

Changes of Myoplasmic Calcium Concentration during Fatigue in Single Mouse Muscle Fibers

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ABSTRACT Measurements of the intracellular free concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) were performed during fatiguing stimulation of intact, single muscle fibers, which were dissected from a mouse foot muscle and loaded with fura-2. Fatigue, which was produced by repeated 100-Hz tetani, generally occurred in three phases. Initially, tension declined rapidly to $\sim 90\%$ of the original tension ($0.9 P_o$) and during this period the tetanic $[\text{Ca}^{2+}]_i$ increased significantly (phase 1). Then followed a lengthy period of almost stable tension production and tetanic $[\text{Ca}^{2+}]_i$ (phase 2). Finally, both the tetanic $[\text{Ca}^{2+}]_i$ and tension fell relatively fast (phase 3). The resting $[\text{Ca}^{2+}]_i$ rose continuously throughout the stimulation period. A 10-s rest period during phase 3 resulted in a significant increase of both tetanic $[\text{Ca}^{2+}]_i$ and tension, whereas a 10-s pause during phase 2 did not have any marked effect. Application of caffeine under control conditions and early during phase 2 resulted in a substantial increase of the tetanic $[\text{Ca}^{2+}]_i$ but no marked tension increase, whereas caffeine applied at the end of fatiguing stimulation (tension depressed to $\sim 0.3 P_o$) gave a marked increase of both tetanic $[\text{Ca}^{2+}]_i$ and tension. The tetanic $[\text{Ca}^{2+}]_i$ for a given tension was generally higher during fatiguing stimulation than under control conditions. Fatigue developed more rapidly in fibers exposed to cyanide. In these fibers there was no increase of tetanic $[\text{Ca}^{2+}]_i$ during phase 1 and the increase of the resting $[\text{Ca}^{2+}]_i$ during fatiguing stimulation was markedly larger. The present results indicate that fatigue produced by repeated tetani is caused by a combination of reduced maximum tension-generating capacity, reduced myofibrillar Ca^{2+} sensitivity, and reduced Ca^{2+} release from the sarcoplasmic reticulum. The depression of maximum tension-generating capacity develops early during fatiguing stimulation and it is of greatest importance for the force decline at early stages of fatigue. As fatigue gets more severe, reduced Ca^{2+} sensitivity and reduced Ca^{2+} release become quantitatively more important for the tension decline.

INTRODUCTION

Repetitive stimulation of skeletal muscle causes a decline in tension production known as fatigue. In intact animals neural factors can be involved in the development of fatigue, but processes within the muscle are of greater importance (Merton, 1954;

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Bigland-Ritchie, Furbush, and Woods, 1986; Vøllestad, Sejersted, Bahr, Woods, and Bigland-Ritchie, 1988). Three main mechanisms have been proposed to explain the impaired function of muscles in fatigue: (a) reduced Ca^{2+} release from the sarcoplasmic reticulum (SR) (e.g., Allen, Lee, and Westerblad, 1989), (b) reduced Ca^{2+} sensitivity of the myofilaments (e.g., Godt and Nosek, 1989), and (c) reduced maximum Ca^{2+} -activated tension (e.g., Edman and Lou, 1990).

It has recently been shown that intact, single fibers dissected from a mouse foot muscle can be used in physiological studies (Lännergren and Westerblad, 1987) and this preparation offers an opportunity to study cellular mechanisms of fatigue in intact mammalian muscle cells. Using this preparation, Lännergren and Westerblad (1991) recently showed that the tension reduction during fatigue produced by repeated tetani occurred in three phases. Initially tension declined rapidly to ~90% of the original (phase 1), followed by a long period of almost stable tension production (phase 2). Finally, there was a rapid tension decline (phase 3).

We have now extended the study of Lännergren and Westerblad (1991) by using fibers loaded with the Ca^{2+} indicator fura-2 to investigate the role of changes in the intracellular free concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$) during fatiguing stimulation. The results show that the tension decline during phase 1 was accompanied by an increase in tetanic $[\text{Ca}^{2+}]_i$, and application of caffeine, which increased tetanic $[\text{Ca}^{2+}]_i$ further, did not result in increased tension production. These findings strongly indicate that the tension decline during phase 1 is caused by a reduction of the maximum tension-generating capacity. Tetanic $[\text{Ca}^{2+}]_i$ remained high during phase 2. During phase 3 tetanic $[\text{Ca}^{2+}]_i$ fell, but for a given tension it was still markedly higher than under control conditions. Thus, the rapid tension reduction during phase 3 appears to be due to the combined effect of reduced Ca^{2+} release from the SR and reduced Ca^{2+} sensitivity.

A preliminary account of some of these results has been presented (Allen and Westerblad, 1990).

METHODS

Fiber Dissection and Mounting

Male mice were killed by rapid neck disarticulation. Large single fibers were dissected from the surface of the flexor brevis muscle of the foot (Lännergren and Westerblad, 1987); these fibers were probably mainly fast-twitch fibers (type IIA or IIB) (Lännergren and Westerblad, 1991). After dissection the tendons were gripped by platinum foil microclips and the preparation was transferred to the stimulation chamber, where it was mounted between an Akers AE 801 force transducer and an adjustable holder, which allowed the fiber to be stretched to the length giving maximum tetanic tension.

Stimulation

The fiber was flanked by platinum electrodes and stimulated by current pulses with a duration of 0.5 ms and an intensity of $\sim 1.2 \times$ threshold. Fatigue was produced by repeated 100-Hz tetani given until tetanic tension was down to $\sim 30\%$ of the original ($0.3 P_0$). Tetani with a duration of 350 ms were initially produced every 4 s and the stimulation was made more demanding every second minute by reducing the tetanic interval to 3.0, 2.5, 2.0, 1.7, 1.5, and 1.2 s and thereafter by increasing the tetanic duration. This progressively more demanding

stimulation protocol was used to produce a similar type of fatigue in fibers with different fatigue resistance. In some experiments 10-s pauses were interspersed during fatiguing stimulation.

A series of contractions at stimulation frequencies ranging from 20 to 200 Hz was generally produced before fatiguing stimulation and between successive fatigue runs; these contractions were elicited to obtain a relation between fura-2 ratios (see below) and tension under control conditions. At lower stimulation frequencies both the tension and the fura-2 ratios became unfused. Frequencies at which the oscillation in tension was $> 10\%$ of the total tension were not used for measurements and for this reason the lowest tension obtained under control conditions ranged from 0.3 to 0.5 P_o .

Solutions

Fibers were dissected in a solution with the following composition (in mM): 136.5 NaCl, 5.0 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.4 NaH₂PO₄, and 11.9 NaHCO₃ (pH \sim 8). After being mounted in the experimental chamber and loaded with fura-2, fibers were superfused with a slightly different solution (in mM): 121 NaCl, 5.0 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.4 NaH₂PO₄, 24 NaHCO₃, and 5.5 glucose. This solution was bubbled with 5% CO₂ + 95% O₂, which gave a pH of 7.3. About 0.2% fetal calf serum was added to both solutions.

In a few experiments fibers were studied with oxidative phosphorylation prevented by addition of 2 mM NaCN to the stimulation Tyrode solution.

A caffeine-containing solution was made up by dissolving 10 mM caffeine in the stimulation Tyrode solution.

All experiments were performed at room temperature (22°C).

Loading of Fura-2 and Fluorescence Measurements

After a few control contractions, the fiber was incubated for 30 min in dissection Tyrode solution containing 20–40 μ M of the acetoxymethyl (AM) ester of fura-2. In some early experiments the temperature was increased to 30°C during the incubation period; this procedure was not found to give better loading than incubation at room temperature. Loading caused no obvious change in tension production.

