Intracellular calcium and force in single mouse muscle fibres following repeated contractions with stretch

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1. The role of the myoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the reduction of muscle force following contractions with stretch was investigated in single fibres from mouse toe muscle. Muscle fibres were either stretched by 25% of their optimum length (L_o) for ten tetani (Protocol I) or stretched by 50% of L_o for between ten and thirty tetani (Protocol II). Indo-1 was used to measure $[Ca^{2+}]_i$.

2. In each protocol the stretch series was compared with isometric controls; the stretch series always resulted in greater changes in muscle properties than in the isometric controls. The observed changes were (i) reduced tetanic force, (ii) reduced tetanic $[Ca^{2+}]_i$, (iii) increased resting $[Ca^{2+}]_i$ and (iv) the greater relative reduction in force at low stimulus frequencies (30 and 50 Hz) compared with high (100 Hz). These changes were maintained for up to 60 min.

3. Stretching a resting muscle fibre had no effect on the subsequent $[Ca^{2+}]_i$ or force.

4. Following Protocol I 10 mM caffeine restored tetanic force to pre-stretch levels. Tetanic $[Ca^{2+}]_i$ *vs.* force curves were constructed pre- and post-stretch and showed that neither the maximum Ca^{2+}-activated force nor the Ca^{2+} sensitivity of the muscle fibres post-stretch was significantly different from control. The force reduction, therefore, appears to be the result of reduced tetanic $[Ca^{2+}]_i$.

5. The more severe stretching regimen of Protocol II resulted in a much greater reduction in force than Protocol I. Ten millimolar caffeine did not restore control force. Comparison of the $[Ca^{2+}]_i$–force relationships pre- and post-stretch showed that the reduction in tetanic force was caused by a combination of a reduced tetanic $[Ca^{2+}]_i$, reduced maximum Ca^{2+}-activated force and reduced Ca^{2+} sensitivity.

6. Following both protocols the resting $[Ca^{2+}]_i$ showed a small rise which persisted for at least 60 min. This elevated $[Ca^{2+}]_i$ was associated with a reduction in the pump rate of the sarcoplasmic reticulum Ca^{2+} pump.

7. This study establishes that reduced Ca^{2+} release and reduced Ca^{2+} sensitivity contribute to the reduction in force generating capacity of single mammalian muscle fibres following active stretches.

Hough (1902) recognized the condition of exercise-induced muscle damage which followed prolonged and unaccustomed exercise and was characterized by muscle pain, tenderness and weakness which took a number of days to recover. In recent years it has become apparent that relatively short periods of exercise can induce a similar condition, particularly when untrained muscles are stretched during contraction. A familiar example is the stiffness, soreness and weakness of the quadriceps muscle that can follow walking down a mountain by an untrained individual. This condition is called stretch-induced muscle injury or eccentric exercise-induced muscle injury (for review see Armstrong, Warren & Warren, 1991). The main consequences of stretch-induced muscle injury are (i) reduced force, particularly at low stimulus frequencies, which occurs immediately after the exercise (Davies & White, 1981), (ii) pain in the affected muscle prominent 1–2 days after the event (Newham, Jones & Clarkson, 1987), and (iii) muscle degeneration which develops over a few days and is characterized by disorganization of the sarcomere pattern, release of intracellular proteins, and infiltration of the muscle by inflammatory cells (Armstrong, Ogilvie & Schwane, 1983; Newham, McPhail, Mills & Edwards, 1983). Everyday experience and

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experiment both show that stretch-induced muscle injury can be dramatically reduced by training (Newham *et al.* 1987; Balnave & Thompson, 1993).

The present study is concerned with the early phase of stretch-induced muscle injury and with the possible mechanisms which lead to the observed reduction in force. It seems likely that the myofibrillar disorganization contributes to the early fall of force and several studies have described the disruption in sarcomere organization which can be observed immediately after stretch-induced muscle injury (Newham *et al.* 1983; Armstrong *et al.* 1983; Wood, Morgan & Proske, 1993). However, several lines of evidence suggest that reduced Ca^{2+} release also contributes to the fall of force. (i) It has been shown that caffeine contractures in muscles injured by stretch can restore the normal force (Warren, Lowe, Hayes, Karwoski, Prior & Armstrong, 1993). (ii) Muscles injured by stretch exhibit a greater relative reduction of force at low stimulus frequencies than at high (low-frequency fatigue) and this pattern of force reduction can be caused by reduced Ca^{2+} release (Edwards, Hill, Jones & Merton, 1977; Westerblad, Duty & Allen, 1993).

In the present study we test the hypothesis that a component of the early contractile changes which occur in stretch-induced injury arises from changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). An important issue is to distinguish between contractile changes due to the repeated activity (fatigue) from those caused by the superimposed stretch. This was done by using isometric tetani as controls; it is established that the energy consumption of isometric tetani is greater than with tetani in which a stretch occurs (Woledge, Curtin & Homsher, 1985) so that isometric tetani should represent a more than adequate control for the metabolic changes associated with activity. Only those changes in contractile performance following the stretch series which were in excess of those following the isometric series are regarded as being a consequence of stretch.

Single mouse muscle fibres were stretched during repeated tetani and force and $[Ca^{2+}]_i$ were measured before, during, and after the period of stretching. Using methods developed in previous studies of fatigue (Westerblad & Allen, 1991) we have analysed the reduced force into three components: (i) a reduction in maximum Ca^{2+}-activated force, (ii) reduced tetanic $[Ca^{2+}]_i$ and (iii) reduced Ca^{2+} sensitivity. The reduced maximum Ca^{2+}-activated force is probably a consequence of myofibrillar damage (e.g. Brown & Hill, 1991). We also found a small, persistent elevation of resting $[Ca^{2+}]_i$ following the stretching regime. Our results support the hypothesis that reduced Ca^{2+} release and reduced Ca^{2+} sensitivity contribute to the impaired mechanical performance which follows stretch.

A preliminary account of these experiments has been communicated to the Physiological Society (Balnave & Allen, 1994*a*).

METHODS

Single muscle fibres were dissected from the flexor brevis muscle of male mice which were killed by rapid cervical dislocation. Details of fibre dissection and mounting have been described previously (Westerblad & Allen, 1991). The fibre was superfused at room temperature (22 °C) with the following solution (mM): 121·0 NaCl, 5·0 KCl, 1·8 $CaCl_2$, 0·5 $MgCl_2$, 0·4 NaH_2PO_4, 24·0 $NaHCO_3$, 5·5 glucose. Fetal calf serum (0·2%) was added to the solution, which was bubbled with 95% O_2 and 5% CO_2, giving a pH of 7·3. The fibre was mounted between an Akers AE 801 force transducer (SensoNor, Horten, Norway) and the arm of a motor (Model 300H, Cambridge Technology, Cambridge, MA, USA). This allowed known length changes to be imposed on the muscle fibre. A ramp generator was used to determine the pattern of the length change. The fluorescent calcium indicator indo-1 was microinjected into the fibre to a concentration of about 100 μM and was used to measure $[Ca^{2+}]_i$. The fibre was illuminated at 360 nm and the resulting fluorescence measured at 400 and 505 nm. The ratio of 400/505 nm was calculated by an analogue divide circuit and the ratio later converted to $[Ca^{2+}]_i$ using the *in vivo* calibration procedure described in Westerblad & Allen (1993).

