcium concentration to the resting value after a tetanus. Our results further show that even after a single twitch the level of calcium is maintained above the resting value for several hundreds of milliseconds (see also Konishi *et al.* 1991). This accords with the slow restoration of the sarcomere pattern after a twitch or a tetanus, as monitored by the laser diffraction spectrum recorded from a single muscle fibre (Edman, 1980; Edman & Flitney, 1982).

The progressive decrease in the rate of decay of the free calcium concentration during repetitive stimulation (Fig. 2) suggests that even after a single twitch the short-term calcium sequestering capacity is strained and becomes less and less effective as stimulation goes on. Parvalbumin is generally thought to act as a temporary sink in the myoplasm before calcium is taken up by the sarcoplasmic reticulum (Gillis, 1985). However, the calcium binding capacity of parvalbumin in the myoplasm is likely to be limited (Hou *et al.* 1991). Thus, by progressively filling up the calcium binding sites of parvalbumin during repetitive stimulation, the rate of decay of the free calcium concentration can be expected to be steadily reduced in accord with the experimental observations reported here. The calcium pump of the sarcoplasmic reticulum is apparently unable to compensate for the accumulation of free calcium in the myoplasm under these conditions. The possibility also exists that the effectiveness of the calcium pump is steadily reduced during repeated stimulation.

The transitory rise of $[Ca^{2+}]_i$ that occurs during force relaxation (Cannell, 1986) has been further elucidated in the present study. Although this component is most clearly visible after tetanic stimulation, it can be demonstrated in a single twitch as well (Fig. 2*B*, trace *a*). Our results show that this component is closely related to the pseudo-exponential phase of relaxation, i.e. the phase associated with non-uniform sarcomere length changes within the fibre (Cleworth & Edman, 1969, 1972). This component is reduced by fatigue, lowered temperature and increased sarcomere length (C. Caputo & K. A. P. Edman, unpublished data). These interventions are all known to reduce the non-uniform sarcomere length behaviour during the same phase of relaxation (Edman & Flitney, 1982; Curtin & Edman, 1989), indicating that the secondary rise of the calcium transient is closely linked with the sarcomere movements. The present results demonstrate that length changes imposed during relaxation, before the tension shoulder, lead to a transitory increase of the intracellular free calcium concentration. Shortening was found to be most effective in this respect, causing a steady increase of the calcium ion concentration throughout the movement. However, sarcomere elongation also had a clear effect by inducing an initial brief hump of the calcium transient. These effects could mean that the relative movement of the thick and thin filaments during relaxation promotes the release of calcium from the troponin binding sites on the thin filaments. In view of the large sarcomere movements that normally occur after the tension 'shoulder', a sizeable amount of calcium is likely to be released into the myoplasm by this mechanism. Due to the limited calcium-buffering capacity of the myoplasm (see earlier), the free calcium concentration will therefore increase temporarily before returning towards the resting level.

Enhanced release of calcium from the actin filament, due to reduced affinity of troponin for calcium, would seem to be a plausible cause of the transient rise of $[Ca^{2+}]_i$ during relaxation. This is in line with the observation that the 'calcium hump' is not associated with a transient increase in tension but, instead, with a more rapid decline of force. Another possibility worth considering would be that the sliding of the filaments during relaxation leads to a more rapid release of calcium bound to the myosin light chains (e.g. Holroyde, Potter & Solaro, 1979). There is no reason to

believe, on the other hand, that the intrinsic fibre movements would mobilize calcium from the sarcoplasmic reticulum. This is inferred from the fact that there was no significant effect on the calcium transient when movements were performed during the tetanus plateau (Fig. 6) or when the fibre was relaxed (K. A. P. Edman, F. Lou & Y.-B. Sun, unpublished observations).

As previously demonstrated (Huxley & Simmons, 1973; Edman, 1980), the non-uniform behaviour of the sarcomeres along the muscle fibre during relaxation leads to a more rapid decline of force than would occur if the entire fibre behaved uniformly. Stronger segments will shorten slowly during the initial, linear phase of relaxation at the expense of weaker segments which are elongated to a point where the cross-bridges start to slip. Once this slippage of cross-bridges has started in a given segment during the relaxation phase, the ability of this segment to resist stretch is drastically reduced, resulting in the fast, exponential decay of force that occurs after the tension shoulder. Thus, on purely mechanical grounds, the non-uniform behaviour of the sarcomeres along the fibre can be presumed to abbreviate the relaxation phase during an isometric twitch or tetanus (Edman, 1980).

The present results point to still another mechanism by which the non-uniform length changes along the fibre may affect the relaxation time. Our data suggest (see above) that shortening and also, to some extent, elongation of the sarcomeres during the relaxation phase enhances the dissociation of calcium from the troponin binding sites on the thin filament. This effect may be regarded as a positive feedback mechanism that is initiated by the non-uniform sarcomere behaviour and that further promotes the decay of force during relaxation.

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