The optical arrangements used to record the fluorescence signal from fura-2 have been described in detail previously (Lee, Westerblad, and Allen, 1991). Briefly, experiments were performed on the stage of a Nikon Diaphot microscope and a $\times 20$ Fluor objective was used. An Ealing Beck 150-W light source with a xenon arc lamp provided UV illumination and a shutter prevented illumination of the preparation except when required. Neutral density filters, which reduced the intensity of the illumination 10–30 times, were used throughout experiments to prevent bleaching and changes in apparent Ca²⁺ sensitivity of fura-2 (Becker and Fay, 1987; Lee et al., 1991). The fluorescence light was guided to a photomultiplier tube and its amplified output at 505 nm (measured in nano- or microamperes of photocathode current) was displayed on a pen recorder (together with tension and stimuli) and also stored on video tape for later analysis. The fluorescence light signal was recorded from $\sim 50\%$ of the total length of the fiber. Measurements of the fluorescent light were made from pen records filtered at 10 Hz; original records in illustrations were prepared from data stored on tape (fluorescence signals filtered at 30 Hz).

The Ca²⁺-dependent signal of fura-2 is usually obtained by illuminating at 340 and 380 nm and dividing the resulting fluorescence signals emitted at 505 nm. We have used this "ratio" method, but instead of illuminating at 340 nm we illuminated at 360 nm. The advantage of using 360 nm is that this wavelength is approximately the isobestic point for fura-2 (the illumination wavelength where the resulting fluorescence is not affected by the Ca²⁺ concentration; Gryniewicz, Poenie, and Tsien, 1985) and consequently the 360-nm signal should remain

almost unchanged during the rapid alterations of $[Ca^{2+}]_i$ which occur during contraction. If the 360-nm signal remains almost unchanged, it is possible to get the Ca^{2+} -dependent signal by dividing a previously determined 360-nm signal by the 380-nm signal during contraction. Thus, only one contraction is required, whereas recordings from two successive contractions are needed to get the Ca^{2+} -dependent signal when 340 and 380 nm are used (cf. Lee et al., 1991). In the present experiments the 360-nm signal, which was checked at frequent intervals, displayed no or very small changes during contraction (the change was in all cases $<10\%$ of the total signal). The changes observed were probably movement artifacts, because they could be diminished by reducing fiber movement during contraction (i.e., by minor alterations in the way the fiber was mounted in the chamber). Tetanic 360/380 ratios are given as the peak ratio and all observable changes of the 360-nm signal during contraction were corrected for in calculations.

The fluorescence light signal at the beginning of experiments was $2.48 \pm 0.31 \mu A$ (mean \pm SEM, $n = 9$). By comparing these signals and signals produced by known concentrations of fura-2 in a glass capillary of approximately the same dimensions (cf. Klein, Simon, Szucs, and Schneider, 1988), it was found that the average fiber contained $\sim 17 \mu M$ fura-2. Thus, buffering of $[Ca^{2+}]_i$ by fura-2 should be minimal (cf. Lee et al., 1991).

Besides the Ca^{2+} -dependent fura-2 signal, four other sources may significantly contribute to the fluorescence light signal: (a) background (i.e., light recorded with the UV illumination on but no muscle cell in the light path); (b) autofluorescence due to endogenous compounds, such as NADH, present in the fiber before loading with fura-2; (c) fura-2 AM in remnants of damaged fibers; and (d) unhydrolyzed fura-2 AM in the muscle fiber.

(a) The background fluorescence was substantial, especially directly after incubation (up to $\sim 50\%$ of the total signal). It then declined to $\sim 25\%$ of the total signal during the first hour and thereafter it declined in parallel with the signal from the fiber. This signal presumably arose from fura-2 AM bound to the chamber base or walls. By moving the fiber sideways out of the UV beam, the background light was determined at frequent intervals throughout the experiments and it has been corrected for in all measurements. The background fluorescence was always determined immediately before and after fatiguing stimulation and it was assumed to decline linearly during the stimulation period.

(b) The autofluorescence could not be measured in the present series of experiments due to the high background light; a small amount of fura-2 AM presumably remained bound to the chamber (see above) from one experiment to another. To measure the autofluorescence we therefore performed a separate experiment at a time when the experimental chamber had been used for other types of experiments for a long period. At this time the background light was reduced ~ 10 -fold. The autofluorescence was ~ 10 and 30 nA at 340 and 380 nm, respectively. Fatigue was produced in this experiment and this resulted in a minor increase of the autofluorescence; maximum signals during fatiguing stimulation were ~ 15 and 40 nA, respectively. Thus the autofluorescence signal was in all cases $<2\%$ of the mean signal after loading with fura-2 and has therefore been ignored.

(c) Damaged fibers could not be completely cleaned away from the fiber and these remnants might take up fura-2 AM, which would result in a substantial fluorescence signal that could not be corrected for in the same way as the background light. In control experiments fura-2 AM was found to give a 360/380 ratio of ~ 1 and this ratio was not affected by changes of the Ca^{2+} concentration. This ratio was similar to the ratio measured in resting fibers and consequently lower than the ratio obtained during contraction. Thus, a substantial loading of damaged fiber with fura-2 AM would reduce tetanic ratios.

Directly after loading the fluorescence signals declined rapidly and the tetanic ratio, which at this stage was much lower than usual, increased in parallel with this decline. This finding is probably caused by fura-2 AM loaded in remnants of damaged fibers being washed away.

Having this in mind, we always left the fiber for at least 1 h after the incubation period and then checked that stable peak and resting ratios were obtained by producing tetani at long intervals.

The relative importance of fluorescence signals from damaged fibers was assessed by observing some fibers in the microscope with UV illumination on. It was then found that the fluorescence came mainly from the only live fiber and the fluorescence from remnants of damaged fibers was just detectable.

(d) The intracellular hydrolysis of fura-2 AM has been found to be incomplete in some cells (for review see Roe, Lemasters, and Herman, 1990). If this occurred in our cells, it would, like mechanism (c), tend to lower the tetanic ratios and this would not be easily corrected for. To study the relative importance of fluorescence signals from intracellular esterified fura-2, three fibers were pressure-injected with the pentapotassium salt of fura-2 (cf. Lee et al., 1991); this procedure also eliminates the problem with loading of remnants of damaged fibers and any possible loading of compartments other than the myoplasm. The amplitudes of the fluorescence signals were similar in injected and loaded fibers. The resting ratio was almost the same in injected (1.05 ± 0.04 , $n = 3$) and loaded (1.08 ± 0.03 , $n = 9$) fibers, whereas the peak ratio during a 100-Hz tetani was slightly higher in injected fibers (3.23 ± 0.25 vs. 2.56 ± 0.13).

As discussed above, Ca^{2+} -independent fluorescence signals will tend to reduce the tetanic ratio. However, if the proportion of Ca^{2+} -sensitive to Ca^{2+} -insensitive fluorescence remains constant during experiments, relative changes of ratios will not be affected. The peak ratio of 100-Hz tetani elicited under control conditions (i.e., not during fatiguing stimulation) generally remained constant within 10% throughout experiments, which often lasted for more than 5 h and during which the amplitude of the fluorescence signals fell to $\sim 50\%$ of the amplitude at the beginning of the experiment (i.e., after the initial rapid decline). Moreover, ratio-tension curves obtained before and after fatiguing stimulation were very similar (see Fig. 9). Thus, the relative contribution to the fluorescence signals from esterified fura-2 appears to be constant throughout experiments.

Neutral density filters were used to minimize photobleaching of fura-2 and periods of UV illumination were kept as short as possible. During fatiguing stimulation the preparation was generally illuminated in three successive contraction-rest periods (the first two at 380 nm and the third at 360 nm), initially at every tenth tetanus and then at longer intervals. In a control experiment a fiber was stimulated to produce one 100-Hz tetanus every 30 s for 10 min and during this period the fiber was illuminated for a period of approximately the same duration as that during a normal fatigue run. Both the resting and the tetanic ratios remained unchanged during this period and consequently photobleaching should not significantly affect our results.

