Reduced maximum shortening velocity in the absence of phosphocreatine observed in intact fibres of *Xenopus* skeletal muscle

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1. ADP inhibits the maximum shortening velocity (V_0) in skeletal muscle. [ADP] may increase considerably during contractions and reduce V_0 in the absence of energy buffering by phosphocreatine (PCr). We have tested this hypothesis by comparing V_0 in long and short tetani produced in situations where PCr buffering is absent.

2. Single, intact muscle fibres were dissected from toe muscles of *Xenopus* and stimulated by current pulses at 20 °C. The test sequence consisted of a 400 ms tetanus, followed after 3 s by a 1400 ms tetanus and after an additional 4 s by a 400 ms tetanus. V_0 was measured with slack tests at 200 and 1200 ms, respectively.

3. The PCr system was inactivated in three ways: (i) fatiguing fibres with repeated short tetani; (ii) inhibition of the creatine kinase (CK) reaction with dinitrofluorobenzene; and (iii) inhibition of energy metabolism with iodoacetic acid and cyanide.

4. Under control conditions V_0 was similar in all three test tetani. With inactive PCr buffering V_0 was about 30% lower in the long tetanus. This slowing recovered fully in the second short tetanus in fatigue and with CK inhibition.

5. Calculations suggest that [ADP] can reach very high levels (about 3 mM) during prolonged contractions in the absence of PCr buffering.

Skeletal muscle normally contains a large concentration of phosphocreatine (PCr). The function of PCr is to replenish ATP via the creatine kinase (CK) reaction:

$$PCr + ADP \rightleftharpoons Cr + ATP.$$

The equilibrium constant for this reaction is about 100, which means that the myoplasmic [ATP] ($[ATP]_i$) will not show any marked decline until the PCr store is almost depleted (Carlson & Siger, 1960). Thus, in brief contractions, PCr will act as an energy buffer preventing any major changes of the concentration of ATP and ADP occurring. During prolonged intense activation (i.e. during fatigue) the PCr store may become depleted and in this situation $[ATP]_i$ has been found to fall (Nassar-Gentina, Passonneau & Rapoport, 1981; Nagesser, van der Laarse & Elzinga, 1993). However, $[ATP]_i$ has to fall to very low levels (about 0·5 mM) before this will have any major impact on muscle function and even in severe fatigue such low levels have not been measured (for review see Fitts, 1994).

A net breakdown of ATP will result in a concomitant increase of the free myoplasmic [ADP] ($[ADP]_i$) and this may affect muscle function; for instance, ADP has been shown to act as a competitive inhibitor (inhibition constant (K_i), 250 μM) of the shortening velocity at zero load (V_0) (Cooke & Pate, 1985). However, a build-up of ADP is prevented not only by the CK reaction but also by the myokinase reaction:

$$2\,ADP \rightleftharpoons ATP + AMP,$$

and AMP is then metabolized to inosine monophosphate (IMP) or adenosine. Thus the increase of $[ADP]_i$ in fatigue is considered to be small and a frequently quoted increase is from 20 μM at rest to 200 μM in fatigue (Dawson, Gadian & Wilkie, 1978).

In skeletal muscle fatigue there is generally a reduction of the shortening speed, which is mainly ascribed to acidosis (for review see Fitts, 1994). In a recent study we found approximately a 50% reduction of V_0 in fatigued, single fibres of *Xenopus* (Westerblad & Lännergren, 1994). After considering the slowing which could be explained by acidosis, a large component remained. We suggested that this component might be due to a net breakdown of ATP and accumulation of ADP; calculations showed that $[ADP]_i$ had to be elevated to about 3 mM (for details see

