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CHANGES OF INTRACELLULAR pH DUE TO REPETITIVE STIMULATION OF SINGLE FIBRES FROM MOUSE SKELETAL MUSCLE

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SUMMARY

1. The performance of skeletal muscle during repetitive stimulation may be limited by the development of an intracellular acidosis due to lactic acid accumulation. To study this, we have measured the intracellular pH (pH_i) with the fluorescent indicator BCECF (2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein) during fatigue produced by repeated, short tetani in intact, single fibres isolated from the mouse flexor brevis muscle.

2. The pH_i at rest was $7{\cdot}33 \pm 0{\cdot}02$ (mean ± S.E.M., $n = 29$, 22 °C). During fatiguing stimulation pH_i initially went alkaline by about 0·03 units (maximum alkalinization after about ten tetani). Thereafter pH_i declined slowly and at the end of fatiguing stimulation (tetanic tension reduced to 30% of the original; $0{\cdot}3 P_o$), pH_i was only $0{\cdot}063 \pm 0{\cdot}011$ units ($n = 14$) more acid than in control.

3. We considered three possible causes of acidosis being so small in fatigue: (i) a high oxidative capacity so that fatigue occurs without marked production of lactic acid; (ii) an effective transport of H^+ or H^+ equivalents out of the fibres; (iii) a high intracellular buffer power.

4. The oxidative metabolism was inhibited by 2 mM-cyanide in three fibres. After being exposed to cyanide for 5 min without stimulation, the tetanic tension was reduced to about $0{\cdot}9 P_o$ and pH_i was alkaline by about 0·1 units. The fibres fatigued faster in cyanide and the pH_i decline in fatigue was more than twice as large as that under control conditions.

5. Inhibition of Na^+–H^+ exchange with amiloride resulted in a slow acidification of rested fibres; resting pH_i was not affected by either inhibition of HCO_3^-–Cl^- exchange with DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid) or inhibition of the lactate transporter with cinnamate.

6. Fibres fatigued in cinnamate displayed a markedly larger acidification (~ 0·4 pH units) and tension fell more rapidly than under control conditions; inhibition of Na^+–H^+ and HCO_3^-–Cl^- exchange did not have any significant effect on fatigue.

7. The intracellular buffer power, assessed by exposing fibres to the weak base trimethylamine, was about 15 mM (pH unit)$^{-1}$ in a HEPES-buffered solution (non-CO_2 or intrinsic buffer power) and about 33 mM (pH unit)$^{-1}$ in a bicarbonate-buffered solution. Somewhat higher values of the intrinsic buffer power was obtained from changes of the partial pressure of CO_2 (P_{CO_2}) of the bath solution. Application of

lactate or butyrate frequently gave an infinite buffer power, which indicates that powerful pH-regulating mechanisms operate in these cases.

8. The dependence of tetanic tension on pH_i of rested fibres was studied by changing the P_{CO_2} of the bath solution. The tension was found to change with a slope of about $0{\cdot}33 P_0$ (pH unit)$^{-1}$).

9. The slowing of relaxation which occurs in fatigue is often attributed to acidosis. In agreement with this, acidification of rested fibres resulted in a marked slowing of relaxation.

10. Relaxation was slower after about ten fatiguing tetani, i.e. at a time when pH_i was increased. Thereafter relaxation slowed further and the greatest slowing was observed in the most acidified fibres. Thus, the slowing of relaxation in fatigue was due to both pH-independent and pH-dependent processes.

11. In conclusion, the acidosis in fatigue was small and this was mainly because of an effective extrusion of H^+ ions by the lactate transporter. Thus neither the tension decline nor the slowing of relaxation in fatigue could be explained by an intracellular acidification. If the lactate transporter was inhibited, the acidification during fatiguing stimulation became larger and probably contributed to fatigue.

INTRODUCTION

The function of skeletal muscle becomes impaired during prolonged activation. This phenomenon of fatigue is generally manifested as (i) reduced isometric tension production, (ii) slowed relaxation, and (iii) reduced shortening velocity. Acidosis due to accumulation of lactic acid has long been suggested to be of major importance for the development of fatigue (e.g. Hill & Kupalov, 1929) and many studies, employing a variety of experimental conditions, have shown a marked acidosis ($\sim 0{\cdot}5$ pH units) in fatigue (for review see Westerblad, Lee, Lännergren & Allen, 1991). It is easy to explain how a reduction of the intracellular pH (pH_i) could contribute to fatigue since acidosis is known to impair several intracellular processes: it reduces both the maximum Ca^{2+}-activated tension and the Ca^{2+} sensitivity of the myofilament (Fabiato & Fabiato, 1978); it slows cross-bridge cycling (Edman & Mattiazzi, 1981); it affects the function of the Ca^{2+} release channels of the sarcoplasmic reticulum (SR) (Ma, Fill, Knudson, Campbell & Coronado, 1988); it impairs the function of the SR Ca^{2+} pumps (MacLennan, 1970).

Assuming that acidosis contributes to the tension decline in fatigue, this means that intracellular pH-buffering and pH-regulating mechanisms will have an important role in minimizing the development of fatigue. In mouse muscles at rest, the main pH-regulating process is Na^+–H^+ exchange, which at 37 °C accounts for about 80% of the H^+ efflux, while Cl^-–HCO_3^- exchange accounts for the remaining 20% (Aickin & Thomas, 1977*b*). When an excess of lactic acid is produced, such as during fatiguing stimulation, another pH-regulating mechanism becomes important: a carrier-mediated co-transport of lactate ions and proton equivalents (Juel, 1988; Mason & Thomas, 1988)

In the present study we have employed single muscle fibres dissected from a mouse foot muscle and fatigue was produced by repeated short tetani (Lännergren & Westerblad, 1991). The fluorescent pH indicator BCECF (2′,7′-bis(carboxyethyl)-

5(6)carboxyfluorescein) was used to measure pH_i. The aim of the study was to answer the following two questions: firstly how much does pH_i change during fatigue and what contribution does this make to the tension decline and the slowing of relaxation? Secondly, how do the various pH-regulating systems affect the pH_i changes observed during fatiguing stimulation?

An abstract of part of this study has been published (Allen & Westerblad, 1991).

METHODS

Fibre dissection, mounting and fatiguing stimulation has been described in detail previously (Lännergren & Westerblad, 1991; Westerblad & Allen, 1991). Briefly, male mice were killed by rapid neck disarticulation and single muscle fibres were dissected from the flexor brevis muscle of the foot. The fibre was mounted between an Akers AE 801 force transducer and an adjustable holder, which allowed the fibre to be stretched to the length giving maximum isometric tension. Fatigue was produced by repeated 350 ms stimulation trains at 100 Hz; the periods of repeated tetani will be referred to as fatigue runs. Tetani were initially given every 4 s and the tetanic interval was reduced every second minute. Fatiguing stimulation continued until tension was reduced to about 30% of the original (throughout the paper, force will be expressed relative to P_o, that is the tension produced in 100 Hz tetani elicited in the rested state). A short pause ($\sim$ 10 s) was given each time the tetanic interval was changed and sometimes also towards the end of fatigue runs.

During fatigue runs some fibres started to show a tension decline during tetani ('sag'). When this occurred, the stimulation frequency was reduced to 70 Hz which mostly reduced the sag and sometimes also led to a small increase of the peak tension during tetanus (e.g. Fig. 3); towards the end of fatiguing stimulation a few fibres displayed a marked sag even at 70 Hz stimulation and in these fibres the stimulation frequency was reduced further. It has previously been shown that fibres generally recover fully after a fatigue run (Lännergren & Westerblad 1991) and hence more than one fatigue run can be produced in each fibre. In the present study we have frequently produced two fatigue runs, one in standard solution and one in the presence of either lactate or inhibitors of various pH-regulating systems. We varied the sequence so that in some experiments fatigue was first produced in the control solution and then in the altered solution, whereas in other experiments the altered solution was used in the first fatigue run. All fibres fatigued in the presence of the lactate transport inhibitor cinnamate (α-cyano-4-hydroxycinnamic acid) eventually went into a contracture and thereafter did not respond to electrical stimulation. Thus with this drug control fatigue runs were always produced first. The cinnamate-induced contractures generally occurred well after the end of fatigue runs, i.e. at a time when tetanic tension had partly or fully recovered.

Measurement of relaxation parameters

Relaxation at the end of a tetanus can usually be divided into two phases: a relatively slow linear force decline, which is homogeneous along the fibre, followed by a faster exponential decline associated with intrafibre movements (Huxley & Simmons, 1970; Curtin & Edman, 1989). We have measured the slope and the duration of the initial linear phase (for details see Westerblad & Lännergren, 1991) and compared changes occurring during fatiguing stimulation with changes in response to acidification of rested fibres. Values of the two relaxation parameters are presented as a percentage of the values obtained in the first fatiguing tetanus or in a tetanus elicited before acidification.

