The effects of repeated active stretches on tension generation and myoplasmic calcium in frog single muscle fibres

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1. A series of contractions with stretches (eccentric contractions) beyond the optimal length for tension generation (optimum) were shown to induce a shift in that optimum in single muscle fibres of frog, as has been previously reported for whole muscles. Shifts averaging 0·129 μm (sarcomere)$^{-1}$ or 6% were found, without apparent damage to the fibre.

2. The stiffness of fibres was found to fall during a stretch, even though tension was rising. In addition, the isometric stiffness fell as a result of a series of eccentric contractions.

3. Calcium-sensitive fluorescent dyes indicated that such contractions did not reduce the amplitude of the intracellular calcium transient, but did increase its duration. A rise in resting [Ca^{2+}] was found to accompany damage, but not necessarily the shift in optimum.

4. The twitch potentiator nitrate was shown to increase myoplasmic [Ca^{2+}] during twitch and tetani, but not to reverse the shift in optimum length due to eccentric contractions. Both eccentric contractions and twitch potentiation reduced the maximum stimulation rate to which a fibre could respond with propagated action potentials.

5. These results exclude reduced myoplasmic [Ca^{2+}] as the cause of the shift in optimum length in this preparation.

At the end of his much quoted paper on the force–velocity relation of muscle during stretch, Katz (1939) reported that frog sartorius muscles subjected to multiple stretches appeared to undergo 'a partial transformation of active contractile into passive elastic tissue'. He concluded this from observing a shift in the length–tension relation to longer lengths, often with little loss of peak tension, a slower development of tension during a tetanus, a smaller twitch, and a greater undamped elastic shortening during a quick release, all expected consequences of increased series compliance. This work was largely ignored for many years, perhaps due in part to the lack of any plausible mechanism.

Morgan (1990) suggested on theoretical grounds that lengthening of active muscle at long length was extremely non-uniformly distributed among the sarcomeres, with most of the lengthening absorbed by a few sarcomeres being lengthened beyond filament overlap. He further suggested that most such sarcomeres re-interdigitate on relaxation, but that a few may become disrupted and fail to re-interdigitate, converting contractile sarcomeres to passive structures. This proposal provided a possible mechanism for Katz's observations.

Wood, Morgan & Proske (1993) repeated Katz's experiments in greater detail with whole toad sartorius, and supplemented them with electron microscopy. They showed a shift in the length–tension relation, an increase in compliance, and a decrease in the yield tension during stretch, all increasing with an increasing number of eccentric contractions. They also found regions of disrupted sarcomeres, similar to those implicated in 'delayed onset muscle soreness', the damage, tenderness and soreness found after unaccustomed eccentric exercise in whole animals and humans (Armstrong, Ogilvie & Schwane, 1983; Newham, McPhail, Mills & Edwards, 1983). We have chosen the shift in the optimal fibre (or mean sarcomere) length for isometric tension generation (L_{opt}) as our prime measure of the accumulated changes due to stretch, as it is relatively easy to quantify, and should provide a near-linear measure of the mean number of disrupted sarcomeres in myofibrils. In addition to toad sartorius, such shifts have been seen in intact rat vastus intermedius muscles (present authors, R. Lynn and J. A. Talbot, unpublished observations) and in human ankle extensors (present authors, C. Jones, T. Allen and U. Proske, unpublished observations). Our first aim is to measure shifts in the length–tension relation of single muscle fibres from the frog.

An alternative hypothesis for the effects of stretch would be that the initial effect of eccentric contractions is to reduce the release of calcium from the sarcoplasmic reticulum. This would account for the observed shift of the frequency–tension relation to higher frequencies (Davies & White, 1981; Newham, Jones & Clarkson, 1987), and the

resulting submaximal activation would account for the apparent shift in the length–tension relation to longer lengths (Endo, 1973). In support of this, Warren, Lowe, Hayes, Karwoski, Prior & Armstrong (1993) showed that a caffeine contracture restored most of the tension deficit in mouse whole muscles after eccentric contractions. Balnave & Allen (1995), using the slowly responding dye indo-1 to measure $[Ca^{2+}]_i$ during a tetanus of single mouse fibres, found a small (<10%) but significant fall in the apparent $[Ca^{2+}]_i$ during submaximal tetani, but not maximal tetani, after eccentric contractions. Indo-1 was unable to resolve individual $[Ca^{2+}]_i$ transients in these experiments, so that the observed fall could be due to reduced release of calcium due to each action potential, or to some stimuli failing to produce action potentials. One aim of the present experiments is to resolve this by examining internal calcium ion concentration ($[Ca^{2+}]_i$) during tetani with a rapidly responding dye.

The twitch : tetanus ratios of frog single fibres, especially at low temperature, are much greater than those of mammalian muscle. Also a number of twitch potentiators are available to reverse any decrease in calcium release, and so any shift that may occur from this submaximal activation. These are traditionally described as potentiating the twitch but not the maximal tetanus (Sandow, 1964), a view supported by work in this laboratory (Julian & Morgan, 1981; Rome, Morgan & Julian, 1985) at least on fibres that have not been subjected to eccentric contractions. By combining frog fibres, low temperatures and potentiator, we can ensure that a twitch is always near to the maximal activation even after eccentric contractions, and so test the submaximal activation hypothesis.

Finally, the resting $[Ca^{2+}]_i$ has been reported to increase after eccentric contractions, but it has not been clear whether this is the cause or result of structural and mechanical changes. We have investigated the relation between raised calcium and shift in the length–tension relation, to determine if one is necessary for the other.