Westerblad & Lännergren, 1994), which is considerably higher than the expected global $[ADP]_i$ in fatigue (Dawson *et al.* 1978; Nagesser *et al.* 1993). The present experiments were directly aimed at studying whether rapid, large changes of ATP/ADP occur with an inactive PCr–CK system. We have measured V_0 after 200 and 1200 ms of tetanic contraction, the idea being that a marked reduction of V_0 would occur with the longer duration in the absence of PCr due to a build-up of ADP, whereas with PCr present no such build-up would develop. The PCr–CK system was inactivated in three ways: (i) fatiguing fibres with repeated short tetani, (ii) inhibition of CK with dinitrofluorobenzene (DNFB), and (iii) inhibition of energy metabolism with iodoacetic acid (IAA) and cyanide (CN). The results showed no substantial reduction of V_0 with prolonged contraction under control conditions. However, at times when the PCr–CK system would be expected not to operate, V_0 was markedly lower in the long tetanus; in fatigue and with DNFB this slowing fully recovered during a 4 s rest period. Thus these results support the idea that contractions are accompanied by high $[ADP]_i$ when the PCr–CK system is not functioning.

METHODS

Fibre dissection, mounting, stimulation and solutions

Adult, female *Xenopus laevis* were killed by stunning followed by decapitation. Intact single fibres were dissected from the lumbrical muscles II–IV of the foot; only easily fatigued, type 1 fibres were used (Westerblad & Lännergren, 1986).

The tendons of an isolated fibre were gripped by platinum foil clips. The preparation was mounted between an Akers AE 801 force transducer and the moveable arm of a galvanometer (G120DT; General Scanning, Watertown, MA, USA). Fibres were stretched to a length giving maximum tetanic tension; this length was 1·95 ± 0·12 mm (mean ± S.E.M.; $n = 12$) and will be referred to as L_0. Fibres were electrically stimulated at 50–70 Hz via platinum plate electrodes flanking the fibre; the duration of standard tetani was 400 ms.

Under control conditions fibres were stimulated at 3 min intervals and with this regime tetanic tension remained stable within a few per cent. The tension of a standard tetanus immediately prior to fatiguing stimulation or pharmacological inhibition was set to 100%.

Fibres were superfused with a standard Ringer solution of the following composition (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; sodium phosphate buffer, 3·0 (pH 7·2). Solution temperature was held at 20·0 ± 0·2 °C (measured close to the fibre) by passing the solution through a temperature-controlled heat exchanger. Energy metabolism was inhibited by adding 2 mM NaCN and 0·1 mM IAA to the Ringer solution and adjusting the pH to 7·2 with HCl. Inhibition of CK was induced by the addition of 10 μM DNFB; in spermatozoa this concentration has been found to effectively block creatine kinase while having no unspecific effects (Tombes, Brokaw & Shapiro, 1987).

Statistics

Values are presented as means ± S.E.M. Paired t tests were used to verify statistical significance; the significance level was set at 0·05 throughout.

Force–velocity measurements

The arm of the galvanometer could be moved rapidly by changing the current passing through the galvanometer coil; a 200 μm length step was completed within 2 ms. The position of the arm and the signal from the force transducer were displayed on a digital oscilloscope and stored in a personal computer.

Under control conditions the shortening velocity at zero load (V_0) was obtained with slack tests (Edman, 1979): the fibre was rapidly shortened to four different lengths in subsequent tetani and the time from the release until tension began to redevelop was measured. The time to take up the slack was plotted against the respective release length. Linear regression was used to draw a straight line through data points (correlation coefficient (r) in all cases > 0·99) and the slope of this line and the intercept on the length axis were noted. V_0 was obtained by dividing the slope of the line with L_0; V_0 obtained in this way was set to 100%. The intercept on the length axis (i.e. the total series compliance; Edman, 1979) was 5·6 ± 0·2% of L_0 ($n = 12$) and was assumed to remain constant throughout individual experiments.

A standard test protocol was used in all experiments. This consisted of a 400 ms tetanus, followed after 3 s by a 1400 ms tetanus and finally, after an additional 4 s pause, by a 400 ms tetanus. After 200 and 1200 ms, respectively, a rapid release was performed with the amplitude set so that, under control conditions, the slack was taken up in 10–15 ms. A line was drawn between data points obtained in this way and the intercept on the length axis (see Fig. 1*C*) and V_0 was established from the slope of this line. The tetani produced with this test protocol will be referred to as test tetani in the following.