Most of the measurements were taken before or after the series of stretched contractions but measurements were also taken during the stretch series to determine whether stretch caused a sudden change in tetanic $[Ca^{2+}]_i$ (see Fig. 1). During such contractions the fraction of the fibre in the measurement window decreases and the signal at both 400 and 505 nm decreases. Control measurements in which the measurement window was suddenly reduced during a contraction showed that, while the signal at each measurement wavelength decreased, the ratio signal was largely unaffected. Thus, movement artifacts are largely eliminated in the ratio signal.

In some experiments the distribution of the elevated resting $[Ca^{2+}]_i$ along the fibre was examined. The measurement window of the photomultiplier tube was reduced so that fluorescent signals from only 100 μm of the fibre were recorded. The muscle chamber was moved so that the whole length of the resting fibre gradually passed under the window while recording the resting indo-1 ratio.

A camera recorded the image of the fibre in red light throughout the experiments. This image was continuously displayed on a monitor so that any non-uniformities which developed during contraction could be observed.

Experimental protocol

The force–frequency relationship of each muscle fibre was established by producing isometric tetani at 30, 50, 70 and 100 Hz with 1 min of rest between each tetanus. In some experiments the maximum force of the fibre was elicited with a 100 Hz tetanus in the presence of 10 mM caffeine. In the first set of experiments (Protocol I) the force–frequency relationship was analysed before and 10 min after a series of ten 100 Hz tetani. Each tetanus was 350 ms in duration with a 4 s interval between tetani. This procedure was performed first at constant length (isometric series) and, second, with a stretch starting 200 ms after the start of the tetanus (stretch series). The fibre was returned to its original length 150 ms after the end of the tetanus (see Fig. 1) and this cycle was repeated for each of the ten tetani. The fibres in the present study usually had some adherent damaged fibres on the surface making it difficult to measure sarcomere spacing. For this reason length changes were determined with respect to the length at which tetanic force was maximal (L_o, typically 800 μm). In Protocol I the fibres were stretched from L_o by 25% L_o over 50 ms,

which is equivalent to five muscle lengths per second. The second set of experiments (Protocol II) was similar in design but the stretching protocol was more severe. To ensure that the preparation was on the descending limb of the length–force curve these experiments were performed at a starting length of $L_o + 100\ \mu m$. The muscle fibre was stretched by 50% L_o over 100 ms, which again corresponds to five muscle lengths per second. In Protocol II two stimulus patterns were used. In one pattern the muscle fibre was given ten tetani in the isometric and the stretch series. In the second pattern tetanic stimulation was continued in the isometric series either until thirty tetani had been performed or until force had fallen to 50%. The same number of tetani were then performed in the stretch series. The force–frequency relationship was analysed before and 10, 30 and 60 min post-stimulation for both the isometric and stretch series.

Terminology

In this study we describe changes in muscle properties following the isometric series as 'fatigue' and changes in muscle properties following the stretch series which are greater than those following the isometric series are described as 'stretch induced'.

Statistics

Data are quoted as means ± S.E.M.; the number of experiments is given as n. Paired or unpaired Student's t tests, as appropriate, were used to verify statistical significance with $P < 0.05$ taken as significant.

RESULTS

Figure 1 illustrates the force and $[Ca^{2+}]_i$ record of a representative single muscle fibre during a tetanus in which a stretch of 25% L_o was imposed on the fibre. In this fibre there was the suggestion of an increase in tetanic $[Ca^{2+}]_i$ following the stretch, but there was no consistent change in the twenty-seven fibres used in this study. Following the stretch series one of the fibres showed obvious localized damage immediately after stretch and rapidly became inexcitable. The remaining twenty-six fibres showed variable reductions in force but the force generally remained more-or-less stable over 1 h and there was no change in the stimulus threshold. When fibres were viewed in the microscope or on the monitor, the isometric tetanic contractions appeared uniform with no signs of localized shortening or stretching.

Protocol I

Previous studies on this preparation (Lännergren & Westerblad, 1991; Westerblad & Allen, 1991; Westerblad *et al.* 1993) have demonstrated that between thirty and several hundred isometric tetani are required to reduce force to 30% of control. Although most of the force recovers quickly, there is a greater reduction in the force at low stimulus frequencies compared with high which persists for at least 1 h. To minimize such effects of fatigue, only ten tetani were performed in Protocol I. Under these stimulating conditions isometric force declined to about 88% of control on the 10th tetanus and exhibited only small reductions in force after a 10 min rest, consistent with earlier findings.

To establish that single fibres of mouse skeletal muscle develop stretch-induced injury following stretch during contraction it was necessary to demonstrate (i) reduced tetanic force and (ii) greater reduction of force at low stimulus frequencies than high and (iii) that any such effects are greater than those occurring during the isometric controls. Considering the latter point first, the isometric series produced only small reductions in force at 50, 70 and 100 Hz (open circles, Fig. 2*B*) and there were no signficant changes in $[Ca^{2+}]_i$ as shown by Fig. 3*B* (open circles). Figure 2*A* shows the force recorded from a representative fibre at various stimulation frequencies before and 10 min

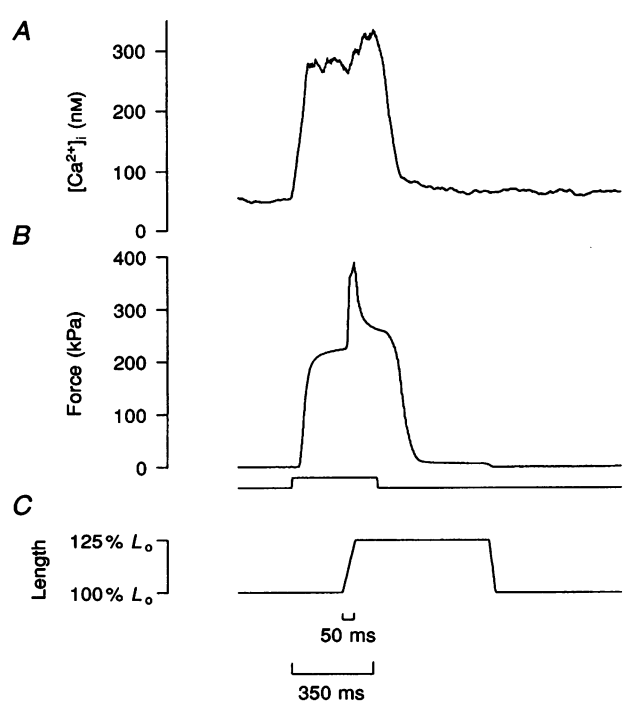

Figure 1. Record from a 350 ms, 100 Hz tetanus with a stretch equivalent to 25% of the optimum fibre length applied 200 ms into the tetanus

