# The effect of intracellular pH on contractile function of intact, single fibres of mouse muscle declines with increasing temperature

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1. The effect of altered intracellular pH ($pH_i$) on isometric contractions and shortening velocity at 12, 22 and 32 °C was studied in intact, single fibres of mouse skeletal muscle. Changes in $pH_i$ were obtained by exposing fibres to solutions with different $CO_2$ concentrations.

2. Under control conditions (5% $CO_2$), $pH_i$ (measured with carboxy SNARF-1) was about 0·3 pH units more alkaline than neutral water at each temperature. An acidification of about 0·5 pH units was produced by 30% $CO_2$ and an alkalinization of similar size by 0% $CO_2$.

3. In acidified fibres tetanic force was reduced by 28% at 12 °C but only by 10% at 32 °C. The force increase with alkalinization showed a similar reduction with increasing temperature. Acidification caused a marked slowing of relaxation and this slowing became less with increasing temperature.

4. Acidification reduced the maximum shortening velocity ($V_0$) by almost 20% at 12 °C, but had no significant effect at 32 °C. Alkalinization had no significant effect on $V_0$ at any temperature.

5. In conclusion, the effect of $pH_i$ on contraction of mammalian muscle declines markedly with increasing temperature. Thus, the direct inhibition of force production by acidification is not a major factor in muscle fatigue at physiological temperatures.

Changes in the intracellular pH ($pH_i$) are generally believed to have a large impact on the function of skeletal muscle. For instance, a reduction of $pH_i$ has repeatedly been shown to reduce the isometric force, shortening velocity and relaxation speed of muscle fibres (e.g. Chase & Kushmerick, 1988; Cooke, Franks, Luciani & Pate, 1988; Westerblad & Allen, 1992). Acidosis also inhibits key enzymes of the energy metabolism of the fibres (e.g. phosphofructokinase; Trivedi & Danforth, 1966) and may therefore indirectly impair muscle function. During strenuous activity of skeletal muscle, $pH_i$ often declines due to accumulation of lactic acid and hence lactacidosis is considered to be an important cause of fatigue, that is, the impairment of muscle function observed during strenuous activity (for recent reviews see Fitts, 1994; Allen, Lännergren & Westerblad, 1995). For instance, studies of human fatigue using nuclear magnetic resonance have shown a good temporal correlation between the decline of muscle pH and the reduction of force (e.g. Miller, Boska, Moussavi, Carson & Weiner, 1988; Cady, Jones, Lynn & Newham, 1989). However, during recovery after human fatigue, force has been found to recover more rapidly than muscle pH, which indicates that the reduction of pH is not the direct cause of the force reduction (Miller *et al.* 1988; Sahlin & Ren, 1989).

Studies of the effect of pH on the contractile function of isolated mammalian muscle fibres have generally been performed at or below room temperature, which is considerably lower than the temperature experienced by the fibres *in situ*. In a recent study employing skinned muscle fibres from the rat, Pate and co-workers showed that the depressive effect of lowered pH on isometric force and shortening velocity is much smaller at 30 °C than at 10 °C (Pate, Bhimani, Franks-Skiba & Cooke, 1995). We have now studied the effects of altered $pH_i$ on isometric contractions and shortening velocity in single, intact fibres of mouse muscle at 12, 22 and 32 °C. The study provides the first measurements of shortening velocity in single intact fibres of mammalian muscle. $pH_i$, measured with the fluorescent pH indicator carboxy SNARF-1, was altered by exposing fibres to solutions with different $CO_2$ concentrations. Under our control conditions (bath solution bubbled with 5% $CO_2$), $pH_i$ was about 7·5 at 12 °C and fell by about 0·15 pH units as the temperature was increased by 10 °C. The results show that altered $pH_i$ has the largest effects on contractile function at 12 °C. At 22 °C the effects of $pH_i$ alterations were intermediate and the smallest effects were observed at 32 °C. Thus, the present results agree with those of Pate *et al.* (1995) and show that pH has little direct influence on the contractile function of mammalian muscle at physiological temperatures.

