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SLOWING OF RELAXATION DURING FATIGUE IN SINGLE MOUSE MUSCLE FIBRES

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SUMMARY

1. In the preceding paper we analysed the force decline in fatigue of isolated mouse muscle fibres. Using the same preparation and stimulation scheme we have now examined another aspect of muscle fatigue, namely slowing of relaxation.

2. Relaxation at the end of a tetanic contraction can usually be divided into two phases, an initial nearly linear force decline, followed by an exponential phase. We have analysed the initial phase in terms of decline rate and duration. In rested fibres the decline rate after a 350 ms tetanus was not affected by a 30 % reduction of tetanic tension (obtained by decreasing the stimulation frequency).

3. Relaxation became gradually slower during fatiguing stimulation with a maximum reduction at the time when tetanic tension was reduced to about 75 % of the original (end of phase 2, see preceding paper). At this time the decline rate of the linear phase had fallen to 55·2 ± 2·9 % (mean ± S.E.M., $n = 25$) and its duration had increased to 151·3 ± 14·2 % of the control (unfatigued 350 ms, 70 Hz tetanus).

4. Short rest periods (duration = 10 s), given at various times during fatiguing stimulation, resulted in a clear, but partial, normalization of the relaxation parameters; at the time of maximum slowing the mean decline rate increased from 50·3 to 58·7 % and the duration decreased from 167·9 to 144·0 % of the control ($n = 14$).

5. The influence of intracellular acidosis on relaxation was studied by exposing rested fibres to 30 % CO_2 instead of the normal 5 %. This resulted in a decline rate of 43·0 ± 2·6 % and a duration of 221·1 ± 13·1 % of the control ($n = 7$).

6. In amphibian muscle relaxation is known to become gradually slower during a single, prolonged tetanus. The existence of such an effect also in the present preparation was studied by giving 'interrupted' tetani with a total duration of about 2 s. In rested fibres the mean rate of relaxation was found to fall from 140·9 to 71·8 % ($n = 11$) of the control (end of 350 ms stimulation) with a time constant of about 0·5 s. Thus, a marked slowing during a long tetanus occurs also in mammalian muscle.

7. A distinct slowing of relaxation during prolonged tetani was observed also in the fatigued and in the acidified state. Under these two conditions the mean rate of

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relaxation fell from 87·0 to 34·0% ($n = 3$) and from 74·0 to 23·0% ($n = 5$) of the control, respectively, with time constants similar to that in the rested state.

8. Protein analysis by one-dimensional gel electrophoresis of whole muscles showed a mean parvalbumin content of $5{\cdot}10 \pm 0{\cdot}27$ g (kg wet weight)$^{-1}$.

9. In conclusion, slowing of relaxation in fatigue is likely to be due to the combined effect of altered cross-bridge kinetics and reduced rate of Ca^{2+} removal from troponin C. The latter appears to be caused exclusively by impaired function of sarcoplasmic reticulum (SR) Ca^{2+} pumps, since Ca^{2+} saturation of parvalbumin, estimated from interrupted tetani, seems to remain constant during fatiguing stimulation.

INTRODUCTION

In fatiguing skeletal muscle several properties change: the tension-generating capacity declines, the shortening speed is reduced and relaxation becomes slower. In the preceding paper (Lännergren & Westerblad, 1991) we focused on the decline of tetanic tension and suggested that it was due both to reduced tension-generating capacity of the contractile elements and to impaired Ca^{2+} handling. This study is instead directed towards the mechanisms behind the slowing of relaxation associated with fatigue.

The slowed relaxation may reflect: (i) reduced rate of cross-bridge cycling (e.g. Edwards, Hill & Jones, 1975); (ii) impaired function of the sarcoplasmic reticulum (SR) Ca^{2+} pump (e.g. Dawson, Gadian & Wilkie, 1980; Allen, Lee & Westerblad, 1989); (iii) Ca^{2+} saturation of parvalbumin, a myoplasmic protein suggested to buffer Ca^{2+} and speed up relaxation after a short period of stimulation (for review see Gillis, 1985). Fatigue is often accompanied by a marked acidosis, which may affect both cross-bridge kinetics (mechanism (i); Edman & Mattiazzi, 1981) and the Ca^{2+} pump (mechanism (ii); MacLennan, 1970), but not the Ca^{2+} buffering by parvalbumin (Pechère, Derancourt & Haiech, 1977). In the present study the role of acidosis was tested by comparing the relaxation of fatigued fibres and rested fibres acidified by exposure to high CO_2. Mechanism (iii) was more specifically addressed by measuring the parvalbumin content in the muscle and by giving 'interrupted' tetani (Abbott, 1951), since relaxation has been found to become progressively slower as a function of tetanus duration; this has been attributed to gradual filling of Ca^{2+} binding sites on parvalbumin (Curtin, 1986; Peckham & Woledge, 1986).

The present results show that the slowing of relaxation in acidified fibres was larger than the maximum slowing during fatiguing stimulation. The parvalbumin content was high and a marked slowing of relaxation as a function of tetanus duration was observed both in rested, in acidified and in fatigued fibres.