A, $[Ca^{2+}]_i$ during the tetanus. *B* shows the force produced. The stimulation period is indicated below the force record. *C*, length of the muscle fibre as a percentage of the optimum fibre length (L_o). The stretch duration was 50 ms and the fibre was held at the longer length for 500 ms.

after the stretch series. Although the force was reduced at all frequencies 10 min post-stretch, the relative reduction was greater at low frequencies of stimulation. For example, at 100 Hz muscle fibre force was reduced to 82% of pre-stretch control, while at 30 Hz force was reduced to 64%. The combined results of 12 experiments are shown in Fig. 2*B* (filled points). The data are presented as the ratio of force before and after the stretch series at each stimulus frequency and show large reductions in force at the low frequencies (30, 50 and 70 Hz) and a moderate reduction at 100 Hz. Since the force generated by the muscle fibres at 100 Hz is not maximal (Westerblad & Allen, 1991), 10 mM caffeine was added to the perfusate and a 100 Hz tetanus was produced. Caffeine was able to restore the maximum Ca^{2+}-activated force of the muscle fibres to a level which was not significantly different from the control.

Figure 3*A* shows the tetanic $[Ca^{2+}]_i$ in the same representative muscle fibre. Tetanic $[Ca^{2+}]_i$ was reduced by about the same relative amount at all frequencies of stimulation following the stretch series. The averaged results of twelve experiments are presented in Fig. 3*B*. Tetanic $[Ca^{2+}]_i$ was reduced at all frequencies following stretch, while there was no significant reduction in tetanic $[Ca^{2+}]_i$ following the isometric series. Stimulation (100 Hz) in the presence of 10 mM caffeine induced a substantial increase in $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ enabled the fibre to produce the same maximum muscle force post-stretch as was obtained pre-stretch (Fig. 2*A*).

We have established that there was no significant change in maximum Ca^{2+}-activated force following Protocol I but that tetanic $[Ca^{2+}]_i$ was reduced. In order to ascertain whether a change in Ca^{2+} sensitivity of the myofibrillar proteins took place following stretch, the $[Ca^{2+}]_i$ *vs.* force curve was plotted using the tetanic $[Ca^{2+}]_i$ and force values of each muscle fibre at 30, 50, 70 and 100 Hz, as well as at rest and at 100 Hz in the presence of 10 mM caffeine (Westerblad & Allen, 1993). These points were then fitted to the following Hill equation:

$$P = P_{max}[Ca^{2+}]_i^N / (Ca_{50}^N + [Ca^{2+}]_i^N), \quad (1)$$

where P is the relative force, P_{max} is the force at saturating $[Ca^{2+}]_i$, Ca_{50} is the $[Ca^{2+}]_i$ giving 50% of P_{max}, and N is a constant related to the steepness of the relationship. Figure 4*A* shows such a curve pre- and 10 min post-

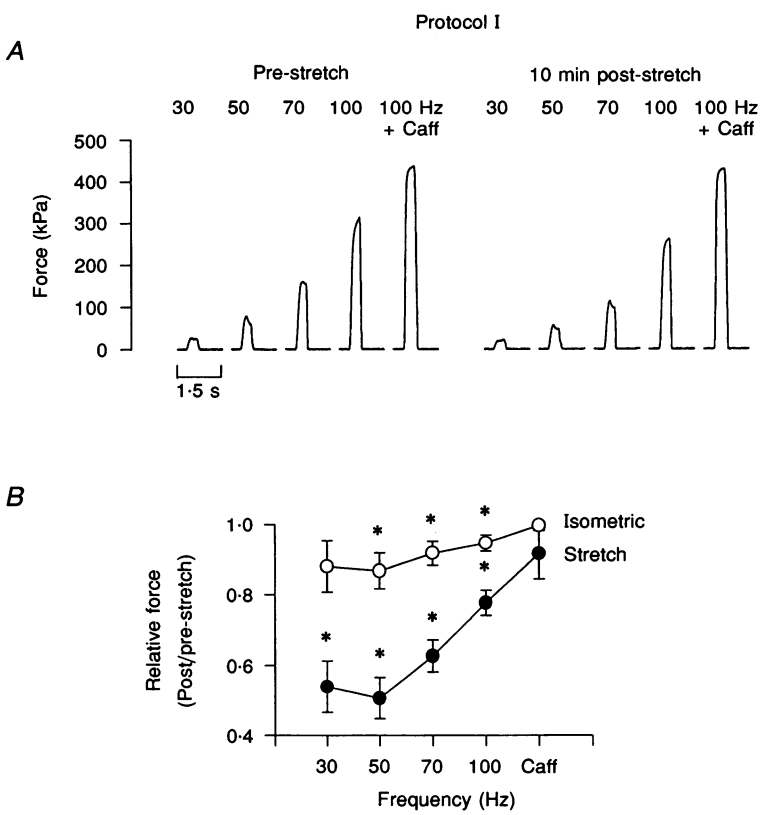

Figure 2. Force at various stimulus frequencies following isometric tetani or stretch

A, force–stimulus frequency relationship from 1 fibre before and 10 min after the stretch series of Protocol I. Post-stretch force was reduced at all frequencies apart from the 100 Hz tetanus in 10 mM caffeine. *B* shows this relationship in 12 experiments plotted as a ratio of 10 min post-stimulation force to pre-stimulation force. The caffeine (Caff) data was only available from 5 experiments. ○, the isometric series; ●, the stretch series; bars, S.E.M.; * points which were significantly different from 1·0 ($P < 0·05$).