# METHODS

### Fibre dissection and mounting

Male mice (NMRI) were killed by rapid neck disarticulation and single fibres were dissected from the flexor brevis muscle of the hindlimb as described previously (Lännergren & Westerblad, 1987). This muscle contains about 90% fast-twitch (type IIX or IIA) fibres and the remaining 10% are slow-twitch (type I) fibres (Allen, Duty & Westerblad, 1993). We always used large fibres dissected from the surface of the muscle, which would belong to the fast-twitch group. The isolated fibre was mounted between an Akers 801 force transducer and the moveable arm of a galvanometer (G120DT; General Scanning, Watertown, MA, USA). The position of the galvanometer arm and the signal from the force transducer were displayed on a strip-chart recorder and stored in a personal computer. Measurements of the force and shortening velocity were made from data stored in the computer. The length of the fibre was adjusted to the length giving maximum tetanic force. At this length, sarcomere lengths, measured at the centre of fibres with an ocular scale, ranged from 2·3 to 2·4 $\mu$m and the total fibre lengths ($L_0$) ranged from 0·53 to 0·95 mm.

### Solutions

Under control conditions, the fibre was superfused with a Tyrode solution of the following composition (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 5% $CO_2$–95% $O_2$ giving a pH of 7·5, 7·4 and 7·3 at 12, 22 and 32 °C, respectively. An intracellular acidification was produced by bubbling the solution with 30% $CO_2$–70% $O_2$, which gave a solution pH of 6·7, 6·6 and 6·7 at 12, 22 and 32 °C, respectively. An alkalinization was produced by superfusing the fibre with the following solution (mM): NaCl, 136·5; KCl, 5·0; $CaCl_2$, 1·8; $MgCl_2$, 0·5; $NaH_2PO_4$, 0·4; $NaHCO_3$, 11·9; glucose, 5·5. This latter solution, which was also used during fibre dissection, was not bubbled and had a pH of 7·9, 7·8 and 7·8 at 12, 22 and 32 °C, respectively; for simplicity, this solution will be referred to as the 0% $CO_2$ solution. Fetal calf serum (about 0·2%; Gibco) was added to the above solutions to help maintain the viability of the fibres (Lännergren & Westerblad, 1987). The temperature was continuously monitored close to the fibre and controlled by passing the solutions through a Peltier heat exchanger which allowed both heating and cooling. During experiments the temperature was kept constant (±0·2 °C) at 12, 22 or 32 °C. The highest temperature used (32 °C) is some degrees lower than the expected temperature of the flexor brevis muscle *in situ*. However, we did not use a higher temperature because 32 °C is the highest temperature at which the present preparation can produce repeated contractions without a decline in force (Lännergren & Westerblad, 1987). Furthermore, by changing the temperature in steps of 10 °C, the $Q_{10}$ (relative change for a 10 °C change of temperature) of various processes can be directly assessed.

In $pH_i$ calibration experiments, fibres were exposed to a solution with a potassium concentration similar to the intracellular concentration (mM): KCl, 175; $MgCl_2$, 0·5; $KH_2PO_4$, 1·2; pH buffer, 10. To make $pH_i$ equal to the extracellular pH, 10 $\mu$M of the proton and potassium ionophore nigericin was added to the solution (Thomas, Buchsbaum, Zimniak & Racker, 1979). The pH of the solution at 22 °C was set (by addition of HCl or KOH) between pH 6·5 and 8 in steps of 0·5 pH units. The pH buffers used were Mes at 6·5, Hepes at 7·0–7·5 and Taps at 8·0 (Good & Izawa, 1972). The pH of these solutions can be expected to change with temperature and we therefore also measured their pH at 12 and 32 °C; the measured changes in pH with temperature (see Fig. 1*A*) agreed within 0·1 pH units with published values of the temperature dependence of the buffers (Good & Izawa, 1972).

### Stimulation

Fibres were stimulated with biphasic current pulses (total duration, 1 ms) delivered via platinum plate electrodes lying parallel to the long axis of the fibre. The amplitude of the current pulses was adjusted to about 120% of the contraction threshold. The stimulation frequency at each temperature was set so that maximum tetanic force was obtained: 30 Hz at 12 °C, 100 Hz at 22 °C and 160 Hz at 32 °C. The tetanus duration was 350 ms at 22 and 32 °C. Force increased more slowly at 12 °C and longer tetanus durations of 450–550 ms were therefore used at this temperature. Fibres were allowed to rest for at least 1 min between tetanic contractions.