METHODS

All protocols used have been approved by the Harvard Medical Area Standing Committee on Animals to comply with Federal, State and Harvard regulations governing the humane care and use of laboratory animals. Frogs were killed by decapitation followed by double pithing after being immersed in ice water for no less than 10 min.

Single muscle fibres were dissected from the tibialis anterior muscles of the frog *Rana temporaria*. Dissection and the experimental chamber were as previously described (Claflin, Morgan, Stephenson & Julian, 1994). For these experiments, the chamber was mounted on an inverted microscope (Nikon Diaphot 300) fitted with a photometer system (Photon Technology International (PTI), South Brunswick, NJ, USA) and an illumination system (PTI Deltascan 4000). All experiments were done at $3{\cdot}0 \pm 0{\cdot}1$ °C. The normal Ringer solution contained (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85; pH 7·2. The potentiating solution substituted $NaNO_3$ for the NaCl. Stimulus strength was set to 1·25 times the threshold value at a mean sarcomere length of 2·2 μm. Sarcomere length was estimated by photographing three points along the fibre at a magnification of 561, measuring the length of a string of thirty sarcomeres at each point, and averaging the three values that resulted. Fibre length was measured with a dial indicator gauge while translating the chamber on the microscope stage. This enabled any desired mean sarcomere length to be set.

Monitoring intracellular $[Ca^{2+}]$

For these experiments, only ratiometric fluorescent dyes were used, specifically fura-2 for measuring resting levels and mag-indo-1 for measuring transients (Molecular Probes). Both were loaded by soaking the fibre for 60 min at 20 °C in a Ringer solution containing the acetoxymethyl ester form of fura-2 at a concentration of 15 μM and mag-indo-1 at 10 μM.

Ratiometric dyes allow correction of the signal for motion artifacts, that is changes in light signal caused by variations in the amount of dye contributing to the signal, either through translation or rotation of the fibre, or through movement of the fibre relative to the focal plane of the microscope. For fura-2, the sample was alternately illuminated with two excitation wavelengths (2 nm bands centred on 380 and 344 nm, set by diffraction grating monochromators) and the fluorescence monitored by a single photomultiplier in a 40 nm band centred at 510 nm, set by a filter (Omega Optical, Brattleboro, VT, USA). In order to minimize the number of contractions, the alternation was done by 'chopping' the incident illumination with the toothed mirror of the Deltascan. The photomultiplier was used in photon counting mode, and the ratio calculated by the PTI software at the requested rate, usually ten ratios per second. This relatively low time resolution measurement was adequate for observing resting $[Ca^{2+}]_i$ levels, though not for resolving the $[Ca^{2+}]_i$ transients.

Mag-indo-1 was chosen for its ability to track rapid $[Ca^{2+}]_i$ transients (Zhao, Hollingworth & Baylor, 1996) and because its emission spectrum shifts on binding of Ca^{2+}. This allows a fluorescence ratio to be formed from a single contraction without compromising time resolution as with chopping. For mag-indo-1, a single excitation wavelength band was used (10 nm centred on 365 nm) and the fluorescence measured at 408 nm (40 nm wide) and 495 nm (20 nm wide) (filters supplied by PTI) using two photomultipliers. Because light is gathered from two different paths using two different photomultipliers, the absolute value of the ratio may change from day to day. This difficulty can be circumvented for mag-indo-1, by expressing all ratio changes as a fraction of the resting ratio. The resting ratio is indistinguishable from the ratio for zero $[Ca^{2+}]_i$, R_{min}, so variations from fibre to fibre in resting ratio must be due to equipment, not variations in resting $[Ca^{2+}]_i$. A further complication arises from the fact that the gain of photomultipliers varies significantly with the position of the image on the cathode. The removal of motion artifact requires that the images of the fibre fall on similar parts of the two photomultipliers, so that movement affects the two signals equally. This was done as accurately as possible. In order to reduce noise in view of the low sensitivity of mag-indo-1 to $[Ca^{2+}]$, the photomultipliers were used in analog mode, at higher light intensities than would have been possible in photon counting mode. In this case analog signals proportional to the light were recorded on a digital oscilloscope (Nicolet 4094 or Pro20, Madison WI, USA) typically using a 0·5 ms sampling interval. The relatively small background- and auto-fluorescence signals, produced by illuminating the chamber and fibre before adding dye, were subtracted, and the ratio (R) of the dye

fluorescence at 408 nm to that at 495 nm was formed. The field of view was defined by a mask in the light path, and was set to be 1·16 mm × 0·3 mm at the fibre. For twitches, nine traces were averaged, while single sweeps were used for tetani.

Finding the optimal mean sarcomere length for isometric tension generation (L_{opt})

Fixed-end contractions (20 stimuli s^{-1} for 500 ms with 2 min rest periods) were performed at nominal mean sarcomere lengths of 2·2, 1·8, 2·0, 2·1, 2·2, 2·3, 2·4, 2·6 and 2·2 μm. Tension was measured as the average value over the last interstimulus interval. A straight line was fitted to the tensions at 2·2 μm as a function of contraction number; all contractions were normalized to this line, and scaled by its mid-point. This corrected for any change in tension during the run, but did not normalize for any changes over longer time scales. These points were then plotted against sarcomere length, and all points above 80% of maximum tension were fitted with a Gaussian curve. The position of the peak of the best fit Gaussian curve was taken as the optimal mean sarcomere length for tension generation.