Solutions

Fibres were dissected in a solution with the following composition (mM): NaCl, 136·5; KCl, 5·0; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 0·4; $NaHCO_3$, 11·9. After mounting in the experimental trough and loading with BCECF, fibres were normally superfused with the following solution (mM): NaCl, 121; KCl, 5·0; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 0·4; $NaHCO_3$, 24·0; glucose, 5·5; this solution was bubbled with either 5 or 30% CO_2 which gave an extracellular pH (pH_o), measured close to the fibre, of 7·30 and 6·55, respectively. In some experiments, where the pH_i response to exposure to weak acid or base was studied, fibres were superfused with a HEPES-buffered solution

(mM): NaCl, 133·5; KCl, 4·0; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 1·2; HEPES, 10·0; glucose, 5·5; this solution was titrated with NaOH to pH 7·3. About 0·2 % fetal calf serum was added to all the above solutions. All experiments were performed at room temperature (22 °C).

Loading of BCECF and fluorescence measurements

After being mounted in the stimulation trough and a few test contractions, the fibre was incubated for 15 min in 5–15 μM-BCECF in the membrane permeant acetoxymethyl ester (AM) form. After being taken up by the cell, BCECF-AM is converted by cytoplasmic esterases to the

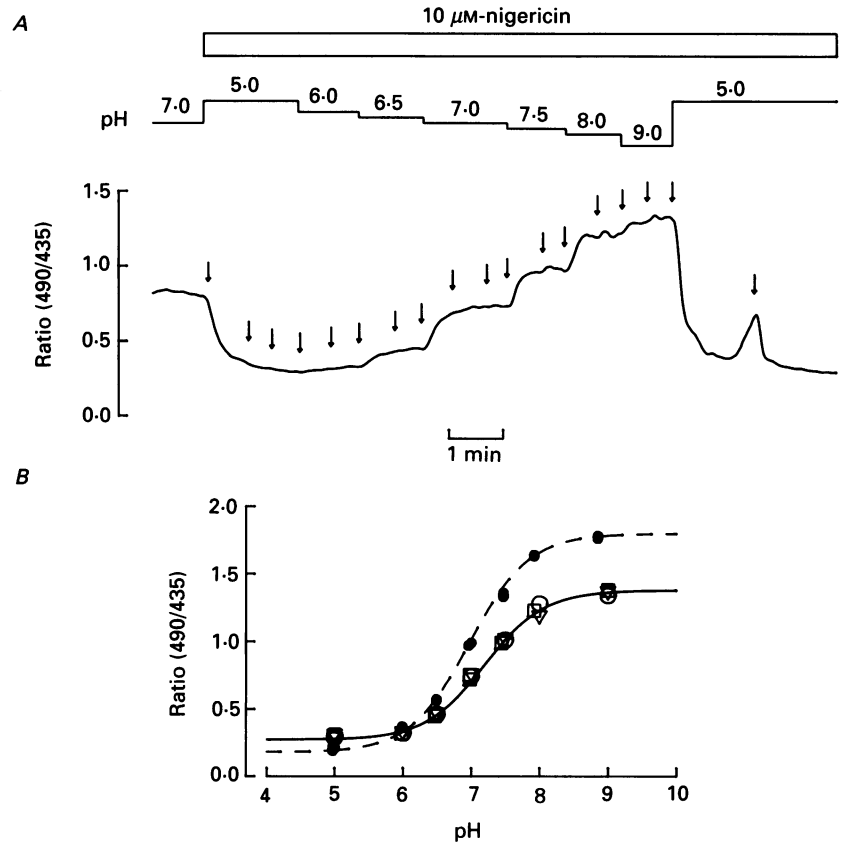

Fig. 1. *A* shows a continuous record of the fluorescence 490/435 ratio signal obtained from an intracellular calibration experiment. The fibre was first exposed to a high-K^+ solution with pH 7·0 and then to a series of solutions containing 10 μM-nigericin and with pH ranging from 5·0 to 9·0. The arrows indicate times when 1 ml solution was introduced into the experimental chamber. In *B* values of the ratio *vs.* pH are plotted. Open symbols obtained in three intracellular calibrations and filled circles obtained in *in vitro* calibrations. Curves were constructed by fitting data points to eqn (1).

impermeant tetracarboxylic acid form of BCECF. The optical arrangement used to record the fluorescence signal have been described in detail previously (Lee, Westerblad & Allen, 1991). Briefly, a Nikon Diaphot microscope with a ×20 Fluor objective was used. The ultraviolet illumination was obtained from an Ealing Beck 150 W light source with a xenon arc lamp and a shutter prevented illumination of the preparation except when required. Neutral-density filters were used throughout experiments to prevent photobleaching. The fluorescence light, recorded

from approximately 50% of the total length of a fibre, was guided to a photomultiplier tube and its amplified output (measured in μA of photocathode current) was displayed on a pen-recorder and stored on videotape.

The pH-dependent signal of BCECF was obtained by illuminating at 490 and 435 nm and dividing the resulting fluorescence signals emitted at 530 nm. To get fluorescence signals of approximately the same size, the intensity of the 490 nm illumination was reduced 300 times with neutral density filter whereas the 435 nm illumination was reduced 30 times. The illuminating wavelength was automatically switched between 435 and 490 nm every 4 s. The emitted light was passed to two sample-and-hold circuits so that the fluorescence signal at one illuminating wavelength was held when the other wavelength was in use. The two continuous signals obtained in this way were passed to an analog-divide circuit, whose output supplied a continuous 490/435 ratio signal. Because of the nature of the divide circuit, the full time resolution was only achieved after 4 s.

Prolonged and intense illumination of fluorescent probes causes photobleaching which may affect measurements (Roe, Lemasters & Herman, 1990; Lee *et al.* 1991). Photobleaching did not significantly affect the present results because: (i) in one control experiment a fibre was continuously illuminated for 7 min (the duration of a normal fatigue run) and during this period the 490/435 ratio signal remained unchanged; (ii) two fibres were reloaded with BCECF approximately 3 h after the initial loading and this resulted in a 2·5-fold increase of the emitted light, whereas the 490/435 ratio was not affected; (iii) the relation between pH_i and 490/435 ratio was similar in six intracellular calibration experiments (see below), despite at least a 3-fold difference in the duration of illumination before the calibration was performed.

Besides the pH-dependent fluorescence signal from the muscle fibre, three other sources may contribute to the fluorescence light: (i) background fluorescence, (ii) autofluorescence, and (iii) light emitted by BCECF-AM.

Background fluorescence. The background signal was measured at regular intervals by moving the fibre out of the field of view; it has been subtracted from all signals. In the present experiments (in contrast to previous experiments with the Ca^{2+} indicator Fura-2; Westerblad & Allen, 1991) the background signal was found to be very small and remained virtually constant throughout experiments.

Autofluorescence. The fibre's autofluorescence was measured before loading with BCECF in thirteen experiments. The average autofluorescence was 0·04 μA at 435 nm and 0·02 μA at 490 nm which should be compared to 0·98 and 0·95 μA, respectively, after loading with BCECF. Thus autofluorescence did not substantially affect the light signal and has been ignored.

Light emitted by BCECF-AM. In an *in vitro* experiment the light emitted by BCECF in the AM form was found to be only 1–2% of the light obtained from BCECF in the tetracarboxylic acid form. Thus the contribution to the *in vivo* light signal by esterified BCECF is likely to be very small and has been ignored.

In an attempt to determine whether BCECF had entered compartments other than the myoplasm, fibres were viewed using a video camera with a coupled image intensifier. Images showed only the expected variations in staining associated with the thickness of the fibre and none of the patchy staining associated with organelle uptake (see e.g. Roe, Lemasters & Herman, 1990). Furthermore, ratio images of the fibre showed a uniform pH throughout the fibre and no discernible gradients occurred during fatigue runs.

The intracellular concentration of BCECF was assessed by comparing the fluorescence of loaded fibres with that from a glass capillary of the same inner diameter as a normal fibre containing a known concentration of BCECF. The mean intracellular BCECF concentration was found to be ~ 100 μM.

Calibration of BCECF signals

An intracellular calibration was attempted in six fibres and Fig. 1*A* shows the record from one such calibration. The fibres were in this case bathed in a solution with a potassium concentration similar to the intracellular (mM): KCl, 175; $MgCl_2$, 0·5; KH_2PO_4, 1·2; HEPES, 10·0 (intracellular K^+ concentration obtained from Juel, 1986). The fibres produced a transient contracture (amplitude 40–84% of the maximum tetanic tension, duration 2–4 s), when exposed to this high-K^+ solution. Nigericin, which acts as a proton and potassium ionophore and makes it possible to set the pH_i equal to the extracellular pH (pH_o) (Thomas, Buchsbaum, Zimniak & Racker, 1979), was

applied after relaxation from the contracture. The fibres were then exposed to a series of solutions with pH ranging from 5·0 to 9·0; pH was adjusted by addition of HCl or KOH. Complete calibrations were performed in the fibre depicted in Fig. 1*A* and in two more fibres and the values are plotted in Fig. 1*B* (open symbols). It can be seen that the values obtained from these three fibres were close to identical. Further, values from the remaining three fibres, from which complete calibrations were not obtained, agree closely with the plotted values.