METHODS

In general the methods were the same as in the preceding paper (Lännergren & Westerblad, 1991). Briefly, single fibres, dissected from the flexor brevis muscle of the mouse, were fatigued by repeated 350 ms tetani. Rested fibres were acidified by exposure to 30% CO_2 instead of the normal 5%.

Interrupted tetanic stimulation

Interrupted tetani were given to evaluate possible changes of relaxation speed developing during prolonged stimulation. These tetani had a duration of about 2 s and consisted of recurring 100 ms periods with 70 Hz stimulation and 50 ms periods without stimulation.

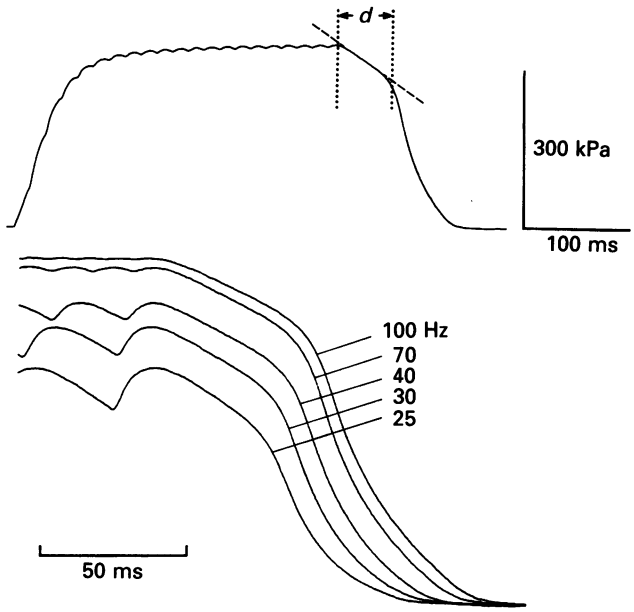

Fig. 1. Tension records from tetani elicited before fatiguing stimulation in one fibre. The upper part shows a 70 Hz, 350 ms tetanic contraction. Measurements were made from the linear phase of relaxation: the duration (*d*) and the tension decline rate (slope of dashed line) has been measured. The lower part shows the relaxation of five consecutive tetanic contractions produced at the frequencies indicated. Note that the rate of tension decline during the linear phase is the same at all frequencies.

Measurement of relaxation parameters

After tetanic stimulation relaxation usually occurs in two phases. Initially tension falls linearly and during this phase the relaxation process is homogeneous along the fibre. This relatively slow linear phase is interrupted by a faster exponential phase during which intrafibre movements start to appear, that is, some parts of the fibre shorten whereas others are being lengthened (Huxley & Simmons, 1970; Cleworth & Edman, 1972). The two phases proved to be unusually well defined in the isolated mouse fibres and we have regarded measurements of the initial phase, where no translations occur, to be easiest to interpret (see Discussion) and have considered this phase only.

Relaxation rate has mostly been estimated from the time taken for tension to decay by a fixed fraction of the maximum tension (e.g. from 100 to 90%). A weakness of this way of measuring is that it will depend on the tension level, which usually decreases during fatiguing stimulation. This point was investigated separately in four fibres. Tetani (350 ms) were given at frequencies ranging from 25 to 100 Hz, giving maximum tensions from 70 to 105%, where 100% by our definition is the maximum force in a 70 Hz tetanus. The lower part of Fig. 1 shows records from one such experiment. It can be seen that the rate of relaxation was nearly the same at all frequencies and thus independent of the tension level. The same result was obtained in the other three fibres and in each experiment the speed of tension fall did not vary by more than 10%; this variation was random and could not be correlated with the tetanic tension. From the records in Fig. 1 it can be measured that tension decreases from 100 to 90% in 32 ms at 100 Hz and in 18 ms at 25 Hz, i.e.

an almost twofold difference. Thus, values describing the same rate of tension fall were markedly lowered when the tension output was reduced. With this in mind we have instead adopted measurements of the absolute slope of the linear phase (kPa s^{-1}), as indicated in the upper part of Fig. 1.

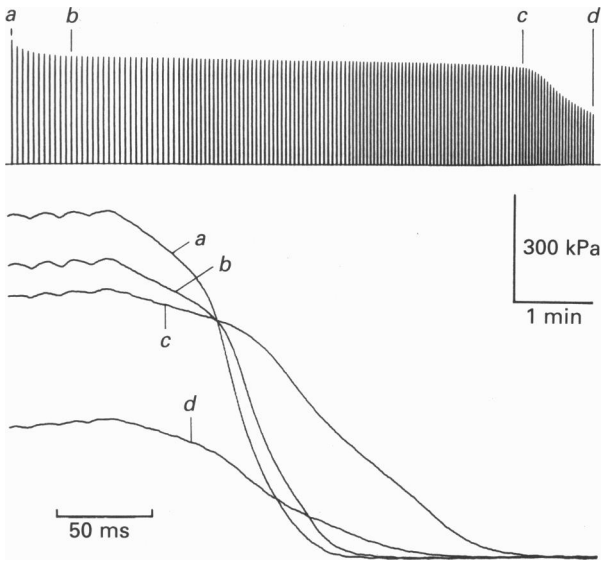

Fig. 2. Slowing of relaxation during fatiguing stimulation. A typical fatigue curve of a single fibre is shown in the upper part. Each tetanus appears as a vertical line. The lower part displays the relaxation of the tetani indicated above the fatigue curve (*a–d*). The tension bar refers to the upper part only.