Eccentric contractions

The standard eccentric contraction consisted of a stretch starting at 2·2 μm mean sarcomere length, at an amplitude of 7·5% of the fibre length at 2·2 μm mean sarcomere length and at a velocity of 1 μm (sarcomere)$^{-1}$ s^{-1}, approximately one-sixth of the unloaded shortening velocity of these fibres at this temperature. This stretch was imposed half-way through a 1 s tetanus with 20 stimuli s^{-1} and 2 min rest periods. The number of such contractions varied from seven to twenty-six, in an attempt to produce measurable shifts in L_{opt} without excessive damage.

Measurement of stiffness

In five experiments, stiffness was measured during some of the eccentric contractions, by superimposing a sinusoidal length change of frequency near 2 kHz and peak-to-peak amplitude approximately 0·5 nm (half-sarcomere)$^{-1}$. Length and tension were measured every 20 μs, and the resonant frequency of the tension transducer was 1·5 kHz. Tension was recovered by low-pass filtering. All filtering was performed by taking a fast Fourier transform of the sampled signal, multiplying this by the chosen filter characteristic, and then taking an inverse Fourier transform. The frequency response of the low-pass filter fell linearly between 400 and 600 Hz, was unity below 400 Hz and zero above 600 Hz. To find stiffness, both tension and length signals were bandpass filtered, using a filter response that was zero below 1400 Hz and above 2600 Hz, unity between 1600 and 2400 Hz, and varied linearly with frequency in the transition regions. Both of these AC components were then squared, low-pass filtered, and the square root taken, to produce an r.m.s. value. The ratio of the r.m.s. values was taken as stiffness. The program Igor (WaveMetrics, Lake Oswego, OR, USA) was used for all data processing.

Varying stimulus frequency

Fixed-end contractions were performed at mean sarcomere length of 2·2 μm with a duration of 1 s, at rates of 20, 5, 8, 12, 30 and 20 pulses s^{-1}. The number of stimuli that produced propagated action potentials was counted from the mag-indo-1 fluorescence ratio signal.

Test sequence

The standard experimental sequence was as follows. After loading the fibre with dye, and checking the stimulation parameters and the dye fluorescence for twitches, the first test sequence followed: averaged twitch, varying stimulus frequency, and a length–tension relation. Potentiator was then introduced, and the test sequence repeated. Normal solution was reintroduced, and eccentric contractions followed. The test sequence was then repeated in normal solution and then again in potentiating solution.

RESULTS

Damage to fibres

Many of the fibres eventually suffered damage due to repeated eccentric contractions and ceased propagating action potentials. In such cases, data from the run in which damage became apparent and from the preceding run were discarded, except for Fig. 7. In other cases, the fibres completed the protocol with no sign of damage.

Response to stretch

Responses of a typical fibre to the first and last stretches are shown in Fig. 1. The following features can be seen, and were noted as being common to all fibres. The tension record of the first stretch showed a sharp yield point, followed by a continued slow increase in tension throughout most of the stretch. Later stretches produced much more rounded tension responses during the stretch, though the peak tension was close to that of the first stretch, the fall being less than that for the isometric tension preceding the stretch. In contrast, the stiffness for the first stretch showed an initial rise to a peak corresponding to the yield point of tension, then a steady fall throughout the stretch. The last contraction showed an isometric stiffness that was reduced, relative to the first contraction, by a substantially greater fraction than was the tension. The initial rise in stiffness during the stretch was still present, but the continued fall during most of the stretch was substantially smaller. The contractions used were too short to explore the isometric stiffness after stretch thoroughly, but it was clear that the first stretch reduced isometric stiffness much more than the last one. That is, the stiffness after the stretch was less than the stiffness before the stretch, but the difference was greater for the first stretch than the last.

Length–tension relations

L_{opt} was always longer after the series of eccentric contractions than before. The maximum tension generated was also less. In one fibre, shown in Fig. 2, the fall was small enough for tension at long length to increase as a result of the stretches, as reported by Katz (1939). In the other fibres, the fall was sufficient that no clear increase in tension was seen at any length. The magnitudes of the shifts in L_{opt} for the various fibres are shown on the abscissae of Fig. 5. Some of the variability is due to variations in the number of eccentric contractions, but it was apparent that the shift seen in a given fibre depended on other factors as well. An estimate of repeatability can be seen from Fig. 3.

The mean increase in L_{opt} due to eccentric contractions was 0·129 ± 0·068 μm (sarcomere)$^{-1}$ (mean ± S.D.; $n = 8$), which was significantly different from zero at $P < 0·001$ (from Student's t test). The substitution of potentiating solution did not reverse the shift in L_{opt}. The mean shift due to

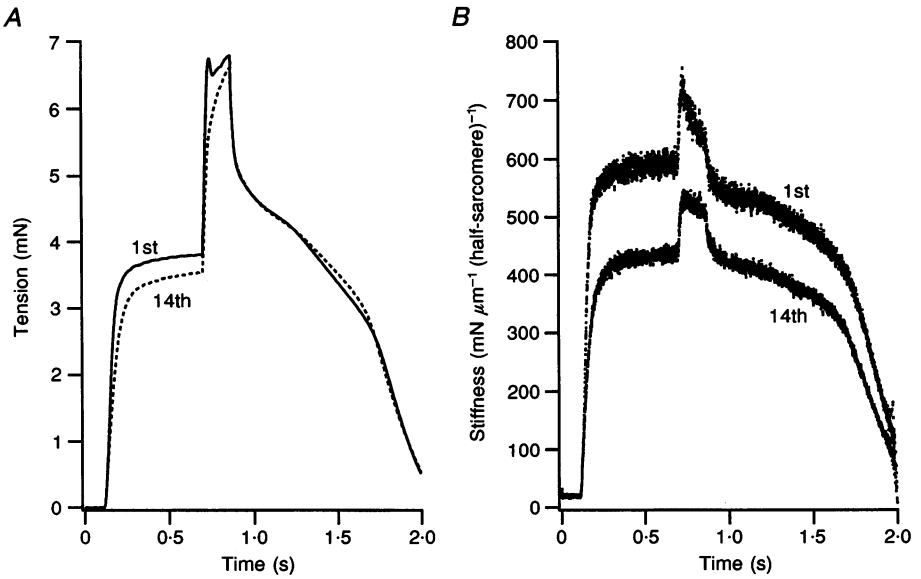

Figure 1. Tension (*A*) and stiffness (*B*) measured during the first and fourteenth stretch applied to a single fibre