In vitro calibrations were also performed (●, Fig. 1*B*). BCECF was in this case added to the same series of high-K^+ solutions which were used for intracellular calibration. The curves in Fig. 1*B* were obtained by fitting values to the following equation (cf. Grynkiewicz, Poenie & Tsien, 1985):

$$\mathrm{pH} = \mathrm{p}K_A - \log[(R - R_{min})/(R_{max} - R)] - \log(S_{f2}/S_{b2}), \quad (1)$$

where $\mathrm{p}K_A$ is $-\log$ dissociation constant, R is the 490/435 ratio at a given pH. R_{min} and R_{max} are the ratios when the dye is predominantly in the H^+-free (i.e. high pH) and H^+-bound (i.e. low pH) state, respectively. S_{f2} and S_{b2} are constants representing the fluorescence levels with 435 nm illumination when all the dye is in the H^+-free and H^+-bound state, respectively. In the intracellular calibration the S_{f2}/S_{b2} was $0{\cdot}88 \pm 0{\cdot}03$ and the data points were best fitted to a curve where $R_{min} = 1{\cdot}38$, $R_{max} = 0{\cdot}27$, and $\mathrm{p}K_A = 7{\cdot}15$. The *in vitro* calibrations gave the following values: $R_{min} = 1{\cdot}80$, $R_{max} = 0{\cdot}18$, $\mathrm{p}K_A = 6{\cdot}81$, and $S_{f2}/S_{b2} = 0{\cdot}68$. Such differences between *in vivo* and *in vitro* properties of indicator dyes have frequently been reported and may for instance be due to binding to cytoplasmic constituents (e.g. Thomas *et al.* 1979; Rink, Tsien & Pozzan, 1982). The present *in vitro* $\mathrm{p}K_A$ is somewhat lower than previously reported (6·97; Rink *et al.* 1982), which could be due to differences in the calibration solutions. The values from the intracellular calibration has been used to convert ratios, which were measured from chart records, into pH_i throughout; the smallest detectable change of pH_i was about 0·01 units.

Intracellular buffer power

The pH_i was measured in rested fibres during exposure to weak acids (lactic acid, butyric acid and increased CO_2 concentration) and weak base (trimethylamine (TMA)). Weak acid and base enter the cell mainly in the uncharged form and dissociate into the charged forms within the cell. The amount of acid or base entering the cell depends on both the extracellular and intracellular pH (for a detailed account see e.g. Roos & Boron, 1981). We have therefore chosen to present our data as buffer power, which takes differences in pH_o and pH_i into account. For addition of an acid, the buffer power (β) was calculated as:

$$\beta = 10^{(\mathrm{pH}_o - \mathrm{pH}_i)}[S]/\Delta\mathrm{pH}_i, \quad (2)$$

where pH_i and pH_o are obtained in the presence of the acid, $[S]$ is the extracellular acid concentration, $\Delta\mathrm{pH}_i$ is the maximum change in pH_i from the mean of the pH_i before and after exposure. The buffer power for addition of a base was obtained in an equivalent way. When the CO_2 concentration was changed, the buffer power was obtained by first calculating the change in internal $[HCO_3^-]$ and this change was then divided by the pH_i change (for details, see e.g. Curtin, 1987). Note that the units for β are mmol l^{-1} (pH unit)$^{-1}$, but we will abbreviate this to mM (pH unit)$^{-1}$.

Statistics

Values are given as means ± S.E.M. or as a range; changes are presented as mean difference ± standard error of the mean difference. Student's *t* test was used to verify statistical significance; the significance level was set at 0·05 throughout.

RESULTS

After being loaded with BCECF, fibres were allowed to rest for at least 30 min in the standard, bicarbonate-buffered solution bubbled with 5% CO_2. After this period of rest, the pH_i of the fibres was $7{\cdot}33 \pm 0{\cdot}02$ ($n = 29$) which is similar to other estimates in mouse muscle, provided correction is made for the known effects of temperature on pH_i (Aickin & Thomas, 1977*a*). During experiments there was a very slow decline of pH_i with time ($\sim 0{\cdot}05$ pH units h^{-1}).

Figure 2 shows continuous tension and pH_i records from a fatigue run. This fibre displayed a very small acidosis: when tension had been brought down to 24% of the original, pH_i was only reduced by about 0·02 units. The stimulation was made progressively more demanding every second minute by a reduction of the tetanus

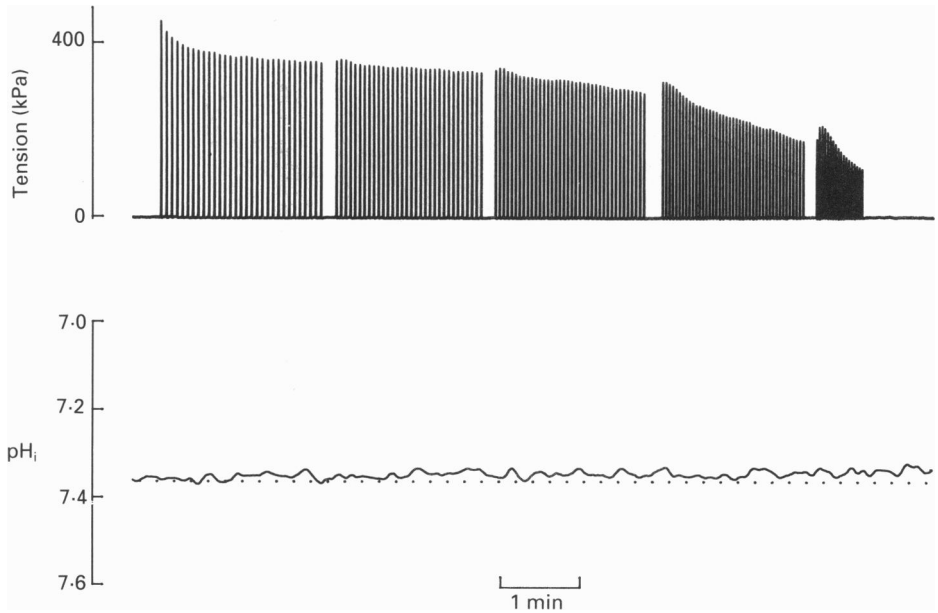

Fig. 2. Records from a representative fatigue run. Upper and lower trace show continuous records of tension and pH_i, respectively. Each vertical line in the tension record represents a tetanic contraction. The dotted line indicates the pH_i at rest.

interval (see Methods) and at these times the fibre was allowed to rest for about 10 s. It has previously been shown that towards the end of fatiguing stimulation there is a marked tension recovery during these 10 s pauses (Lännergren & Westerblad, 1991). Such a recovery can be seen in the tension record in Fig. 2; note that the tension increase was not accompanied by any obvious change of pH_i.

Standard fatigue runs were produced in fourteen fibres. At the beginning of fatiguing stimulation ten out of the fourteen fibres displayed a small alkalinization (e.g. dashed line in Fig. 3). This pH_i increase ranged from 0·01 to 0·06 units and the maximum occurred after five to twelve tetani. After this early phase, pH_i declined in all preparations and at the end of fatiguing stimulation fibres were slightly,but significantly, acidified in comparison to before stimulation: the pH_i reduction was $0·063 \pm 0·011$ units.

Cyanide experiments

The small size of the pH_i reduction in fatigue might be explained by a high oxidative capacity so that fatigue occurs without any marked production of lactic acid. To test this hypothesis, three fibres were fatigued in the presence of 2 mM-cyanide which will inhibit the oxidative metabolism. Before being fatigued, fibres

were exposed to cyanide for about 15 min without stimulation. During this time tetanic tension was reduced to about $0{\cdot}9\,P_0$ and pH_i went alkaline by $0{\cdot}10 \pm 0{\cdot}04$ units. All three fibres were more rapidly fatigued in cyanide and the acidosis developing during fatiguing stimulation was significantly larger ($0{\cdot}16 \pm 0{\cdot}03$ *vs.*

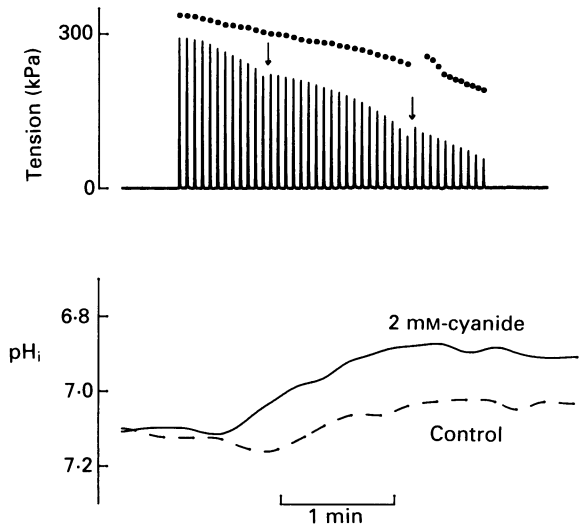

Fig. 3. Tension and pH_i records obtained from a fibre during a fatigue run in the presence of 2 mM-cyanide. The arrows above the continuous tension record indicate times when the stimulation frequency was reduced (first from 100 to 70 Hz and then from 70 to 60 Hz). The filled circles in the tension panel and the dashed line in the pH_i panel indicate peak tetanic tension and pH_i obtained from the same fibre during a fatigue run under control conditions. Note that the fibre fatigued more rapidly and became more acidified in the presence of cyanide.