Measurements were made from data stored in the computer. From the linear portion of relaxation of standard 350 ms tetani a very good fit to a straight line could be obtained over periods of 20 to 100 ms ($R > 0{\cdot}99$), except for a few cases during the final part of fatiguing stimulation. As shown in the upper part of Fig. 1 the duration of the linear phase was also measured. In contrast to the rate of decline, duration did not remain unchanged as the stimulation frequency was altered (lower part of Fig. 1). Values for the two relaxation parameters are for each fibre given as percentages of values obtained from an unfatigued 350 ms 70 Hz tetanus. Values are presented as means ± S.E.M. or as a range. Paired *t* tests were used to test the significance of differences; the significance level was set at 0·05 throughout.

During interrupted tetani the decline rate of the linear part of relaxation was measured for each pause. In the first pause the linear portion was often very short, or even missing, and therefore reliable measurements could frequently not be obtained at this point.

Parvalbumin analysis

Whole flexor brevis foot muscles, from which the single fibres have been isolated, were dissected out and carefully cleaned of connective tissue. The muscles were quickly frozen in liquid nitrogen and then freeze-dried. The parvalbumin content was determined for each muscle as described by Simonides & van Hardeveld (1989). Briefly, the muscles were homogenized in 150 μl buffer with 87·5 mM-Tris (pH 7·4), 2 mM-dithiotreitol and 10% glycerol. The homogenate was heated to 80 °C and then centrifuged at 10000 *g*; 2% sodium dodecyl sulphate (SDS) and 0·001% Bromophenol Blue was added to the supernatant which was then heated to 60 °C for 10 min. Samples were electrophoresed on SDS polyacrylamide gels according to Laemmli (1970) with a 6·5% stacking gel and a 15% separating gel. Rabbit parvalbumin (molecular weight = 12100; Sigma) at various dilutions was used as a reference. After fixing, staining and drying the gel, absorption profiles were

obtained using a laser densitometer. Comparison with profiles representing known amounts of parvalbumin yielded values for the samples. A factor of 3·7 was used to convert muscle dry weight to muscle wet weight.

RESULTS

The upper part of Fig. 2 shows tension records from a fibre displaying the typical pattern of force decline during fatiguing stimulation (described in detail in the

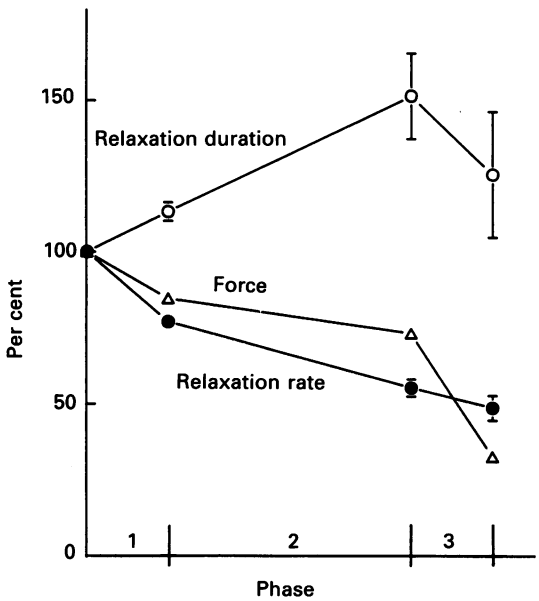

Fig. 3. Summary of changes of relaxation in fatigue. Measurements were made at the times indicated in Fig. 2; at the beginning of the fatigue run, at the end of phase 1 and of phase 2, respectively, and at the end of fatiguing stimulation. Values were obtained from twenty-five fibres, of which seventeen could be used for measurements at the end of fatiguing stimulation (a linear phase could not be discerned in three fibres and in five experiments stimulation was stopped before tension was down to 0·3 P_0). ○ represent the duration of the linear part of relaxation, ● the rate of decline during this part and △ the tetanic tension. Means ± S.E.M.; 100 % refers to values obtained in the rested state in each fibre.

preceding paper; Lännergren & Westerblad, 1991). Briefly, fatigue development can be divided into three phases: initially there is a clear tension fall to about 0·85 P_0 (original tension) over the first eight to fourteen tetani (phase 1), then follows a much longer period of almost stable tension production (phase 2) and finally there is a rapid tension decline (phase 3). The lower part of Fig. 2 illustrates the slowing of relaxation developing during fatiguing stimulation. It can be seen that the rate of relaxation became successively slower during the stimulation period. Further, the duration of the linear phase of relaxation was markedly increased and this increase was found to have its maximum at the transition between phase 2 and 3, whereafter it decreased. Similar observations were made in all other experiments and in Fig. 3 the results from twenty-five fibres are summarized. Tetani (350 ms, 70 Hz) elicited in the rested

state gave a mean tension decline during the linear part of relaxation of 1640 kPa s^{-1} (range = 1200–2450 kPa s^{-1}) and this part had a mean duration of 39·1 ms (range = 23–54 ms); 100% in Fig. 3 (and in the following) refers to values obtained in the rested state in each experiment.

