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INTRACELLULAR CALCIUM AND TENSION DURING FATIGUE IN ISOLATED SINGLE MUSCLE FIBRES FROM *XENOPUS LAEVIS*

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SUMMARY

1. Single muscle fibres were dissected from *Xenopus* lumbrical muscles and microinjected with the photoprotein aequorin in order to measure the myoplasmic free calcium concentration ($[Ca^{2+}]_i$). Fatigue was produced by repeated intermittent tetanic stimulation continued until tension had declined to approximately 50% of the initial level. Fibres were then allowed to recover by giving tetani at less frequent intervals. Aequorin light (a measure of $[Ca^{2+}]_i$) and tension were measured during fatiguing stimulation and recovery.

2. During fatiguing stimulation, tetanic tension decined steadily, but peak aequorin light first increased before declining substantially. The largest light signal was about 155% of initial control while at the end of fatiguing stimulation the tetanic light fell to about 14% of control.

3. Fibres showed a characteristic slowing of relaxation in the fatigued state. This was associated with a slowing of the rate of decline of the aequorin light signal.

4. Intracellular acidosis produced by equilibrating the Ringer solution with either 5 or 15% CO_2 caused an increase in the light signal associated with a tetanus. Carbon dioxide also caused a reduction of tension and a slowing of relaxation.

5. *In vivo* pCa–tension curves were constructed by exposing the fibres to a series of K^+ concentrations which produced contractures of different sizes. Light and tension were measured during periods when both were relatively stable and the light signal was subsequently converted to pCa.

6. Exposure of fibres to 5 or 15% CO_2 caused the pCa–tension curve to be shifted to the right of the control curve. This indicates a reduced Ca^{2+} sensitivity of the contractile proteins, which is in agreement with results from skinned fibre studies.

7. The pCa–tension points obtained from tetani during the early part of fatiguing stimulation also deviated to the right of the control pCa–tension curve, suggesting a reduced Ca^{2+} sensitivity of the contractile proteins. At the end of fatiguing stimulation, however, pCa–tension points did not differ greatly from the control pCa–tension curve, suggesting that Ca^{2+} sensitivity was approximately normal. Thus the reduced $[Ca^{2+}]_i$ during tetani at the end of fatiguing stimulation (when tension was reduced to approximately 50%) could explain all of the reduction in tension.

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8. After fatiguing stimulation, tension and light recovered monotonically in some fibres; however, in the majority of fibres, tension and light showed a secondary decline followed by a slower recovery (post-contractile depression).

9. During post-contractile depression, caffeine contractures or tetani in the presence of caffeine gave increased aequorin light signals and the tension developed was close to that produced in an unfatigued tetanus.

10. The results of this study show that changes of $[Ca^{2+}]_i$ during fatigue can account for much of (i) the tension reduction during fatigue, (ii) the slowing of relaxation, and (iii) the tension reduction during post-contractile depression.

INTRODUCTION

Repeated tetanic contractions of skeletal muscle eventually lead to a reduction in the developed tension, i.e. fatigue develops. In intact animals, this familiar phenomenon is known to have contributions from the central nervous system, the neuromuscular junction and the muscle itself (Bigland-Ritchie & Woods, 1984). However, many features of fatigue may be seen in isolated muscle preparations, suggesting that processes within the muscle make a large contribution.

In isolated muscles, the intracellular mechanisms which cause the various manifestations of fatigue are not well understood. One hypothesis to explain reduced tension in fatigue is that it is due to the changes in intracellular metabolites which accompany prolonged activity. These include reduced concentrations of phosphocreatine (PCr) and ATP, and increased concentrations of creatine (Cr), inorganic phosphate (P_i), ADP, lactic acid and hydrogen ions (i.e. a reduced pH) (Dawson, Gadian & Wilkie, 1978; Nassar-Gentina, Passonneau, Vergara & Rapoport, 1978). Further, it is established from skinned fibre studies that increased P_i and H^+ have marked tension-reducing properties when applied to isolated myofibrils (Fabiato & Fabiato, 1978; Cooke & Pate, 1985; Kentish, 1986; Godt & Nosek, 1989).

An alternative hypothesis suggests that changes in excitation–contraction coupling may underlie the fall in tension. This was first suggested by Eberstein & Sandow (1963) on the basis that the tension developed by fatigued muscles could be substantially increased by exposure to high potassium or caffeine, procedures which bypass certain steps in the excitation–contraction sequence. While these observations have been confirmed, and it is widely accepted that changes in excitation–contraction coupling have some role in fatigue (Grabowski, Lobsiger & Lüttgau, 1972; Lännergren & Westerblad, 1989), there is little information on the precise step in excitation–contraction coupling which might be affected.

Several other features of fatigued muscle may also be related to changes in intracellular Ca^{2+} movements resulting from abnormal excitation–contraction coupling. Slowing of relaxation is a characteristic feature of fatigue and has been postulated to be due to a reduced rate of uptake of Ca^{2+} by the sarcoplasmic reticulum (Dawson, Gadian & Wilkie, 1980). Also, recent studies have shown that following stimulation there is often a reversible secondary decline of tension (post-contractile depression), which has been attributed to failure of excitation–contraction coupling (e.g. Lännergren & Westerblad, 1989). However, direct evidence on both these points is lacking.

In this study, we have investigated the role of excitation–contraction coupling in

fatigue by using the Ca^{2+}-sensitive photoprotein aequorin to measure the myoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Single muscle fibres were fatigued with intermittent tetanic stimulation which continued until tension was reduced to about 50% of control. The results show that there are changes in $[Ca^{2+}]_i$ during fatigue and recovery which can explain much of (i) the tension reduction during fatigue, (ii) the slowing of relaxation, and (iii) the tension reduction during post-contractile depression. Preliminary accounts of some of these results have been presented (Allen, Lee & Westerblad, 1988, 1989*a*).

METHODS

Single fibre dissection and mounting

Experiments were performed on single muscle fibres from adult female *Xenopus laevis*. The frogs were killed by stunning followed by decapitation. Single fibres were dissected from any of the lumbrical muscles II–IV under dark field illumination. The trimmed tendons of the fibres were held with platinum foil micro-clips. After dissection, the largest and smallest diameters of the fibres were measured using an ocular scale and the cross-sectional area of the fibre was calculated. Fibres were then transferred to the perfusion channel of the experimental chamber. One end was attached to a horizontally mounted tension transducer (Akers AE 801, SensoNor, Norway) via a glass extension with a fine hook at the end. The other end was attached to a movable hook which allowed the length of the fibre to be adjusted so that maximum tetanic tension was obtained. Fibres were superfused with a standard Ringer solution containing (mM): Na^+, 120; K^+, 2·5; Ca^{2+}, 1·8; Cl^-, 121; HPO_4^{2-}, 2·15; $H_2PO_4^-$, 0·85; pH, 7·0. All experiments were performed at 21 °C.

Microinjection of aequorin

Fibres were injected with aequorin essentially as described by Blinks, Rüdel & Taylor (1978). Briefly, the procedure was as follows. Aequorin solution (approximately 50 μM in 140 mM-KCl, 5 mM-HEPES, pH 7) was placed at the end of a conventional microelectrode (resistance 30–50 MΩ). The microelectrode was inserted into the fibre under visual control and while monitoring the electrode potential. When the microelectrode was in the fibre, pulses of pressure from a gas cylinder were applied. After several pressure pulses, the electrode was removed and a photomultiplier tube with an attached acrylic light guide was placed over the fibre. The light emitted by the fibre was then monitored during a test stimulus. Often several impalements with different microelectrodes were necessary to achieve an acceptable aequorin signal. In some experiments the external $[Ca^{2+}]$ was raised to 5 mM during the injection period because this was found to decrease the risk of damage to the fibre during injection.

Experimental protocol

Fibres were stimulated with platinum electrodes using alternating current pulses with a duration of 0·5–1·0 ms and an intensity of approximately 1·2 × threshold. The stimulation frequency was 40 or 50 Hz. Fatigue was produced using a protocol similar to that of Westerblad & Lännergren (1986). Initially, 500 ms tetani were given every 4 or 5 s and the inter-tetanus interval was then successively decreased every 2 min to 4·0, 3·0, 2·5, 2·0, 1·7 and 1·5 s. Stimulation was continued until the tension was reduced to about 50% of control. To study recovery from fatigue, test tetani were given at various times after fatiguing stimulation. These times were usually 1, 5, 10, 20, 30 and 40 min into recovery and then every 20 min until tension was stable.

The perfusion channel of the experimental chamber was provided with a side arm which allowed injection of solutions. In most experiments K^+ contractures were produced before fatiguing stimulation by rapid injection, via this side arm, of solutions containing varying concentrations of K_2SO_4. These solutions were constructed by mixing 75 mM-K_2SO_4 (150 mM-K^+) with Ringer solution in various proportions. In some experiments 150 mM-K^+ was applied at various times during the recovery period. Applications of Ringer solution containing high doses of caffeine (10 mM) were performed in a few experiments. These caffeine contractures generally resulted in irreversible fibre damage. In other experiments low doses of caffeine (3 mM) were instead combined with tetanic stimulation; this procedure decreased the risk of fibre damage.

In some experiments the effects of acidosis were examined before fatiguing stimulation. Fibres were exposed to Ringer solution which had been equilibrated with either 5 or 15% CO_2, which reduced the external pH to 6·3 and 5·9, respectively.

Tension, aequorin light and stimuli were displayed on a pen recorder and also recorded on tape for later analysis. Values in the results are presented as mean ± S.E.M.

Interpretation and calibration of aequorin light signals

During fatiguing stimulation there are a number of metabolic changes inside muscle fibres which might affect aequorin light emission. Decreased pH is known to occur in muscle fibres exposed to repeated tetani, due to lactic acid production (Dawson *et al.* 1978). In the fibre types used in these experiments, intracellular pH fell during fatiguing stimulation by about 0·6 pH units in easily fatiguable fibres and by 0·3 pH units in fatigue-resistant fibres (Westerblad & Lännergren, 1988). Acidosis reduces aequorin light emission, but this effect is small at intracellular levels of $[Ca^{2+}]$; an acidosis of 0·5 pH units at a $[Ca^{2+}]$ of 10 μM caused a 30% (0·15 log unit) reduction in light emission, but had no effect at $[Ca^{2+}] = 0{\cdot}1$ μM (Allen & Orchard, 1983). Others have found no change in aequorin light emission for a 0·5 pH unit change at $[Ca^{2+}]$ of 2–6 μM (e.g. Fabiato, 1985).