### Force and shortening velocity measurements

Tetanic force under isometric conditions was measured as the maximum force during the tetanus. Changes in the rate of relaxation were assessed by measuring $1/t_{70}$, where $t_{70}$ is the time from the last stimulus pulse until force had declined to 70% of the force at the end of the tetanus. This measure of the rate of relaxation is dominated by the initial, linear phase of relaxation, where fibres have been shown to relax isometrically (Huxley & Simmons, 1970).

Fibre shortening during tetanic stimulation was achieved by changing the current passing through the galvanometer coil which moved the galvanometer arm. The galvanometer coil current was controlled by a ramp generator so that the arm could be moved at various velocities. With the fastest ramp, a 200 $\mu$m step was completed within 1·2 ms.

The force–velocity relationship was assessed by producing ramp releases at different velocities and measuring the force during these ramps; the ramp velocities were set so that forces during the ramps ranged between about 5 and 80% of the isometric force. Each ramp was preceded by a small, rapid shortening step to discharge the series elasticity of the fibre (see upper panel of Fig. 4*A*). Releases frequently resulted in a minor reduction of the resting tension and to deal with this, force during releases was always measured relative to the resting level after the tetanus.

The shortening velocity at zero load ($V_0$) was obtained with slack tests (Lännergren, 1978; Edman, 1979). Rapid releases of at least four different amplitudes were then given in subsequent tetani and the time to take up the slack was measured. The release amplitudes were plotted against the time to take up the slack. Linear regression was then used to draw a straight line through the data points and $V_0$ was obtained by dividing the slope of this line by $L_0$. The exact starting point of force redevelopment was often difficult to appraise (see Fig. 5*B*) and at the higher temperatures (22 and 32 °C), where the shortening velocity was fast, even small errors in measurements of the starting point can have a relatively large impact on the estimate of $V_0$. To obtain an objective measure we therefore fitted a single exponential function to the initial 40 ms of force redevelopment (starting at a point at which force was clearly above the baseline) and the time to take up the slack was obtained by extrapolation to zero force (i.e. the force level after the tetanus; see above). The curve fit was generally very good at 22 and 32 °C (see Fig. 5*B*), which indicates that the precision in our measurements of the time to take up the slack is adequate. At 12 °C the curve fits were less good, especially at large shortening steps where the amplitude of the force redevelopment was small. However, at 12 °C the time to take up the slack was relatively long and a small error in the measurement will have little impact on the estimate of $V_0$.

### Measurements of intracellular pH

$pH_i$ was measured with the fluorescent pH indicator carboxy SNARF®-1 (Molecular Probes Europe, Leiden, The Netherlands) in a separate series of experiments. In these experiments we gave priority to performing as many measurements of $pH_i$ as possible from each preparation and hence no systematic testing of the force response at different $pH_i$ was performed. Fibres were incubated in 10 $\mu$M of the membrane-permeable acetoxymethyl ester form of the dye for 10 min. Before the onset of $pH_i$ measurements, fibres were allowed to rest for about 30 min and were then stimulated to check that normal tetanic contractions could be obtained; fibres which did not produce healthy contractions were discarded. After the rest period, we first measured the $pH_i$ response to altered $CO_2$ at the three different temperatures and thereafter followed measurements for the intracellular calibration.

The fluorescence of carboxy SNARF-1 was measured with a system consisting of a xenon lamp, a monochromator and two photomultiplier tubes (PTI; Photo Med GmbH, Wedel, Germany). The excitation light was set to 540 ± 5 nm and the light emitted at 580 ± 5 and 640 ± 5 nm was measured. The $pH_i$-dependent signal was obtained as the ratio of the signal at 580 nm to that at 640 nm. Intracellular calibration experiments were performed in order to translate fluorescence ratios into $pH_i$ values. Fibres were then exposed to a high-$K^+$ solution (see above), which resulted in a brief contracture. Nigericin (10 $\mu$M) was added to the solution when the contracture had ended. Fibres were then exposed to solutions with a pH ranging from about 6·5 to 8 at 12, 22 and 32 °C (see above). To achieve a fast solution exchange in the calibration experiments, the perfusion chamber was drained with a syringe as soon as the new solution arrived and the chamber was then rapidly filled with the new solution. The fluorescence light declined with time during the calibration experiments and changes in pH or temperature were therefore performed as soon as a stable ratio was obtained in each solution. It was generally possible to obtain at least six measurements in one fibre before the signals had declined to a level at which measurements became uncertain. In some experiments we exposed fibres to a solution with the pH set to 8·5 (Taps used as pH buffer), but this resulted in a very fast decline of the fluorescence signals, which made measurements unreliable.