$0{\cdot}07 \pm 0{\cdot}03$ pH units). This is illustrated in Fig. 3 where tension and pH_i records from one fibre exposed to cyanide are compared with records obtained from the same fibre under control conditions. It may be noted in Fig. 3 that the clear early alkalinization in the control fatigue run was much less marked in the presence of cyanide; the other two fibres exposed to cyanide did not display any early alkalinization.

Inhibition of transport processes

The small size of the pH_i reduction in fatigue may be caused by an effective transport of H^+ or H^+-equivalents out of the fibres. This hypothesis was tested by pharmacological inhibition of the three major pH-regulating transport processes in skeletal muscle: (i) the Na^+–H^+ exchanger was inhibited by 0·1–0·5 mM-amiloride; (ii) the Cl^-–HCO_3^- exchanger was inhibited by 0·05 mM-DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid); (iii) the lactate transporter was inhibited by 4 mM-cinnamate. These drugs are fluorescent themselves and thus may affect measurements. With the concentrations and excitation wavelengths used, amiloride and DIDS did not have any noticeable effect on the fluorescence light. Application of cinnamate, on the other hand, caused a stepwise increase of the fluorescence ratio signal; this increase has been corrected for in all measurements.

Inhibition of Na^+–H^+ exchange with amiloride in six fibres resulted in a slow acidification of about 0·02 pH units min^{-1} (see lower trace Fig. 11). Three fibres were fatigued in the presence of amiloride, two of which were also fatigued in the standard solution. During fatiguing stimulation neither the tension decline nor the pH_i

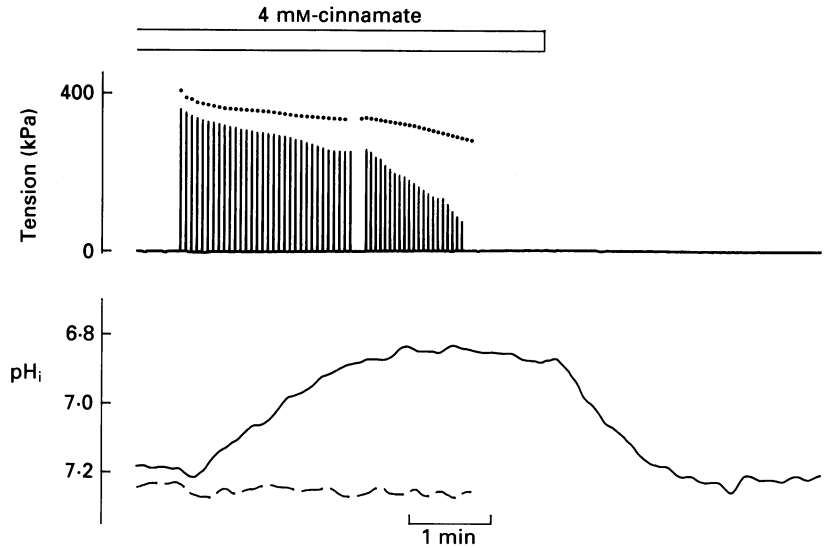

Fig. 4. Fatigue in the presence of 4 mM of the lactate transport inhibitor cinnamate. Filled circles (peak tetanic tension) and dashed line (pH_i) obtained from the same fibre during a control fatigue run. Period of cinnamate exposure indicated above the tension record.

changes were significantly different in the presence of the drug and under control conditions; the paired controls revealed that one fibre became slightly more fatigue resistant and displayed a larger pH_i decline than its control, whereas the other fibre became less fatigue resistant and less acidified. These findings suggest that Na^+–H^+ exchange is important for the pH_i regulation at rest, which agrees with previous results from mouse skeletal muscle (Aickin & Thomas, 1977*b*), whereas it plays no significant role in the present type of fatigue.

Application of the Cl^-–HCO_3^- exchange inhibitor DIDS did not change pH_i in the rested state in any of the three fibres exposed to this drug. Further, DIDS did not significantly alter any fatigue properties. A control fatigue run in the standard solution was produced in two of the three fibres exposed to DIDS: the pH_i decline was similar with and without the drug and in the presence of DIDS one fibre became more fatigue resistant whereas the other fatigued more rapidly. Thus, inhibition of the Cl^-–HCO_3^- exchange did not have any noticeable effect in the rested state or during fatiguing stimulation.

The lactate transport inhibitor cinnamate did not noticeably influence the resting pH_i in six fibres which were exposed to the drug. In contrast to amiloride and DIDS, cinnamate exerted a marked effect on fatigue and a typical example is shown in Fig. 4. An early alkalinization was observed in three out of five fibres fatigued in the presence of cinnamate; this small alkalinization was at its maximum after five

tetani, which is slightly earlier than under control conditions. After the first few tetani there was a marked acidification in all fibres and in the fatigued state pH_i was reduced by $0{\cdot}35 \pm 0{\cdot}04$ units. A paired comparison with standard fatigue runs could be made in three fibres and the pH_i reduction in fatigue was significantly larger in

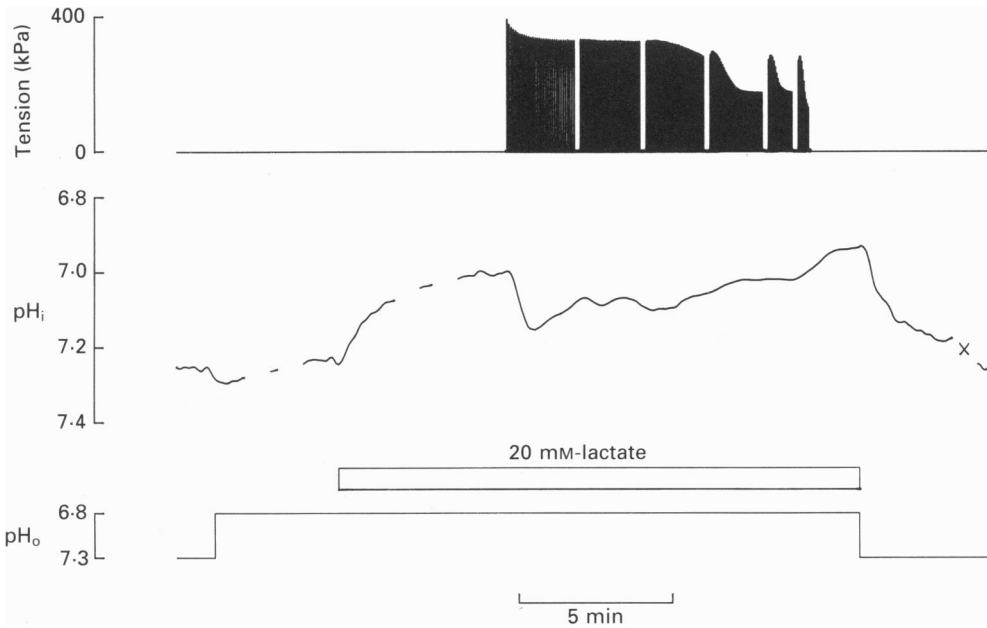

Fig. 5. Tension and pH_i records from a fatigue run in a solution with 20 mM-lactate and reduced pH. The timing of pH_o changes and of lactate exposure are shown below the pH_i record. The interruptions in the pH_i record are due to periods when the fibre was not illuminated; the duration of the last interruption (marked ×) was 5 min.

the presence of cinnamate ($0{\cdot}38 \pm 0{\cdot}03$ *vs.* $0{\cdot}04 \pm 0{\cdot}02$ pH units). These three fibres also fatigued more rapidly in the presence of cinnamate; the mean time to depress tetanic tension to about $0{\cdot}3 P_0$ was approximately 4 min in cinnamate and 10 min in the standard solution (see Fig. 6*B*).

Recovery of pH_i could be followed in three fibres exposed to cinnamate during fatiguing stimulation and the fibre depicted in Fig. 4 displayed a typical pattern: initially there was a very slow pH_i recovery as the exposure to cinnamate continued and pH_i recovered rapidly after a delay of about 30 s when the fibre was returned to the normal solution.

The reduction of the maximum tension-generating capacity at the end of a fatigue run in the presence of cinnamate was assessed in one experiment by application of 10 mM-caffeine. The pH_i was in this fibre reduced by 0·4 units and application of caffeine resulted in a tension increase from 27 to 62% of the control. In another experiment a fibre was exposed to the dissection solution (0% CO_2; see Methods) at the end of a fatigue run in cinnamate. This resulted in a rapid increase of pH_i from 6·97 to 7·03 and a tension increase from 34 to 51% of the control.