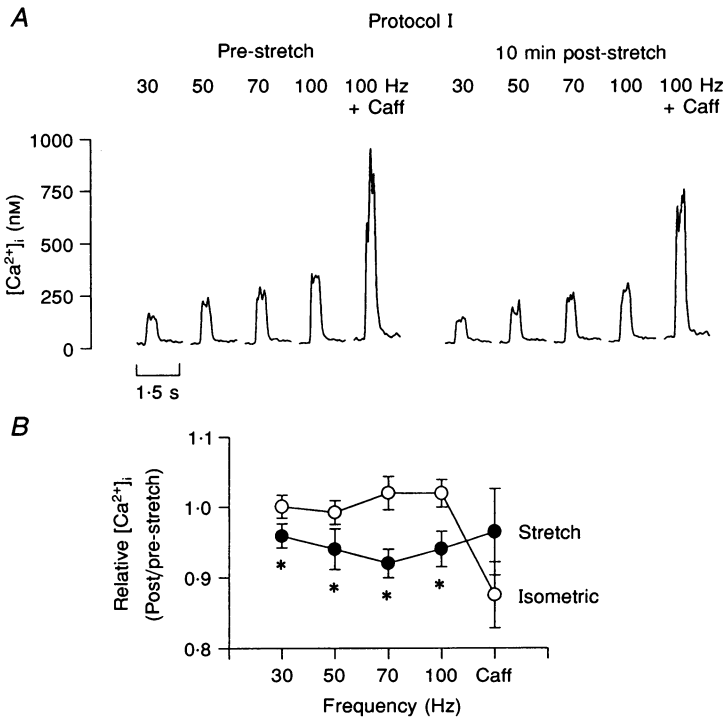

Figure 3. $[Ca^{2+}]_i$ during tetani at various stimulus frequencies before and after the stretch series

A, tetanic $[Ca^{2+}]_i$–stimulus frequency relationship from the same preparation as Fig. 2. Post-stretch $[Ca^{2+}]_i$ was reduced at all frequencies. *B* shows this relationship in 12 experiments plotted as a ratio of 10 min post-stimulation $[Ca^{2+}]_i$ to pre-stimulation $[Ca^{2+}]_i$. The caffeine data was only available from 5 experiments. ○, the isometric series; ●, the stretch series; bars, S.E.M.; * points which were significantly different from 1·0 ($P < 0·05$).

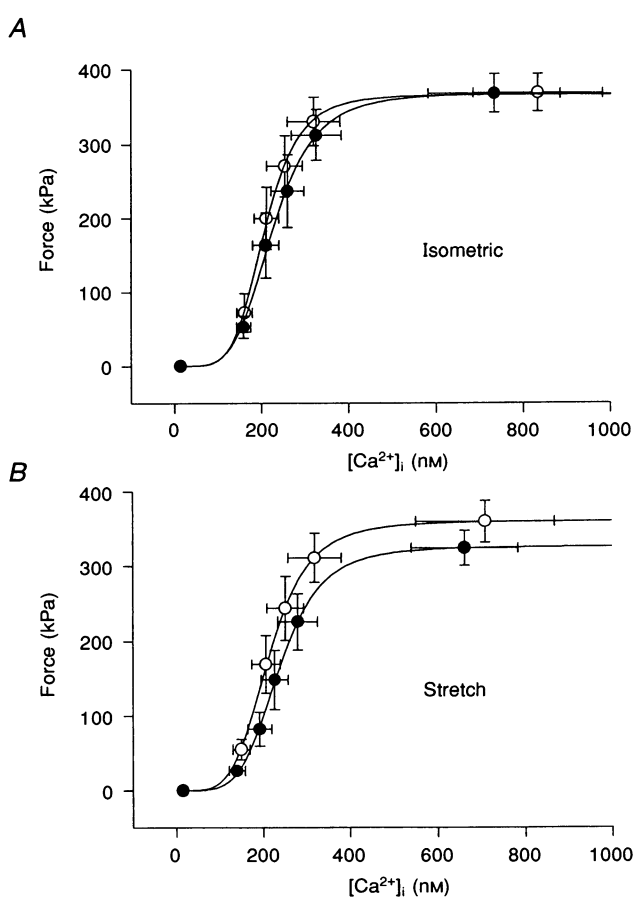

Figure 4. Force *vs.* $[Ca^{2+}]_i$ relation pre-stimulation (○) and post-stimulation (●) for the isometric series (*A*) and the stretch series (*B*)

Data points are the means from 5 experiments and bars represent S.E.M. Curves were obtained by fitting the data points to eqn (1). The fitting parameters for the stretch series are given in Table 1.

Table 1. Changes in the $[Ca^{2+}]_i$–force relationship following the stretch protocol

$[Ca^{2+}]_i$ –force relation	Pre-Stretch	Post-Stretch
Protocol I		
P_{max} (kPa)	359 ± 27	326 ± 20
Ca_{50} (nM)	217 ± 29	234 ± 19
N	6·38 ± 0·78	5·98 ± 0·34
Protocol II		
P_{max} (kPa)	298 ± 6	179 ± 15*
Ca_{50} (nM)	219 ± 16	273 ± 17*
N	4·86 ± 0·28	3·27 ± 0·19*

Parameters describing the $[Ca^{2+}]_i$–force relationship pre-stretch and 10 min post-stretch for Protocol I ($n = 5$) and pre-stretch compared with the combined 10, 30 and 60 min post-stretch for Protocol II ($n = 4$). * Significantly different from pre-stretch level ($P < 0·05$).

isometric stimulation for Protocol I. There was no significant difference between these curves. Similarly, Fig. 4*B* shows that no significant difference existed between the $[Ca^{2+}]_i$ *vs.* force curves pre- and 10 min post-stretch (for mean data from five fibres see Table 1). This indicates that the reduction in muscle fibre force following ten active stretches of 25% L_o appears to be exclusively due to the reduction in tetanic $[Ca^{2+}]_i$.

Protocol II

Since there was no significant decrease in maximum Ca^{2+}-activated force following stretch in Protocol I (Fig. 4*B*) despite evidence of myofibrillar disruption from other studies (Newham *et al.* 1983; Armstrong *et al.* 1983; Wood, Morgan & Proske, 1993), a more severe stretching protocol was performed in Protocol II. When only ten tetani were used in the isometric series, the tetani after 10 min of recovery showed no significant change from control (open circles, Fig. 5*B*). When the longer series of tetani were used, an average of twenty-eight tetani were generated and on the last of these tetani the isometric force was reduced to about 58% of control. Most of this fatigue recovered within the first 10 min but there was a small but significant reduction in the force at 50 Hz after 10 min recovery in the isometric series (not shown). This is consistent with our earlier study of low frequency fatigue following isometric contractions (Westerblad *et al.* 1993).

As expected, force loss was greater following severe stretching than it was after Protocol I. The force record of