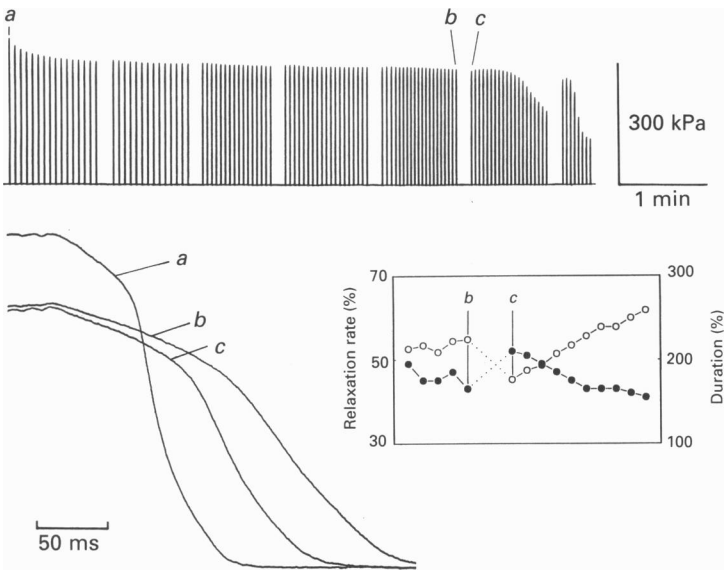

Fig. 4. A substantial normalization of the relaxation occurs during a 10 s pause. The upper part shows a typical fatigue run with 10 s pauses given every minute. The lower part displays the relaxation of the first tetanus (*a*) and tetani immediately before (*b*) and after a pause (*c*). In the inset, values of the relaxation rate (●) and the duration (○) are plotted for five tetani before and ten tetani after the puase. Observe that the marked recovery of relaxation was transient.

When fatiguing stimulation was started, the relaxation was affected without delay and both the rate of tension decline and the duration of the linear portion were significantly changed at the transition from phase 1 to 2, that is, during approximately ten tetani (Fig. 3). During the more long-lasting phase 2 the slowing of relaxation increased progressively. During phase 3 the mean rate of relaxation became slightly more reduced, whereas the duration decreased again in most fibres.

Short-rest experiments (10 s pauses)

In one group of experiments fatiguing stimulation was stopped for 10 s each minute. This experimental approach was primarily used to investigate the possible role of failing T-tubule function for the reduction of tetanic tension (for more details see the preceding paper). As illustrated in the upper part of Fig. 4, the tetanic tension was markedly higher after a 10 s rest period given during phase 3, whereas no change of the tension output was observed during phase 2. Pauses during phase 2 had, however, a clear effect on relaxation. This is shown in the lower part of Fig. 4 where it can be seen that both the speed of relaxation and the duration recovered substantially during the rest period. As shown by the inset in Fig. 4, the recovery of

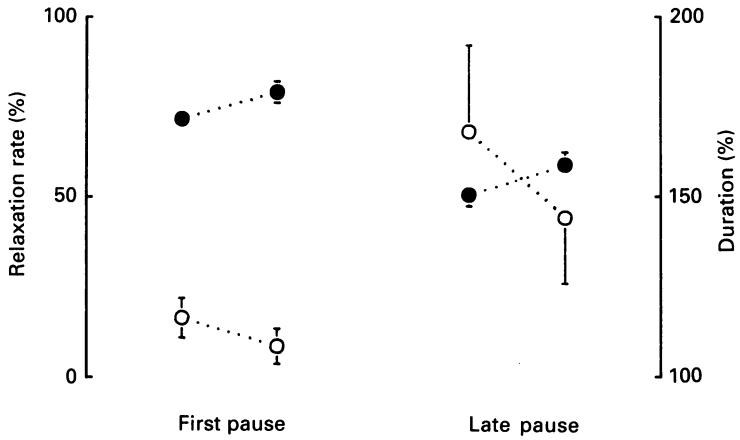

Fig. 5. Summary of the recovery of relaxation occurring during 10 s pauses. The first pause was given after 1 min of stimulation and values for the late pause were obtained from the last rest period of phase 2. ● represent the relaxation rate and ○ the duration. Data from fourteen fibres.