Magnesium (Mg^{2+}) is another ion which can compete with Ca^{2+} for aequorin and hence cause a reduction in light emission. Its concentration might be expected to rise during fatigue because of release from ATP and possibly also because of displacement from parvalbumin if the average $[Ca^{2+}]_i$ during fatiguing stimulation is increased. However, to our knowledge, $[Mg^{2+}]_i$ has not been measured during fatigue. Thus the changes in the intracellular environment of fatigued fibres may cause a small reduction in aequorin light for a given $[Ca^{2+}]$. In view of these uncertainties, it is important to note that the Ca^{2+} signals during fatigue have now been measured with Fura-2 (Allen, Lee & Westerblad, 1989*b*), a fluorescent Ca^{2+} indicator which is not sensitive to $[Mg^{2+}]$ and has quite different properties to aequorin. The Fura-2 signals confirmed all the major features observed in the present study, making the presence of major artifacts due to changes in intracellular metabolites most unlikely.

Consumption of aequorin will reduce the aequorin light signals during fatigue. To quantify this effect we have compared the integrated light in one tetanus with the light produced when all the remaining aequorin was discharged at the end of an experiment with Triton X-100. On average, a normal tetanus (50 Hz, 0·5 s, unfatigued) consumed 0·07% (range 0·04–0·13%, $n = 6$) of the aequorin, while a maximal K^+ contracture (150 mM-K^+) consumed 3·3% (range 1–8%). In the six experiments examined, the train of tetani during fatigue consumed 5·9% (range 1–13%) of the aequorin, the variability arising both from the size in aequorin transients and the number of tetani required for fatigue. Correction for this small effect is made in the Discussion. Potassium contractures were never produced during a fatigue run so that, although they produce significant consumption, they do not affect our estimate of the fall of light during fatigue.

In some experiments (see Figs 5, 6 and 10) the light signals have been converted to $[Ca^{2+}]$ using a calibration procedure, details of which are given in Smith & Allen (1988). In principle this involves normalizing the light signal for the amount of aequorin in the preparation and then converting the normalized light signal to $[Ca^{2+}]$ by means of an appropriate *in vitro* calibration curve. It should be noted that this calibration has a number of well-recognized weaknesses (e.g. Blinks, Wier, Hess & Prendergast, 1982; Smith & Allen, 1988) and provides an indication of $[Ca^{2+}]$ rather than a precise calibration.

RESULTS

It is well recognized that single muscle fibres vary substantially in their susceptibility to fatigue. Following previous studies with *Xenopus* single fibres (Westerblad & Lännergren, 1986) we have defined easily fatiguable fibres as those in which tetanic tension declined to less than 50% at stimulation intervals of 2·5 s or longer, and fatigue-resistant fibres as those in which shorter interstimulus intervals than 2·5 s were needed for tension to fall to 50%. In this study we report results from seven easily fatiguable and from three fatigue-resistant fibres.

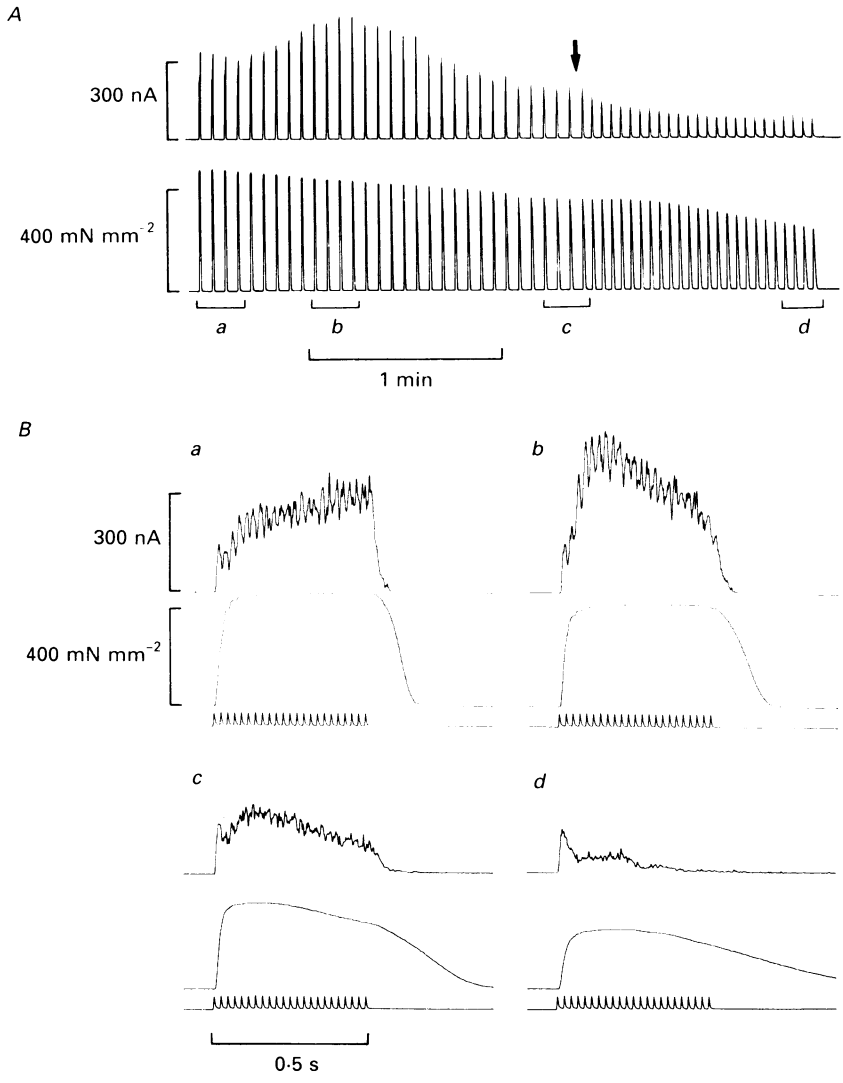

Fig. 1. Records of aequorin light and tension from an easily fatiguable fibre during fatiguing stimulation (fifty-five tetani). *A*, continuous record of aequorin light (above) and tension (below). Arrow marks the point at which stimulation was changed from 4 to 3 s interval. *B*, averaged records ($n = 4$) of aequorin light (above) and tension (below) from the periods shown in *A*. The stimulus marker is also shown. Note the changes in the form of the aequorin light transient and the slowing of tension relaxation in fatigue.

The amplitude of aequorin signals during fatigue

Figure 1 shows a representative example of aequorin light and tetanic tension from an easily fatiguable fibre. Figure 1*A* shows a continuous record, while Fig. 1*B* shows averaged records from the periods indicated in *A*. It can be seen that while tetanic tension declined more or less steadily during fatiguing stimulation, the amplitude of the aequorin signal showed a more complex pattern, increasing over the first twelve

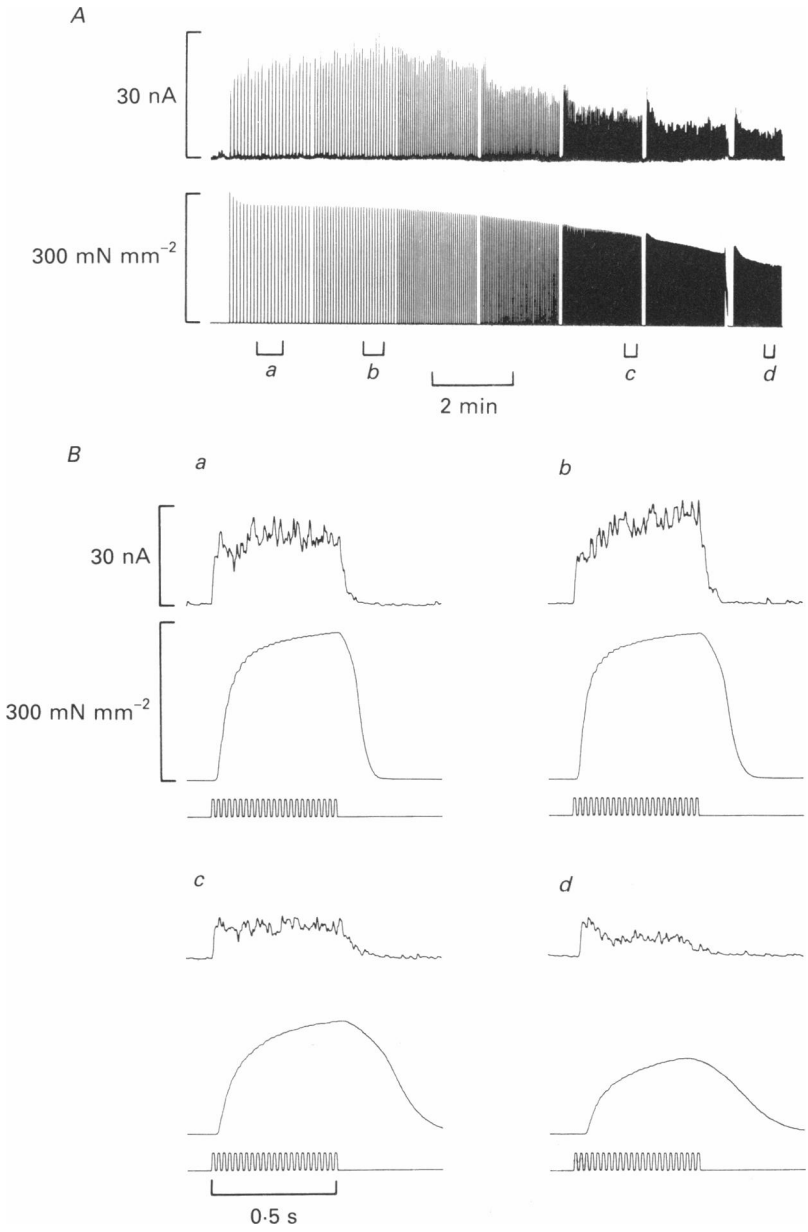

Fig. 2. Records of aequorin light and tension from a fatigue-resistant fibre during fatiguing stimulation (311 tetani). *A*, continuous record of aequorin light (above) and tension (below). *B*, averaged records ($n = 8$) of aequorin light (above) and tension (below) from the periods shown in *A*. The stimulus marker is also shown. Note the changes in the form of the aequorin light transient and the slowing of tension relaxation in fatigue.