Calibration of Fura-2 Signals

The relation between fura-2 ratios and $[Ca^{2+}]_i$ was assessed according to Grynkiewicz et al. (1985) and using the equation:

$$[Ca^{2+}]_i = K_D(R - R_{\min}/R_{\max} - R)\beta \quad (1)$$

Since in vitro calibration has proven to be inaccurate (e.g., Konishi, Olson, Hollingworth, and Baylor, 1988), we tried to perform an intracellular calibration at the end of each experiment. The fiber was first exposed to a solution containing 10 μ M ionomycin and 20 mM EGTA (pH = 8.0; the calibration solutions were made alkaline to increase the Ca^{2+} transport efficiency of ionomycin [Liu and Hermann, 1978]). In successful experiments this resulted in a transient contracture and ratio increase, which was followed by a ratio decline to a level slightly lower than the resting ratio. A reliable measurement of R_{\min} was obtained in this way in five fibers, giving a mean of 0.93 (range 0.90–1.00).

After measuring R_{\min} , the fiber was exposed to a solution containing 20 mM Ca^{2+} instead of EGTA to determine R_{\max} . In many cases this procedure resulted in an irreversible contracture

and a rapid decline of the fluorescence signals. Reliable values of R_{\max} could therefore be obtained in only three fibers and these had a mean of 3.34 (range 3.17–3.46).

The relation between the resting ratio under control conditions and R_{\min} on the one hand, and the peak ratio in a 100-Hz control tetanus and R_{\max} on the other hand, was almost constant; R_{\min} was $88 \pm 1\%$ ($n = 5$) of the resting ratio and R_{\max} was $136 \pm 2\%$ ($n = 3$) of the peak ratio. We have assumed that the same relation occurred in fibers where R_{\min} and/or R_{\max} were not obtained and thus R_{\min} and R_{\max} were in these cases calculated as 88% of the resting ratio and 136% of the peak ratio, respectively.

K_D was assumed to be 150 nM, which is an approximate mean of values previously given for skeletal muscle cells (Klein et al., 1988; Konishi et al., 1988; Lee et al., 1991). β is the fluorescence at 380-nm excitation of Ca^{2+} free divided by Ca^{2+} bound fura-2. Since R_{\min} almost equals 1, which means that the 360- and 380-nm signals for the Ca^{2+} free dye were almost the same, β has been given the same value as R_{\max} .

Statistics

Data are presented as mean \pm SEM or range; changes are given as mean difference \pm SE of the mean difference. Student's *t* test was used for determination of statistical significance; the significance level was set at 0.05 throughout.

RESULTS

Fig. 1 shows records from two consecutive 100-Hz tetani elicited before fatiguing stimulation in a fiber loaded with fura-2 AM. During the first tetani the fiber was excited at 380 nm and during the second at 360 nm. As can be seen in the second panel, the 360-nm signal was not noticeably affected by stimulation, while the 380-nm signal showed a marked decrease. The third and fourth panels show the 360/380 ratio and the $[\text{Ca}^{2+}]_i$, respectively. A comparison between these two panels reveals that small alterations of the high ratio during stimulation become augmented when converted to $[\text{Ca}^{2+}]_i$, whereas alterations of the resting ratio become less marked.

Under control conditions the calculated resting $[\text{Ca}^{2+}]_i$ of the nine fibers loaded with fura-2 AM was 30 ± 3 nM and the $[\text{Ca}^{2+}]_i$ during 100-Hz stimulation was 967 ± 89 nM.

Original records from a typical fatigue run are shown in Fig. 2. The continuous tension record shows the way in which tension generally declined: an initial rapid fall down to $\sim 90\%$ of the original tension (phase 1), followed by a long period of almost stable tension production (phase 2), and finally a relatively fast tension decline (phase 3). Fig. 2 *B* shows fluorescence ratio and tension records from the times marked above the continuous tension record. The ratio records display a typical pattern; that is, the resting ratio increased monotonically throughout the stimulation period, whereas the peak ratio initially increased, remained high during phase 2, and finally decreased.

The results from all nine fibers loaded with fura-2 AM are summarized in Fig. 3. During phase 1, where tension fell to $\sim 0.9 P_o$, the peak fluorescence ratio increased significantly, by $12.2 \pm 1.9\%$. Then followed the period where both tetanic ratio and tension remained almost stable (phase 2), and finally both parameters declined rapidly (phase 3). In the fatigued state, when tension had been reduced to $35.0 \pm 2.2\%$ of the original, the tetanic ratio had fallen significantly by $14.1 \pm 2.4\%$ of the

original. The resting ratio rose continuously throughout the stimulation period and in the fatigued state it increased significantly, by $27.9 \pm 3.4\%$.

The ratio- $[Ca^{2+}]_i$ relation is steep at ratios near R_{min} and it becomes successively less steep as the ratio increases. Consequently, similar alterations of the peak and resting ratios reflect quantitatively larger changes of peak $[Ca^{2+}]_i$ than of resting $[Ca^{2+}]_i$ (see Fig. 1). This can be made clear by comparing similar changes of the resting and peak ratios in Fig. 3; for example, the increase of the resting ratio developing during the whole fatiguing stimulation period is similar to the increase in the peak ratio occurring during phase 1, while the absolute increase in $[Ca^{2+}]_i$ is about eight times larger in the latter case.

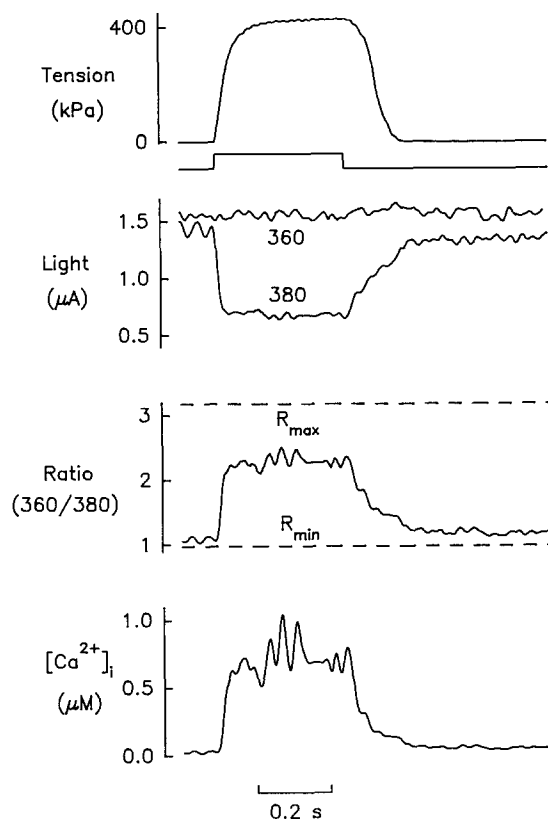


FIGURE 1. Records from two successive 350-ms, 100-Hz tetani elicited under control conditions in a fiber loaded with fura-2 AM. The top panel shows the tension produced; the stimulation period is indicated below the tension record. The second panel shows the fluorescence signals emitted at 505 nm when the fiber was illuminated at 360 and 380 nm, respectively. These fluorescence signals were used to produce the 360/380 ratio signal, which is displayed in the third panel. In this preparation both R_{min} and R_{max} could be established at the end of the experiment, and they are indicated by the dashed lines. In the bottom panel the ratio signal has been converted to $[Ca^{2+}]_i$ using Eq. 1 and the following values: $K_D = 150$ nM; $R_{min} = 0.97$; $R_{max} = 3.17$; $\beta = 3.17$.

Recovery could be followed in eight of the nine fibers loaded with fura-2 AM. After 5 min of recovery tetanic tension as well as peak and resting fluorescence ratios had recovered to within 5% of the original. Recovery of the resting ratio increase in fatigue was followed after the end of fatiguing stimulation in five fibers and it had a half-time of 11.6 ± 2.5 s. In unfatigued tetani we mostly observed a small elevation of the resting ratio after tension had declined to zero (e.g., Fig. 2 B, a). In this case the ratio returned to the resting level with a half-time of 1–2 s, more than five times faster than in the fatigued state.