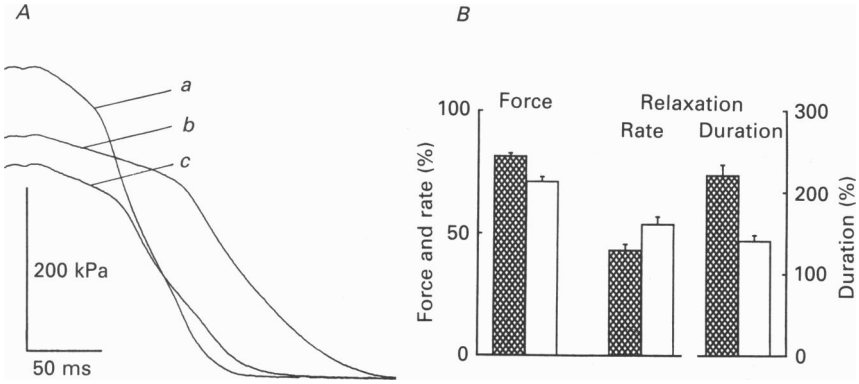

Fig. 6. The maximum slowing of relaxation in fatigue (end of phase 2) is compared with the slowing due to acidosis. *A*, relaxation of a fibre in the rested state (*a*, 5% CO_2), in the acidified state (*b*, 30% CO_2) and at the end of phase 2 (*c*, 4 min stimulation, 5% CO_2). Results from seven fibres are summarized in *B*. Cross-hatched bars, acidosis; open bars, end of phase 2. Note that relaxation was markedly more affected in the acidified state.

relaxation was only temporary and in this fibre relaxation characteristics were back to the pre-pause level at about the sixth tetanus after the pause. Figure 5 shows a plot of mean values of the relaxation parameters obtained immediately before and after pauses at 1 min of stimulation and at the end of phase 2. At both these instances the two relaxation parameters recovered significantly during the pause.

Experiments with high CO_2

Intracellular acidosis, produced in rested fibres by increasing the CO_2 content of the bath solution, had a marked influence on relaxation. Figure 6*A* shows tension records from one such experiment in which the slowing of relaxation due to acidosis

can be compared with the slowing caused by fatiguing stimulation. In this particular fibre the 4 min record represents conditions at the transition between phase 2 and 3, where changes in relaxation parameters (rate and duration) were most prominent. From the records it is clear that relaxation was markedly slower in the acidified than

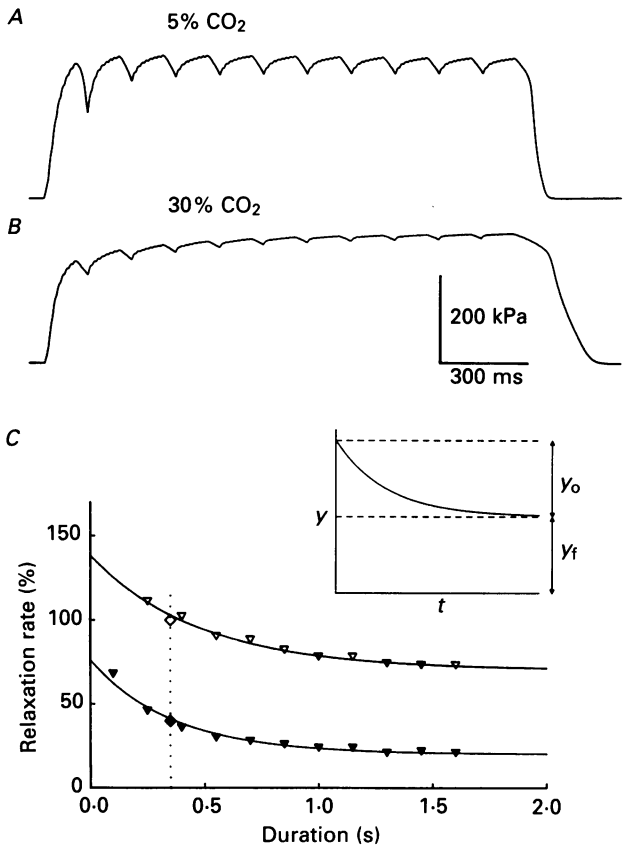

Fig. 7. Interrupted tetani elicited in a fibre in the rested (*A*) and the acidified (*B*) state. Stimulation periods were 100 ms and rest periods 50 ms. In *C* the relaxation rate is plotted against the time from the onset of contraction: ▽ rested state, ▼ acidified state. The dotted line indicates the duration of standard tetani (350 ms) and the diamonds give the relaxation rate of these. The inset shows the relaxation rate parameters in eqn (1), which were used to construct the curves in *C*.

in the fatigued state. The same observation was made in six other fibres exposed to high CO_2 and the results from these experiments are summarized in Fig. 6*B*.