Experimental procedures

Fatigue was produced by repeated 400 ms tetani given every 3 s. The normal fatiguing stimulation pattern was interrupted by test tetani every tenth tetanus. Fatiguing stimulation continued until isometric tension was reduced to about 0·5 of the maximal isometric tension (P_0).

In experiments with DNFB test tetani were produced every 2 min. In experiments with inhibition of energy metabolism (CN + IAA) test tetani were produced every minute.

RESULTS

Under control conditions there was a small, but significant, reduction of V_0 during the test sequence. As compared with V_0 obtained from tetani with various release lengths, the first short tetanus had a V_0 of 100 ± 1·6%. In the subsequent long tetanus V_0 was reduced to 97·3 ± 1·5% and V_0 of the final short tetanus was 95·9 ± 1·4%.

Fatigue

During fatigue runs changes of isometric tension and V_0 followed the same pattern as we have observed previously (Westerblad & Lännergren, 1994). Initially tension fell rapidly to about 80% while V_0 (as measured in the first test tetanus) showed minor changes (phase 1). Then followed a period with almost stable tension and a marked reduction of V_0 (phase 2). Finally both force and V_0 displayed a fast decline (phase 3).

Figure 1 shows typical results from test tetani produced in one fibre in control and at the end of phase 2. It can be seen that the time required to take up the slack was similar in all three tetani under control conditions (Fig. 1*A*). At the end of phase 2 , on the other hand, the shortening speed was markedly lower in the long tetanus than in the first short tetanus and this slowing recovered almost fully during the 4 s pause between the long tetanus and the second short tetanus (Fig. 1*B*).