Fatigue in high extracellular lactate

Attempts to inhibit lactate extrusion during fatiguing stimulation were also performed by adding lactate ions to the bathing solution. At the normal extracellular pH (7·3) addition of lactate had no marked effect on pH_i in rested fibres (see below). Three fibres were fatigued in 20 mM-lactate ($pH_o = 7·3$) and this did not markedly affect the fatigue properties: the acidification during fatiguing stimulation was $0·093 \pm 0·023$ pH units; paired comparisons with control conditions could be made in two of the three fibres and both these fibres became slightly more fatigue resistant in the presence of lactate and the pH_i decline was similar.

Three fibres were exposed to 20 mM-lactate at reduced pH_o; one of these experiments is shown in Fig. 5. These fibres were bathed in a solution containing 7·6 mM-sodium bicarbonate instead of the normal 24 mM ($NaHCO_3$ replaced by NaCl) which gave a pH_o of 6·8. Exposure to this low-bicarbonate solution without added lactate resulted in a slow acidification (0·02–0·06 pH units in 5 min). After addition of 20 mM-lactate, pH_i declined rapidly and after 5 min it was $0·32 \pm 0·04$ units lower than in the standard solution. During fatiguing stimulation these fibres initially showed a marked alkalinization (mean increase 0·11 pH units with a maximum after thirteen to seventeen tetani); this pH_i increase was significantly larger than that in the standard solution. After the early alkalinization, pH_i declined slowly so that in fatigue the mean pH_i was similar to that at the beginning of fatiguing stimulation. During the initial 1–2 min after fatiguing stimulation, the fibres became slightly more acid (mean reduction 0·02 pH units) and after the subsequent return to the standard solution ($pH_o = 7·3$) pH_i recovered rapidly.

Correlation between pH_i decline and fatigue resistance

If reduced pH_i is the dominant cause of fatigue, it would be expected that fatigue occurred at a similar pH_i in all fibres or alternatively after a similar pH_i reduction. In the present study neither of these two expectations were fulfilled: in the fatigued state pH_i ranged from 6·79 to 7·35 and the acidification during fatigue runs ranged from zero to 0·57 pH units. If, on the other hand, intracellular acidosis is one factor among several which contributes to fatigue, it might be expected that fibres displaying a rapid acidification also fatigue rapidly. There was a general tendency for fast pH_i reductions to be associated with short endurance times (i.e. the time taken to reach the standard fatigue level ($0·3\ P_o$)) and this was particularly clear in fibres exposed to cyanide and cinnamate. Figure 6 compares values obtained from fibres exposed to cyanide (●) and cinnamate (▼) with their respective controls (○). In the presence of these two inhibitors, the pH_i reduction during fatiguing stimulation was faster and this was in all cases associated with reduced fatigue resistance. Thus these results support the hypothesis that acidosis directly or indirectly can contribute to fatigue.

The effect of pH_i on tension production

To study the effect of altered pH_i on tension production in rested fibres, pH_i was changed either by increasing or decreasing the CO_2 concentration of the bath solution or by adding 20 mM-lactate at reduced pH_o (see above). Original tension records from

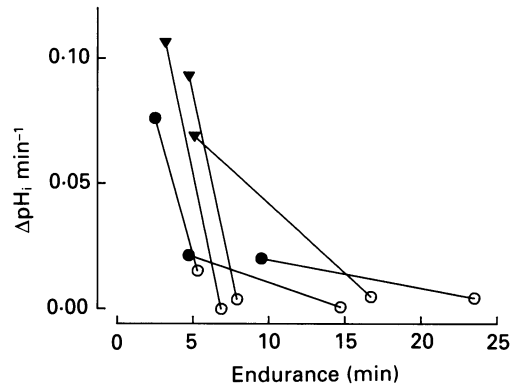

Fig. 6. The relation between endurance and rate of acidification illustrated by paired comparisons obtained from fibres fatigued both under control conditions (○) and in the presence or either cyanide (●) or cinnamate (▼). The endurance is defined as the time taken for tetanic tension to decline to the standard fatigue level ($\sim 0{\cdot}3P_0$); the rate of acidification during fatigue runs is taken as the ratio between the total ΔpH_i and the duration of the fatigue run.

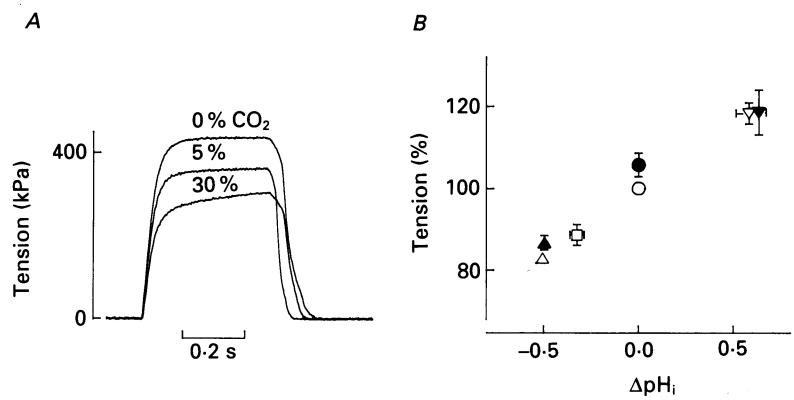

Fig. 7. The effect of pH_i on tension production of rested fibres; pH_i was changed by altering the CO_2 concentration or by adding 20 mM-lactate at pH_o 6·8. *A* shows tension records from tetani elicited at three CO_2 concentrations: pH_i was 7·96, 7·35, and 6·84 with 0, 5, and 30 % CO_2, respectively. *B* shows mean values (±S.E.M.) of peak tetanic tension *vs.* pH_i. The tension and pH_i at 5 % CO_2 are 100 % and 0·0, respectively. ○, 5 % CO_2; △ = 30 % CO_2 ($n = 8$), ▽ = 0 % CO_2 ($n = 7$), and □ = 20 mM-lactate ($n = 3$); filled symbols, CO_2 concentrations as for open symbols with 10 mM-caffeine added ($n = 4$).

a fibre exposed to 0, 5 and 30 % CO_2 are shown in Fig. 7*A* and the results from all these experiments are summarized in Fig. 7*B*. Under normal conditions (no caffeine added; open symbols in Fig. 7*B*) there was a linear relation between tension and pH with a slope of about $0{\cdot}33P_0$ (pH unit)$^{-1}$. Four fibres were also exposed to 10 mM-caffeine at the three CO_2 concentrations (filled symbols). The idea was that since caffeine causes the free myoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) during tetani to increase above saturation for troponin-C, the tension in caffeine would represent the maximum Ca^{2+}-activated tension which may be directly compared with results from skinned fibre experiments (Westerblad & Allen, 1991). It can be seen that the tetanic

tension with and without caffeine was similar at all three CO_2 concentrations, which indicates that the tetanic $[Ca^{2+}]_i$ was close to saturation even without addition of caffeine.

Slowing of relaxation

Fatigue is generally associated with a slowing of relaxation and this slowing has often been attributed to a reduction of pH_i (e.g. Edman & Mattiazzi, 1981). In the

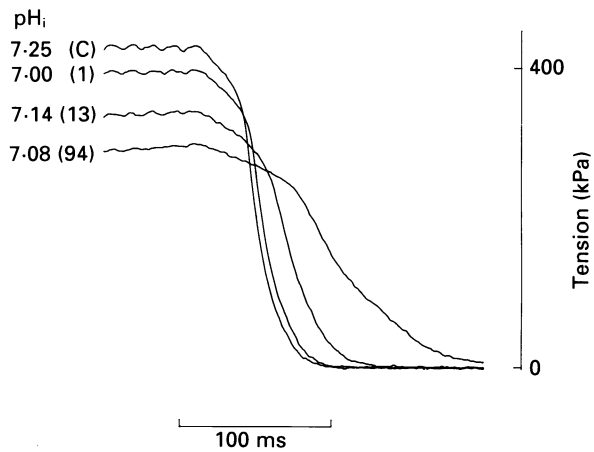

Fig. 8. Slowing of relaxation during fatigue in 20 mM-lactate and pH_o reduced to 6·8. Tension records obtained from a control tetanus (C) before application of lactate, from the first tetanus of the fatigue run (1), and from the fatigue run at the time of maximum early alkalinization (thirteenth tetanus, 13) and maximum slowing of relaxation (ninety-fourth tetanus, 94); the pH_i at the time of each tetanus is indicated. The last stimulus was given at leftmost edge of the timebar. Observe that during the fatigue run relaxation becomes markedly slower despite the increase of pH_i.

present preparation relaxation is already markedly slowed after about ten fatiguing tetani (Westerblad & Lännergren, 1991), i.e. at a time when pH_i has increased rather than decreased in most fibres (see above). Thus the slowing down of relaxation in fatigue cannot be solely due to acidosis. This is clearly illustrated in Fig. 8, which shows tension records obtained from a fibre fatigued in a solution with 20 mM-lactate and pH reduced to 6·8. When this fibre was exposed to the lactate-containing solution, pH_i was reduced by 0·25 units and the linear phase of relaxation was slowed. Fatiguing stimulation then started and after thirteen tetani pH_i had increased by 0·14 units and relaxation had become markedly slower. In this fibre maximum slowing of relaxation occurred at the ninety-fourth tetanus and pH_i was at this stage still higher than at the beginning of fatiguing stimulation.