Interrupted tetani

In rested amphibian muscles the rate of relaxation becomes progressively slower as a function of tetanus duration (e.g. Abbott, 1951). In order to investigate if such a progressive slowing also occurs in mammalian muscle, interrupted tetani were elicited. Figure 7*A* shows one such tetanus produced in the rested state and a progressive slowing of relaxation is obvious in this case. Figure 7*B* shows an

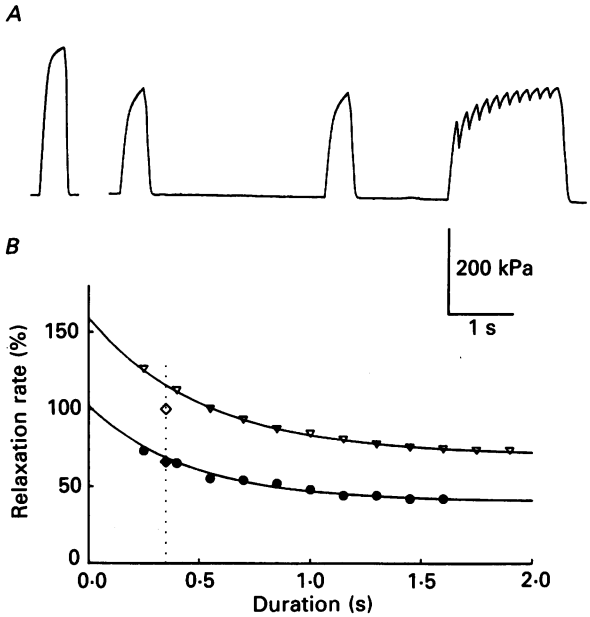

Fig. 8. *A* shows tension records from the first tetanus of a fatigue run and from two tetani followed by an interrupted tetanus given at the end of phase 2. The plot in *B* shows the slowing of relaxation during interrupted tetani in the rested (▽) and in the fatigued state (●). The diamonds and the dotted line have the same meaning as in Fig. 7.

TABLE 1. Mean values of relaxation parameters obtained from interrupted tetani

	n	y_f(%)	y_0(%)	τ(s)
Rested	11	71·8±1·8	69·1±5·1	0·54±0·03
Acidified	5	23·0±1·5	51·0±3·9	0·43±0·02
Fatigued	3	34·0±3·1	53·0±7·2	0·48±0·04

In each experiment y_f, y_0 and τ were obtained by fitting data points with eqn (1).

interrupted tetanus elicited when the fibre was exposed to 30% CO_2. Even though the relaxation is markedly slowed in this case, it is still substantially faster at the beginning than at the end of the contraction.

The values of relaxation speed obtained during interrupted tetani have been fitted with the equation:

$$y = y_0 e^{-t/\tau} + y_f, \quad (1)$$

where y is the rate of relaxation, t is the time from the onset of contraction, y_0 is the rate of relaxation at $t = 0$, y_f is the rate of relaxation at $t = \infty$ and τ is the time constant.

The occurrence of a progressive slowing was also investigated during fatiguing stimulation. Interrupted tetani were produced approximately 1 s after a standard 350 ms tetanus at the end of phase 2, where the slowing of relaxation has been found to have its maximum (see above). One such experiment is illustrated in Fig. 8. This fibre had been fatigued by 103 tetani and the tetanic tension was reduced to 71% when an interrupted contraction was elicited. It can be seen that the relaxation was

considerably faster at the beginning of the contraction also in this case with the initial decline rate ($y_o + y_f$) being more than twice as big as the final rate (y_f). Similar results were obtained in two more fibres.

The results from the interrupted tetanus experiments are summarized in Table 1. This shows that mean values of y_f, y_o and τ were similar in the three states with one major exception: y_f was markedly higher in the rested state than in the other two states.

Parvalbumin analysis

The parvalbumin content of whole flexor brevis muscles was determined by gel electrophoresis using rabbit parvalbumin as a reference. Nine muscles from six animals were analysed and the parvalbumin content was found to be $5{\cdot}10 \pm 0{\cdot}27$ g (kg wet weight)$^{-1}$. This value is somewhat higher than previously reported for fast-twitch mouse muscles (range 4·4–4·9 g kg^{-1}; Heizmann, Berchtold & Rowlerson, 1982) and also higher than most values for frog muscle (see Simonides & van Hardeveld, 1989). Assuming that mouse and rabbit parvalbumins have the same molecular weight, the content (5·1 g kg^{-1}) corresponds to a concentration of 0·43 mM.

DISCUSSION

The experiments in this study have dealt with the slowing of relaxation associated with muscle fatigue. The relaxation generally occurred in two phases: a slow, linear tension fall, followed by a more rapid, nearly exponential decline. Both phases became slower during fatiguing stimulation, but we have focused on the initial linear phase on the following grounds. (i) In unfatigued fibres the relaxation has been shown to be isometric during the linear phase and intrafibre movements start to appear at the transition to the exponential phase (Huxley & Simmons, 1970; Cleworth & Edman, 1972); this has recently been shown to occur also in fatigued and acidified fibres (Curtin & Edman, 1989). (ii) During the linear phase of relaxation the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) falls rapidly, whereas it declines slowly, remains more or less constant or even transiently increases during the exponential phase (Cannell, 1986). Thus, measurements from the linear phase appear to be easier to interpret in terms of both cross-bridge kinetics and Ca^{2+} handling.