tetani and thereafter showing a pronounced decline. For the easily fatigued fibres, if the amplitude of the first aequorin transient is defined as 100%, the largest transient was 162 ± 28% and occurred, on average, on the eighth tetanus (range three to twelve). By the time tension had declined to 50%, which required, on average, fifty-

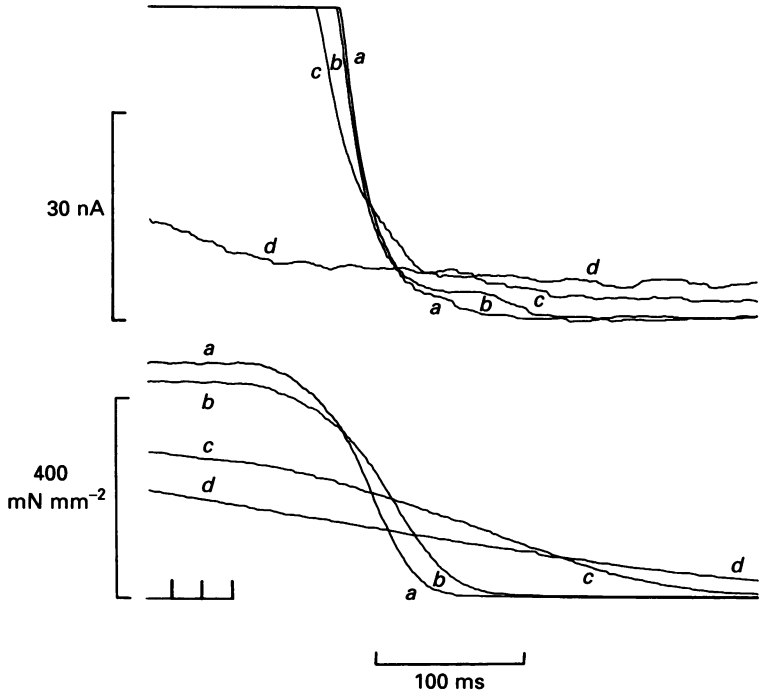

Fig. 3. Slowing of tension relaxation during fatigue is associated with slowing of the rate of fall of aequorin light. The averaged records of Fig. 1 are displayed here with higher gain and time resolution. It can be seen that as tension relaxation slows, the rate of fall of aequorin light is also slowed. Also, in the later tetani, the minimum level of the light signal is higher than in the earlier tetani, i.e. there is a raised resting light level.

nine tetani (range twenty-five to ninety-four tetani), the light signal had declined to $13 \pm 2\%$.

Figure 2 shows an example of aequorin light and tetanic tension from a fatigue-resistant fibre. Figure 2*A* shows a continuous record, while Fig. 2*B* shows averaged records from the periods indicated in *A*. In these fibres the pattern was similar to that seen in the easily fatiguable fibres, with tension declining steadily throughout fatiguing stimulation, while the light signal first showed an increase in amplitude and then a substantial decrease. In fatigue-resistant fibres the maximum aequorin light was $140 \pm 15\%$. In comparison with easily fatiguable fibres, the increase in aequorin light occurred somewhat later in fatigue-resistant fibres (maximum, on average, at thirty-third tetanus, range fourteen to forty-three). The reduction of tension to 50% required 340 tetani (range 216–495) and at that time the peak aequorin signal was $18 \pm 6\%$.

Panels *B* of Figs 1 and 2 show that there were also changes in the form of the aequorin signals as fatigue developed. The light signals often changed from a form which was increasing throughout stimulation (as in Fig. 1*Ba*) or flat, to one which was largely decreasing (as in the other panels in Fig. 1*B*). Also, the early peak in the transient at the later stage of fatigue was characteristic (e.g. Figs 1*Bd* and 2*Bd*).

The slowing of relaxation and the decline of light

Slowing of relaxation is a characteristic feature of fatigue and is clear in the later tetani shown in Figs 1*B* and 2*B*. This could arise either because the rate at which Ca^{2+} is removed from the myoplasm is slowed during fatigue or because of changes in cross-bridge cycling. If slowed relaxation is due to $[Ca^{2+}]_i$ falling more slowly in a fatigued fibre, then it should be possible to observe a slower fall of the aequorin light signal at the end of a tetanus.

The fall in aequorin light at the end of a tetanus in an unfatigued fibre has been described as falling in three phases (Cannell, 1986). There is a rapid exponential phase, which includes more than 95% of the signal, followed by a period in which the fall of light slows or even reverses slightly and finally a long slow tail of light. We did not regularly observe the second phase described by Cannell (1986), probably because this phase is most obvious in tetani of 1 s or greater in duration.

The simplest way to determine whether the rapid phase of decline of the aequorin signal was altered in the fatigued state and contributed to the slowing of relaxation, would be to measure the time for the light signal to decline to, say, 25% of its level after the last stimulus (t_{25}(light)) and to compare this with the time taken for tension to decline to some fraction of its level at the last stimulus. There are, however, several problems with this approach. Towards the end of fatiguing stimulation both tension and light start to decline in individual tetani well before the last stimulus. This can lead to an artificial reduction in the apparent rate of decline of tension when measured by this criterion. Furthermore, in some preparations (e.g. Fig. 3, trace *d*) the fast phase of decline of light is completely absent by the end of fatigue, so that measurement of t_{25}(light) as defined above probably measures the characteristics of the slow phase of light decline. To minimize these problems, we measured t_{25}(light) and t_{50}(tension) (the time taken for the tension to decline to 50% of its level after the last stimulus) for the first tetanus of fatiguing stimulation and the last tetanus which still had a fast component of decline of light and in which t_{50}(tension) was still increasing. In eight experiments t_{50}(tension) for the first tetanus was 106 ± 6 ms and t_{25}(light) was 43 ± 1 ms. Towards the end of fatiguing stimulation t_{50}(tension) had increased to 246 ± 30 ms while t_{25}(light) had increased to 103 ± 13 ms. Both increases were highly significant ($P < 0{\cdot}01$ on a paired t test).

Within individual experiments there was a clear correlation between the gradual slowing of relaxation and the decline of light. This is obvious when records at various stages of fatiguing stimulation are superimposed as in Fig. 3. In this record the aequorin signals are displayed at high gain so that only the lowest 10% of the control signal is visible. It is clear that the aequorin signals slowed progressively throughout fatigue and that, while there is some slowing of the rapid phase, there is in addition a large increase in the amplitude of the slow phase. The similarity of the slow mechanical relaxation in the last tetanus (*d* in Fig. 3) and the slowed decline of the accompanying aequorin signal suggests that a reduced rate of fall of $[Ca^{2+}]_i$ was making a substantial contribution to the reduced rate of relaxation. A similar tendency for slowing of the fall of light to accompany slowing of the fall of tension was seen in all experiments. There was no obvious difference between easily fatiguable and fatigue-resistant fibres in this respect, except that more tetani were required to produce these changes in fatigue-resistant fibres.

Towards the end of fatiguing stimulation, the aequorin light was still detectably elevated in some fibres when the next tetanus started (see figure in Allen, Lee & Westerblad, 1988). This could occur either because the steady state resting $[Ca^{2+}]_i$ was elevated, or because the tail of decline of aequorin light was now so long that it

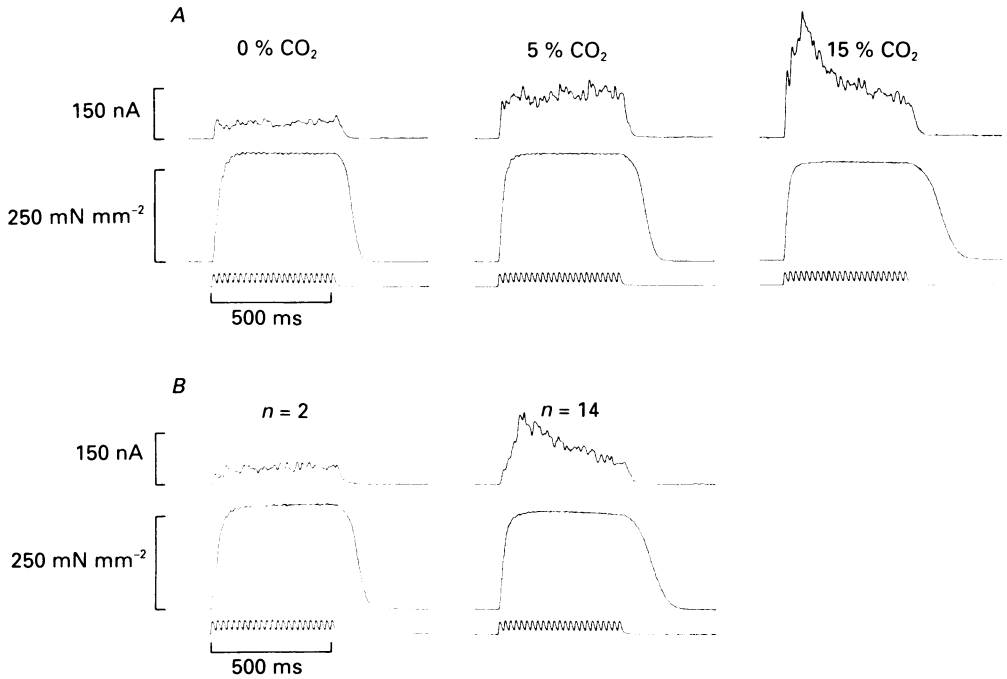

Fig. 4. Comparison of the effects of acidosis and fatigue on aequorin light and tension in the same muscle fibre. Each panel shows aequorin light (above), tension (middle) and stimulus marker (below) from a single contraction. *A*, the three panels show from left to right the effects of 0, 5 and 15% CO_2. *B*, the two panels show the second and fourteenth tetani of fatiguing stimulation in the same muscle fibre and illustrate the early rise in tetanic $[Ca^{2+}]$.

merged with the next tetanus (i.e. had the inter-tetanus interval been long enough, the light would have declined to the original resting level). Unfortunately, the relation between $[Ca^{2+}]$ and light for aequorin is such that low calcium levels are very difficult to detect (Blinks *et al.* 1982), and so it is not an ideal tool with which to distinguish between these possibilities. Nevertheless, in three fibres which had a large light signal, it was established by photon counting that the resting light 30 s after the last tetanus was elevated. Thus, although it was not visible in all fibres, this suggests that a raised resting $[Ca^{2+}]_i$ is present towards the end of fatiguing stimulation and in the early part of recovery.