Stimulation at 20 pulses s^{-1} was applied from 0·1 to 1·1 s. Temperature, 3 °C. Cumulative changes in the response are discussed in the text. (Fibre 951220, runs 4B and 4N.)

potentiator after one or more series of eccentric contractions was +0·021 ± 0·017 μm (sarcomere)$^{-1}$ (mean ± S.D.; $n = 5$ measures from 4 fibres; fibre 951215 shown in Fig. 3, giving two values, not shown in Fig. 5 as twitches were not recorded). That is, the shift due to potentiation was in the same direction as that due to the eccentric contractions and was just statistically significantly different from zero ($P = 0·05$ from Student's two-tailed t test). The probability of the true mean of the potentiator shifts compensating for the eccentric shifts, that is being more negative than −0·146, or minus the average value of the eccentric shifts for those fibres, is less than 0·0001.

Potentiation before eccentric contractions also produced a slight average shift to longer lengths, with the mean of six fibres being +0·016 μm (sarcomere)$^{-1}$, not significantly different from zero ($P = 0·26$).

Figure 3 shows ten measurements of L_{opt} from one fibre. The first three values were measured on one day, the entire chamber and fibre were then stored overnight at 1 °C, and subsequent measurements were taken the next day. Eccentric contractions were imposed between the first and second, and between the fourth and fifth length–tension relation measurements. This fibre was still functioning well 8 h and more than fifty tetanic contractions after sustaining a shift in optimal length of more than 10%. Note that in both cases, potentiation produced no decrease in L_{opt}, and that the five measures of L_{opt} in normal solution taken after the second set of eccentric contractions were very consistent.

Calcium signal during twitches

The dye fluorescence ratio signals from mag-indo-1 showed some residual signal with many of the characteristics of motion artifact. We were unable to show whether this was

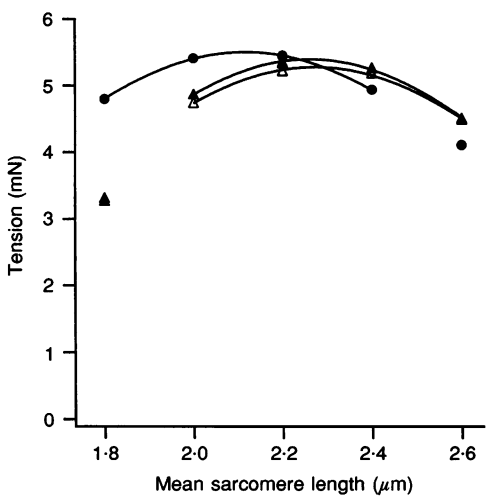

Figure 2. Changes in the length–tension relation due to eccentric contractions

Length–tension relations from a fibre in normal solution before stretches (●, optimum tension at a mean sarcomere length of 2·117 μm), in normal solution after 15 eccentric contractions (▲, optimum at 2·261 μm), and in potentiating solution after the stretches (△, optimum at 2·273 μm). The curves are Gaussian best fits to tensions above 80% of maximum. (Fibre 951103.)

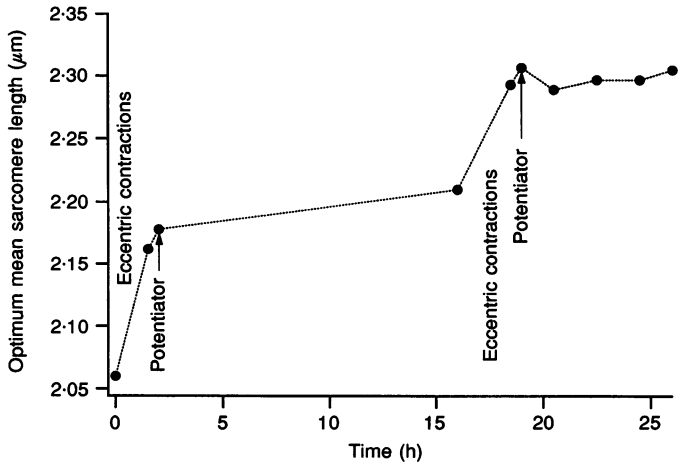

Figure 3. Repeated measurements of L_{opt} from a single fibre over a period of 26 h

Eccentric contractions were imposed between the first and second, and between the fourth and fifth determinations of optimum length. Note the repeatability of measurements, the increase in L_{opt} caused by each series of 13 eccentric contractions, the small change produced by potentiating solution, and the lack of any apparent damage despite a substantial shift in optimum length. (Fibre 951215.)

due to imperfect matching of the variations of photomultiplier gain with fibre position, or other factors. This is unlikely to affect measured parameters from twitches, as the peak $[Ca^{2+}]_i$ occurs before tension generation (Claflin *et al.* 1994).