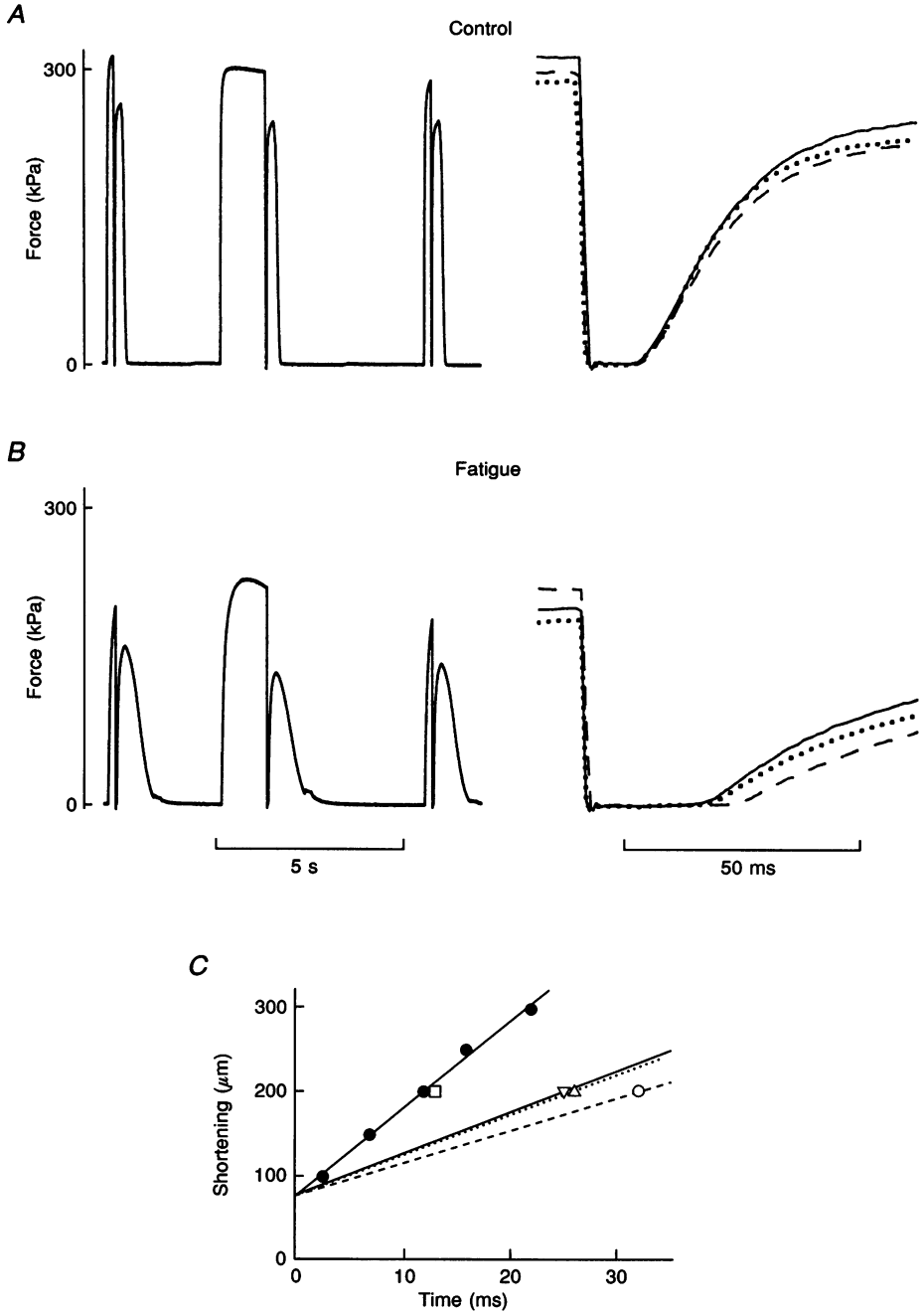

Figure 1. Slack tests in fatigue

A shows tension records of test tetani produced in control (left) and superimposed records on an expanded time base from times of fibre shortening (right). Continuous line, first tetanus; dashed line, second (long) tetanus; dotted line, third tetanus. *B* shows records as in *A* obtained in fatigue. In *C*, the amplitude of the shortening step is plotted against the time required to take up the slack. V_0 in control was established from tetani with different shortening amplitudes (records not shown). Data points from these tetani (●) were connected by a straight line and the intercept on the abscissa gave the series compliance, which was considered to be constant and was used when calculating V_0 for test tetani. V_0 in control was similar in all three test tetani (□), whereas in fatigue, V_0 of the long tetanus (○) was markedly lower than in the short (▽ and △) tetani.

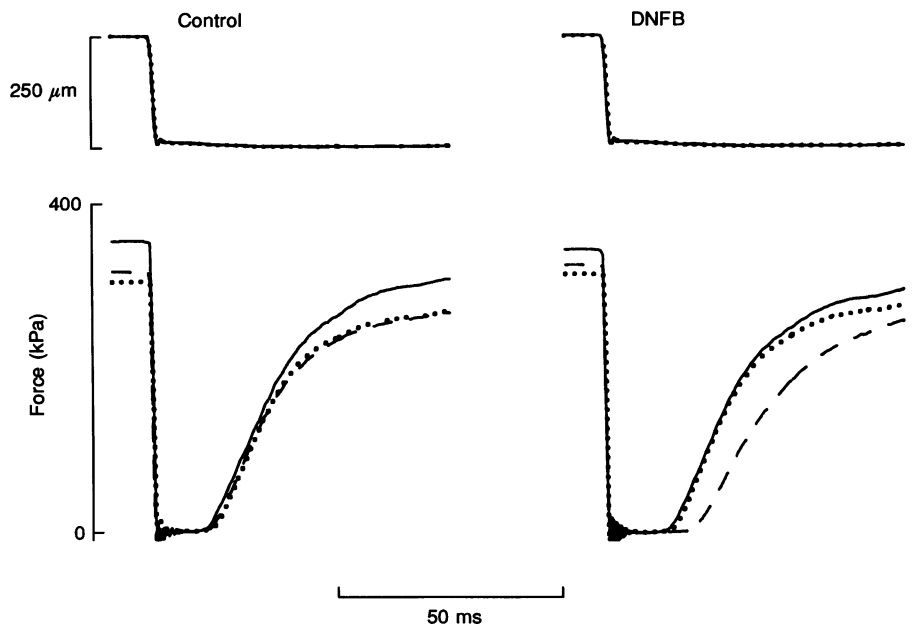

Figure 2. Slack tests in the presence of dinitrofluorobenzene
Tension records (lower traces) from periods of shortening in control and with DNFB. Upper traces show the position of the galvanometer arm. Lines as in Fig. 1. Observe that the shortening velocity was markedly reduced in the long tetanus with DNFB.