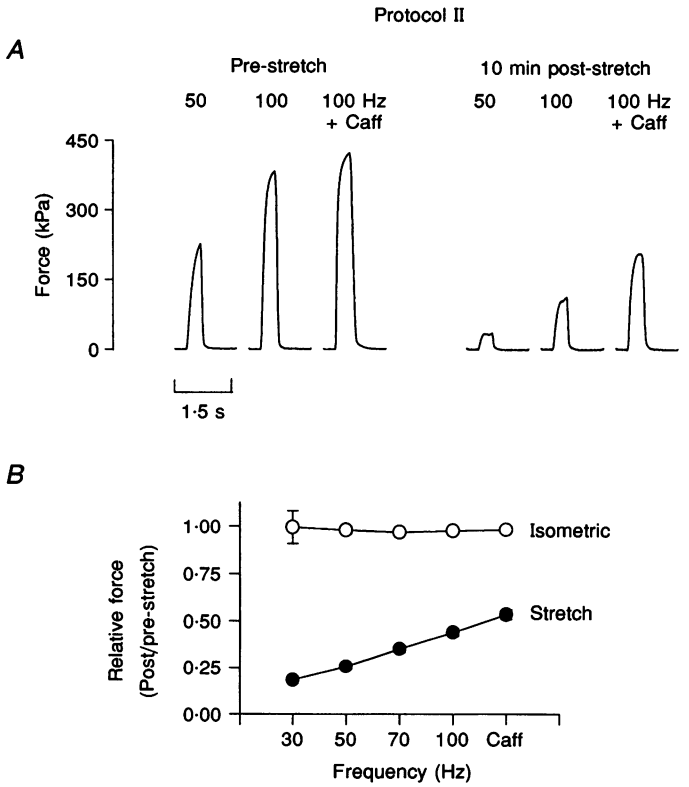

Figure 5. Tetanic force before and after the stretch series of Protocol II

A, force–stimulus frequency relationship from one fibre before and 10 min after the stretch series. This fibre had 10 tetani during the isometric controls and the stretch series. Post-stretch force was reduced at all frequencies, including 100 Hz in the presence of caffeine. *B* shows this relationship in 6 experiments plotted as a ratio of 10 min post-stimulation force to pre-stimulation force. ○, isometric series; ●, stretch series; bars (often not visible) ± one S.E.M.; * points which were significantly different from 1·0 ($P < 0·05$).

the representative fibre (Fig. 5*A*) shows that the 100 Hz force was reduced to 28%, 10 min post-stretch. However, when a tetanus at 100 Hz was elicited in the presence of 10 mM caffeine, maximum force was only restored to 48% of the pre-stretch force. This reduction in maximum Ca^{2+}-activated force is shown in relation to the relative force of six muscle fibres at different stimulus frequencies in Fig. 5*B*.

The reduction in tetanic $[Ca^{2+}]_i$ was also greater after the larger stretch in Protocol II and Fig. 6*B* shows that the tetanic $[Ca^{2+}]_i$ during a 100 Hz tetanus fell to about 60% of the prestretch control. Note that the caffeine-induced tetanic $[Ca^{2+}]_i$ also declined after the stretch series. The tetanic $[Ca^{2+}]_i$ and force generating capacity of the muscle fibres were followed for 60 min after stretch and Fig. 6 shows that both reductions were maintained over 1 h.

In five experiments we tested whether the stretch regime of Protocol II had any effect on resting fibres. Ten stretches were imposed on resting fibres and there was no significant effect on subsequent $[Ca^{2+}]_i$ or force.

To investigate whether the Ca^{2+} sensitivity of the contractile proteins was altered by the stretching regime of Protocol II, $[Ca^{2+}]_i$ *vs.* force curves were again employed. As expected, there was no difference between the curves pre- or 10, 30 and 60 min post-isometric stimulation (Fig. 7*A*). However, the curves 10, 30 and 60 min post-stretch were all significantly different from the pre-stretch curve (Fig. 7*B*). Table 1 gives the average values of the parameters of eqn (1) for both the pre-stretch $[Ca^{2+}]_i$ *vs.* force curves and the post-stretch $[Ca^{2+}]_i$ *vs.* force curves. Note that the Ca_{50} was increased, indicating a decrease in

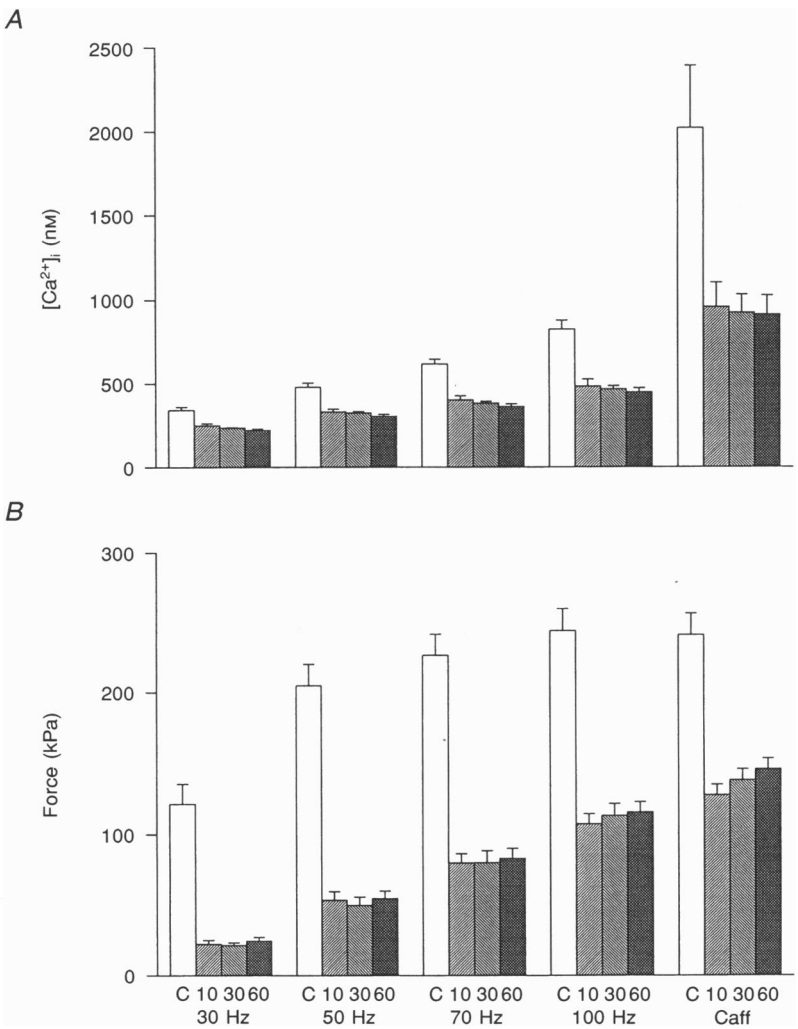

Figure 6. Tetanic $[Ca^{2+}]_i$ (*A*) and force (*B*) at various stimulus frequencies before and after the stretch series

Each group of bars (and S.E.M.) shows first the control (pre-stretch) value (C), then values at 10, 30 and 60 min after the stretch series of Protocol II. Ten tetani were used for the control isometric series and the for the stretch series. All post-stretch values of $[Ca^{2+}]_i$ and force were significantly reduced compared with the pre-stretch values ($P < 0{\cdot}05$); none of the recovery values were statistically different from each other. There was no significant change in $[Ca^{2+}]_i$ or force after the corresponding isometric series.