Under control conditions the rate of tension decline during the linear phase of relaxation was 1700 ± 130 kPa s^{-1} ($n = 20$) and the duration of this relaxation phase was 37 ± 3 ms. In Fig. 9 relaxation measurements during fatiguing stimulation (filled symbols) are compared with measurements in acidified rested fibres (i.e. fibres exposed to 30% CO_2, 20 mM-lactate at pH_o 6·8, or amiloride; open symbols). The fatigue data points were obtained from fibres fatigued both in the normal solution

and, to make it possible to study relaxation over a wider range of pH_i, in solutions altered as described above (i.e. inhibitors or lactate added). During fatiguing stimulation relaxation was measured at two stages: (i) at the time of maximum early alkalinization or at the tenth tetanus in fibres which did not display any early

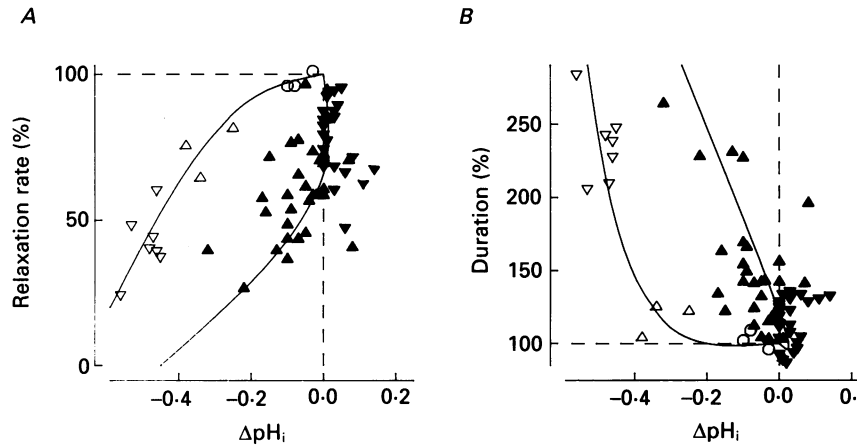

Fig. 9. Summary of changes of the relaxation rate (*A*) and duration (*B*) of the linear phase of relaxation; 100% (relaxation rate and duration) and 0·0 (pH_i) refer to control values obtained in the first tetanus of fatigue runs and in a tetanus elicited before acidification of rested fibres. During fatiguing stimulation data points were obtained at the time of early alkalinization (▼) and maximum slowing of relaxation (▲); acidification of rested fibres was produced by exposure to amiloride (○), 20 mM-lactate at pH_o 6·8 (△), and 30% CO_2 (▽). Curves drawn by eye; dashed lines indicate no difference from control values.

alkalinization and (ii) at the time of maximum slowing of relaxation which generally occurs before the rapid tension decline at the end of fatiguing stimulation (see Westerblad & Lännergren, 1991). At the first stage, pH_i had increased significantly ($0·031 \pm 0·007$ units, $n = 26$) and both relaxation parameters were significantly slowed (rate $77·6 \pm 2·5$% and duration $115 \pm 3·1$% of the original). Thereafter pH_i declined in all fibres and this was accompanied by a further slowing of relaxation.

The data points obtained from fibres acidified in the rested state lie to the left of points obtained during fatiguing stimulation, i.e. in the rested state a larger pH_i reduction was required to produce a given slowing of relaxation. It can also be seen in Fig. 9 that in the rested state relaxation appeared to be progressively more affected by acidosis as the pH_i reduction became larger.

Fibres exposed to 2 mM-cyanide displayed an alkalinization of about 0·1 pH units before fatiguing stimulation (see above). This alkalinization was not accompanied by any significant change of the relaxation speed.

Application of weak acids and bases to rested fibres

In addition to the rate of production *vs.* extrusion of hydrogen ions, the degree of acidification in fatigue depends on the intracellular buffer power. We have assessed the buffer power by applying weak acid and base to rested fibres and Fig. 10 shows

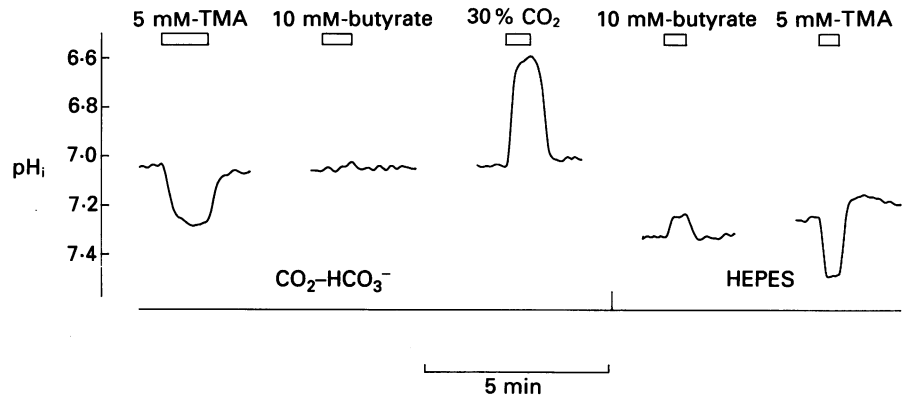

Fig. 10. The pH_i response to application of weak base (trimethylamine (TMA)) and weak acid (butyrate and increased P_{CO_2}) in the standard bicarbonate-buffered solution and in the HEPES-buffered solution. Note the absent or very small acidification caused by butyrate application.

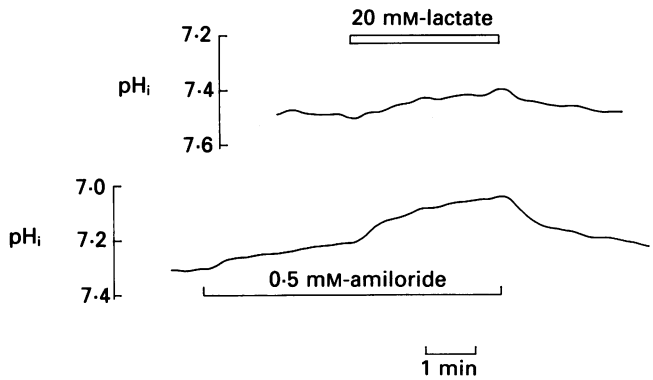

Fig. 11. The acidification caused by lactate exposure in bicarbonate-buffered solution with (lower trace) and without (upper trace) amiloride. The bar indicating period of lactate exposure refers to both traces.

records from one such experiment. Application of 5 mM of the weak base TMA resulted in a marked alkalinization in the standard bicarbonate-buffered solution and the response to TMA was somewhat larger in HEPES-buffered solution. In this experiment weak acid was applied either by exposing the fibre to butyrate or increased CO_2 concentration. The response to these two manoeuvres was markedly different: exposing the fibre to a solution bubbled with 30 % instead of 5 % CO_2 (pH_o reduced from 7·30 to 6·55) resulted in marked pH_i reduction, whereas application of 10 mM-butyrate had no noticeable effect in the bicarbonate solution and produced only a small pH_i reduction in the HEPES solution. The small or absent pH_i response to butyrate was a consistent finding and a similar result was obtained when fibres were exposed to lactate at constant pH_o (Fig. 11, upper trace). If, on the other hand, fibres were exposed to lactate at reduced pH_o (see Fig. 5), a sizeable pH_i reduction was obtained.