The twitch tension and dye fluorescence ratio signals for one fibre at the four stages of the experiment are shown in Fig. 4. The twitch tension was decreased by eccentric contractions, but was increased by potentiator both before and after the

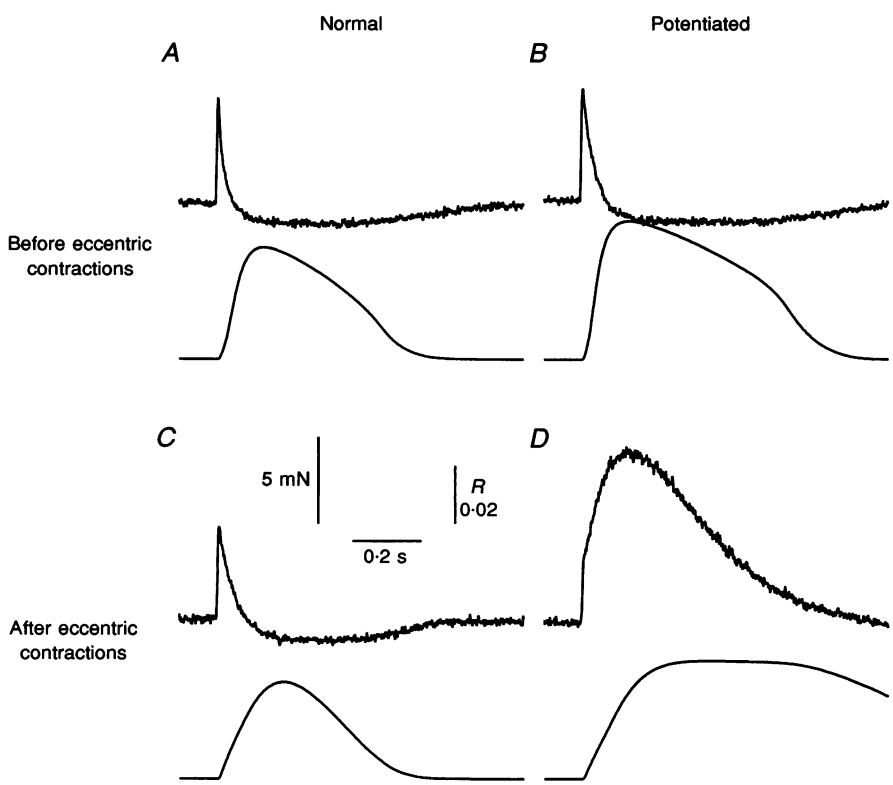

Figure 4. Examples of twitches at the four stages of an experiment

The upper trace of each panel is the ratio of mag-indo-1 fluorescence (R, see Methods for description), assumed to be proportional to $[Ca^{2+}]_i$. The lower traces are tension. A and B were recorded before the series of eccentric contractions, and C and D after. A and C were recorded while the fibre was in normal Ringer solution, while B and D were recorded in nitrate potentiating solution. All records are an average of 9 sweeps; 18 eccentric contractions.

eccentric contractions. In normal solution, the eccentric contractions led to a slight decrease in the amplitude of the dye fluorescence signal but a greater increase in width, not consistent with the idea that reduction in the twitch tension is due to reduced calcium release. Potentiation after eccentric contraction led to a very large and long transient, suggestive of a dying fibre. However fifteen more tetani were recorded from this fibre without signs of damage being apparent in the tension trace.

Changes in the amplitude and half-width of the intracellular calcium transient are summarized in Fig. 5. The logarithmic ordinates are simply to accommodate the larger changes without crowding the smaller ones. In the left panel, the amplitudes were substantially unaffected by stretches, and in most cases by potentiator. The two exceptions with near-doubling of the amplitude are from different fibres, the large increase due to potentiator after eccentric contractions being from the experiment shown in Fig. 4. The half-width showed much greater variation, especially when potentiated. Eccentric contractions also produced an increase in half-width, with the increase tending to be greater for fibres with a greater shift of L_{opt} (correlation of +0·565), again inconsistent with any suggestion that the shift is due to reduced calcium release.

Mean values and standard deviations of changes were calculated. Due to eccentric contractions, the peak decreased to 96 ± 16%, and the half-width increased to 190 ± 110% of control ($n = 7$). Potentiator, before eccentric contractions, increased the peak to 121 ± 30% and the half-width to 253 ± 118% of control ($n = 6$). Potentiator after eccentric contractions increased the peak to 131 ± 52% and half-width to 644 ± 517% of values in normal solution after eccentric contractions ($n = 3$). Of these, only the change in half-width due to potentiator before eccentric contraction was statistically significantly different from 100% ($P = 0·025$ from Student's two-tailed t test), with the change in half-width for eccentric contractions attaining $P = 0·076$.

Calcium signal during tetani

Typical dye fluorescence ratio signals during tetani are shown in Fig. 6. The stimulation rate was 20 pulses s^{-1}, leading to a just discernible ripple in tension in normal solution. The contraction in potentiating solution had a much larger apparent $[Ca^{2+}]_i$, and a reduced tension ripple, but only a small increase in tension. After the eccentric contractions, though the tension was less, the apparent $[Ca^{2+}]_i$ was clearly greater than before, apparently due to increased summation, in turn due to the increased duration of each stimulus response. Note also the slower rate of rise of tension. In potentiator after the eccentric contractions, only half of the stimulus pulses led to pulses of apparent $[Ca^{2+}]_i$, though those pulses were much prolonged, as in the twitch records in Fig. 4. Note that again potentiator did not increase tension significantly, despite the increase in apparent $[Ca^{2+}]_i$. If the stimulation rate was raised to 30 pulses s^{-1}, some stimuli failed to elicit a $[Ca^{2+}]_i$ response after eccentric contractions, in both normal and potentiating solution.