The rate of tension fall during the linear phase of relaxation declined throughout the period of fatiguing stimulation. A reduced rate of Ca^{2+} uptake by the SR is likely to contribute to this decline: Allen *et al.* (1989) have shown in *Xenopus* fibres that the slowing of relaxation developing during fatiguing stimulation is accompanied by a reduced rate of decline of $[Ca^{2+}]_i$; the affinity for ATP hydrolysis decreases in fatigue and Dawson *et al.* (1980) have shown theoretically that this can have large effects on active transport systems such as the SR Ca^{2+} pumps; the function of the SR Ca^{2+} pumps is affected by the intracellular pH (pH_i) which generally falls during fatiguing stimulation (see below). A reduced rate of cross-bridge cycling may also be of importance for the declining rate of relaxation and a correlation has been shown between a reduction of shortening velocity, which more directly reflects cross-bridge kinetics, and slowed relaxation (Edman & Mattiazzi, 1981; Lännergren & Westerblad, 1989).

The duration of the linear phase of relaxation displayed a progressive increase during most of the fatiguing stimulation period (phase 1 and 2). This increase can be explained by a reduced rate of Ca^{2+} removal from troponin C and/or by altered cross-bridge kinetics. A slowing of Ca^{2+} removal from troponin C is likely to occur during fatiguing stimulation (discussed above) and this will increase the time for $[Ca^{2+}]_i$ to fall to a level where the contractile elements in some regions of the fibre are so poorly activated that they will 'give', resulting in segment movements and a faster fall of tension. Altered cross-bridge function probably also contributes to the prolongation of the linear phase of relaxation. Fatigue affects isometric force more than force during stretching (Curtin & Edman, 1989) and poorly activated regions may, by being slowly stretched, resist the force by stronger regions and thus prolong the time until 'giving'.

During the final part of fatiguing stimulation (phase 3) the duration of the linear phase of relaxation decreased significantly. This reduction is most probably due to impaired activation of the contractile elements, since it is unlikely that alterations which affect cross-bridge kinetics (e.g. reduced pH_i; see below) would be reversed during the final part of the stimulation period. The tetanic $[Ca^{2+}]_i$ has been shown to be markedly reduced in fatigue (Allen *et al.* 1989) and thus, although the actual rate of $[Ca^{2+}]_i$ decline may be low, the $[Ca^{2+}]_i$ level where the contractile elements in some regions of the fibre start to 'give' might be reached more rapidly.

Fatigue vs. acidosis

Both the duration and the rate of decline of the linear phase of relaxation were markedly affected in the acidified state. In the preceding paper we concluded that the tension reduction in acidified fibres was mainly due to impaired cross-bridge function. It is then likely that a direct inhibition of cross-bridge activity is also of major importance for the slowing of relaxation. This hypothesis is supported by results from frog fibres, where acidosis, besides reducing the tetanic tension and the relaxation speed, also reduces the maximum shortening velocity (Edman & Mattiazzi, 1981). Moreover, it has been shown in *Xenopus* fibres that, in contrast to the situation in fatigue, the slowing of relaxation in acidified fibres is not accompanied by a reduced rate of $[Ca^{2+}]_i$ decline (Allen *et al.* 1989). However, since it is known that acidosis impairs the function of the SR Ca^{2+} pump (MacLennan, 1970) a reduced rate of Ca^{2+} removal from troponin C may still contribute to the slowing of relaxation due to acidosis in the present preparation.

Relaxation was more affected in the acidified state than in the fatigued state. The most straightforward explanation of this finding is that the fibres became less acidified during fatiguing stimulation. We chose to use 30 % CO_2 to produce acidosis because this has been shown to reduce pH_i from 7·0 to 6·6 (Meyer, Kushmerick & Dillon, 1983), which is similar to the reduction of pH_i observed in mammalian muscle fatigued by intermittent contractions (Sahlin, Edström, Sjöholm & Hultman, 1981; Cady, Jones, Lynn & Newham, 1989). It is, however, possible that pH_i falls less in the present single-fibre preparation, for example by lactic acid being more effectively transported out of the cell.

A comparison of the changes of the relaxation parameters in the fatigued and in the acidified state reveals that the difference in duration is markedly larger than the

difference in rate (Fig. 6*B*). The tetanic $[Ca^{2+}]_i$ has been found to be increased in fibres acidified by exposure to high CO_2, whereas it is reduced in fatigued fibres (Allen *et al.* 1989). Thus it might take longer for $[Ca^{2+}]_i$ to fall to a level where some regions of the fibre start to 'give' in the acidified state, although the actual rate of $[Ca^{2+}]_i$ decline appears to be faster in acidified than in fatigued fibres (Allen *et al.* 1989).