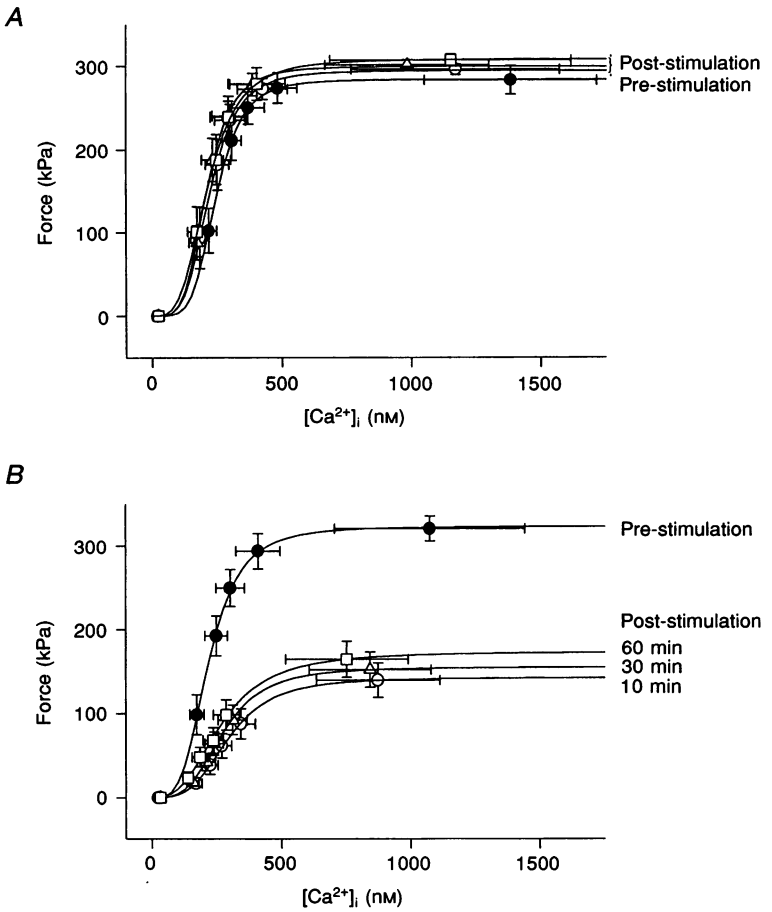

Figure 7. The force–$[Ca^{2+}]_i$ relationship before and after Protocol II

Force *vs.* $[Ca^{2+}]_i$ relation pre-stimulation (●), 10 min post-stimulation (○), 30 min post-stimulation (△) and 60 min post-stimulation (□) for the isometric series (*A*, $n = 4$) and the stretch series (*B*, $n = 5$). Bars represent S.E.M.

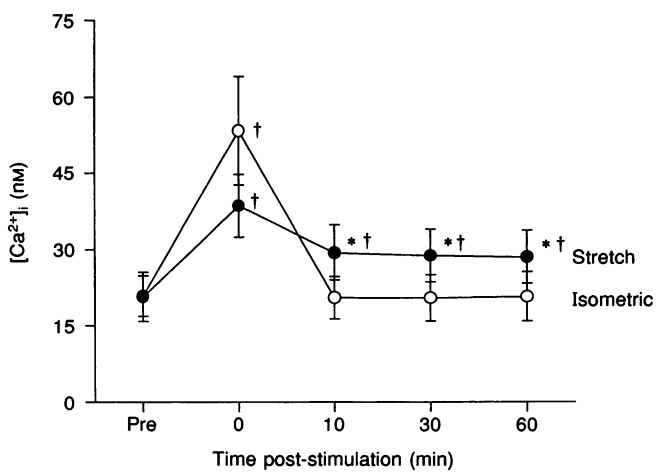

Figure 8. Resting $[Ca^{2+}]_i$ in the 60 min post-stimulation following Protocol II

○, isometric series; ●, stretch series; bars represent S.E.M. †significantly different from pre-stimulation level at $P < 0{\cdot}05$; * significantly different from corresponding isometric level at $P < 0{\cdot}05$.

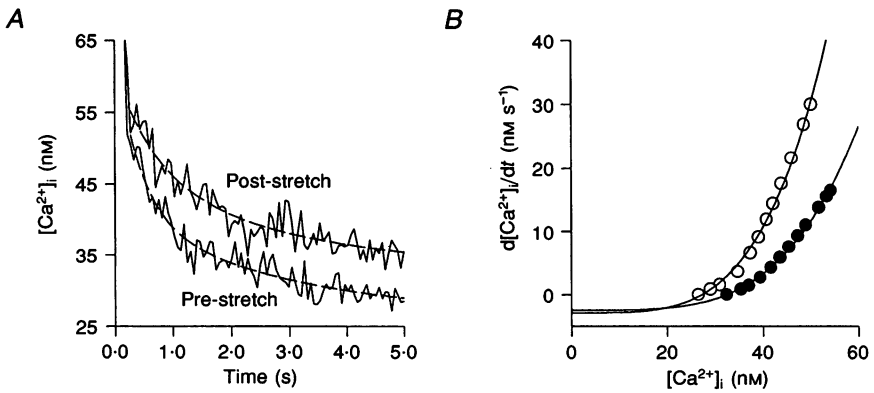

Figure 9. Analysis of the function of the SR Ca^{2+} pump in Protocol II

A, tails of the 100 Hz tetanic $[Ca^{2+}]_i$ signals averaged from four fibres pre-stretch and 10 min post-stretch. Elevated $[Ca^{2+}]_i$ is plotted against time after the final stimulus. The dashed lines represent the records fitted to the sum of two exponentials and a constant, which was then used to obtain the relation between $d[Ca^{2+}]_i/dt$ and $[Ca^{2+}]_i$. *B* shows this relation plotted for both the pre-stretch (○) and post-stretch (●) conditions by fitting data points to eqn (2).

Ca^{2+} sensitivity, and the N was decreased, indicating that the curve was less steep. The reduction in P_{max} confirms that the maximum Ca^{2+}-activated force was reduced following this severe stretching protocol.

In Protocol I there was a small, yet significant, elevation in resting $[Ca^{2+}]_i$ 10 min after stretch which was not evident after isometric stimulation. A similar observation was made after Protocol II (Fig. 8) but in this case the increase in resting $[Ca^{2+}]_i$ was larger. Immediately after stimulation the resting $[Ca^{2+}]_i$ was significantly greater than the pre-stimulation level for both the isometric and stretch conditions. Resting $[Ca^{2+}]_i$ had returned to the pre-stimulation level 10 min after isometric stimulation, consistent with the findings of Westerblad & Allen (1991). However, 10 min after Protocol II was completed, resting $[Ca^{2+}]_i$ remained almost 50% higher than the pre-stretch level. The elevation in resting $[Ca^{2+}]_i$ was maintained significantly higher than both the pre-stretch level and the corresponding isometric levels for 60 min post-stretch.

In five fibres the distribution of elevated resting $[Ca^{2+}]_i$ along the fibre was investigated (see Methods) in an attempt to determine whether the elevation of $[Ca^{2+}]_i$ was localized or uniform. No consistent deviations from the mean $[Ca^{2+}]_i$ were found at any point along the fibre.