Resting calcium signal

In three experiments, the dye fura-2 was used to estimate resting $[Ca^{2+}]_i$. These experiments are summarized in Fig. 7.

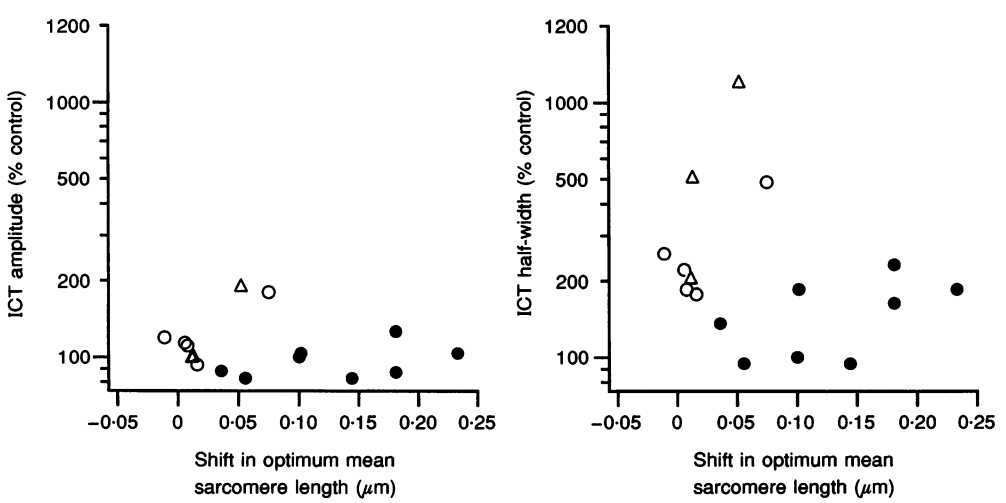

Figure 5. Summary of changes in parameters of the twitch dye signals

Amplitude and half-width of the mag-indo-1 fluorescence ratio signal, assumed to be proportional to the intracellular calcium transient (ICT) are expressed as a percentage of their value before the relevant treatment. ○, changes due to nitrate before stretch – parameters in potentiator before eccentric contractions as a percentage of control plotted against the difference in L_{opt} between potentiated and normal contractions before the eccentric contractions; ●, changes due to stretch; △, changes due to nitrate after stretch – difference between potentiated after eccentric contractions and normal solution after eccentric contractions.

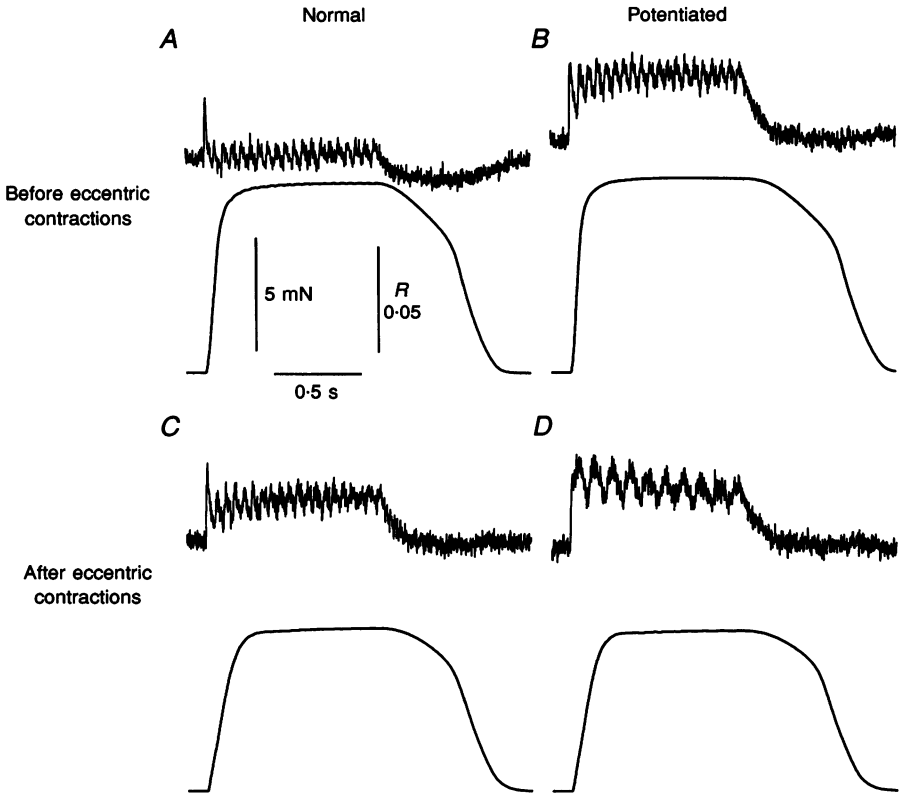

Figure 6. Examples of tetani at the four stages of an experiment

The upper trace of each panel is the ratio of mag-indo-1 fluorescence (R, see Methods for description), assumed to be proportional to $[Ca^{2+}]_i$. The lower traces are tension. A and B were recorded before the series of eccentric contractions, and C and D after. A and C were recorded while the fibre was in normal Ringer solution, while the B and D were recorded in nitrate potentiating solution. All records are single sweeps; 13 eccentric contractions.

In the first experiment, the fibre was not exposed to potentiator before the eccentric contractions, and survived the entire protocol without damage and with only small increases in resting $[Ca^{2+}]_i$ mainly during the eccentric contractions. The shift in optimal mean sarcomere length for tension generation for this fibre was 0·10 μm (sarcomere)$^{-1}$.