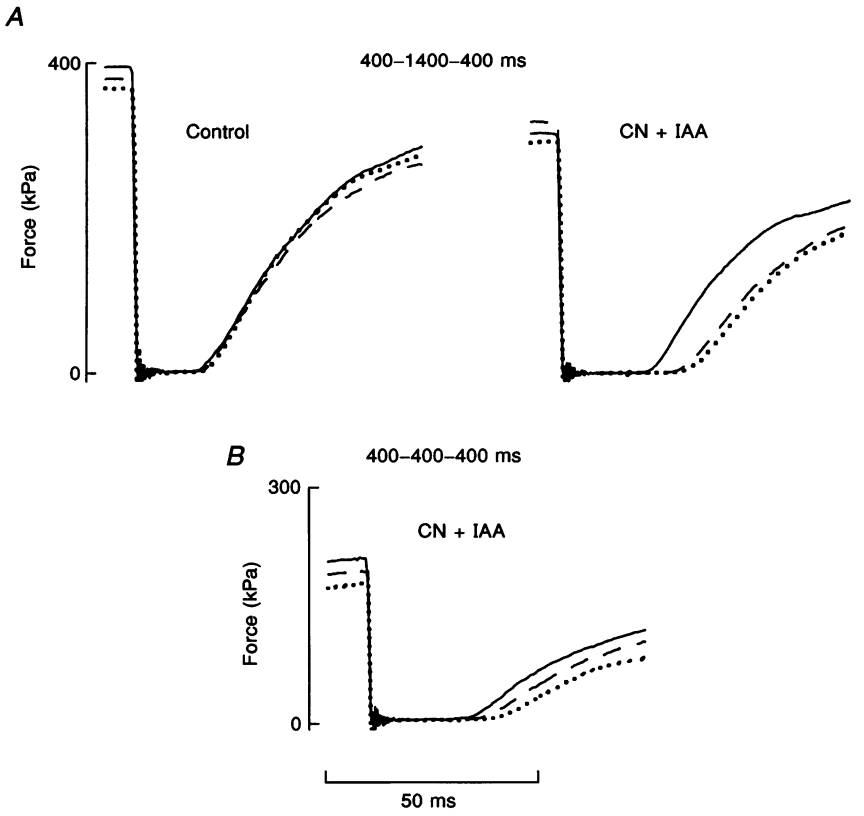

Figure 3. Slack tests with inhibition of energy metabolism
A shows tension records obtained from normal test tetani (lines as in Fig. 1). *B* shows records from another fibre where the test sequence was altered so that the tetanus duration was kept constant. Note that while the slowing was similar in the long and second short tetanus in *A*, there was a gradual slowing with time in *B*.

Test tetani during fatigue runs were produced in four fibres. Initially there was no significant difference between V_0 in long and short tetani. The first significant slowing in long tetani was observed when isometric tension was reduced to 75%. When tension had fallen to 70%, the difference in V_0 between short and long tetani had increased, V_0 in the long tetanus being about 75% of V_0 in the first short tetanus. As fatigue progressed V_0 in both short and long tetani fell but the relation between them remained virtually unchanged.

During the 4 s pause after the long tetanus the additional slowing recovered, so that there was no significant difference between V_0 in the first and second short tetanus.

Inhibition of creatine kinase

Figure 2 shows typical results from an experiment with 10 μM DNFB. In this fibre 10 min exposure to DNFB caused a minor reduction of tetanic tension and shortening speed in short tetani, while there was a marked slowing of shortening in the long tetanus.