It has been shown that thorough washing is required to remove all nigericin from the superfusion system and that residual nigericin may affect $pH_i$ measurements (Richmond & Vaughan-Jones, 1993). We carefully cleaned our superfusion system with distilled water at the end of each experiment. Under our experimental conditions no further washing seemed to be required, because measurements in intact fibres were not systematically altered during the series of experiments.

At each temperature studied, the following equation was fitted to mean values of ratios at different pH:

$$pH_i = pK_{app} - \log((R - R_{min})/(R_{max} - R)), \quad (1)$$

where $R_{min}$ is the ratio at high pH (where the dye is in the non-protonated form) and $R_{max}$ is the ratio at low pH (where the dye is in the protonated form). The apparent dissociation constant of carboxy SNARF-1 ($K_{app}$) is the real dissociation constant ($K_a$) multiplied by $S_{f2}/S_{b2}$, where $S_{f2}$ and $S_{b2}$ represent the 640 nm signal when the dye is in non-protonated and protonated form, respectively. This means that $pK_a$ (−log of dissociation constant) equals $pK_{app}$ plus $\log(S_{f2}/S_{b2})$. No direct calibration experiments were performed to establish $S_{f2}/S_{b2}$, but while the 580 nm signal showed a marked pH dependence, changes in the 640 nm signal were small and hence the difference between $pK_a$ and $pK_{app}$ will be small (cf. Blank *et al.* 1992). An estimate of $S_{f2}/S_{b2}$ was obtained by measuring the 640 nm signal in experiments where the extracellular $CO_2$ concentration was directly changed from 0 to 30% or vice versa; the loss of total fluorescence from the dye was minimal during this manoeuvre while a similar change in pH during the calibration experiments was associated with some loss of fluorescence which affected measurement of the 640 nm signal. Changing between 0 and 30% $CO_2$ resulted in a change in $pH_i$ of about 1 pH unit in the region where the ratio to pH relationship is rather steep (see Fig. 1). The ratio of the 640 nm signal in 0% $CO_2$ (i.e. the dye mainly in the non-protonated form) to that in 30% $CO_2$ (i.e. mainly protonated form) ranged between 1·07 and 1·20 ($n = 7$). This result indicates that $S_{f2}/S_{b2}$ is only about 1·3 or less and $\log(S_{f2}/S_{b2})$ would then be about 0·1.

### Statistics

Values are presented as means ± S.E.M. or as a range. Statistical significance was determined with Student's paired $t$ tests or one-way repeated measures ANOVA followed by Dunnett's test for multiple comparisons (SigmaStat, Jandel, San Rafael, CA, USA). The significance level was set at 0·05 throughout.

## RESULTS

### Measurements of $pH_i$

Figure 1*A* shows collected data from experiments where carboxy SNARF-1 was calibrated using the nigericin method. This plot clearly shows that calibrations have to be performed at the relevant temperature, because the fluorescence ratios obtained were clearly different at the different temperatures. The main difference is that the fluorescence ratio obtained at a given $pH_i$ fell markedly with increasing temperature. Curve fitting of eqn (1) to mean values gave a good fit at all three temperatures. However, it must be noted that the pH range of the calibration solutions was rather limited and, especially, the estimate of $R_{min}$ is uncertain because the highest pH of the calibration solutions was about 8 (solutions with a higher pH gave a rapid loss of fluorescence signal; see Methods). Nevertheless, it seems clear that the dynamic range increased with increasing temperature: $R_{max}/R_{min}$ was 1·76 (3·88/2·21) at 12 °C and 3·26 (3·62/1·11) at 32 °C.

Estimates of $pK_{app}$ were also uncertain mainly due to the fact that no measurements were performed above ~pH 8. The values of $pK_{app}$ were similar at the different temperatures: 7·8 at 12 °C and 7·6 at 22 and 32 °C. Since $\log(S_{f2}/S_{b2})$ was estimated to about 0·1 (see Methods), $pK_a$ ($pK_{app} + \log(S_{f2}/S_{b2})$) would be 7·5 to 7·7, which is in good agreement with previous estimates obtained *in vitro* and intracellularly in cardiac myocytes (Buckler & Vaughan-Jones, 1990; Blank *et al.* 1992).