One potential explanation for the elevated resting $[Ca^{2+}]_i$ may be a decreased ability of the SR Ca^{2+} pumps to remove Ca^{2+} from the myoplasm. To investigate this possibility, the function of the SR Ca^{2+} pumps were analysed using the procedure described by Klein, Kovacs, Simon & Schneider (1991), which has previously been applied to the present preparation (Westerblad & Allen, 1993; Westerblad *et al.* 1993). To reduce noise the 100 Hz tetanic $[Ca^{2+}]_i$ records from four fibres were averaged both pre-stimulation and 10 min post-stimulation. The $[Ca^{2+}]_i$ signals for 5 s after the last stimulus were fitted to the sum of two exponential functions and a constant (Fig. 9*A*). The initial 200 ms of the $[Ca^{2+}]_i$ tails were not used in the fitting procedure because $[Ca^{2+}]_i$ is declining rapidly during this period so the SR Ca^{2+} pump rate may not be in equilibrium with $[Ca^{2+}]_i$. The rate of $[Ca^{2+}]_i$ decline ($d[Ca^{2+}]_i/dt$) was then calculated at various points along these fits and then plotted against $[Ca^{2+}]_i$ (Fig. 9*B*). The curves in Fig. 9*B* were obtained by fitting the records to the following equation:

$$d[Ca^{2+}]_i/dt = A[Ca^{2+}]_i^N - L \quad (2)$$

where A is a factor which corresponds to the rate of Ca^{2+} uptake by the SR, N is the power function, and L represents the SR Ca^{2+} leak.

From eqn (2) the values of N obtained pre- and post-stretch were 4·1 and 3·6, respectively, but to compare values of A pre- and post-stretch N was set to 4. This gave a good fit in both situations. The post-stretch pump function curve was shifted to the right of the pre-stretch curve (Fig. 9*B*), which corresponded to a pre-stretch pump rate ($5{\cdot}3 \times 10^{-6}$ nM^{-3} s^{-1}) which was more than twice as high as the post-stretch pump rate ($2{\cdot}2 \times 10^{-6}$ nM^{-3} s^{-1}). The values of L were similar pre- and post-stretch (2·9 and 2·5 nM s^{-1}, respectively). The $[Ca^{2+}]_i$ tail was also analysed before and 10 min after the isometric series in the same four fibres. In this situation the pump rate had returned to the pre-stimulation value ($5{\cdot}0 \times 10^{-6}$ nM^{-3} s^{-1}) after 10 min recovery ($5{\cdot}3 \times 10^{-6}$ nM^{-3} s^{-1}).

DISCUSSION

An important issue is whether a single muscle fibre which is stretched during contraction represents an adequate model of stretch-induced injury. In favour of this assertion is that following repeated stretched contractions there was an

immediate fall of force which was maintained at the reduced level for at least 1 h. In addition, our preparations showed a greater reduction in force at low frequencies, which also showed little recovery over 1 h. These responses are very similar to those in human and animal studies (e.g. Davies & White, 1981; Faulkner, Jones & Round, 1989). In single fibres, as in intact muscle studies (Jones, Newham & Torgan, 1989; McCully & Falkner, 1985), isometric contractions did not produce a comparable effect so it is possible to distinguish the effects of fatigue from the additional effects associated with stretch. Furthermore, stretch of resting muscle fibres was without effect as previously shown in animal and human studies (Faulkner *et al.* 1989; Jones *et al.* 1989).

One respect in which the single fibre model seems to differ from studies on whole muscle preparations is that the single fibre seems to be relatively resistant to the immediate consequences of stretch. For instance, our Protocol I is similar to that of Warren *et al.* (1993) who used whole mouse soleus muscles; in both cases ten tetani in which the muscle was stretched were used and they observed a larger fall of force (reduced by 36% compared with our 18%) although our stretch velocity was much higher (5 L_0 s^{-1} compared with 1 L_0 s^{-1}). This result is suprising since it might have been predicted that the relative lack of connective tissue in a single fibre would have made it more susceptible to stretch-induced injury. One outcome which we had initially thought might prove common, namely that a small number of stretches caused obvious local damage leading to contracture and cell death, in fact only occurred once in twenty-seven experiments. In other experiments, even when force was drastically reduced after Protocol II, there were no visible signs of localized damage or inhomogeneous contraction.

Reduced maximal Ca^{2+}-activated force

Reduced maximal Ca^{2+}-activated force was assessed by the effect of a 100 Hz tetanus in the presence of 10 mM caffeine; the $[Ca^{2+}]_i$–force curves suggest that this produced a $[Ca^{2+}]_i$ which was adequate to maximally activate the myofibrils. In Protocol I there was no fall in maximal force whereas in Protocol II there was a pronounced fall to around 50%. There are a variety of possible causes of this drastic reduction in maximum force.

(i) The most likely explanation is that it is a consequence of structural damage to the myofibrils. Many histological studies have shown severe myofibrillar disorganization of various types but most of these studies were performed several days after the period of stretching and it could have been caused by longer term degenerative changes. However, several studies have been performed immediately after a period of stretch and presumably show the kinds of damage from which our fibres might suffer (Newham *et al.* 1983; Armstrong *et al.* 1983; Wood *et al.* 1993). In light micrographs these studies show focal widening of I bands while electron micrographs show localized displacement and distortion of Z-lines, usually with disruption of the sarcomeres on either side. Of particular interest is the study by Brown & Hill (1991) in which single fibres were fixed during a single tetanus immediately after the fibre had been stretched. They showed overstretch of single or half-sarcomeres which were scattered throughout the fibre in such a way that there was no obvious abnormality by light microscopy.

(ii) Changes in sarcomere length away from the optimum could cause reduced maximum force. Such changes have been observed by Wood *et al.* (1993) who showed that following stretches during contraction, the length at which maximum force was achieved was increased. They proposed that, as a consequence of the kind of myofibrillar disruption observed by Brown & Hill (1991), the active sarcomeres in series with the over-stretched sarcomeres will have a shorter than expected sarcomere length. This would lead the length–force relation of such a muscle to be shifted towards longer lengths as observed. Although we did not systematically check this point, in one experiment we found that the peak of the length–force curve had shifted to L_0 + 100 μm after Protocol II.

(iii) There are also various metabolic changes which could have reduced maximum force, e.g. intracellular acidosis and increased intracellular inorganic phosphate. The fact that metabolic changes recover quickly after activity (Cady, Elshove, Jones & Moll, 1989) and that there were no such changes in the isometric controls make this explanation unlikely.

(iv) Localized damage to the surface membrane leading to local contracture is a further possibility. This seems unlikely due to our observation that the resting fibre remained uniform in shape, had no localized elevation of resting $[Ca^{2+}]_i$, and that tetani following stretch-induced injury remained homogeneous with no obvious localized shortening and stretching.