Four fibres were exposed to DNFB. After 4–6 min exposure, V_0 of the long tetanus started to become reduced and the most marked difference in V_0 between long and short tetani occurred after 8–10 min exposure. At this time V_0 of the long tetanus was less than 70% of that in short tetani. When the exposure to DNFB exceeded 10 min, there was a general decline of shortening speed and also of tetanic tension. Recovery from this state was slow and incomplete.

Inhibition of glycolysis and oxidative phosphorylation

During the first 5–7 min in 2 mM CN and 0·1 mM IAA both V_0 and isometric tension fell slowly to about 75%. At this stage no significant difference between V_0 in short and long tetani had developed. During the following minute there was a marked reduction of V_0 in the long and second short tetani and also an accelerated decline of isometric tension. Figure 3*A* shows representative records from tetani produced at this stage. In the following test, tetanic tension was very low and rigor tension developed within 1 min after the test.

In contrast with the situation in fatigue and with DNFB, there was no recovery of V_0 between the long and second short tetanus after 6–8 min in CN + IAA (Fig. 3*A*). This may be caused by a rapid depletion of the energy stores of the fibres (the duration between the occurrence of additional slowing in the two latter tetani and rigor development was only 2 min) and hence V_0 of the second short tetanus may be reduced just because it occurred later. This assumption was confirmed by exposing fibres to CN + IAA and changing the test protocol so that the tetanus duration was kept constant in all three tetani. Records from one of these experiments are shown in Fig. 3*B* and it can be seen that the shortening speed showed a gradual decline in the successive tetani; similar results were obtained in two more fibres. Thus, when comparing the two protocols, the main difference is that, with the standard protocol, V_0 is similarly reduced in the long tetanus and second short tetanus (Fig. 3*A*), whereas

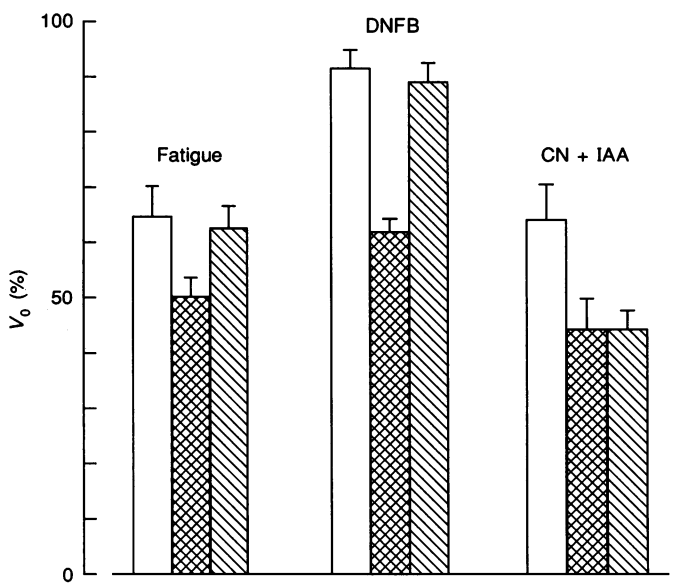

Figure 4. Summary of the changes of V_0

Mean values (± S.E.M.) of V_0 from test tetani in fatigue, with inhibition of CK (DNFB), and with inhibition of energy metabolism (CN + IAA) ($n = 4$ in all three conditions). □, first tetanus; ▩, second (long) tetanus; ▨, third tetanus.

with constant tetanic duration V_0 was reduced most in the last tetanus (Fig. 3*B*).

Figure 4 summarizes the results from all three interventions: fatigue, DNFB and CN + IAA. In all three situations V_0 of the long tetanus was highly significantly lower than in the first short tetanus (P always $< 0{\cdot}005$). V_0 recovered fully in the second short tetanus in fatigue and with DNFB, while no recovery occurred with CN + IAA.