10 s pauses

One possible mechanism for the marked recovery of relaxation during 10 s pauses might involve changes of the affinity for ATP hydrolysis since even small such changes can have large effects on active transport systems (Dawson *et al.* 1980). It is possible that the ATP/ADP ratio in the vicinity of the SR Ca^{2+} pumps becomes reduced during fatiguing stimulation and that this reduction shows some recovery during the 10 s pauses (cf. Rossi, Eppenberger, Volpe & Cotrufo, 1990), which may then result in a higher relaxation speed.

Changes in relaxation rate during single tetani: Ca^{2+}–parvalbumin interaction

Apart from transport of Ca^{2+} into the SR by an active pumping process, another mechanism for lowering of myoplasmic $[Ca^{2+}]$ has been suggested to operate in fast-twitch muscle, namely a temporary binding of Ca^{2+} to parvalbumin. The muscle used in the present study contained a relatively high concentration of parvalbumin and we have used large surface fibres with contractile properties representative of fast-twitch (type II) fibres, which generally have larger amounts of parvalbumin than slow-twitch (type I) fibres (Heizmann *et al.* 1982). Thus, it seems reasonable to assume that the fibres studied here contain parvalbumin at a concentration at least as high as that measured for the whole muscle (0·43 mM).

The possible action of parvalbumin as a Ca^{2+} buffer has previously been evaluated by measuring relaxation rate after tetani of various durations (Curtin, 1986) or by briefly interrupting stimulation at regular intervals during a long tetanus (Abbott, 1951; Peckham & Woledge, 1986). We used the latter method and found an approximate halving of relaxation rate during a 1·5 s tetanus of rested fibres, which is in accord with results in amphibian fibres. A corresponding slowing was found in acidified fibres, although the initial $(y_o + y_f)$ and final (y_f) values were lower by 60–70 % in agreement with the CO_2 results discussed above. This result is compatible with Ca^{2+} buffering by parvalbumin, since this reaction is pH insensitive (Pechère *et al.* 1977).

Previous studies in amphibian muscle (Curtin, 1986; Peckham & Woledge, 1986) have indicated that recovery of the maximum relaxation rate $(y_o + y_f)$ is a relatively slow process and requires many minutes for completion at 0 °C. It would thus be expected that with repeated tetani parvalbumin would soon be filled with Ca^{2+} and remain saturated during continued stimulation. This assumption was recently tested in single *Xenopus* fibres (22·5 °C) and in these it was found that in the fatigued state relaxation rate was uniformly low during an interrupted tetanus elicited a few seconds after an ordinary 350 ms tetanus (Westerblad & Lännergren, 1990). It was therefore a surprise to find that there was a marked slowing of relaxation during an interrupted tetanus elicited during fatiguing stimulation in the present preparation (Fig. 8). At first sight, this indicates that the common interpretation of

Ca^{2+}–parvalbumin interaction is not valid for mammalian fibres and that some other mechanism is responsible for slowing of relaxation during a long tetanus. However, the rate constant for Ca^{2+} dissociation is relatively fast (about 1 s^{-1} at 20 °C; Gillis, 1985; Hou, Johnson & Rall, 1990). Thus, in the present study a substantial repriming would be possible during the 0·8–1·5 s intervals preceding the interrupted tetani, provided that the SR Ca^{2+} pumps work at a sufficiently high rate. Also, with the stimulation scheme used to induce fatigue (tetanus intervals of 2–3 s) repriming would be possible.

Why then does repriming of maximum relaxation rate take so much longer in amphibian muscle? A possible explanation would be that Ca^{2+} pumps work at a lower rate at a corresponding temperature. An indication that this is the case may be obtained by comparing relaxation rates in amphibian and mouse fibres at the end of a long tetanus, when parvalbumin should be Ca^{2+} saturated. Using our data from *Xenopus* fibres (Westerblad & Lännergren, 1990) and the present ones and taking into account the difference in temperature (22·5 *vs.* 25 °C) we find that the relaxation rate is approximately 50 % lower in *Xenopus* fibres. Assuming Ca^{2+} uptake by the SR to be the main determinant of relaxation speed at this stage, this would support the idea of a lower pumping rate in amphibian fibres.

There is an uncertainty with this reasoning, however, since it cannot be excluded that cross-bridge kinetics are altered during the interrupted, 1·5 s tetani. During fatiguing stimulation we find a rapid decline in tension during the first ten tetani (phase 1), corresponding to a total stimulation time of 3·5 s. This decline could not be overcome by caffeine and was therefore attributed to a depression of cross-bridge force-generating capacity (see preceding paper). If this interpretation is correct it is conceivable that the turnover rate of cross-bridges may change as well and that this may influence measurements already at the end of a 1·5 s tetanus.

In conclusion, in the present model of fatigue the slowing of relaxation is likely to be due to the combined effect of altered cross-bridge kinetics and a reduced rate of Ca^{2+} removal from troponin C. The latter appears to be caused exclusively by impaired function of the SR Ca^{2+} pumps, since Ca^{2+} saturation of parvalbumin, estimated from interrupted tetani, seems to remain constant throughout fatiguing stimulation.

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