Fig. 4 shows selected records from a fatigue run of a fiber injected with fura-2. The typical pattern obtained during fatiguing stimulation in loaded fibers was also obtained in the three injected fibers: the tetanic fluorescence ratio initially increased and thereafter declined, while the resting ratio rose continuously.

10-s Pauses

Lännergren and Westerblad (1991) have shown that tetanic tension was markedly larger after a 10-s rest period during phase 3, whereas tension was not changed after a 10-s pause during phase 2. Fig. 5 shows original records from an experiment where

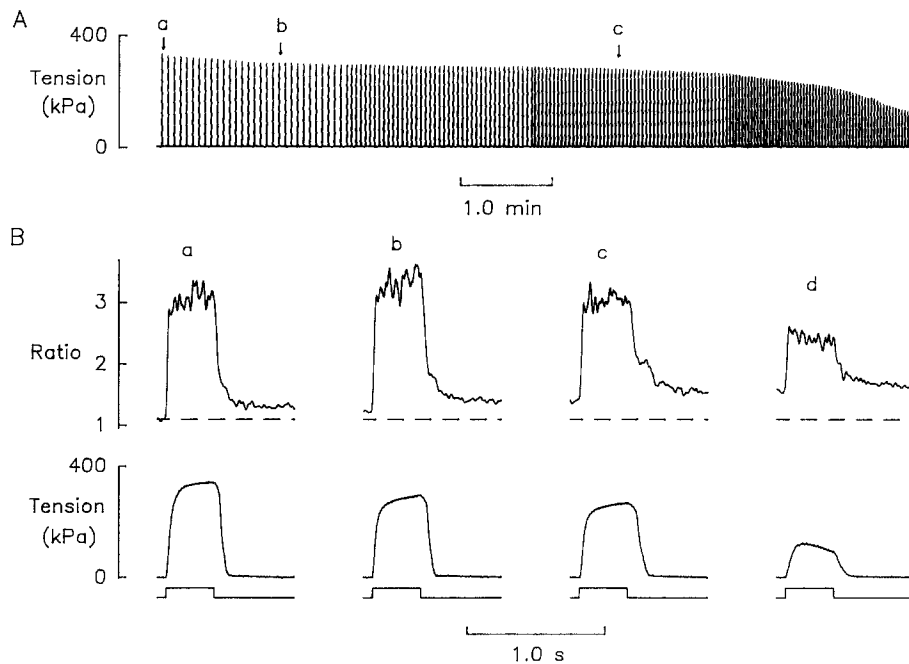


FIGURE 2. Original records of tension and 360/380 ratio signals obtained from a fatigue run of a fiber loaded with fura-2 AM. (A) Continuous tension record; each tetanus appears as a vertical line. (B) Tension and fluorescence ratio signals from times indicated above the continuous tension record in A. The dashed lines represent the resting ratio before fatiguing stimulation. Stimulation periods are shown below the tension records.

the fiber was allowed to rest for ~ 10 s initially every second minute and eventually at shorter intervals. The continuous tension record in A is in agreement with the results of Lännergren and Westerblad (1991): tension was markedly affected by a 10-s pause during phase 3 but not during phase 2. It can also be noted that maximum tension occurred on the third or fourth tetanus after a pause. In B, records from the first tetanus and tetani before and after the last pause are shown in detail. The substantial tension increase, which occurred during the last pause, was accompanied by a marked increase of the tetanic fluorescence ratio; the small additional tension

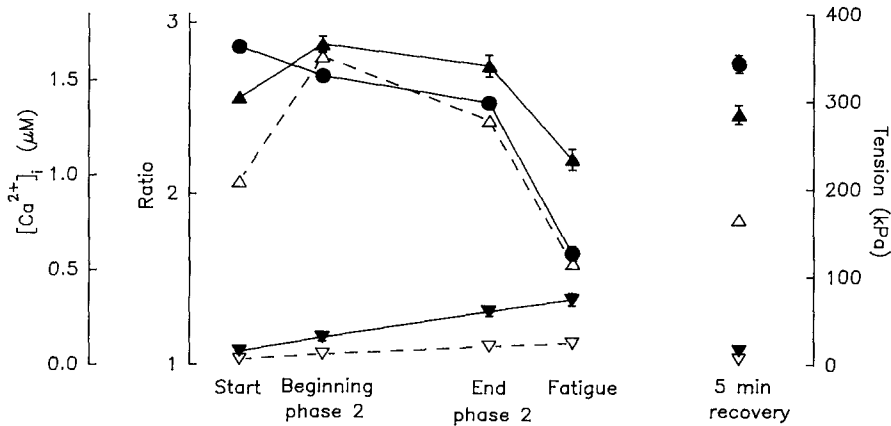


FIGURE 3. Summary of the changes in tension and fluorescence ratios ($[Ca^{2+}]_i$) which occurred during fatiguing stimulation in the nine fibers loaded with fura-2 AM. Measurements were made at the times indicated in Fig. 2A: the first tetanus in a fatigue run = start; 20th tetanus = beginning phase 2; a tetanus produced immediately before the final rapid tension decline = end phase 2; the last fatiguing tetanus = fatigue. Measurements were also performed after 5 min of recovery in eight of the nine fibers. Values of tension and ratios are given as mean \pm SE of the mean difference from control values (error bars in most cases smaller than symbols). ●, Tetanic tension; ▼, resting ratio; ▲, tetanic ratio. The open triangles connected by dashed lines show mean values of $[Ca^{2+}]_i$.

increase over the following two tetani also coincided with a minor increase of the tetanic ratio.

Fig. 6 summarizes the results from five experiments where 10-s pauses were interspersed. Neither the tetanic tension, the resting ratio, nor the tetanic ratio was markedly changed during the first pause, which occurred after 2 min of stimulation. During the last pause, on the other hand, all three parameters were significantly changed: tetanic tension directly after the pause was substantially larger and it increased further over the next two or three tetani, resulting in an increase from $\sim 0.4 P_0$ before the pause to a maximum of $\sim 0.7 P_0$ after the pause; the peak ratio

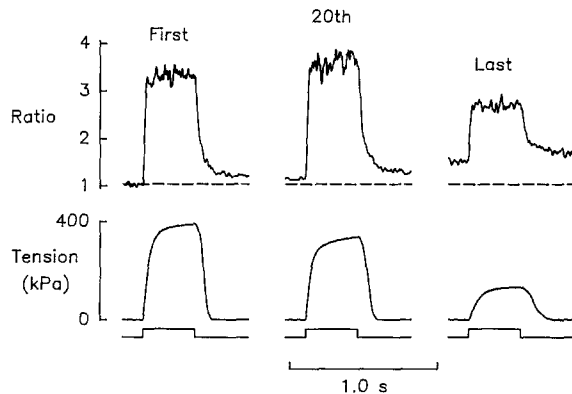


FIGURE 4. Selected tetani from a fatigue run produced in a fiber injected with fura-2; records of the fluorescence ratio and tension obtained from the first, the 20th, and the last tetanus of the fatigue run. The dashed line represents the resting ratio before fatiguing stimulation. The stimulation period is shown below each tension record.