In summary, we believe that the reduction of maximum $[Ca^{2+}]_i$-activated force which occurred after Protocol II was probably caused by myofibrillar disorganization and this needs to be confirmed by structural studies in our model of stretch-induced injury.

Reduced tetanic $[Ca^{2+}]_i$

Tetanic $[Ca^{2+}]_i$ was reduced by about 6% following the stretch series in Protocol I and by 30–40% in Protocol II. These findings suggest that reduced activation may have contributed to the reduced force observed after stretched contractions and this conclusion was supported by two other approaches. Firstly, the addition of 10 mM caffeine increased the tetanic $[Ca^{2+}]_i$ in a 100 Hz tetanus by more than twofold and was able to overcome all (Protocol I) or some (Protocol II) of the reduced force. This result confirms the observation of Warren *et al.* (1993) using isolated

mouse soleus muscle; a minor proviso is that some of the potentiation of force could have arisen from the Ca^{2+}-sensitizing action of caffeine (Wendt & Stephenson, 1983). Secondly, plotting the $[Ca^{2+}]_i$–force relation in Protocol I showed no significant change in maximum force or Ca^{2+} sensitivity implying that the reduced tetanic $[Ca^{2+}]_i$ was the sole cause of the reduced force. However, in Protocol II all three possible mechanisms appear to operate.

It has been previously demonstrated that a uniform reduction in tetanic $[Ca^{2+}]_i$ at all stimulus frequencies, as we have observed here, causes a greater reduction in force at low stimulus frequencies than at high (Westerblad *et al.* 1993). This is a simple consequence of the shape of the relation between $[Ca^{2+}]_i$ and force. At high stimulus frequencies the tetanic $[Ca^{2+}]_i$ is relatively close to saturation so that a moderate fall in $[Ca^{2+}]_i$ has relatively little effect on force. Conversely at low stimulus frequencies the $[Ca^{2+}]_i$ is on the steep part of the $[Ca^{2+}]_i$–force relation so a moderate fall in $[Ca^{2+}]_i$ produces a substantial fall of force. Thus, it seems that in the present experiments the fall in tetanic $[Ca^{2+}]_i$ contributes to the greater reduction in force observed at low frequencies compared with high following the stretch series.

The mechanism of the reduced Ca^{2+} release is not known. One hypothesis is that sarcomere disorganization leads to surface membrane disruption and an increase in Ca^{2+} entry into the fibre. This possibility is consistent by the increase in resting $[Ca^{2+}]_i$ which we observed and with the increase in mitochondrial Ca^{2+} (Duan, Delp, Hayes, Delp & Armstrong, 1990). The elevated resting $[Ca^{2+}]_i$ could activate proteases and might lead to partial inactivation of SR Ca^{2+} release channels as demonstrated by Gilchrist, Wang, Katz & Belcastro (1992) and might therefore explain the reduced tetanic $[Ca^{2+}]_i$. Comparable findings, namely that brief periods of elevated $[Ca^{2+}]_i$ lead to a subsequent reduction in SR Ca^{2+} release, have recently been reported in skinned fibres with intact T-tubules and SR (Lamb, Junankar & Stephenson, 1994). A problem with this hypothesis is that Ca^{2+} concentrations of the order of 10–100 μM were required for this effect in the above two studies whereas we observed a resting $[Ca^{2+}]_i$ of only 30 nM. Another possibility is that stretch-induced injury causes membrane potential depolarization, a reduced action potential amplitude and reduced Ca^{2+} release. Although we did not investigate this possibility, Warren *et al.* (1993) found no evidence for changes in the resting membrane potential following contractions involving stretch. A further possibility is that some fraction of the SR is damaged after the stretch and no longer able to store or release Ca^{2+}; if this were the case it would be expected that mitochondrial Ca^{2+} would increase (Duan *et al.* 1990). This hypothesis is then consistent with our three key findings, i.e. reduced tetanic $[Ca^{2+}]_i$, increased resting $[Ca^{2+}]_i$ and slowing of the SR Ca^{2+} pump rate.

Reduced Ca^{2+} sensitivity

There was no significant change in Ca^{2+} sensitivity in Protocol I, but a moderate reduction in Protocol II (increase in Ca_{50}). This provides a further mechanism for the reduced force production observed in Protocol II; in addition, the reduced sensitivity may also contribute to the greater reduction in force at low frequencies compared with high. We have no direct evidence for the mechanism involved but metabolic changes seem unlikely, as discussed above. One possible explanation for the reduced Ca^{2+} sensitivity, which is compatible with our results, is that it is a consequence of reduced sarcomere length in the active sarcomeres. It is recognized from studies on skinned fibres that Ca^{2+} sensitivity falls with reduced sarcomere length (Endo, 1972; Stephenson & Williams, 1982) and we have confirmed this in the present intact preparation (Balnave & Allen, 1994*b*). As noted earlier, it is possible that overstretch of some sarcomeres leads the active sarcomeres to have a reduced sarcomere length and the reduced Ca^{2+} sensitivity could be a reflection of this. Other possibilities are that Ca^{2+} activated proteases (Busch, Stromer, Goll & Suzuki, 1972) might have reduced Ca^{2+} sensitivity, either by damage to Z-lines leading to reduced force production (Fridén, Sjöström & Ekblom, 1981) or by attacking one of the regulatory proteins.

Raised resting $[Ca^{2+}]_i$

We have suggested that the elevated resting $[Ca^{2+}]_i$ resulted from increased Ca^{2+} entry through damaged regions in the surface membrane. If such membrane tears were to seal over, one would expect that the SR Ca^{2+} pump would rapidly lower $[Ca^{2+}]_i$ to close to the original resting level. The persistent rise in resting $[Ca^{2+}]_i$ suggests either that the damaged membranes have not sealed over or, alternatively, that Ca^{2+} uptake has been so large that the SR and myoplasmic $[Ca^{2+}]$ have reached a new equilibrium at a substantially elevated levels.

The finding that the SR Ca^{2+} pump rate is reduced, however, suggests a different explanation for the elevated resting $[Ca^{2+}]_i$. It is possible that stretch damages the SR in such a way as to reduce Ca^{2+} pumping ability and this would elevated the resting $[Ca^{2+}]_i$.

Conclusions

This study shows that a single fibre model of stretch-induced injury can be produced with properties closely similar to intact muscles. Measurements of $[Ca^{2+}]_i$ establish that the reduction of force in this model of stretch-induced injury involves reduced Ca^{2+} release and reduced Ca^{2+} sensitivity. An increase in resting $[Ca^{2+}]_i$ was observed which is compatible either with damage to the surface membrane or with reduced SR pumping. The resulting increase in resting $[Ca^{2+}]_i$ may be the trigger for some of the longer term changes which trigger muscle degeneration (e.g. Duncan, 1978).

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