Figure 1*B* shows representative $pH_i$ records from one fibre which was first exposed to 5% $CO_2$, then 0% $CO_2$, and finally 30% $CO_2$. After solution changes, it took 1–2 min for the $pH_i$ to reach a new level where it remained stable until the solution (or temperature) was changed. Mean results of $pH_i$ measurements performed during periods of stable $pH_i$

in the different solutions and at the different temperatures are shown in Fig. 1*C*. It can be seen that $pH_i$ in the three solutions fell in a similar way with increasing temperature and $pH_i$ was about 0·34 to 0·4 pH units lower at 32 °C compared with 12 °C. It is worth noting that this decline in $pH_i$ with temperature closely followed the neutrality of water ($[H^+] = [OH^-]$) at different temperatures (curve in Fig. 1*C*). At 12 °C, $pH_i$ was 7·90 ± 0·05, 7·47 ± 0·07 and 6·97 ± 0·14 in 0, 5 and 30% $CO_2$, respectively. The corresponding $pH_i$ values at 22 °C were 7·81 ± 0·03, 7·38 ± 0·04 and 6·84 ± 0·07 and at 32 °C, 7·49 ± 0·02, 7·17 ± 0·05 and 6·67 ± 0·12.

### Isometric force and relaxation

Figure 2 shows original records from one fibre exposed to the control (5% $CO_2$), acidic (30% $CO_2$) and alkaline (0% $CO_2$) solutions at 12, 22 and 32 °C. It can be seen that the effect of altered $pH_i$ on tetanic force became smaller as the temperature was increased. Similar results were obtained in an additional three fibres and a summary of the results from all four fibres is shown in Fig. 3. Tetanic force increased by about 60% when the temperature was increased from 12 to 22 °C under control conditions, whereas the increase when the temperature was raised from 22 to 32 °C was only about 10% (Fig. 3*A*). While alkalinization gave the largest and acidification the smallest tetanic force at all three temperatures, the effect of altered pH clearly became less with increasing temperature (Fig. 3*B*). However, tetanic force both with alkalinization and acidification was still significantly different from the control at 32 °C.

Under control conditions, the $Q_{10}$ for the rate of relaxation was 3·3 when the temperature was increased from 12 to 22 °C and 2·5 when it was increased from 22 to 32 °C (Fig. 3*C*). The pH dependence of the relaxation rate showed a more complex pattern than that of tetanic force. The pH optimum of relaxation speed seemed to be close to the $pH_i$ obtained in control and the relaxation rate declined both in the alkaline and acidic direction, although the decline with alkalinization was only significant at 22 °C. Figure 3*D* shows that the reduction of the relaxation rate under acidic conditions became smaller with increasing temperature, but at 32 °C it was still about 25% lower than the rate under control conditions.

### Force–velocity relationship

The effect of altered pH on the force–velocity relationship was only studied at 22 °C. Figure 4*A* shows a series of contractions with releases at different ramp speeds produced under control conditions. Force–velocity data points from this series are plotted in Fig. 4*B* together with points obtained in the same way with alkalinization and acidification. It can be seen that the difference between the data points obtained at different $pH_i$ is relatively small but