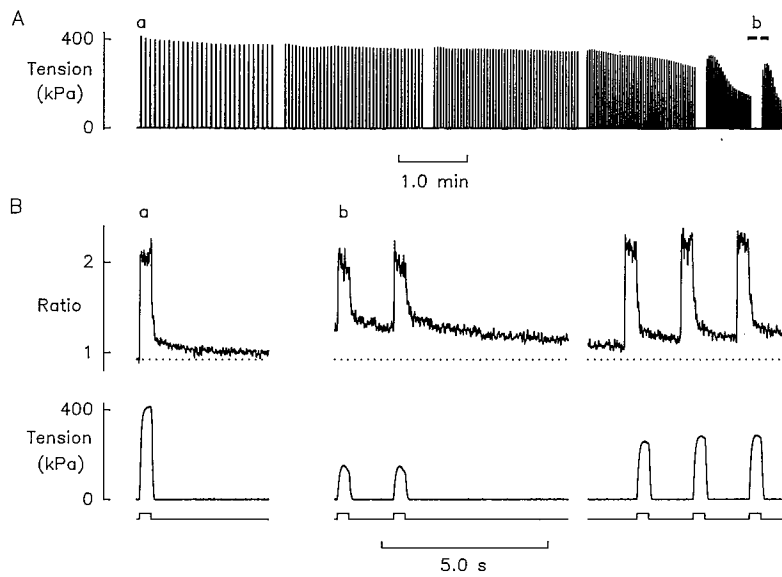


FIGURE 5. A fatigue run with 10-s rest periods. (A) Continuous tension record. (B) Records of tension and fluorescence ratio from the first tetanus (a) and from the last 10-s pause (b). Observe the marked recovery of both tension and ratio that occurred during the pause. The dotted lines represent the resting ratio in control. Stimulation periods are indicated below tension records.

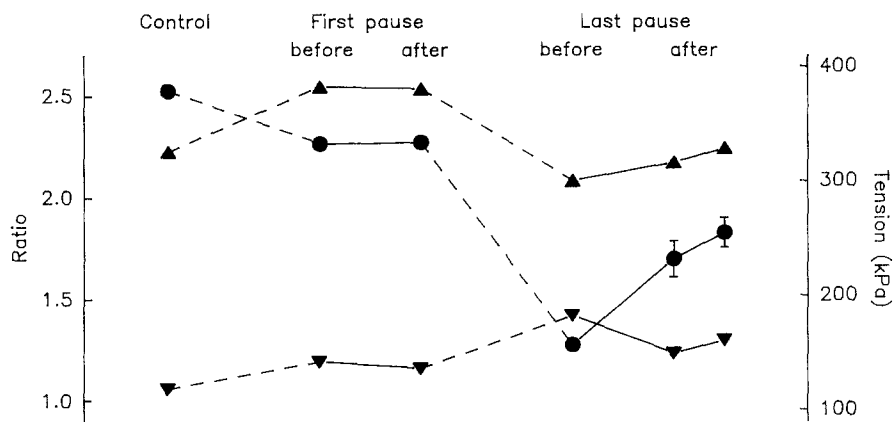


FIGURE 6. Summary of the results from 10-s pause experiments; values obtained from five fibers. Values are given as mean \pm SE of the mean difference between measurements before and after each pause. ●, Tetanic tension; ▼, resting fluorescence ratio; ▲, tetanic fluorescence ratio. Values after the last pause were obtained from the first tetanus after the pause and from the tetanus during which maximum tension was produced (second to fourth tetanus after the pause).

was $3.2 \pm 1.1\%$ higher after the pause and it also increased further over the following two or three tetani; the resting ratio increase, which was present at this stage of fatigue, was approximately halved during the pause.

Caffeine Experiments

Caffeine has frequently been used to activate the myofilaments of muscle fibers. This drug has been shown to have several modes of action and these have recently been discussed by Fryer and Neering (1989). The most important effect of caffeine in the

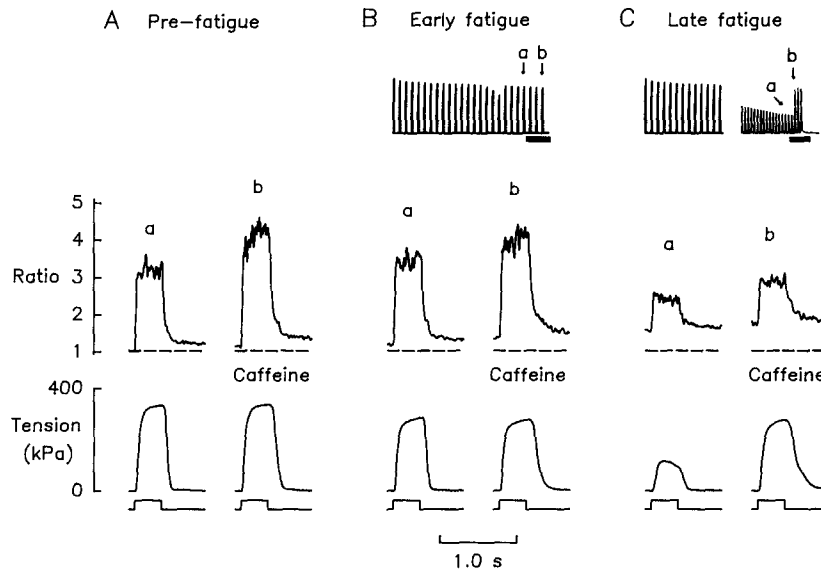


FIGURE 7. Application of 10 mM caffeine in control (A) and during two successive fatigue runs (B and C). Bars below tension records in the top panels indicate caffeine exposure during fatiguing stimulation; caffeine was applied after 22 fatiguing tetani (B) and when tetanic tension was depressed to $0.36 P_0$ by 187 tetani (C). Fluorescence ratio and tension records from tetani elicited before application of caffeine (a) and in the presence of caffeine (b) are shown in the two lower panels. Dashed lines represent resting ratio in control; stimulation periods are displayed below tension records. Note that the tetanic ratio increase induced by caffeine in late fatigue was accompanied by a substantially enhanced tension production, whereas tension was not markedly affected by the increased ratios in the other two states.

present context is its ability to facilitate SR Ca^{2+} channel opening (Rousseau, LaDine, Liu, and Meissner, 1988), and we have combined tetanic stimulation and application of 10 mM caffeine to assess the maximum tension-generating capacity. It may be noted that while 10 mM caffeine causes a maximum contracture in frog muscles (Kanaya, Takauji, and Nagai, 1983), it does not affect resting tension in the present mammalian preparation (Lännergren and Westerblad, 1991).

Most fibers were exposed to caffeine under control conditions and after ~ 20 fatiguing tetani and/or at the end of fatiguing stimulation. Fig. 7 shows original records from a fiber that was exposed to caffeine at all these three occasions. In the

prefatigue state caffeine application gave a marked increase in the tetanic fluorescence ratio, whereas the tension remained virtually unchanged. Caffeine was then applied early during fatiguing stimulation and this also resulted in a marked increase in the tetanic ratio and unchanged tension production. (The reduced tetanic tensions just before caffeine application were due to a reduction of the stimulation frequency; see below.) The fiber was finally exposed to caffeine at the end of a complete fatigue run. At this stage both the peak ratio and the tetanic tension increased substantially.

The results from all caffeine experiments are summarized in Fig. 8. Under control conditions, an increase of the tetanic fluorescence ratio of $\sim 20\%$ resulted in a mean tension increase of only 3%. Thus, the tension produced in a standard 100-Hz tetanus appears to be very close to the maximum tension that can be obtained (see also Fig. 9A). The highest observed tetanic ratios were obtained when fibers were exposed to caffeine early during fatiguing stimulation (in these cases the ratios were

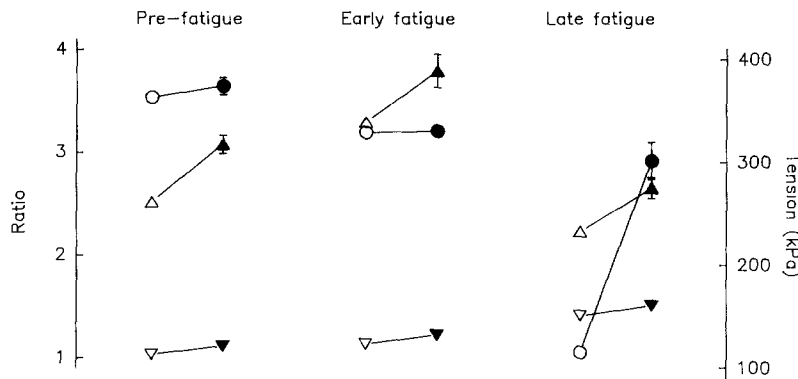


FIGURE 8. Summary of the results from caffeine application under control conditions (pre-fatigue; $n = 6$), after ~ 20 fatiguing tetani (early fatigue; $n = 3$), and in the fatigued state (late fatigue; $n = 4$). \circ, \bullet , Tension; $\nabla, \blacktriangledown$, resting fluorescence ratio; Δ, \blacktriangle , tetanic fluorescence ratio; open and filled symbols represent values obtained before and during caffeine exposure, respectively. Values are given as mean \pm SE of the mean difference between measurements before and during caffeine exposure.

close to the R_{\max}), and despite these high ratios the mean tension was only 93% of the original. At the end of the standard fatiguing stimulation period, however, the increased peak ratio in the presence of caffeine was accompanied by a tension increase from ~ 0.3 to $0.8 P_o$.