The effect of intracellular acidosis on aequorin light and tension

One possible explanation for the early increase in the light transients during fatigue is that it is due to an intracellular acidosis caused by lactic acid production. An increased hydrogen ion concentration could reduce the effectiveness of intracellular calcium buffers, leading to a greater free $[Ca^{2+}]$ inside the cell for a given release of calcium. It has been shown in cardiac muscle that acidosis, due to either

increased CO_2 or lactic acid accumulation, may increase the calcium transients (Allen & Orchard, 1983). Evidence supporting the role of hydrogen ions in the early increase in aequorin light during fatiguing stimulation is shown in Fig. 4. Panel *B* shows that a substantial increase in aequorin light had occurred by the fourteenth tetanus in this experiment. Panel *A* shows the effects of increasing the CO_2 concentration in the same fibre. This series of contractions preceded the fatigue series; the muscle fibre was perfused with Ringer solution equilibrated with 5 or 15% CO_2. Several tetani at 2 min intervals were given until the light and tension response at the new CO_2 level was stable. Carbon dioxide causes an extracellular acidosis (see Methods) and, more important, also causes an intracellular acidosis (Bolton & Vaughan-Jones, 1977; Curtin, 1986, 1987). It can be seen that changing the CO_2 in this way had marked effects on the light signals. The light signal was increased by 5% CO_2, while 15% CO_2, in addition to causing a further increase, also changed the shape of the signal so that it exhibited an early peak and subsequent decline. The form of this transient is similar to that seen in the fourteenth tetanus shown in panel *B*. In four experiments 5% CO_2 caused light to increase to a peak of 218 ± 31% of control, whereas the maximum light signal during fatiguing stimulation was 188 ± 38% of control in these fibres.

At both CO_2 levels there was obvious slowing of relaxation and also a small reduction in tension, in agreement with previous results (Edman & Mattiazzi, 1981; Curtin, 1986). In 5% CO_2 t_{50}(tension) (as defined above) was increased by 22 ± 3 ms ($n = 5$) while in 15% CO_2 t_{50}(tension) increased by 52 ± 6 ms. However, the rate of decline of light was not significantly affected by either level of CO_2; t_{25}(light) decreased by $1{\cdot}8 \pm 2{\cdot}9$ ms in 5% CO_2 and increased by $3{\cdot}7 \pm 2{\cdot}2$ ms in 15% CO_2.

The contribution of reduced tetanic $[Ca^{2+}]_i$ *to reduced force during fatigue*

In a previous study of aequorin signals in frog skeletal muscle (Blinks *et al.* 1978), it was pointed out that the aequorin signal during a tetanus could change over a considerable range with little change in tension. It was suggested that this arose because myoplasmic $[Ca^{2+}]$ under their conditions was considerably above the level required to saturate troponin binding sites. Thus, in order to show that the reduced tetanic $[Ca^{2+}]_i$ in fatigued muscle is the cause of reduced tension, it was desirable to (i) work under conditions in which the $[Ca^{2+}]_i$ in a control tetanus was only just maximal and (ii) to determine the relation between $[Ca^{2+}]_i$ and tension for submaximal levels of tension. To achieve (i), the stimulus frequency was set close to the fusion frequency. To achieve (ii) we attempted to construct an *in vivo* pCa–tension curve using submaximal K^+ contractures. The basis of this method was to produce a series of K^+ contractures which gave different levels of light and tension. Suitable values of light and tension were then plotted so as to obtain a pCa–tension curve under control conditions.

We identified two potential problems with using K^+ contractures in this way. First, because $[Ca^{2+}]_i$ was changing continuously throughout a K^+ contracture, it is difficult to be sure that $[Ca^{2+}]_i$ and tension had reached a steady-state relationship. Second, if there was inhomogeneity of activation, the non-linearity of the relation between aequorin light and $[Ca^{2+}]_i$ would lead to aequorin signals which would exaggerate the spatially averaged $[Ca^{2+}]_i$ (e.g. Blinks *et al.* 1982). Our approach to the

first problem was to select periods of K^+ contractures when both tension and light were relatively constant. After some experimentation we chose periods of 200 ms where tension changed by less than 10% of its maximum value and where light was not rising or reached a peak. (Examples of the method are shown in Fig. 5*B* and *C*

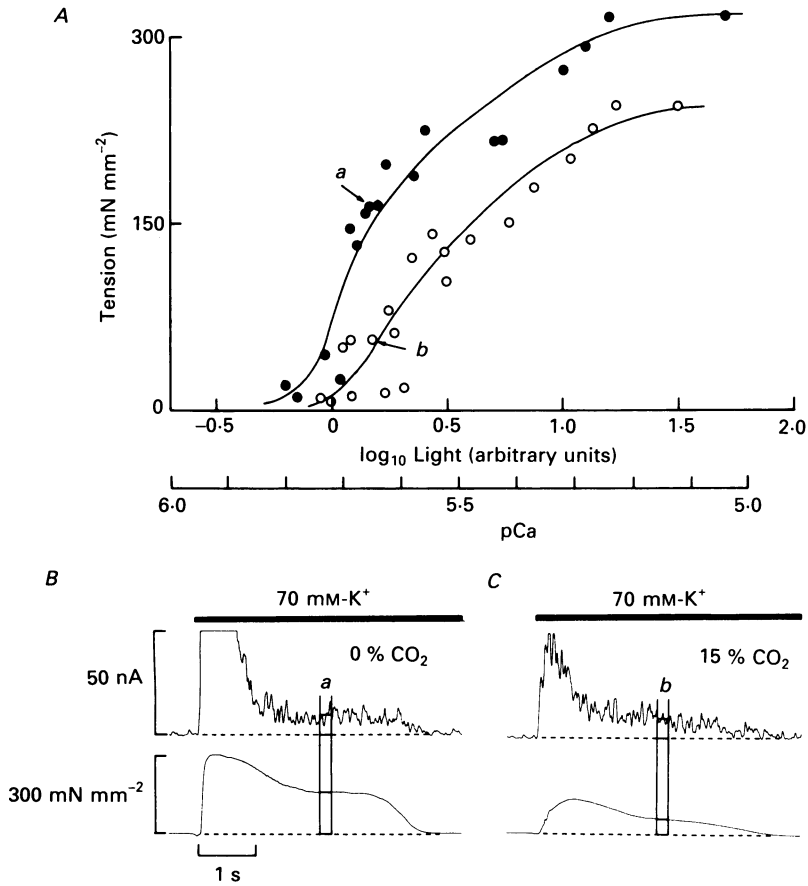

Fig. 5. *In vivo* pCa–tension curves were constructed with the use of K^+ contractures. *A*, *in vivo* pCa–tension curves under control conditions, pH_o 7·1 (●) and in the presence of 15% CO_2, pH_o 6·3 (○). *B* and *C*, examples of records from which the pCa–tension curves were constructed. Both of these records were obtained with a K^+ contracture using 70 mM-K^+. Note that for a similar light level, the contracture in 15% CO_2 produces considerably less tension. Measurements at *a* and *b* are shown on the curves in *A*.

and Fig. 6*B*, *C* and *D*.) Tension and light were averaged over periods fulfilling these criteria, and in this way a sufficient number of points to define a pCa–tension curve were obtained from four or five K^+ contractures. Our evidence that the problem of inhomogeneity cannot be large is that the scatter on these graphs is reasonably small. In addition, in several experiments, points with similar tension levels were measured from contractures at different levels of K^+, but the light signals proved reasonably comparable.

Figure 5 illustrates an attempt to validate this method by examining the effects of increasing the concentration of CO_2 in the Ringer solution. In panel *A*, the filled

circles represent contractures produced by 50, 60, 70, 80 and 150 mM-K^+. Panel *B* shows one such record, from a K^+ contracture at 70 mM-K^+, and illustrates how the measurements were made. This record is particularly satisfactory, since tension and light were virtually constant over about 1 s, giving a high degree of confidence that

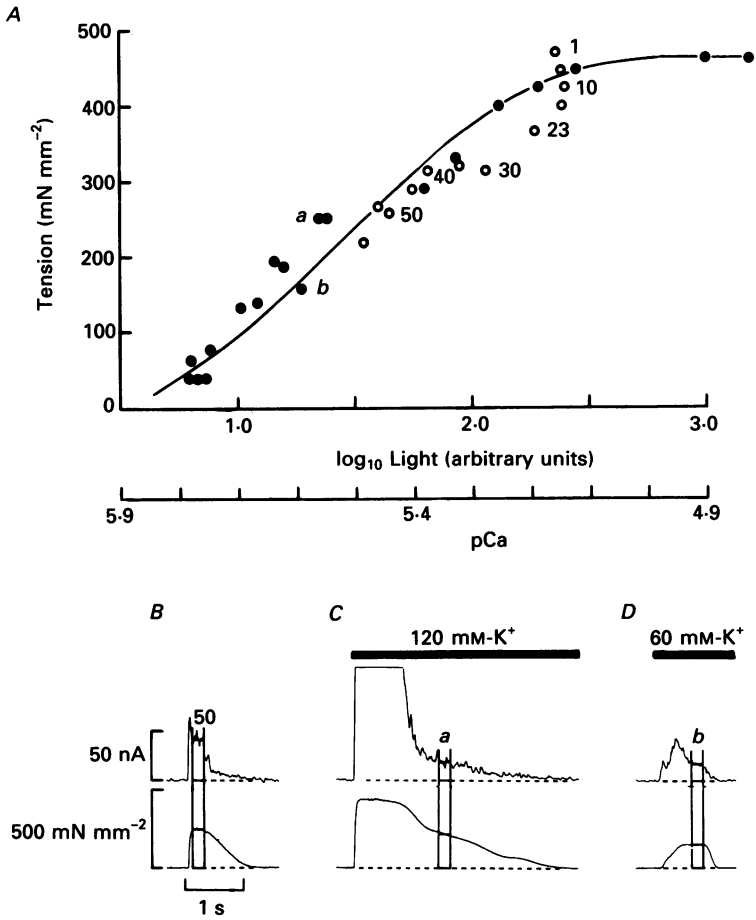

Fig. 6. Comparison of pCa–tension curve under control conditions with points obtained from tetani during fatigue. *A*, filled circles represent control pCa–tension points obtained with K^+ contractures. Open circles are those measured from records of light and tension obtained during fatiguing stimulation. Numbers indicate tetani during the stimulation period. *B*, *C* and *D*, examples of records from which the curves in *A* were constructed. *B* shows the calcium transient and tension from the fiftieth tetanus. *C* and *D* show the K^+ contractures from which the points (*a*) and (*b*) on the curve in *A* were measured.

tension and $[Ca^{2+}]_i$ were close to steady state. Potassium contractures were then examined in the same muscle fibre after the Ringer solution had been equilibrated with 15% CO_2. Panel *C* illustrates another K^+ contracture at 70 mM-K^+ during exposure to CO_2. By chance this gave a stable light level which was very similar to that before exposure to CO_2 (panel *B*), but it can be seen that the steady tension is less than half of that in the absence of CO_2. The curve constructed from such measurements in the presence of CO_2 is shown by the open circles in panel *A*. It can

be seen that it is shifted to the right, in agreement with studies on skinned muscle (Fabiato & Fabiato, 1978). The acidosis produced by CO_2 reduced the maximum developed tension by 25% in this experiment, and shifted the pCa giving 50% maximum tension by 0·11 pCa units. A curve constructed in the same muscle for an exposure to 5% CO_2 (not shown) lay in between the other two.