The buffer power was calculated for each of the above interventions and the results are summarized in Table 1. We have separated the results of experiments where the fibres were bathed in the bicarbonate-buffered solution at constant P_{CO_2} from those where either the P_{CO_2} was changed or the HEPES solution was used. In the latter case the buffering depends on intracellular factors, whereas in the former case CO_2 may

TABLE 1. Values of intracellular buffer power

	Total buffer power (mM (pH unit)$^{-1}$)	Non-CO_2 buffer power (mM (pH unit)$^{-1}$)
TMA	33·0 ± 6·2 (6)	15·4 ± 2·7 (5)
Increased P_{CO_2}	—	33·5 ± 3·9 (8)
Butyrate	∞ (4)	88·3 ± 2·4 (2)
Lactate (pH_o 7·3)	142–∞ (10)	135 ± 58·0 (3)
Lactate (pH_o 6·8)	115 ± 14·8 (3)	—

Values are given as means ± S.E.M. when appropriate. Number of measurements in parentheses.

enter or leave the cell and thus CO_2–HCO_3^- will act as an open buffer system (for details see Roos & Boron, 1981). In Table 1 it can be seen that the buffer power calculated from the responses to application of butyrate and lactate is unrealistically high, which suggests that extrusion of H^+ affects these results. To investigate this point, fibres were exposed to 20 mM-lactate in the presence of H^+-transport inhibitors. Two fibres were studied in the presence and absence of DIDS (0·05 mM) and this drug had no noticeable effect on the degree of acidification caused by application of lactate. Two other fibres were bathed in lactate in the presence of 0·5 mM-amiloride and one of these experiments is shown in Fig. 11. In the standard solution this fibre displayed the normal small pH_i response to lactate, but after addition of amiloride the pH_i reduction caused by lactate application was substantially larger. As described above, amiloride causes a progressive acidification in rested fibres and this must be taken into account when calculating the buffer capacity. If it is assumed that the rate of pH_i decline due to amiloride was the same during as before lactate exposure, the buffer power in the presence of the drug was 107 mM (pH unit)$^{-1}$ as compared to 322 mM (pH unit)$^{-1}$ in the control. A similar result was obtained in the other fibre exposed to lactate in the presence of amiloride. The fibre depicted in Fig. 11 was also bathed in a solution containing both 0·5 mM-amiloride and 0·05 mM-DIDS (results not shown). This resulted in a somewhat faster decline of pH_i before application of lactate, whereas the additional pH_i reduction in response to lactate was similar. Thus the lactate-induced pH_i reduction was larger in the presence of amiloride, but the buffer power was still markedly higher than that obtained in response to TMA and increased P_{CO_2}.

DISCUSSION

Role of pH_i in the decline of tension during fatigue

Previous studies of mouse single fibres fatigued by intermittent tetani have shown that tension declines in three phases (Lännergren & Westerblad, 1991) and that the small decline in the first phase is due to a reduction of maximum Ca^{2+}-activated force

(Westerblad & Allen, 1991). Only two of the metabolic changes which are likely to occur in fatigue are known to have a substantial effect on maximum Ca^{2+}-activated force: the fall in pH_i (Fabiato & Fabiato, 1978; Godt & Nosek, 1989) and the rise in inorganic phosphate (P_i) (Cooke & Pate, 1985; Godt & Nosek, 1989). The present study shows that pH_i changed very little over this period; if anything pH_i was slightly alkaline at the end of phase 1 (after about ten tetani). Thus we can unequivocally state that in this preparation the early decline of force is not due to an acidification; by exclusion it seems likely to be caused by P_i accumulation. There are no suitable measurements of $[P_i]$ in single fibres to allow an accurate assessment of this hypothesis, but it is worth noting that at low $[P_i]$ even small changes in $[P_i]$ lead to substantial changes in tension (Millar & Homsher, 1990), so the hypothesis is certainly feasible.

Towards the end of fatigue runs, there is a rapid decline in tension (phase 3) and this is thought to be caused by the combined effect of reductions of myofibrillar Ca^{2+}-sensitivity and tetanic $[Ca^{2+}]_i$ (Westerblad & Allen, 1991). However, because pH_i hardly changes during fatigue (average acidosis 0·06 pH units) it is clear that acidosis is not the sole cause of either mechanism.

On the other hand, the present study clearly shows that when intracellular acidosis does occur, it makes an additional contribution to the decline of force. Thus an acidosis of 0·5 pH units from the resting pH_i decreased maximum Ca^{2+}-activated force by 0·15–0·2 P_0 in an intact fibre (Fig. 7) which is somewhat less than the tension decline generally reported for skinned fibres (e.g. Cooke & Pate, 1985; Godt & Nosek, 1989). When fatigue runs were performed under circumstances which caused an acidosis to develop, the rate of tension decline was greater (Figs 3 and 4) and the end point of fatigue runs was achieved quicker (Fig. 6*B*). Examination of Fig. 3 shows that the extra component of decline of force in the presence of cyanide (0·4 P_0 (0·15 pH units)$^{-1}$) is much greater than could be accounted for solely by the effect of pH on maximum Ca^{2+}-activated force. A similar phenomenon is observed in Fig. 4 when increased acidosis was produced by blocking the lactate transporter. Furthermore, when a CO_2-free solution was applied at the end of a fatigue run in cinnamate (see Results), a substantial force recovery for a very small pH_i recovery was observed. All these results suggest that acidosis, in addition to reducing maximum Ca^{2+}-activated force, also reduces force by other mechanisms, presumably by reducing myofibrillar Ca^{2+}-sensitivity and SR Ca^{2+} release. Furthermore, acidosis will partially inhibit glycolysis (e.g. Amorena, Wilding, Manchester & Roos, 1990) which would cause more severe changes of energy metabolites in fatigue.

Role of pH_i in the slowing of relaxation during fatigue

After a few fatiguing tetani the fibres generally displayed a small alkalinization and both relaxation parameters measured were significantly slowed. We attribute the tension reduction at this stage to an increased P_i concentration which reduces the maximum Ca^{2+}-activated tension (see above). Can the slowing of relaxation also be explained by increase $[P_i]$? A comparison between results obtained from early fatigue and from rested fibres exposed to cyanide may give an answer to this question. In both these states (i) the tetanic tension is reduced to about 0·9 P_0, (ii) the tetanic $[Ca^{2+}]_i$ is higher than in control (Westerblad & Allen, 1991) and (iii) the pH_i is

alkaline by up to 0·15 pH units (discussed below); the relaxation is, however, slowed in early fatigue but not in rested fibres exposed to cyanide. Thus, while increased $[P_i]$ may explain factors (i) to (iii) above, the slowing of relaxation is likely to be caused by some other mechanism.

After the initial alkalinization, pH_i declined as fatiguing stimulation continued and this was accompanied by a progressive slowing of relaxation which reached a maximum immediately before the final rapid tension decline. The largest prolongation of the relaxation was obtained in fibres displaying the largest acidosis, i.e. fibres bathed in cyanide or cinnamate. Thus when an acidosis occurred in fatigue, this was associated with slowing of the relaxation. Furthermore, the relaxation was markedly slowed down when fibres were acidified in the rested state.

Based on the present results it can be concluded that the slowing of relaxation in fatigue is due to at least two processes: one pH dependent and one pH independent. This conclusion is in agreement with recent results from fatigued human muscle (Cady, Elshove, Jones & Moll, 1989). The mechanisms by which these processes cause slowed relaxation are not fully understood. Measurements of $[Ca^{2+}]_i$ in isolated *Xenopus* muscle fibres have shown that the rate of $[Ca^{2+}]_i$ decline during relaxation is substantially slowed in fatigue, whereas it is not significantly affected in rested fibres acidified by exposure to CO_2 (Allen, Lee & Westerblad, 1989; Lee *et al.* 1991). The slowing of relaxation due to acidosis is accompanied by decreased shortening speed indicating a reduced rate of cross-bridge cycling (Edman & Mattiazzi, 1981). It may then be proposed that the pH-independent process slows relaxation in fatigue mainly by affecting Ca^{2+} handling, while the pH-dependent process primarily acts on cross-bridge cycling.

Mechanism of pH_i regulation during fatigue

Aickin & Thomas (1977*b*) showed that in resting mammalian muscle hydrogen ions were extruded against their electrochemical gradient principally by a Na^+–H^+ exchange mechanism. Our results support this, in that application of the Na^+–H^+ exchange inhibitor amiloride to a resting fibre led to a slowly developing acidosis (0·02 pH units min^{-1}). The other inhibitors we used did not affect resting pH_i.

During fatigue runs neither Na^+–H^+ exchange nor HCO_3^-–Cl^- exchange appeared to have any significant role in pH_i regulation. However, inhibition of the lactate transporter with cinnamate led to a dramatic increase in acidosis during fatigue, suggesting that lactic acid is produced but normally removed by the lactate transporter at a rate almost equal to its production (see below). These results appear to differ substantially from those of Juel (1988), who measured pH_i from surface fibres in a whole mouse soleus muscle. After 2 min of repeated tetani, Juel observed a 0·5 pH-unit acidosis which recovered with a half-time of 8 min (25 °C). The much greater acidosis observed by Juel (0·5 pH units compared to our 0·06 pH units) presumably represents the accumulation of lactic acid both intracellularly and extracellularly within the muscle and much of the recovery must represent the diffusion time for this lactic acid to leave the muscle. This interpretation is supported by Juel's measurement of total muscle lactate which declined with a half-time of 13 min. Juel found that both amiloride and cinnamate slowed the recovery of pH_i suggesting that the Na^+–H^+ exchanger and the lactate transporter were significant routes for H^+ extrusion during fatigue. Our results show that amiloride had no effect

on pH_i during fatigue, suggesting that the contribution of the Na^+–H^+ exchanger is very small under our conditions. However, there is not necessarily any discrepancy between our result and that of Juel. He found that in muscles which had become acid, amiloride blocked recovery. Since our fibres generally became much less acid, our experiments did not explore this point. However, experiments in cinnamate are relevant to this point (Fig. 4). In the continued presence of cinnamate, these fibres showed a slow pH_i recovery ($\sim$ 1 pH unit (30 min)$^{-1}$) which might be a contribution from the Na^+–H^+ exchanger. Thus, with our experimental conditions the lactate transporter was by far the most active proton-extruding mechanism.