In all three states application of caffeine resulted in only a small increase in the resting fluorescence ratio and generally no observable increase in the resting tension.

Ca²⁺ Sensitivity of the Myofilaments

To study the relation between $[Ca^{2+}]_i$ and tension under control conditions, tetani at various stimulation frequencies were produced before and after fatiguing stimulation. The fluorescence ratios obtained in this way were then compared with ratios obtained when tension declined during fatiguing stimulation. The diagram in Fig. 9A shows a typical example of such a comparison. At tension levels lower than $\sim 0.8 P_o$, there was

an almost linear relation between ratio and tension in both control and fatigue and the slope of this relation was approximately the same in the two cases. The ratio for a given tension was always markedly higher during fatiguing stimulation, which indicates that the myofilaments were less sensitive to Ca^{2+} . The original records in Fig. 9 *B* illustrate the reduced Ca^{2+} sensitivity during fatiguing stimulation. A 50-Hz control tetanus is here compared with tetani elicited at the end of phase 2 and during phase 3. The tensions in the first two tetani are approximately the same, while the tetanic ratio is much higher during fatiguing stimulation than in the control. The peak ratio of the tetanus produced during phase 3 is similar to the peak ratio of the control, but the accompanying tension is markedly smaller. Fura-2 ratios obtained in

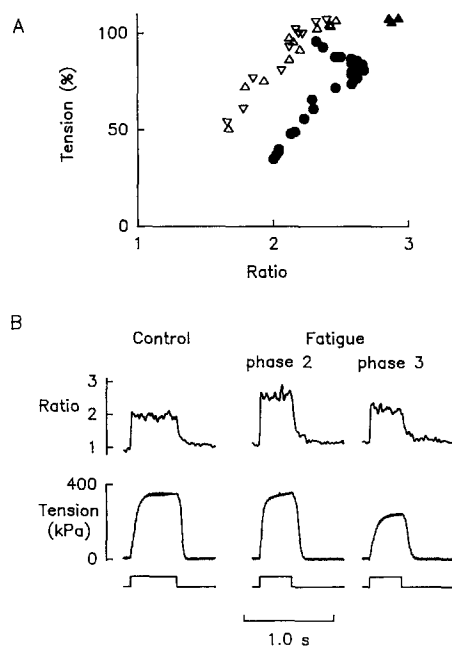


FIGURE 9. Comparison of the fluorescence ratio-tension relation in control and during fatiguing stimulation. (A) Ratio-tension values obtained from one fiber before (∇), after (Δ), and during (\bullet) fatiguing stimulation; measurements performed under control conditions in the presence of caffeine are also shown (\blacktriangle). Observe that the ratio for a given tension was markedly higher during fatiguing stimulation, which indicates a reduced Ca^{2+} sensitivity. (B) Original records selected to illustrate the reduced Ca^{2+} sensitivity during fatiguing stimulation; a 50-Hz control tetanus is shown together with two tetani produced during fatiguing stimulation. Stimulation periods shown below tension records.

control and during fatiguing stimulation could be compared in seven fibers, and at $0.5 P_o$ the ratio was found to be 0.39 ± 0.02 higher during fatiguing stimulation.

In studies of skinned muscle fibers the Ca^{2+} sensitivity is often described by quoting the $[Ca^{2+}]$ required for half-maximal activation (Ca_{50} ; e.g., Godt and Nosek, 1989). If the tetanic tension in the presence of caffeine is assumed to represent the maximum Ca^{2+} -activated tension of our fibers, the Ca_{50} would be the $[Ca^{2+}]$, giving $0.5 P_o$ under control conditions and $0.4 P_o$ in late fatigue (see above). Values of Ca_{50} obtained in this way in seven fibers were $61 \pm 13\%$ higher in late fatigue than in control (mean 529 vs. 329 nM).

In a few experiments we tried to assess the Ca^{2+} sensitivity early during fatiguing stimulation by reducing the stimulation frequency. The continuous tension record in Fig. 7 *B* shows an example of one such experiment; the tension reduction before caffeine application is caused by reduced stimulation frequencies. Fig. 10 shows

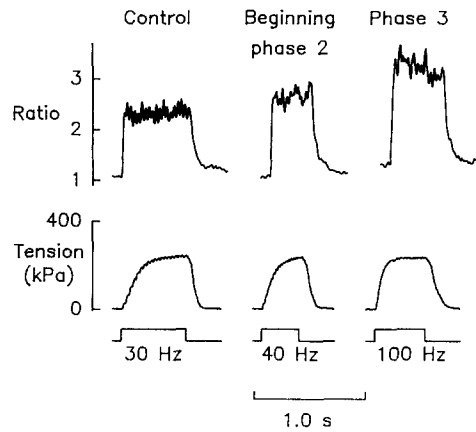


FIGURE 10. Original records illustrating the gradual reduction of Ca^{2+} sensitivity during fatiguing stimulation. Stimulation periods and frequencies are indicated below tension records.

records from another of these experiments. A 30-Hz control tetanus is here compared with a 40-Hz tetanus elicited after ~ 20 fatiguing tetani and a 100-Hz tetanus from the final part of fatiguing stimulation. The tension produced is almost the same in these three tetani, whereas the peak fluorescence ratio is low in the control, somewhat higher in early fatigue, and highest in late fatigue. In three experiments the ratio giving $0.7 P_0$ was 0.24 ± 0.06 higher after 20 fatiguing tetani than in control, and this represents an increase in $[\text{Ca}^{2+}]_i$ of $\sim 30\%$.

During phase 3 the tetanic tension was markedly higher after a 10-s rest period than before (see above). To investigate if a partial recovery of the Ca^{2+} sensitivity contributed to this tension rise, the increase of fluorescence ratio vs. tension associated with a 10-s pause was compared with the line obtained from the ratio-tension relation during fatiguing stimulation. A summary of the results of this comparison is shown in Fig. 11. The data points represent ratio vs. tension values obtained from tetani before and after the last pause, and the dashed and dotted lines represent the mean relations between ratio and tension in control and during

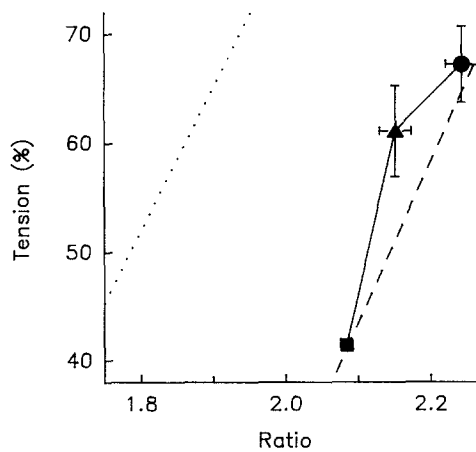


FIGURE 11. Fluorescence ratio-tension relation before and after the last 10-s rest period. Values represent mean \pm SE of the mean difference between measurements of ratio and tension before (■) and after the pause ($n = 5$); the two values after the pause refer to the first tetanus after the pause (▲) and the tetanus with the maximum tension (●) (see Fig. 5). The dashed and dotted lines indicate the mean ratio-tension relation during fatiguing stimulation and under control conditions, respectively.

fatiguing stimulation, respectively. The tetanic ratio immediately after the pause is slightly lower than that obtained for the same tension during fatiguing stimulation, which suggests that a small increase in the Ca^{2+} sensitivity has occurred during the pause, but the value obtained still lies much closer to the fatigue line than to the control line. The maximum tension after the pause was produced in the second to fourth tetani and at this stage the ratio vs. tension value lies very close to the fatigue line. Thus, the small increase of the Ca^{2+} sensitivity associated with a pause was only transient.