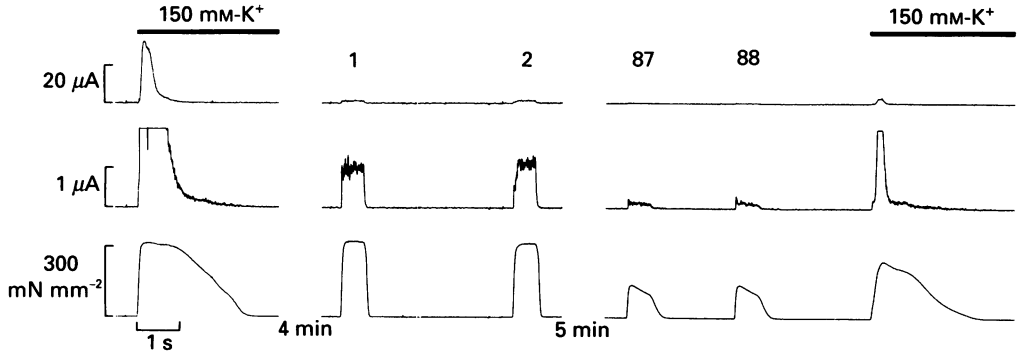

Fig. 7. The effects of maximal depolarization with 150 mM-K^+ before and after fatiguing stimulation. Records show low gain light (above), standard gain light (middle) and tension (below). The control K^+ contracture gave a very large peak light and maximum tension. Fatigue was then produced by repeated tetanic stimulation (numbers refer to tetani). The K^+ contracture given in place of the eighty-ninth tetanus produced a much reduced peak light and 79% of maximum tension.

Figure 6*A* shows a further example of an *in vivo* pCa–tension curve constructed from K^+ contractures (filled circles). The open circles represent points measured in a similar manner from tetani during fatiguing stimulation (panel *B* shows an example). In this experiment the first tetanus appears to lie above the constructed line, but this was not generally the case (e.g. Fig. 10). As described above, during the first ten to twenty tetani tension declined, but light increased, so the points move down and to the right of the constructed curve. In subsequent tetani, both tension and light declined, so the points fall more or less parallel to the constructed curve. However, towards the end of fatiguing stimulation, the points return towards the curve, so that the final tetani do not differ from it. Similar plots were performed for five other preparations and it was generally the case that the first tetani lay close to the constructed line, that tetani during the early part of fatiguing stimulation had a tendency to lie to the right of the curve, and that tetani towards the end of fatiguing stimulation lay close to the curve again. Thus it appears that towards the end of fatiguing stimulation the tension produced is what would be expected from the $[Ca^{2+}]_i$ observed, suggesting that the fall of $[Ca^{2+}]_i$ is making the major contribution to the reduced tension in fatigue.

Figure 7 shows the effects of exposure to 150 mM-K^+ before and after fatiguing stimulation. Before fatigue, this produced a tension similar to tetanic tension, while peak light was approximately twenty times greater than the light obtained from a tetanus at 50 Hz. By the end of fatiguing stimulation the light signal in a tetanus had declined to about 10% of its pre-fatigue level in this experiment. The potassium contracture at the end of fatiguing stimulation produced 79% of maximum tension, and the light signal was reduced to ~ 8% of its pre-fatigue level and its duration was

considerably reduced. Note that although the light in the K^+ contracture is considerably reduced by fatigue, its peak remains substantially larger than the light in a normal tetanus before fatiguing stimulation. On average a maximal K^+ contracture immediately after fatiguing stimulation produced 77 ± 3 % of the tension

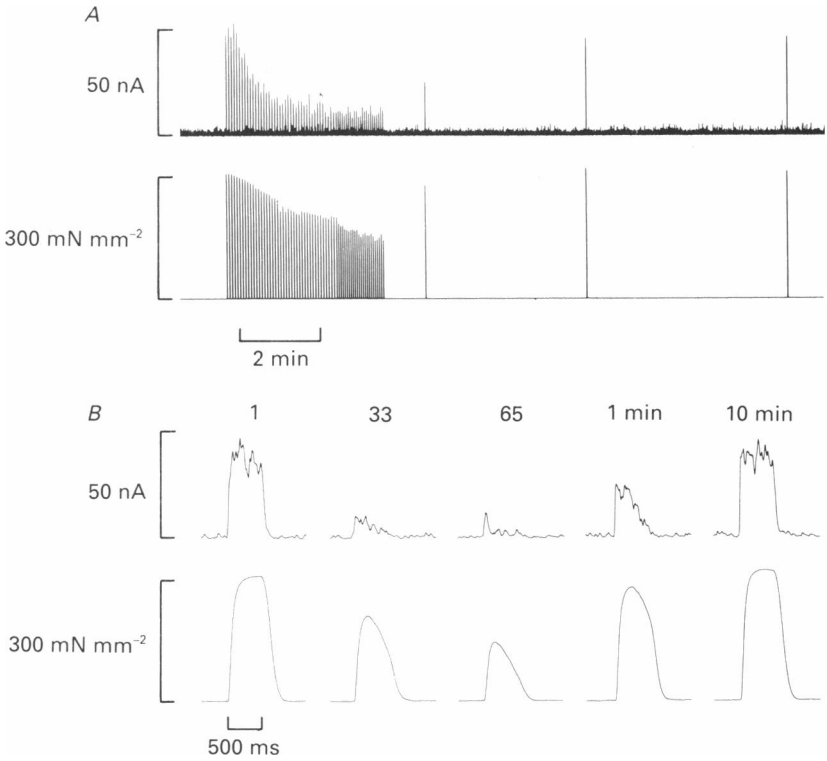

Fig. 8. Recovery from fatiguing stimulation in a fibre which recovered monotonically. *A*, continuous record of aequorin light (above) and tension (below) during and after fatiguing stimulation. By the end of the stimulation period light had declined substantially. Test tetani at 1, 5 and 10 min after the end of fatiguing stimulation show a smooth recovery of both light and tension to levels which are similar to control. *B*, individual tetani from the same experiment displayed on a faster time base. The first, thirty-third and last (sixty-fifth) tetani are shown, together with the tetani after 1 and 10 min of recovery.

given by one before stimulation, while the peak light was reduced to 16 ± 7 %. Despite the reduction of peak light in K^+ contractures in the fatigued state, the light signal was markedly greater than in the preceding tetani. This result shows that a substantial recovery of tension can be produced in a fatigued muscle by a procedure which increases the $[Ca^{2+}]_i$. Possible reasons for the decline of light in a K^+ contracture during fatigue will be considered in the Discussion.

Recovery from fatiguing stimulation

Of the ten fibres studied during fatigue development, eight were followed throughout recovery; the other two fibres showed considerable recovery before the experiment was terminated prematurely by a caffeine contracture or technical

problems. Two patterns of tension recovery were observed. In some fibres tension recovered monotonically, while in others a further reduction of tension after the end of fatiguing stimulation (post-contractile depression) was followed by a gradual recovery to pre-fatigue tensions (Westerblad & Lännergren, 1986).

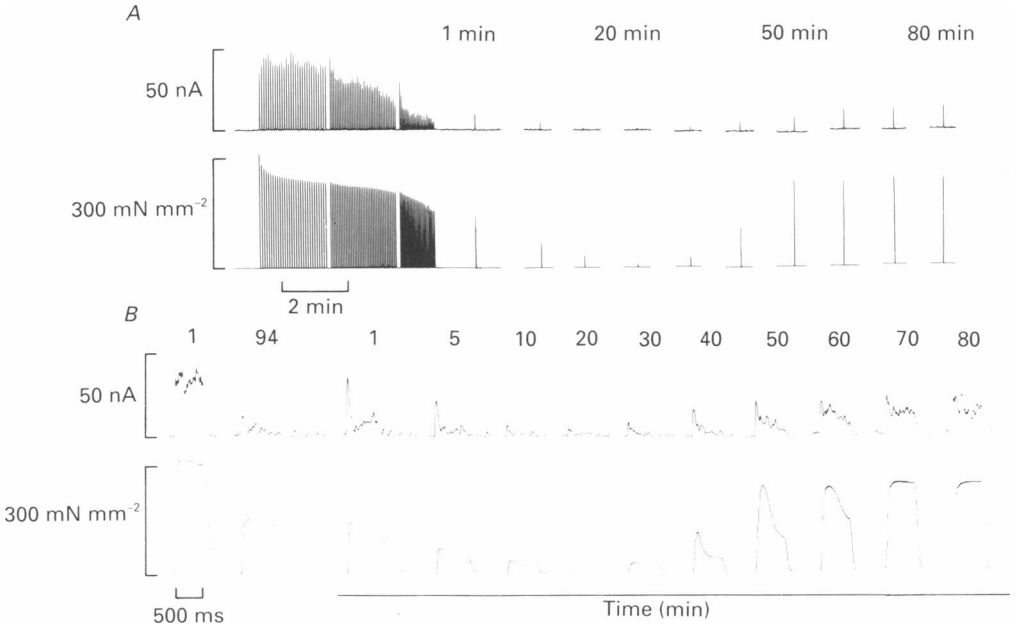

Fig. 9. Recovery from fatiguing stimulation in a fibre which showed post-contractile depression. *A*, continuous record of aequorin light (above) and tension (below) during and after fatiguing stimulation. Following the end of fatiguing stimulation, tension showed a further decline to a minimum at 20 min before finally recovering to a value which was similar to control. Light showed a small recovery at the 1 min test, but then also declined to a minimum at 20 min before recovering to a value which was substantially smaller than the control even after 80 min of recovery. *B*, individual tetani from the same experiment displayed on a faster time base. The first and last tetani of fatiguing stimulation are shown, together with test tetani at 1, 5, 10, 20, 30, 40, 50, 60, 70 and 80 min. The slight recovery of the light signal at 1 min and the sagging form of the tetanus during recovery are typical.