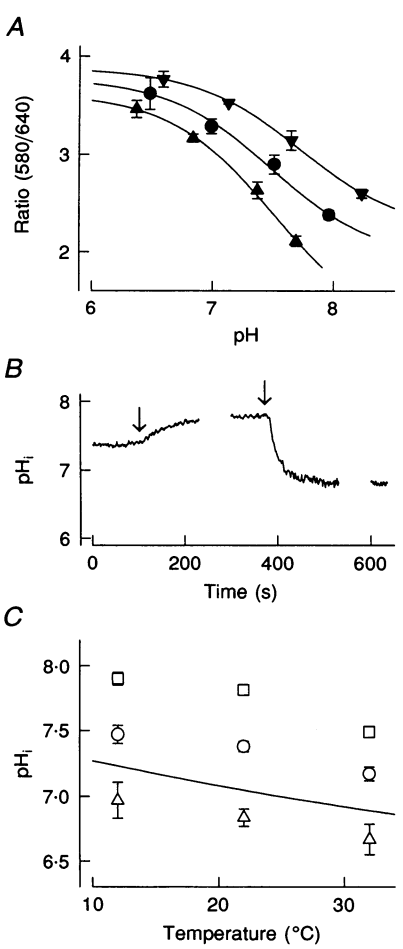


**Figure 1. Measurements of $pH_i$ at different temperatures**

*A*, intracellular calibration of carboxy SNARF-1 with the nigericin method at 12 °C (▼), 22 °C (●) and 32 °C (▲). Each value is the mean ± S.E.M. of 3–5 measurements. The curves were drawn by fitting eqn (1) to mean data points of the 580/640 nm fluorescence ratio *vs.* pH. *B*, original $pH_i$ records from one fibre studied at 22 °C. Downward arrows indicate solution change from 5 to 0% $CO_2$ and from 0 to 30% $CO_2$, respectively. The excitation light was switched off at the interruptions to minimize bleaching of dye. *C*, measurements of $pH_i$ at 12, 22 and 32 °C in intact fibres bathed in Tyrode solution with 0% $CO_2$ (□; alkalinization), 5% $CO_2$ (○; control) and 30% $CO_2$ (△; acidification). Each data point represents the mean ± S.E.M. of 5–7 measurements. The line shows the temperature dependence of neutral water.

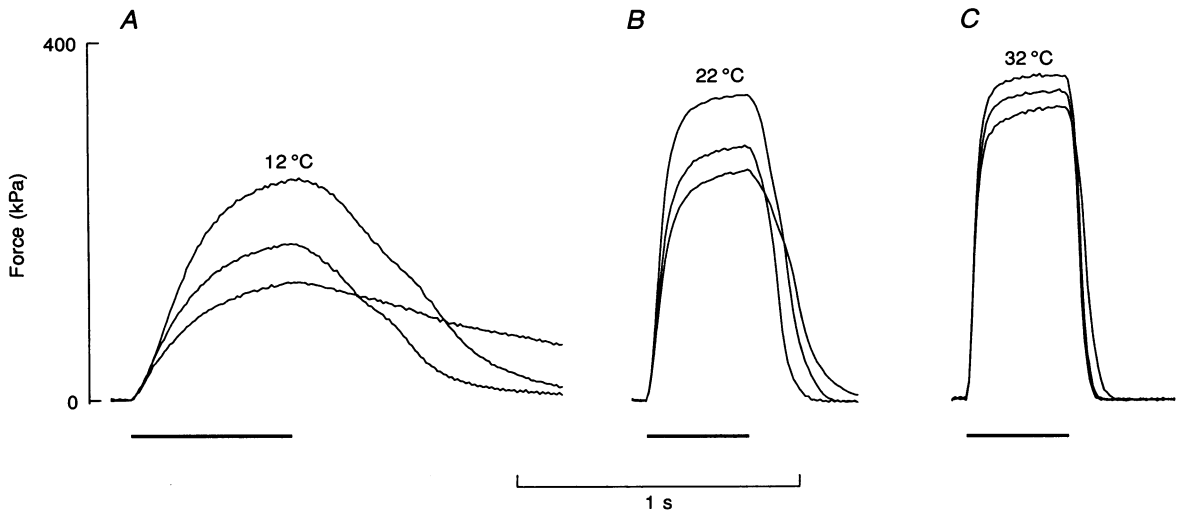


**Figure 2. The effect of altered $pH_i$ on isometric tetani is less at higher temperatures**
Tetanic contractions of one fibre at 12 °C (*A*), 22 °C (*B*) and 32 °C (*C*) with alkalinization (0% $CO_2$), in control (5% $CO_2$) and with acidification (30% $CO_2$). Tetani with highest and lowest force were obtained with alkalinization and acidification, respectively, at all temperatures. Periods of stimulation are shown below the force records.