Cyanide Experiments

Fatigue under anaerobic conditions was studied in three fibers; an additional fatigue run was produced in the presence of 2 mM cyanide. The fatigue resistance in the

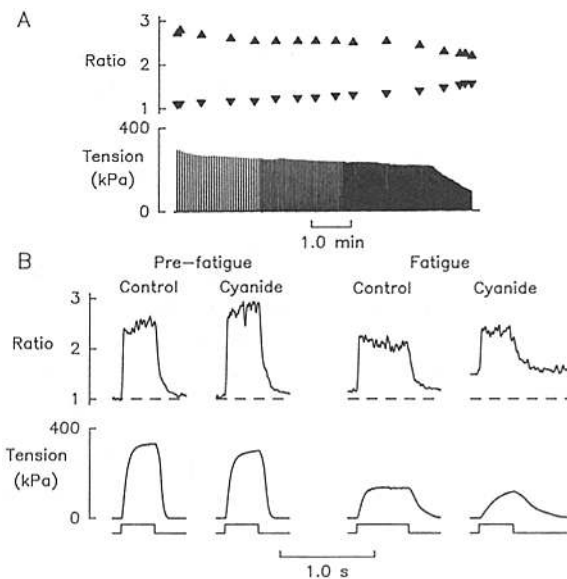


FIGURE 12. Tension and fluorescence ratio signals obtained from a fiber exposed to 2 mM cyanide. (A) A plot of resting and tetanic ratios during fatiguing stimulation and the original tension record. Observe that there was no significant early increase of the peak ratio. (B) Records selected to illustrate that exposure of rested fibers (prefatigue) to cyanide caused a small tension reduction accompanied by an increased tetanic ratio, and the resting ratio in the fatigued state was substantially higher when the fiber had been fatigued in the presence of cyanide.

presence of cyanide was markedly reduced in all three fibers: the duty cycle (= stimulation time/tetanic interval) at the end of the stimulation period was about one-third of that in the standard solution. Except for the reduced fatigue resistance, three prominent differences between control and anaerobic conditions were observed and these are illustrated in Fig. 12. First, after a rest period of ~ 10 min in cyanide the tetanic tension declined to $\sim 0.9 P_0$ and the tetanic fluorescence ratio increased by 5–10%. Second, there was no significant increase of the tetanic ratio during the first part of fatiguing stimulation. Third, the increase in the resting ratio in fatigue was markedly larger in the cyanide solution than under control conditions (mean increase 0.47 and 0.11, respectively). Also, the recovery of the resting ratio after fatiguing stimulation was very slow in cyanide and it became markedly faster when the fibers were returned to the standard solution.

DISCUSSION

We have reported here the first combined measurements of $[Ca^{2+}]_i$ and tension in intact single fibers from mammalian muscle. The values given for $[Ca^{2+}]_i$ are subject to some uncertainty because the intracellular K_D for fura-2 was not established, while R_{max} and β were only obtained in three of nine fibers. In this context it should be noted that if the K_D and/or β which we have used are proven to be wrong, the estimate of $[Ca^{2+}]_i$ is simply changed by the same factor as the change in K_D and/or β . In addition, the relative changes in $[Ca^{2+}]_i$ which we report during fatiguing stimulation are not affected by the value of K_D or β . Our mean value of $[Ca^{2+}]_i$ at half-maximal activation (Ca_{50}) under control conditions (329 nM) is lower than the Ca_{50} often reported for skinned fibers (e.g., Brandt, Cox, Kawai, and Robinson, 1982; Godt and Nosek, 1989), but values similar to ours have also been found (Millar and Homsher, 1990).

We have used a single fiber preparation, which is advantageous in that recordings of tension can be directly compared with measurements of other cell properties, such as $[Ca^{2+}]_i$. It must, however, be remembered that the present experimental conditions differ in some ways from the *in vivo* situation; for example, the composition of the extracellular fluid will change during fatiguing stimulation *in vivo*, whereas it was kept constant in the present experiments. Thus, while single fiber preparations are useful to elucidate basic cellular mechanisms of fatigue, the results from single fibers may not always be directly transferable to the *in vivo* situation.

The present measurements of $[Ca^{2+}]_i$ during fatiguing stimulation have revealed three main features: (a) an early increase of the tetanic $[Ca^{2+}]_i$ associated with a small tension depression; (b) a late tension reduction accompanied by declining tetanic $[Ca^{2+}]_i$; and (c) an increase of the resting $[Ca^{2+}]_i$. These three features will be discussed in turn.

Early Changes of Tetanic $[Ca^{2+}]_i$ and Tension

During the initial 20 tetani of fatiguing stimulation the tetanic fluorescence ratio increased by $\sim 12\%$ (corresponding to an almost doubling of $[Ca^{2+}]_i$), while tetanic tension fell by $\sim 10\%$. The early increase of the tetanic $[Ca^{2+}]_i$ might be due either to increased Ca^{2+} release from the SR or to reduced myoplasmic Ca^{2+} buffering capacity. We consider it unlikely that the Ca^{2+} release would increase during fatiguing stimulation and we therefore believe reduced Ca^{2+} buffering to be a more likely explanation. The increase in tetanic $[Ca^{2+}]_i$ is accompanied by elevated resting $[Ca^{2+}]_i$. Although the cause of the elevation of resting $[Ca^{2+}]_i$ is uncertain (see below), it must lead to an increased filling of myoplasmic Ca^{2+} buffers and consequently contribute to the increase in tetanic $[Ca^{2+}]_i$.

The early tension decline could not be overcome by application of 10 mM caffeine, which increased the tetanic $[Ca^{2+}]_i$ further and it thus appears to be caused by a reduction of the maximum tension-generating capacity. This reduction, as well as the increase in tetanic $[Ca^{2+}]_i$, may be explained by metabolic changes that occur in fatigue. Oxidative metabolism is activated relatively slowly in skeletal muscle (Hill, 1965; Elzinga, Langewouters, Westerhof, and Wiechmann, 1984), so that the initial increase in energy consumption could come either from anaerobic glycolysis or from

net breakdown of phosphocreatine. Lactic acid may accumulate as a consequence of increased anaerobic glycolysis and this will produce an intracellular acidosis, which can reduce maximum Ca^{2+} -activated tension (Fabiato and Fabiato, 1978; Edman and Lou, 1990). Alternatively, if phosphocreatine breakdown occurs, inorganic phosphate ions (P_i) will accumulate and this may also reduce maximum Ca^{2+} -activated tension (Cooke and Pate, 1985; Kentish, 1986). Further, both an acidosis and an accumulation of P_i may explain the increased tetanic $[Ca^{2+}]_i$. Protons are known to compete with Ca^{2+} for binding sites on troponin (Blanchard and Solaro, 1984), so that an acidosis will reduce the amount of Ca^{2+} buffered by troponin. Although P_i does not have any direct effect on the binding of Ca^{2+} by troponin C (Kentish and Palmer, 1989), it might still reduce Ca^{2+} binding by troponin due to the observed cooperativity between crossbridge attachment and Ca^{2+} binding by troponin (Bremel and Weber, 1972; Brandt et al., 1982). The existence of such a mechanism suggests that any agent that reduces tension, such as P_i , will also reduce Ca^{2+} binding by troponin and consequently the Ca^{2+} buffering.