Sources and sinks of protons during fatigue

We observed a very small alkalosis (mean 0·03 pH units) after about ten tetani. Breakdown of phosphocreatine (PCr), which will start during this period, acts as a proton sink because of the different proton-binding constants of PCr and P_i. The stoichiometry (S) of proton absorption due to PCr breakdown can be calculated as:

$$S = (10^{(pH_i - pK)} + 1)^{-1}, \tag{3}$$

where pK is the log proton-binding constant for P_i which equals 6·8 (cf. Amorena *et al.* 1990). At a resting pH_i of 7·3, S = 0·24, Thus if all PCr were to break down, the expected alkalosis would be [PCr] S/buffer power. Taking [PCr] as 35 mM (see Godt & Maughan, 1988) and buffer power as 33 mM (pH unit)$^{-1}$ (total buffer power measured with TMA) the alkalosis would be 0·25 pH units. The much lower figure we report presumably indicates that PCr breakdown is incomplete after ten tetani and partially countered by lactic acid production. However, when an intracellular acidosis was produced by application of lactate at reduced pH_o (Fig. 5), a much larger early alkalosis of up to 0·15 pH units was produced in fatigue runs. Part of the explanation for the larger alkalosis is that the stoichiometry (S; see above) increases to 0·4 at the lower initial pH_i (7·0) in this case. In addition glycolysis was probably partially inhibited by the intracellular acidosis (e.g. Amorena *et al.* 1990), which means that more of the energy required has to come from PCr breakdown. A slower rate of lactic acid production would also mean that the alkalosis produced by PCr breakdown was not counteracted so effectively by lactic acid production.

The acidosis normally observed in fatigue is usually attributed to lactic acid accumulation. We attribute the lack of acidosis in our preparation to rapid extrusion of lactate and proton equivalents by the lactate transporter and to the simultaneous alkalinization produced by PCr breakdown. A rough estimate of the magnitude of lactate production in our experiments can be obtained as follows. For the purpose of this calculation, we make the following simplifications: (i) production of lactic acid and breakdown of PCr are the only important H^+ producing or consuming reactions; (ii) all PCr is broken down by the end of a fatigue run; (iii) the only significant route for efflux of H^+ is by the lactate transporter which is completely blocked by cinnamate. Then at the end of a fatigue run,

$$\Delta pH_i = (\Delta[L^-]_i - \Delta[PCr]S)/\beta, \tag{4}$$

where $[L^-]_i$ = the intracellular lactate concentrations, S = the stoichiometry of proton absorption by PCr breakdown which is pH_i dependent (see above), and β = total buffer power (assumed to be 33 mM (pH unit)$^{-1}$, see above). Taking first a

fatigue run in the presence of cinnamate (Fig. 4): since $\Delta pH_i = 0{\cdot}35$ pH units, $S = 0{\cdot}45$ and $\Delta[PCr] = 35$ mM (see above), then $\Delta[L^-]_i = 27$ mM. Since this fatigue run lasted 3·5 min, the rate of lactate production was $27/3{\cdot}5 = 7{\cdot}7$ mM $(min)^{-1}$ which is similar to that observed after intense exercise in humans (e.g. Juel, Bangsbo, Graham & Saltin, 1990). If we now consider a representative fatigue run (median duration ~ 6 min) in the absence of cinnamate, where $\Delta pH_i = 0{\cdot}06$ pH units and $S = 0{\cdot}25$ which gives a $\Delta[L^-]_i$ of 11 mM. If we assume that the rate of lactate production was the same as in cinnamate, then 46 mM-lactate was produced and consequently $46 - 11 = 35$ mM must have been exported by the lactate transporter. This gives an efflux rate of $35/6 = 5{\cdot}8$ mM $(min)^{-1}$ which seems reasonable since the maximum rate of efflux by the transporter has been shown to be 12 mM $(min)^{-1}$ in mouse muscle (Juel & Wibrand, 1989).

Finally we consider the question of whether the gradients of protons and lactate which are likely to exist are great enough to achieve the flux estimated above. If the lactate transporter is an electroneutral co-transporter, then efflux will only occur when $[H^+]_i\,[L^-]_i$ is greater than $[H^+]_o[L^-]_o$. Under both resting conditions and in normal fatigue $[H^+]_i \sim [H^+]_o$ and $[L^-]_o \sim 0$, so that the required driving force is simply $[L^-]_i$. The above equation shows that at the end of a fatigue run $[L^-]_i$ is about 11 mM and this has to be capable of achieving a lactate efflux of 5·8 mM $(min)^{-1}$. Estimates of the Michaelis–Menten constant (K_m) of the transporter for lactate vary considerably (see Juel & Wibrand, 1989), but the value obtained by Juel & Wibrand for intact mouse muscle (3·5 mM) seems most appropriate to the present study. On this basis, the intracellular lactate concentration in our fatigued fibres (~ 11 mM) would give an efflux of about 90% of the maximum or 11 mM $(min)^{-1}$. This is larger than we estimate so it seems that the lactate transporter under our conditions is capable of the efflux which occurs.

pH buffering

Muscle fibre buffering will have a role in minimizing the pH_i changes associated with lactic acid production. Measurements with TMA gave an estimate of 15 mM (pH unit)$^{-1}$ for the intrinsic (or non-CO_2) buffer power and 33 mM (pH unit)$^{-1}$ for the total buffer power. The former is substantially less than the value often reported for skeletal muscle (e.g. 45 mM (pH unit)$^{-1}$; Aickin & Thomas, 1977*a*), but a value similar to ours has recently been reported for small fibre bundles (18 mM (pH unit)$^{-1}$; Amorena *et al.* 1990). The difference between our value and those obtained from whole muscles may reflect the fact that weak acids or bases can be applied more quickly to a single fibre than to a whole muscle so that regulatory mechanisms have less time to affect the results (cf. Bountra, Powell & Vaughan-Jones, 1990). The total buffering power is less than that expected theoretically ($2{\cdot}3[HCO_3^-]_i$ + intrinsic buffer power; for details see Roos & Boron, 1981) which would amount to about 70 mM (pH unit)$^{-1}$ at pH_i of 7·3. A discrepancy of this sort has previously been observed in both skeletal (Aickin & Thomas, 1977*a*) and smooth muscle (Aickin, 1988), but the cause remains obscure. When P_{CO_2} changes were used to determine the intrinsic buffer power, a value of 33 mM (pH unit)$^{-1}$ was obtained. We do not know why this is so much larger than the buffer power determined with TMA; only a small part of the discrepancy can be explained by the fact that the determination with

changed P_{CO_2} was over a more acid pH range and buffer power increases as pH_i decreases (Amorena *et al.* 1990).

Application of external lactate (pH_o constant) led to a very small intracellular acidosis and a correspondingly large apparent buffering. A similar observation has been made in smooth muscle (Aickin, 1988; especially the panel discussion after Aickin's presentation deals with possible causes of this finding). We have considered the following explanations for this finding.

Firstly, lactate and proton equivalents enter the fibre relatively slowly so that regulation of pH_i has time to occur. Juel & Wibrand (1989) measured the pH_i change in mouse soleus when lactate (30 mM, pH_o 6·2) was applied and it occurred with a half-time of about 2 min, which would be slow enough to allow time for regulation.

Secondly, the lactate and protons which enter the fibre are consumed in metabolic pathways. There is evidence that intracellular lactic acid can be both converted to glycogen and consumed by the citric acid cycle at an overall rate of around 0·5 mM min^{-1} (Bangsbo, Gollnick, Graham & Saltin, 1991).

Thirdly, the protons which enter the fibre with lactate are extruded by another pathway. To assess this possibility we inhibited the main proton extrusion pathways. Figure 11 shows that the inhibition of the Na^+–H^+ exchanger led to a somewhat larger ΔpH_i suggesting that some of the protons which enter the fibre are extruded by Na^+–H^+ exchange.

In summary, we consider the buffer power obtained with TMA to be the most reliable. The mechanism(s) underlying the very large apparent buffering with lactate application remains obscure. However, these uncertainties are unlikely to affect our main conclusions regarding the role of pH_i in fatigue.

Conclusions

The acidosis during fatigue in single mouse muscle fibres is very small mainly because of an effective extrusion of H^+ by the lactate transporter. Thus neither the tension decline nor the slowing of relaxation in fatigue can be explained by an intracellular acidification. If the lactate transporter is inhibited, pH_i becomes more acid during fatiguing stimulation and acidosis is then likely to contribute to both the tension reduction and the slowed relaxation. Thus with the present preparation it is possible to produce fatigue under conditions where there is either no change in pH_i or a change similar to that which occurs in intact muscles. This ability has allowed us to identify with greater precision the contribution of pH_i change to fatigue.

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