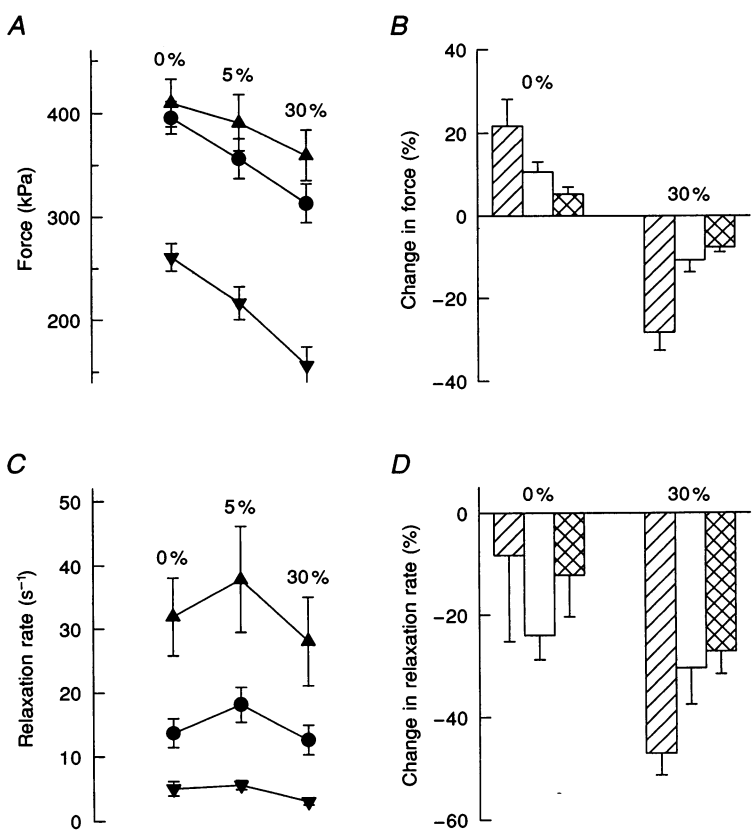


**Figure 3. The effects of altered $pH_i$ on force and relaxation at different temperatures**
Mean values (± S.E.M.) of tetanic force and rate of relaxation obtained in four fibres. *A*, tetanic force produced at 12 °C (▼), 22 °C (●) and 32 °C (▲) with alkalinization (0% $CO_2$), in control (5% $CO_2$) and with acidification (30% $CO_2$). *B*, the relative change in force in each fibre with alkalinization (0% $CO_2$) and acidification (30% $CO_2$) compared with control. ▨, 12 °C; □, 22 °C; ▩, 32 °C. *C*, the rate of relaxation ($1/t_{70}$) at different temperatures and $pH_i$; format as in *A*. *D*, the relative change in the relaxation rate; format as in *B*.

**Table 1. The effect of $pH_i$ on the force–velocity relationship**

| | $V_{max}$ ($L_0$ s$^{-1}$) | $V_0$ ($L_0$ s$^{-1}$) | $P_0$* (%) | $a/P_0$* |
|---|---|---|---|---|
| Control | 6·73 ± 0·87 | 6·95 ± 0·74 | 115 ± 2 | 0·182 ± 0·012 |
| Alkalinization | 6·81 ± 0·78 | 6·77 ± 0·75 | 111 ± 3 | 0·185 ± 0·027 |
| Acidification | 5·73 ± 0·91 | 6·38 ± 0·73 | 110 ± 3 | 0·254 ± 0·023 |

Data (means ± S.E.M.) from five experiments with ramp releases at 22 °C. Control, 5% $CO_2$; alkalinization, 0% $CO_2$; acidification, 30% $CO_2$. The shortening velocity at zero load was obtained from force–velocity curves ($V_{max}$) and slack tests ($V_0$). $P_0$*, the force at zero velocity obtained by extrapolation of force–velocity curves, is given as a percentage of the respective isometric force. $a/P_0$* describes the curvature of the force–velocity relationship.

that there is a clear trend for points under alkaline conditions to lie to the right and points under acidic conditions to the left of the control points. The curves in Fig. 4*B* were drawn by fitting the following hyperbolic function to each set of data points:

$$P = \frac{b(P_0^* + a)}{(V + b)} - a, \quad (2)$$

where $P$ and $V$ are the measured force and velocity, respectively, $P_0$* is the extrapolated force at zero velocity, and $a$ and $b$ are constants. Compared with the control curve, the curve obtained with acidification shows a reduction of both $P_0$* and the shortening velocity at zero load ($V_{max}$), whereas the curve obtained with alkalinization only shows a small increase in $P_0$*. In Fig. 4*C* the curves have been replotted with $P_0$* and $V_{max}$ obtained at each $pH_i$ set to 100%. With this method of plotting it can be seen that the force–velocity relationship is less curved with acidification (dotted line) than in control (continuous line) or with alkalinization (dashed line).