After being exposed to cyanide for ~ 10 min, fibers displayed an increase in the tetanic $[Ca^{2+}]_i$ and a tension reduction similar to those observed in early fatigue. When the mitochondria are blocked by cyanide, it is possible that a small excess of lactic acid will be produced although the energy demand is low at rest. Alternatively, in cyanide-exposed fibers the small energy consumption at rest may be met by phosphocreatine breakdown. Thus, it appears that the alteration causing reduced tetanic tension and increased tetanic $[Ca^{2+}]_i$ in early fatigue under control conditions may occur already at rest under anaerobic conditions.

Late Changes in Tetanic $[Ca^{2+}]_i$ and Tension

After the long period of almost constant tetanic $[Ca^{2+}]_i$ and tension (phase 2), both these parameters started to fall relatively fast (phase 3). The tension decline during phase 3 appears to be mostly due to impaired activation of crossbridges and not to reduced maximum tension-generating capacity, because caffeine applied at this stage can bring tension back up to $\sim 0.8 P_0$. The impaired activation of the myofilaments seems to be caused by a combination of (a) reduced Ca^{2+} sensitivity and (b) reduced Ca^{2+} release from the SR.

(a) In addition to their depressive effect on the crossbridge force-generating capacity, an increase in P_i and a reduction of the intracellular pH (pH_i) have been found to reduce the Ca^{2+} sensitivity of the myofilaments (e.g., Godt and Nosek, 1989). About half of the Ca^{2+} sensitivity reduction appeared to occur already during the first 20 fatiguing tetani. As judged from caffeine tetani, the reduction of the maximum tension-generating capacity followed a similar time course: the maximum tension was reduced by $\sim 10\%$ after 20 tetani and by 20% at the end of fatiguing stimulation (see also Fig. 7 of Lännergren and Westerblad, 1991). Thus, we suggest that the reduced Ca^{2+} sensitivity and depressed maximum tension have the same underlying cause.

In a recent study Godt and Nosek (1989) bathed skinned fibers in solutions mimicking the intracellular milieu that is thought to occur in fatigue. They obtained a reduction of maximum Ca^{2+} -activated tension similar to ours in a solution mimicking fatigue without change of pH (their test solution 1), while the tension

reduction was greater than ours in a solution where the pH was reduced from 7.0 to 6.65 (solution 2). The Ca_{50} was markedly higher in their preparation than in ours (see above), but a comparison between relative changes in Ca_{50} may still be performed. We report an increase in Ca_{50} of ~60% in late fatigue, which is similar to the increase in the solution with reduced pH (solution 2) of Godt and Nosek, whereas the increase is smaller in their solution 1. Based on this comparison, we suggest that the pH_i reduction in our fatigued fibers is between 0 and 0.35 pH units and that the other changes in the intracellular milieu are similar to those in the fatigue solutions of Godt and Nosek (1989).

(b) Impaired function at many sites may contribute to the reduced tetanic $[Ca^{2+}]_i$ in fatigue; for example (i) failing propagation of action potentials into the t-tubular system, (ii) inhibition of SR Ca^{2+} channel opening, and (iii) reduced SR Ca^{2+} content. The marked recovery of tetanic $[Ca^{2+}]_i$ observed after a 10-s rest period in late fatigue may then be explained by reversal of any of these mechanisms.

(i) Impaired signal transmission in the t-tubules may be caused by accumulation of potassium ions and/or depletion of sodium ions, and these ionic changes may be reversed during a 10-s pause. The gradual increase in tension and tetanic $[Ca^{2+}]_i$ over two to four tetani after a pause can be taken as an argument against this mechanism, since the ionic composition in the t-tubules would be most favorable in the first tetanus after the pause before getting worse again.

(ii) The opening of the SR Ca^{2+} release channels is inhibited by changes that might occur in fatigue; for example, reduced concentration of adenine nucleotides (e.g., Meissner, Darling, and Eveleth, 1986), increased Mg^{2+} concentration (e.g., Lamb and Stephenson, 1991), and reduced pH (e.g., Ma, Fill, Knudson, Campbell, and Coronado, 1988).

(iii) The function of the SR Ca^{2+} pumps is likely to be impaired in fatigue due to acidosis (MacLennan, 1970) and/or reduced affinity for ATP hydrolysis (Dawson, Gadian, and Wilkie, 1980). Impaired SR Ca^{2+} pumping in fatigue is suggested by the much slower decline of the resting $[Ca^{2+}]_i$ after the end of fatiguing stimulation than after a single tetanus. A reduced Ca^{2+} pumping rate may result in the SR becoming depleted of Ca^{2+} in late fatigue, and a 10-s rest period may allow it to be at least partially refilled. The tetanic $[Ca^{2+}]_i$ in the presence of caffeine was higher under control conditions than in the fatigued state, which might be taken as evidence of a reduced SR Ca^{2+} content in fatigue. This statement, of course, is based on the assumption that application of 10 mM caffeine causes maximum Ca^{2+} release. In addition to direct effects of SR Ca^{2+} depletion, the $[Ca^{2+}]_i$ in the SR has recently been found to regulate the function of the SR Ca^{2+} release channels (Ikemoto, Ronjat, Mészáros, and Koshita, 1989).

Increase in the Resting $[Ca^{2+}]_i$

The resting $[Ca^{2+}]_i$ increased monotonically throughout fatiguing stimulation, and in the fatigued state it was about four times higher than in control. In the fatigued state the resting fluorescence ratio declined with a half-time of ~12 s, which is ~10 times slower than the decline under control conditions. Thus, the increase in resting $[Ca^{2+}]_i$ appears not to be caused by a simple addition of the small “tails” of $[Ca^{2+}]_i$ which follow after each tetanus.

Although highly significant, the elevation of the resting $[Ca^{2+}]_i$ in the present preparation is quantitatively much smaller than that previously observed in *Xenopus* fibers fatigued in a similar way (Lee et al., 1991). Another difference between the present preparation and *Xenopus* fibers was the markedly slower recovery of the resting $[Ca^{2+}]_i$ after fatiguing stimulation in *Xenopus*; in the present experiments recovery was completed after ~ 1 min, whereas up to 30 min was needed in *Xenopus* fibers. In this study the increase in the resting $[Ca^{2+}]_i$ became much larger when fibers were fatigued in the presence of cyanide, and the recovery was also much slower in this case. When the high energy demand induced by fatiguing stimulation has to be met exclusively by anaerobic metabolism, it is likely that fatigue will be associated with a more severe intracellular acidosis and the increased resting $[Ca^{2+}]_i$ thus seems to be related to reduced pH_i . A direct link between intracellular acidosis and elevated resting $[Ca^{2+}]_i$ could not, however, be established in *Xenopus* fibers; rested fibers acidified by exposure to CO_2 only displayed a minor increase in the resting $[Ca^{2+}]_i$ (Lee et al., 1991).

Conclusions

We suggest that the tension reduction during fatigue produced by repeated tetani is caused by a combination of reduced maximum tension-generating capacity, reduced Ca^{2+} sensitivity of the myofilaments, and reduced Ca^{2+} release from the SR. A large fraction of the reduction of maximum tension becomes manifested already after a few tetani. The relatively fast tension decline in late fatigue appears to be caused by a combination of reduced myofibrillar Ca^{2+} sensitivity, which develops during phases 1 and 2, and reduced Ca^{2+} release from the SR. Thus, in our preparation reduced maximum tension is of greatest importance for the tension reduction observed during phases 1 and 2, while reduced Ca^{2+} sensitivity and reduced Ca^{2+} release become quantitatively more important during phase 3.

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