Neither of the other fibres survived the second run in potentiating solution, and both showed a larger rise in the reported resting $[Ca^{2+}]_i$ during the eccentric contractions. Both of these fibres were examined at five points along the fibre, but in neither case was a clear 'hot spot' found, the increase in resting ratio being near uniform along the fibre.

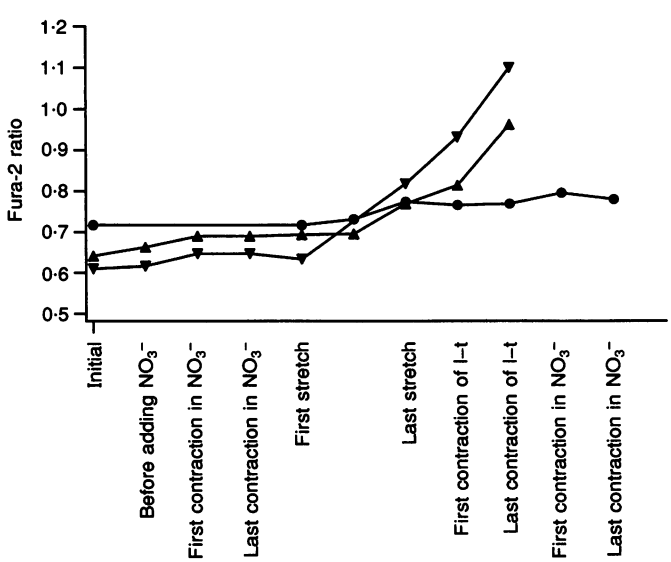

Figure 7. Resting fura-2 ratios, representing resting $[Ca^{2+}]_i$

●, fibre 951220 (13 eccentric contractions) showed a shift in L_{opt} of 0·09 μm (sarcomere)$^{-1}$, with little rise in $[Ca^{2+}]_i$ and no apparent damage. ▲, fibre 951228 (13 eccentric contractions) died in the run following determination of shift, and so no shift figure is available. ▼, fibre 951229 (7 eccentric contractions) showed 0·12 μm (sarcomere)$^{-1}$, and survived 10 contractions after the determination of optimum, but died when stimulated in potentiating solution. l–t, length–tension relation.

DISCUSSION

Hypothesis

These results will be interpreted within the framework of the hypothesis put forward by Morgan (1990), which can be summarized as follows. In any eccentric contraction on the plateau or descending limb of the length–tension relation, lengthening of a myofibril is distributed very unevenly among its half-sarcomeres, with half-sarcomeres rapidly overextending or 'popping', one at a time, in order of their yield tensions. These half-sarcomeres are extended beyond contractile filament overlap, and carry the tension in their passive structures. Thus during a stretch the tension rises, representing a succession of yield points of sarcomeres, while stiffness falls as sarcomeres are converted to more compliant passive elements. On relaxation most sarcomeres resume their pattern of interdigitating filaments, but some suffer disruption of the pattern, and fail to resume interdigitation. These then act as series elasticity in subsequent contractions, leading to the mechanical changes reported. The number of such disrupted half-sarcomeres increases with each contraction, leading to the cumulative nature of the changes. The disrupted half-sarcomeres will in general be at different points in different myofibrils, so that they are difficult to see in optical microscopy. They may also be present in different numbers in different myofibrils, possibly contributing to the fall in peak tension, as different myofibrils then have different optimal lengths. The term 'damage' is not used for such fibres, but is reserved for fibres that are progressing towards failure. The processes by which disruption becomes damage are important, but beyond the scope of the current investigation.

Direct evidence supporting the hypothesis comes from rapid fixation experiments, in which muscle is stretched while tetanized, and then fixed without relaxing. Brown & Hill (1991) reported overstretched half-sarcomeres in single fibres under such conditions and Talbot & Morgan (1996) showed that overstretched sarcomeres can account for most of the applied stretch.

Mechanical measurements

As suggested by Katz (1939), all the mechanical changes can be explained by progressive conversion of active elements to passive elements. In the current hypothesis, this becomes conversion of normal, active half-sarcomeres to disrupted half-sarcomeres, carrying the tension in structural proteins within and around the myofibril. During subsequent isometric contractions, the disrupted sarcomeres will be stretched to long lengths, adding to the fibre length necessary to bring the active sarcomeres to optimal length, explaining the shift in the length–tension relation. The smaller twitches result from increased internal motion and reduced active sarcomere length. The reduction in tension in a partially fused contraction is likely to have a similar basis, though that could not be extensively tested in the current investigation, due to the large twitch : tetanus ratios of these fibres. A disrupted sarcomere is likely to lengthen the corresponding sarcomere in neighbouring myofibrils, and shorten neighbouring sarcomeres in the same myofibril. This increased spread of sarcomere lengths and thus strengths, as well as the increased fraction of the stretch being absorbed by disrupted sarcomeres, can explain the rounding of the tension rise in response to later stretches. These results have all been reported previously in whole muscles, and confirmed here in single fibres. The absence of tendons in this preparation eliminates the possibility of changes in those structures being responsible for the shift.

The theory predicts that stiffness will fall during the progress of a single stretch, even though tension rises, as active sarcomeres are overstretched and converted from a high active stiffness to a lower passive stiffness (Morgan, 1994). The theory also predicts a cumulative fall in isometric stiffness during the sequence of eccentric contractions, as the number of disrupted sarcomeres increases. Both of these were seen, and both are inconsistent with proposals that stiffness changes are due to changes in the number of cross-bridges, due in turn to changes in myofilament overlap (Sugi & Tsuchiya, 1988; Tsuchiya & Sugi, 1988). In particular, the isometric stiffness fell during the series of eccentric contractions much more than the tension. In the simplest case reduction in cross-bridge numbers should decrease both proportionately, and the complications are expected to make the stiffness fall less than tension. These possible complications include compliant filaments (Huxley, Stuart, Hernando & Iving, 1994), series compliance (Morgan, 1977), and shifts to shorter active sarcomere lengths (Julian & Morgan, 1981).