Figure 8 illustrates a fibre which recovered monotonically. In this fibre the tension recovered within 10 min and the light signal had almost fully recovered in the same time. In fibres which showed this pattern of recovery ($n = 3$), tension was $99 \pm 1\%$ of control and light was $89 \pm 14\%$ of control within 30 min after the end of fatiguing stimulation.

Figure 9 shows records from a fibre in which post-contractile depression developed. Tetani identical to those used during the control period and fatiguing stimultion were given during the recovery period. It can be seen that the tension fell to a minimum and then gradually recovered. In this fibre tension reached a minimum of 4% of control 20 min after the end of fatiguing stimulation. The fall of tension during post-contractile depression was accompanied by a fall of the light signal, which in Fig. 9 reached 2% of the control value before it started to recover. In the fibres which showed post-contractile depression, a minimum tension of $7{\cdot}4 \pm 3{\cdot}3\%$ ($n = 5$) of

control was produced at 10–20 min of recovery. The light signal at this time was 2·8±0·7% of control.

After reaching a minumum, tension gradually increased until a relatively stable level was attained after 60–140 min of recovery. The tension produced was then 91±3% of control. The light signal also increased, but it did not recover as completely, being 36±3% of control in the same fibres. (Correction for aequorin

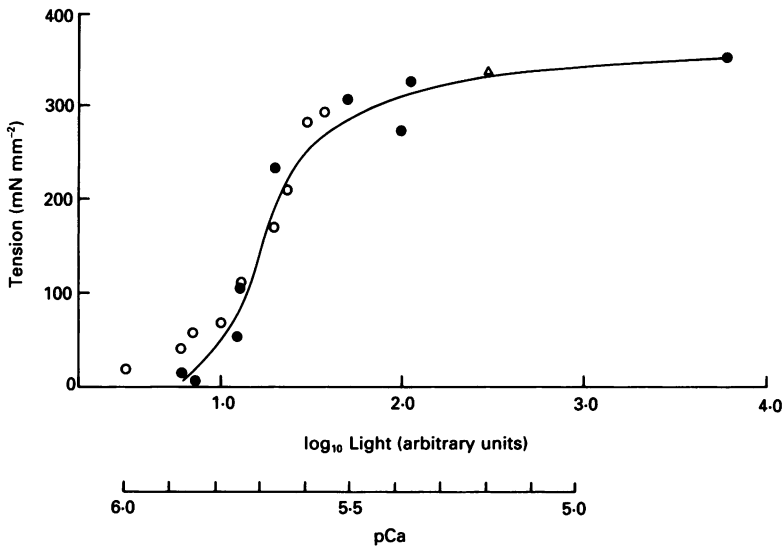

Fig. 10. The relationship between light and tension during recovery. Filled circles represent a control pCa–tension curve measured from K^+ contractures. Open circles represent measurements from records of light and tension obtained during recovery. The points lie very close to the control curve. Open triangle represents results obtained from control tetani before fatiguing stimulation.

consumption increases this figure only to about 40% of control). This lower level of $[Ca^{2+}]_i$ caused the tetani to be less fused after recovery from fatigue, as can be seen in the 60–80 min tests shown in Fig. 9*B*. In some experiments test tetani at higher stimulation frequencies were given. These caused a substantial increase of the light signal and also the contraction became more fused (not shown).

In order to determine whether the $[Ca^{2+}]_i$ during the recovery period could account for the reduced tension during post-contractile depression, we plotted tetani during recovery on pCa–tension curves. One such plot is shown in Fig. 10. The line plotted through the filled circles is a control pCa–tension curve, which was measured from K^+ contractures before fatiguing stimulation. The open circles represent measurements from tetani during recovery. It can be seen that these lie very close to the curve; in other words the tension generated for the level of $[Ca^{2+}]_i$ present during the tetanus is what would be expected from the control pCa–tension curve. Also, it should be noted that when a stable level had been reached in this fibre, the light level was close to the shoulder of the curve. This contrasts with the control, unfatigued tetanus (triangle) which has a light level which is above saturation.

While it is known that the action potentials during post-contractile depression appear normal (Westerblad & Lännergren, 1986), it remains possible that action potentials are no longer able to release an adequate amount of Ca^{2+} from the

sarcoplasmic reticulum and that this causes the decline in $[Ca^{2+}]_i$ and tension. We therefore examined the relation between light and tension produced by K^+ contractures and caffeine during post-contractile depression. Figure 11 shows the peak light and tension in tetani (panel *A*) and K^+ contractures (panel *B*) before,

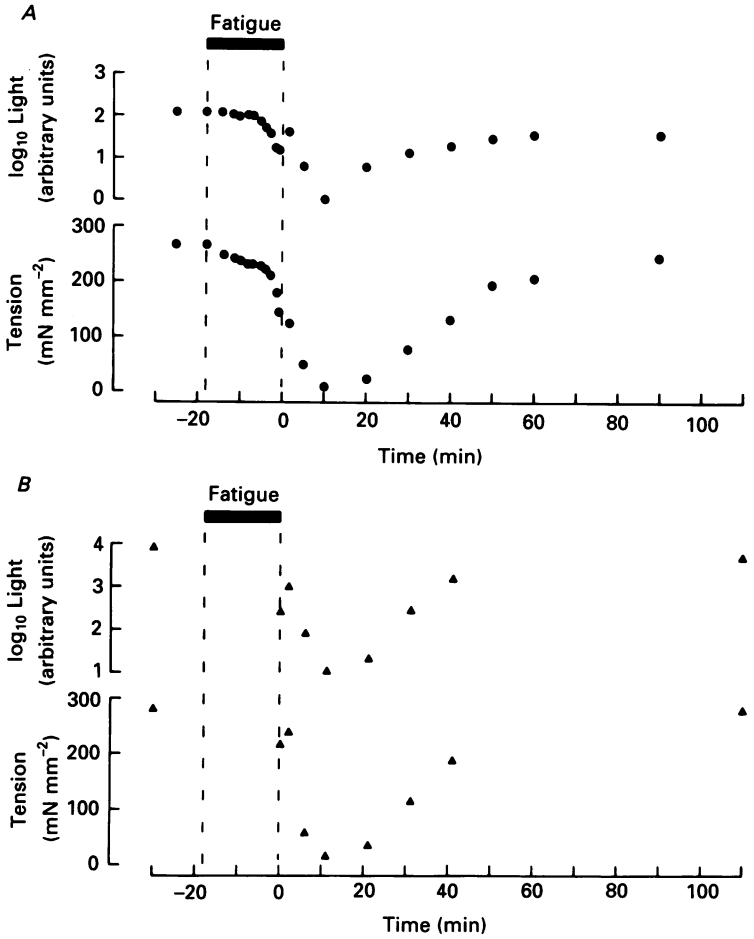

Fig. 11. Aequorin light and tension measured from tetani and maximal K^+ contractures during and after fatiguing stimulation in a fibre which showed post-contractile depression. *A*, light and tension measured from tetani. Note the similarity in the pattern shown by tension and light. *B*, peak light and tension measured from maximal K^+ contractures with 150 mM-K^+ during the same experiment. The light and tension show a similar pattern both to each other and to the pattern observed with tetani.

during and after a period of fatigue. As described above, tetanic light and tension fell during fatigue, and this was then followed by a secondary decline of light and tension, and finally by recovery. Panel *B* shows that peak light and tension elicited by maximal K^+ contractures in the same fibre also showed a decline during post-contractile depression, followed by a recovery. Thus tension produced by K^+ contractures, which cause a continuous depolarization of the membrane, is affected in a similar way to tetanic tension, and so also is the peak light caused by this manoeuvre. (Note that the K^+ contractures caused a much larger peak light signal

than tetani, presumably because they cause continuous depolarization, and hence the ordinate is at least an order of magnitude greater in panel *B*.) Thus the process causing post-contractile depression also affects Ca^{2+} release by continuous depolarization of the membrane, suggesting either that the sarcoplasmic reticulum is