DISCUSSION

Possible causes of a lower V_0 in long tetani

The hypothesis that generated the present experiments was that, when the PCr–CK system is inactivated, $[ATP]_i$ will rapidly fall, leading to an increase of $[ADP]_i$ and this will cause a reduction of V_0 especially in long tetani. We observed a marked slowing in long tetani, in agreement with the hypothesis. However, other factors than increased $[ADP]_i$ may contribute to the slowing. It is well established from skinned fibre experiments that acidosis reduces V_0 (e.g. Cooke, Franks, Luciani & Pate, 1988). The maximum rate of H^+ production in the present fibres can be obtained from Table 2 of Nagesser *et al.* 1993 (in all calculations based on this paper we will translate micromole per gram dry wt to mole per litre (M), assuming that 1 mm^3 fibre corresponds to 0·28 μg dry wt). This gives a rate of H^+ production of about 0·2 mM s^{-1}, which will give an insignificant acidosis during tetani because the buffer power is about 40 mM (pH unit)$^{-1}$ (Curtin, 1987).

During prolonged tetani there will be a large build-up of inorganic phosphate (P_i). Increased $[P_i]$ has a large effect on isometric tension but a marked effect on shortening velocity has only been observed at very low $[ATP]_i$ (Pate & Cooke, 1989). Pate & Cooke explained their shortening velocity results by assuming that P_i was acting as a competitive inhibitor of V_0 with a K_i of 80 mM and a Michaelis constant (K_m) for ATP of 150 μM (Cooke & Pate, 1985). Using these values and the calculated reductions of $[ATP]_i$ given below (and concomitant increases of P_i), we get a maximum additional reduction of V_0 in long tetani of 2%, which is small in comparison to the observed reduction of about 30%.

Skinned fibre results have shown that a reduction of the $[Ca^{2+}]$ may cause a decline in V_0 (Moss, 1986). During prolonged high frequency stimulation myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) falls (Westerblad, Lee, Lamb, Bolsover & Allen, 1990) which might result in reduced V_0. However, a decline of $[Ca^{2+}]_i$ results in reduced isometric force and during the first seconds of stimulation both $[Ca^{2+}]_i$ and force fall slowly (Lännergren & Westerblad, 1986; Westerblad *et al.* 1990). At the time of release in long tetani (i.e. after 1200 ms), no substantial decline of $[Ca^{2+}]_i$ would be expected and in accordance with this there was never any significant difference between isometric force in short and long tetani at times when differences in V_0 were maximal (results not shown but see Figs 1–3).

Thus neither acidosis, P_i accumulation nor reduced $[Ca^{2+}]_i$ can explain the marked reduction in V_0 in long tetani with an inactive PCr–CK system and hence the most likely cause is a net breakdown of ATP leading to ADP accumulation. There is also other evidence indicating that $[ADP]_i$ may reach high levels during intense activity; for instance, a substantial production of IMP has been observed during fatigue, indicating a significant flux through the myokinase reaction which would not occur if $[ADP]_i$ remained low (for review see Katz & Sahlin, 1990). Furthermore, an intact PCr–CK system is required for normal contractile performance of skinned fibres (e.g. Godt & Nosek, 1989).

Quantitative considerations of the postulated increase of $[ADP]_i$

A quantitative analysis is most easily performed on the results with DNFB, because, in these experiments, the PCr–CK system would be inactive while no other major changes would be expected; an indication of this is that the isometric tension was close to normal with DNFB whereas it was reduced in fatigue and with CN + IAA. If we assume that an accumulation of ADP, with a concomitant reduction of $[ATP]_i$, is the sole cause of the decline of V_0, changes of these metabolites required to give the observed slowing can be calculated, assuming that ADP acts as a competitive inhibitor for V_0 with a K_i of 250 μM and a K_m for ATP of 150 μM (Cooke & Pate, 1985). Taking a resting $[ATP]_i$ of 7 mM and a very low $[ADP]_i$ (Nagesser *et al.* 1993), we get an $[ADP]_i$ of 0·9 mM (V_0 reduced to 92%) after 200 ms in the first short tetanus and 3·6 mM (V_0 reduced to 62%) after 1200 ms, giving an additional increase of 2·7 mM in the long tetanus.