In later eccentric contractions, more of the stretch is predicted to be absorbed by further extension of overstretched sarcomeres as the tension continues to rise, so that fewer sarcomeres are 'popped', leading to a smaller fall in stiffness. It is likely that non-linearities in the elastic properties of disrupted sarcomeres will also lead to an increasing stiffness as the tension rises, countering the decrease due to the increasing number of overstretched sarcomeres.

The rise of stiffness at the beginning of the stretch is not predicted by our hypothesis. It has been reported by Sugi & Tsuchiya (1988), Tsuchiya & Sugi (1988) and Piazzesi, Francini, Linari & Lombardi (1992), and interpreted as an increase in the number of cross-bridges. The original cross-bridge model of Huxley (1957) does in fact predict a moderate increase in cross-bridge numbers at moderately slow lengthening velocities (Harry, 1988). Such changes should attain a steady state when stretch has reached a few cross-bridge strokes per half sarcomere, and persist as long as the stretch continues, making them unlikely to be the basis of the slower fall seen as the stretch progressed.

Calcium measurements

The calcium measurements were undertaken primarily to rule out calcium-based explanations for the shift in L_{opt}, but proved very interesting in their own right. Eccentric contractions did not reduce either twitch or tetanic $[Ca^{2+}]_i$ in

this preparation, and in fact increased the duration of the apparent $[Ca^{2+}]_i$ transient during a twitch and increased the mean value during a tetanus. Potentiating the twitch with nitrate failed fully or partially to reverse the shift in L_{opt} due to eccentric contractions, but instead tended to produce further shift in the same direction. Taken together, these observations rule out any model that ascribes shifts in the length–tension relation in this preparation, and by implication other similar preparations, to changes in transient $[Ca^{2+}]_i$.

The measurements of resting $[Ca^{2+}]_i$ support the suggestion that a rise is an early indicator of impending failure (Armstrong, 1984), while also showing that such a rise is not necessary for the observation of cumulative changes in mechanics. No support was found for a focal nature of the rise seen in damaged fibres, though considering the time scale of the observations, a single focus of calcium release with diffusion along the fibre could not be ruled out.

The profound changes in the apparent $[Ca^{2+}]_i$ transient in the presence of potentiator after eccentric contractions suggest that eccentric contractions do impair the calcium sequestering capacity of the sarcoplasmic reticulum, even though their effect on calcium release seems to be minor.

Comparisons with the literature

We know of no previous reports of such shifts in single frog fibres, and only one brief report in mouse single fibres (Balnave & Allen, 1995). The measurements of shift are similar to those reported previously in whole muscle (see Introduction). The smaller fall in tension seen in single fibres is probably due to the presence, in whole muscles, of some fibres that are damaged to the point of not propagating action potentials. Such fibres could still develop a caffeine contracture, consistent with the results of Warren *et al.* (1993). The rise in tension at long lengths in some fibres is consistent with Katz's report, but inconsistent with proposals that the shift in L_{opt} is due to submaximal activation.

The measurements of stiffness during a stretch are similar to those of Sugi & Tsuchiya (1988) and Tsuchiya & Sugi (1988), but those authors did not report the cumulative decrease in isometric stiffness seen here during a series of eccentric contractions.

Changes in stiffness after a series of eccentric contractions were measured by Warren *et al.* (1993) in whole mouse soleus muscles. They reported a stiffness drop after eccentric contractions that was larger than the tetanic tension drop, but not significantly so. Given the large contribution of tendon to compliance in whole muscle preparations, this is indicative of fibre stiffness falling substantially more than tension (Morgan, 1977). Furthermore, their ratio of stiffness to tension after eccentric contractions was $1{\cdot}56\ mm^{-1}$ compared with $1{\cdot}98\ mm^{-1}$ after isometric contractions, suggesting that even whole muscle stiffness fell more than tension when the changes due to isometric contractions are taken into account. This is in accord with our findings.

The increase in apparent $[Ca^{2+}]_i$ after eccentric contractions is in contrast to the report of Balnave & Allen (1995) that apparent $[Ca^{2+}]_i$ fell in submaximal tetani. The large number of differences, including species, temperature, dye, twitch : tetanus ratio and level of activation will need to be investigated to resolve this difference. Despite this, it is now clear that neither a rise in resting $[Ca^{2+}]_i$ nor a fall in apparent tetanic $[Ca^{2+}]_i$ are a precondition for a shift in optimum length.

The shifts in L_{opt} seen in single fibres are comparable to those reported in whole toad muscles (Wood *et al.* 1993), despite very different twitch : tetanus ratios.

We conclude then that the shift in L_{opt} and other mechanical changes resulting from eccentric contractions do occur in single fibres, and that they do not result from disturbances to the intracellular Ca^{2+} dynamics, but probably cause them, for the following reasons. Shifts were not accompanied by decreased intracellular calcium transient, nor always by a significant rise in resting calcium levels. Twitch potentiators did not reverse the shift, even when the resulting twitch : tetanus ratio approached unity. The tension at long lengths sometimes rose, and on other occasions fell very little compared with the shift in L_{opt}.

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Acknowledgements

This work was supported in part by National Institutes of Health grant HL35032 to F.J.J., and an Outside Studies Programme grant from Monash University to D.L.M.

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Received 31 May 1996; accepted 11 September 1996.