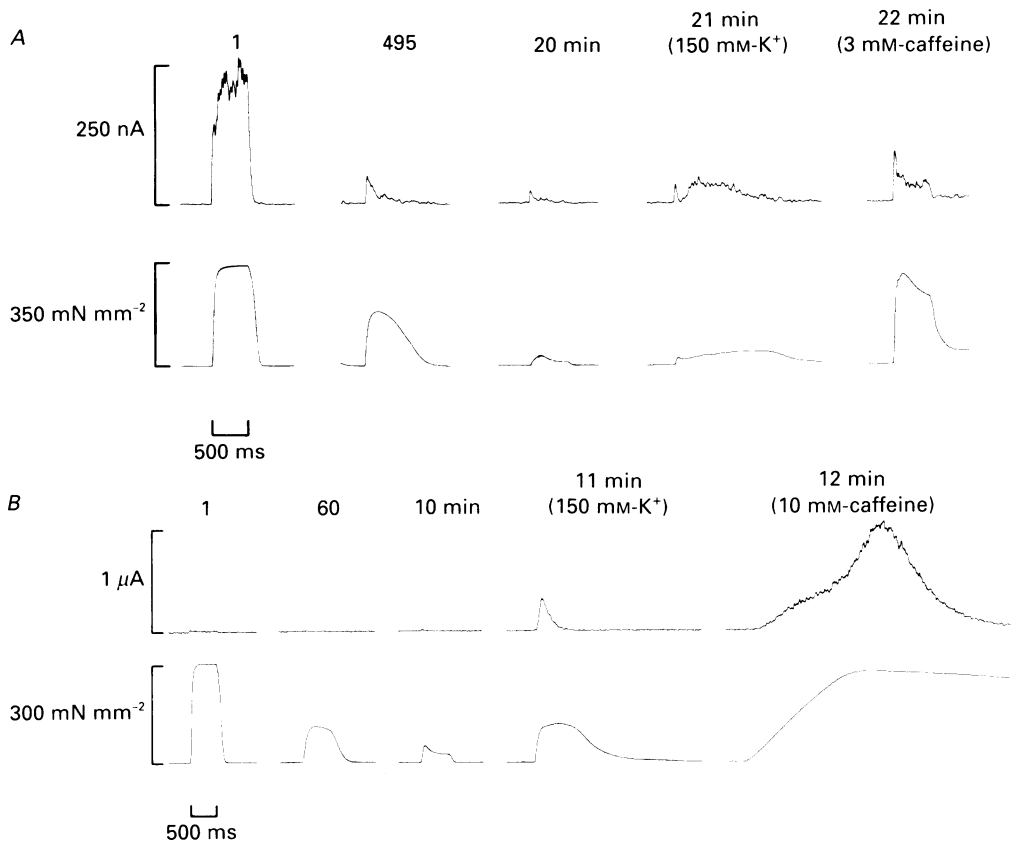

Fig. 12. Effect of high K^+ and caffeine on aequorin light and tension during post-contractile depression. *A*, leftmost panels show the first and last tetani of fatiguing stimulation. Panel marked 20 min shows a tetanus given 20 min after the end of fatiguing stimulation. Panel marked 21 min (150 mM-K^+) shows a K^+ contracture caused by 150 mM-K^+ given 1 min after the tetanus. One minute later still (panel marked 22 min (3 mM-caffeine)) a tetanus after 20 s exposure to 3 mM-caffeine was given. Note that the fibre did not relax completely after the tetanus in 3 mM-caffeine and subsequently went into a contracture. *B*, different experiment. The first four panels are similar to those in *A*, except that in this muscle tension was greatly reduced after 10 min. Final panel, marked 12 min (10 mM-caffeine), shows a contracture provoked by 10 mM-caffeine applied at the start of the twelfth minute.

depleted of Ca^{2+} or that the release mechanism initiated by T-tubular depolarization has failed.

The hypothesis that reduced tetanic $[Ca^{2+}]_i$ (myoplasmic free calcium concentration during a tetanus) is the cause of post-contractile depression is further supported by the finding that caffeine can produce similar tension in unfatigued fibres and during post-contractile depression (Lännergren & Westerblad, 1989).

Figure 12 shows two experiments illustrating this result and, in addition, shows that the $[Ca^{2+}]_i$ can be very large under these conditions. Figure 12*A* shows a control tetanus and the last tetanus of fatiguing stimulation. Post-contractile depression then occurred, as shown by a tetanus at 20 min, when tension was much reduced. One minute later a K^+ contracture produced a slightly larger tension and a moderate increase in light. After another minute a tetanus was given in 3 mM-caffeine and produced a large tension (90% of control), with a light signal which was similar to the preceding K^+ contracture, but only one-fifth of the initial control tetanus. Figure 12*B* shows a similar protocol. When the fibre was in post-contractile depression, as indicated by the reduced tetanic tension, a K^+ contracture was followed by a contracture produced with 10 mM-caffeine. Although the K^+ contracture increased light and tension somewhat, the most dramatic increase was produced by caffeine, which gave a very large light signal (40 × control) and a tension which was 91% of control. Thus, in this experiment there was clearly a large releasable store of Ca^{2+}. Both these experiments show that caffeine was able to increase $[Ca^{2+}]_i$ and tension in post-contractile depression, and was more effective than K^+ contractures. More puzzling is the large variability of the peak light produced by these procedures during post-contractile depression. Thus in the K^+ contracture of Fig. 12*A*, peak light was about one-sixth of that during a control tetanus, while in that of Fig. 12*B* the peak light was about 10 × bigger than the control tetanus. It is possible that spatial gradients of $[Ca^{2+}]_i$ may be produced by K^+ contractures during post-contractile depression (hence exaggerating the light signal with respect to the tension), while caffeine contractures, acting directly on the sarcoplasmic reticulum, give a more accurate picture of the releasable pool of Ca^{2+}.

DISCUSSION

Isolated single fibres show at least two distinct kinds of fatigue. When continuously stimulated at high frequencies (e.g. 100 Hz) there is a rapid and often irregular decline of tension (high frequency fatigue) which is characterized by failure of action potentials (Lüttgau, 1965; Lännergren & Westerblad, 1986) and rapid recovery. Our conditions of intermittent tetani stimulated at low frequency largely avoids this kind of fatigue and leads to a slowly developing fatigue in which action potentials show only minor changes in form (Grabowski *et al.* 1972; Westerblad & Lännergren, 1986).

In principle the tension reduction during fatigue could be due to (i) reduced maximum Ca^{2+}-activated tension i.e. reduced tension production per cross-bridge or a reduced number of cross-bridges participating in contraction, (ii) reduced myoplasmic $[Ca^{2+}]$ during a tetanus, (iii) reduced Ca^{2+}-sensitivity at the contractile proteins or a combination of these effects. We will discuss each of these possibilities in turn.

Reduced maximum Ca^{2+}-activated tension

A variety of metabolic changes occur during fatiguing stimulation, but the tension produced by the contractile proteins is known to be particularly sensitive to the increased inorganic phosphate (P_i) and the decreased pH_i (Cooke & Pate, 1985; Godt & Nosek, 1989). In fact, if the concentrations of P_i and pH_i which are thought to occur in fatigue are applied to skinned fibres, then reductions in tension equal to or

greater than those observed in our fatigued fibres can be obtained. For instance, Godt & Nosek (1989) used solutions with 1 mM-P_i and pH 7·0 to mimic normal conditions and 17 mM-P_i and pH 6·65 to mimic fatigue and observed a 33% reduction in maximum Ca^{2+}-activated tension and a much greater reduction at submaximal $[Ca^{2+}]$.

In opposition to this view is the observation that caffeine can largely or completely reverse the decline of tension in fatigue (Eberstein & Sandow, 1963; Grabowski *et al.* 1972; Länngergren & Westerblad, 1989; present study). Since caffeine directly releases Ca^{2+} from the sarcoplasmic reticulum (SR), raises the myoplasmic $[Ca^{2+}]$ (Fig. 12), and also increases the Ca^{2+} sensitivity of the contractile proteins (Wendt & Stephenson, 1983), there is no difficulty in explaining its action, but this observation does seem to exclude a substantial role for reduction in maximum Ca^{2+}-activated tension in the reduced tension seen in fatigued fibres.

The discrepancy between skinned fibre experiments, which suggest a major role for reduction in maximum Ca^{2+}-activated tension, and application of caffeine in intact fibres, which suggests little or none, could have several explanations. (i) Metabolic changes and tension have not yet been simultaneously measured in individual fibres with known fatigue characteristics. (ii) The P_i sensitivity and the pH sensitivity of different muscles are known to vary (Fabiato & Fabiato, 1978; Godt & Nosek, 1989). Preliminary experiments by R. E. Godt & J. C. Kentish (personal communication) suggest that the P_i sensitivity of the fibres used in this study may be lower than that of some other skeletal muscles. (iii) The observed effects of P_i and pH on skinned fibres may somehow be masked in intact fibres. (iv) The possibility that caffeine may overcome the inhibitory effects of P_i and pH on maximum Ca^{2+}-activated tension has been eliminated by direct experiment. R. E. Godt & J. C. Kentish have shown in preliminary experiments (personal communication) that caffeine applied to skinned fibres does not reverse the inhibition of tension by P_i or pH.

Changes in $[Ca^{2+}]_i$ during fatigue and their contribution to the decline of tension

At the stage of fatigue when tension is reduced to about 50%, the evidence that reduced $[Ca^{2+}]_i$ contributes to the decline of tension is now very strong. We find that the tetanic light signal is reduced to ~ 15%. Correction for aequorin consumption increases this figure to ~ 16% (i.e. by 6% of 15; see Methods). Because of the non-linearity of the relation between aequorin light and $[Ca^{2+}]$, this represents a fall in $[Ca^{2+}]_i$ to 40–50% of control levels (0·3–0·4 pCa units). Our pCa–tension curves show that this fall in $[Ca^{2+}]_i$ was capable of explaining all the fall in tension at the end of fatiguing stimulation. This conclusion is further supported by the fact that application of caffeine or high K^+ during fatigue was capable of substantially increasing $[Ca^{2+}]_i$ and produced increased tension, which was close to control levels in the case of caffeine.

Tetanic $[Ca^{2+}]_i$ changed in a complex way during fatigue and we will discuss separately possible mechanisms for the early rise in tetanic $[Ca^{2+}]_i$ and the late fall.

Possible mechanisms for the early rise in $[Ca^{2+}]_i$

The first obvious change during fatiguing stimulation is an increase in tetanic $[Ca^{2+}]_i$. Two possible mechanisms for this increase are (i) an effect of acidosis on $[Ca^{2+}]_i$ and (ii) an effect of repeated tetani on the distribution of Ca^{2+} between various

compartments within the cell. In support of hypothesis (i), we found that application of extracellular CO_2, which causes an intracellular acidosis, increased tetanic $[Ca^{2+}]_i$. Five percent CO_2, which increased $[Ca^{2+}]_i$ to a similar extent to fatiguing stimulation, caused an intracellular acidosis of between 0·15 and 0·25 pH units (Bolton & Vaughan-Jones, 1977; Curtin, 1987). Rough calculations from the acidosis measured at the end of fatiguing stimulation suggest that a pH_i reduction of 0·05–0·1 units should have been present when the maximum light signals occurred (Westerblad & Lännergren, 1988). Thus quantitative considerations of this sort suggest that acidosis may not be the only mechanism involved.

The second hypothesis above suggests that repeated activity may lead to a rise in tetanic $[Ca^{2+}]_i$ due to redistribution of Ca^{2+} within the fibre. Such redistributions were invoked in a model (Cannell & Allen, 1984) which reproduced the rise in $[Ca^{2+}]_i$ during a tetanus (Blinks *et al.* 1978; present study). In the model, as a tetanus progressed, Ca^{2+} was gradually transferred from calsequestrin sites in the SR to troponin and parvalbumin binding sites in the myoplasm. Thus, although Ca^{2+} release from the SR gradually declined throughout a tetanus, the consequence of increased saturation of Ca^{2+} binding sites in the myoplasm was that the $[Ca^{2+}]_i$ gradually rose.