The maximum rate of ATP consumed (and hence ADP produced) by cross-bridges can be calculated from Elzinga, Lännergren & Stienen (1987) and Nagesser *et al.* (1993) and yields a value of about 5 mM s^{-1}. The study by Nagesser *et al.* (1993) gives a maximum rate of ATP production (excluding the PCr system) of about 0·3 mM s^{-1}. Taken together these values provide an absolute maximum increase of $[ADP]_i$ of 4·7 mM during 1 s stimulation, a value higher than that obtained from analysis of V_0. However, the rate of ATP consumption would fall during prolonged stimulation (as judged from the decline of V_0) and gradients may develop (see below), hence reducing the expected accumulation of ADP. Thus these analyses show that the basis for a significant increase of $[ADP]_i$ during contractions does exist.

The next question is if $[ADP]_i$ could be lowered to the resting value in the 4 s rest period following long tetani. ATP production from glycolysis and oxidative phosphorylation can only reduce $[ADP]_i$ by about 1·2 mM (0·3 mM s^{-1} × 4 s; Nagesser *et al.* 1993) and therefore some other mechanisms

must operate in parallel. One additional way to reduce $[ADP]_i$ would be via the myokinase reaction and subsequent deamination of AMP to IMP (e.g. Katz & Sahlin, 1990). Another possible route for $[ADP]_i$ recovery is that gradients develop during contractions, with [ADP] being higher at sites of rapid energy consumption (e.g. in the vicinity of cross-bridges), and these gradients disappear during the rest period. In agreement with this, Meyer, Sweeney & Kushmerick (1984) showed theoretically that large $[ADP]_i$ gradients are likely to develop in fast twitch fibres with an inactive PCr–CK system. Thus combined action of ATP resynthesis, the myokinase reaction and disappearance of $[ADP]_i$ gradients should be sufficient to restore $[ADP]_i$ in the rest period.

Similar calculations can be performed for the results from fatigue, though in this situation an acidosis of about 0·6 pH units (Westerblad & Lännergren, 1988) will contribute to the markedly reduced V_0 already in the first short tetanus. This general slowing of cross-bridge cycling, and hence reduced rate of energy consumption, can explain a smaller extra accumulation of ADP in long tetani in fatigue; the calculated difference in $[ADP]_i$ between the first short tetanus and the long tetanus was 1·4 mM, which compares with 2·7 mM with DNFB. This smaller accumulation may also explain the recovery of $[ADP]_i$ despite a markedly reduced rate of ATP production in fatigue (Nagesser *et al.* 1993).

A quantitative analysis of the results with CN + IAA is complicated by the fact that the contractile function was rapidly declining. Also, in this situation, there was a general slowing of cross-bridge cycling, as judged from a reduced V_0 in the first short tetanus, and the extra accumulation of ADP in long tetani would be smaller than that with DNFB; an accurate calculation cannot be performed because V_0 was also gradually reduced in successive 400 ms tetani (Fig. 3*B*). With CN + IAA the only routes available for recovery would be the myokinase reaction and disappearance of $[ADP]_i$ gradients and clearly this is not sufficient to produce any net recovery of V_0 between the long and second short tetanus. However, some recovery mechanism was still operating because V_0 was not further reduced in the last short tetanus, which was the case with successive 400 ms tetani.

The present experiments indicate that, with an inactive PCr–CK system, large changes of ATP and ADP develop. These changes are markedly larger than those obtained by direct measurements of metabolites during fatigue (e.g. Dawson *et al.* 1978; Nagesser *et al.* 1993). The reason for this discrepancy is probably methodological limitations. Nuclear magnetic resonance (NMR) (e.g. Dawson *et al.* 1978) provides a time-averaged measure of metabolites and therefore transient changes during contractions cannot be detected. Nagesser *et al.* (1993) performed a biochemical analysis on single *Xenopus* fibres frozen at various degrees of fatigue and found an increase of $[ADP]_i$ of up to 1 mM. However, the fibres were frozen after contractions (within 3 s) and hence changes during tetani would have had time to recover; the present results obtained during fatigue show full recovery of V_0 in 4 s. Furthermore, neither NMR nor biochemical analysis on frozen samples allows detection of intracellular gradients with changes of ATP–ADP being larger in the vicinity of cross-bridges.

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