Possible mechanisms for the late fall in tetanic $[Ca^{2+}]_i$

When tetanic $[Ca^{2+}]_i$ falls, this could arise either because of reduced Ca^{2+} release from the SR or because of increased Ca^{2+} buffering in the myoplasm. Since acidosis *reduces* Ca^{2+} buffering by troponin (Blanchard, Pan & Solaro, 1984) but has no effect on Ca^{2+} binding to parvalbumin (Pechere, Derancourt & Haiech, 1977), we assume that the reduced tetanic $[Ca^{2+}]_i$ represents a reduction in Ca^{2+} release from the SR. Three possible causes of the late fall in tetanic $[Ca^{2+}]_i$ are as follows.

Gradients of Ca^{2+} *release.* The action potential changes in form during fatigue (Westerblad & Lännergren, 1986) and this might be associated with impaired action potential conduction in the T-tubule network leading to reduced Ca^{2+} release in the centre of the fibre. There is some evidence for this theory from observations of wavy myofibrils in the centre of single fibres during fatigue (Gonzalez-Serratos, Garcia, Somlyo, Somlyo & McClellan, 1981). However, we have recently measured the spatial distribution of Ca^{2+} in *Xenopus* fibres using identical protocols to those used in this study and found no inhomogeneities across fibres as tension was reduced to 50% (Allen, Bolsover, Lamb, Lee, Silver & Westerblad, 1989).

Ca^{2+} is present in the SR but is not being released by action potentials. This hypothesis can explain the reduced tetanic $[Ca^{2+}]_i$ and the fact that high K^+ or caffeine applied immediately after fatiguing stimulation increases $[Ca^{2+}]_i$ with recovery of all or most of the tension. The fact that caffeine, which directly opens Ca^{2+} channels in the SR (Suarez-Isla, Orozco, Heller & Froehlich, 1986), is more effective than high K^+ (Lännergren & Westerblad, 1989), which causes continuous depolarization of the T-tubules, suggests that the defect in Ca^{2+} release during fatigue lies in the coupling between T-tubule depolarization and opening of SR Ca^{2+} channels. In support of this theory, it has been found, using electron microprobe techniques, that the SR contained Ca^{2+} even during severe fatigue (Gonzalez-Serratos, Somlyo, McClellan, Shuman, Borrero & Somlyo, 1978).

Ca^{2+} in the SR is greatly reduced during fatigue. This is an attractive hypothesis

because if SR Ca^{2+} uptake is impaired, this can explain all the main changes in $[Ca^{2+}]$ which we observe. Thus a slowed SR Ca^{2+} uptake would reduce the rate of fall of $[Ca^{2+}]_i$ after a tetanus and would increase resting $[Ca^{2+}]_i$. If the mean $[Ca^{2+}]_i$ is elevated, more Ca^{2+} will bind to parvalbumin and, in addition, there will be increased mitochondrial calcium uptake. As a consequence, SR Ca^{2+} will be reduced, leading to reduced tetanic $[Ca^{2+}]_i$. However, this hypothesis does not naturally explain why caffeine can still produce a maximal contracture during fatigue. Perhaps the amount of Ca^{2+} in the SR *is* reduced, but caffeine can still release sufficient to produce a full contracture when coupled with the increase in Ca^{2+} sensitivity which it also produces (Wendt & Stephenson, 1983).

Changes in Ca^{2+} sensitivity during fatigue

It is known that pH_i falls and P_i accumulates during fatigue and that both of these lead to reduction in the Ca^{2+} sensitivity (Fabiato & Fabiato, 1978; Kentish, 1986; Godt & Nosek, 1989) and would therefore be expected to contribute to reduced tension during fatigue. If this reduced Ca^{2+} sensitivity was present, then the pCa–tension points from tetani during fatigue ought to lie to the right of the control curves, as was seen when acidity was induced with CO_2 (Fig. 5). However, our data during fatigue show a shift to the right for tetani early in fatigue, but by the end of fatigue this shift was no longer apparent. Yet the combination of acidosis and P_i accumulation (and changes in ATP and ADP, see below) in skinned fibres led to a desensitization of between 0·25–0·3 pCa units (Godt & Nosek, 1989). Here again there exists an unexplained discrepancy between our results and those that we might expect on the basis of skinned fibre data. Some possible explanations have been discussed in the section on maximum Ca^{2+}-activated tension. Other possible explanations are as follows. (i) It could arise artificially as a consequence of reduced Ca^{2+} sensitivity of aequorin. As discussed in the Methods it is possible that acidosis and changes in $[Mg^{2+}]$ reduce the aequorin sensitivity. It is impossible to quantify this effect with data available, but we do not believe that the true $[Ca^{2+}]_i$ is underestimated by more than 0·1 pCa units and if such an error were present there would remain a substantial discrepancy to explain. (ii) There may be a genuine resensitization to Ca^{2+} superimposed on the desensitization caused by acidosis and P_i. For instance, large increases in ADP (e.g. to 5 mM) have been shown to increase Ca^{2+} sensitivity by about 0·6 pCa units (Hoar, Mahoney & Kerrick, 1987) but it seems unlikely that sufficient change in ADP occurs in fatigue to have much effect. Alternatively, a fall in ATP is known to sensitize the myofibrils to Ca^{2+} (Godt, 1974), but again measured falls in ATP are small (Dawson *et al.* 1978; Nassar-Gentina *et al.* 1978) and are likely to have only a small effect. Furthermore in the study of Godt & Nosek (1989), a realistic increase in ADP (to 0·7 mM) and decrease in ATP (from 6 to 5 mM) was incorporated in the 'fatigue' solution (in addition to the changes in pH and P_i) but a net decrease in Ca^{2+} sensitivity was still observed.

Slowing of relaxation

It seems clear that the slowing of relaxation observed in fatigue has more than one cause. Edman & Matiazzi (1981) and Curtin (1986) have shown that intracellular acidosis slows the rate of relaxation. We confirmed this finding but did not observe

any effect of pH on the rate of decline of $[Ca^{2+}]_i$ after a tetanus. Thus our conclusion is that pH causes a moderate slowing of relaxation, which is caused by a direct effect of pH on cross-bridge cycling and detachment rates. This conclusion is supported by the reduction of V_{max} which Edman & Matiazzi (1981) observed in acid solutions. Our conclusion differs from that of Curtin (1986) who suggested that, at least for long tetani, the effects of pH might be mediated by a slowing of SR Ca^{2+} uptake.

A further Ca^{2+}-independent slowing of relaxation may be caused by the rise in ADP. Cooke & Pate (1985), using maximally Ca^{2+}-activated skinned fibres, found that 1–4 mM-ADP slows V_{max} and Lännergren & Westerblad (1989) showed that V_{max} was reduced during fatigue. Although the ADP levels used by Cooke & Pate (1985) were above those likely to occur in fatigue it seems probable that some of the reduced shortening velocity and the slowing of relaxation arises from this source.

Our experiments suggest that there may also be a Ca^{2+}-dependent component to the slowing of relaxation. We found that the rate of fall of $[Ca^{2+}]_i$ was reduced substantially during fatiguing stimulation and this is likely to cause a further slowing of relaxation. Dawson *et al.* (1980) showed a close correlation between the free energy of ATP hydrolysis and the slowing of relaxation throughout a period of fatigue and suggested that this arose because of a slowing of the SR Ca^{2+} pump. The free energy of ATP sets an upper limit on the concentration gradient of $[Ca^{2+}]$ which the SR Ca^{2+} pump can achieve. Dawson *et al.* (1980) argued that, as the free energy of ATP fell, the rate of pumping of the SR Ca^{2+}pump would also decline. Our finding of a slower rate of fall of $[Ca^{2+}]_i$ supports their hypothesis but it seems likely that direct effects of pH_i and ADP on crossbridge cycling also contribute to slowing of relaxation.

Events during recovery

In some fibres, tension and light recovered quickly and fully, while in others, which exhibited post-contractile depression, full recovery of tension could take more than 2 h and light only recovered partially. This delayed recovery is not due to either a prolonged acidosis (Westerblad & Lännergren, 1988), or to membrane inexitability (Westerblad & Lännergren, 1986). Phosphocreatine has been found to recover five times faster than pH after fatigue (Kushmerick & Meyer, 1985), so it seems unlikely that this or other phosphorus metabolites contribute to the phenomenon. Furthermore, application of caffeine at the time of maximum tension depression (about 20 min after the end of fatiguing stimulation), was able to produce close to full tension (Lännergren & Westerblad, 1989; present study, Fig. 12*B*) and the reduced V_{max} had also almost fully recovered at this time (Lännergren & Westerblad, 1989). These results suggest that post-contractile depression is due to a failure of excitation–contraction coupling, rather than to an inability of the cross-bridges to generate tension and to cycle. This hypothesis is supported by the present finding that tetanic $[Ca^{2+}]_i$ is greatly reduced in post-contractile depression. Comparison of the $[Ca^{2+}]$ with a pCa–tension curve (Fig. 10), suggests that the reduced tension may be entirely explained by the reduced $[Ca^{2+}]_i$.

The reason for the reduced tetanic $[Ca^{2+}]_i$ during post-contractile depression is not clear, but it appears that recovery processes may under certain circumstances lead to transient block of the coupling process. This block may be related to the appearance of vacuoles during fatigue (Gonzales-Serratos *et al.* 1978), which could

conceivably contribute to mechanical disruption of connections between the T-tubules and the SR. The observation that light only recovered partially after more than 2 h indicates that fatiguing stimulation can cause a long-lasting depression of excitation–contraction coupling which persists even when tension has largely recovered.

Conclusions

In this model of fatigue, complex changes in $[Ca^{2+}]_i$ occur which make a major contribution to the changes in mechanical performance observed in fatigue. A substantial part of (i) the slowing of relaxation, (ii) the tension reduction during fatiguing stimulation, and (iii) the secondary tension depression during the recovery period can be ascribed to altered handling of Ca^{2+}. The most probable cause of this alteration is either abnormal coupling between the T-tubules and the SR or Ca^{2+} depletion in the SR.

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