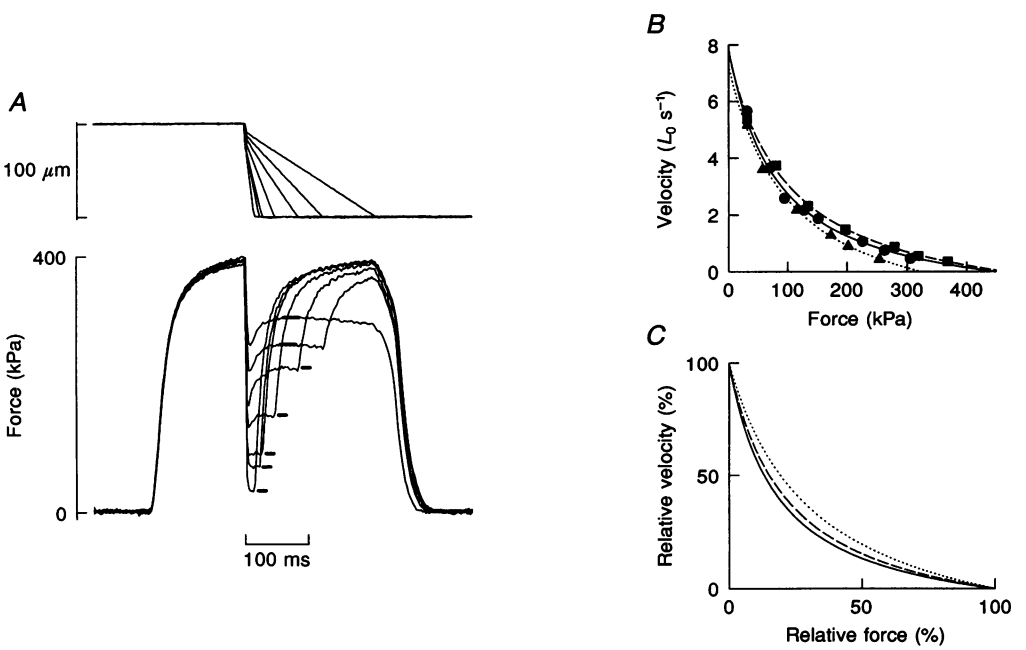


**Figure 4. The force–velocity relationship at different $pH_i$**

*A*, original records of length changes (upper panel) and force (lower panel) in consecutive tetani with ramp releases. Experiment performed at 22 °C under control conditions (i.e. 5% $CO_2$). Note that each ramp shortening is preceded by a short, rapid release which was introduced to discharge the series elasticity of the fibre. *B*, plot of shortening velocity *vs.* force data points obtained in *A* (●, measurements indicated by thick, horizontal lines in *A*) and in the same fibre with alkalinization (■) and acidification (▲). Curves were drawn by fitting eqn (2) to the data points. Dashed line, alkalinization; continuous line, control; dotted line, acidification. *C*, the force–velocity curves in *B* have been redrawn with the maximum force and velocity of each curve set to 100%. Note the markedly reduced curvature with acidification.

Some important properties of the force–velocity relationships are summarized in Table 1. $V_{max}$ was similar in control and with alkalinization but was significantly reduced with acidification. Values of $P_0$* in Table 1 are expressed relative to the maximum isometric force at each $pH_i$, and $P_0$* is then 10–15% higher than the isometric force under all three conditions.

$a/P_0$* is a measure of the curvature of the force–velocity relationship and an increase in this ratio reflects a less curved relationship (e.g. Woledge, Curtin & Homsher, 1985). $a/P_0$* was similar in control and with alkalinization whereas it was significantly higher with acidification, and thus the relationship is more curved in control and with alkalinization than with acidification. Table 1 also gives values of $V_0$ obtained with slack tests in the same fibres. A comparison of $V_{max}$ and $V_0$ reveals that these are similar in control and with alkalinization, but with acidification $V_{max}$ is significantly lower than $V_0$.

### Maximum shortening velocity

$V_0$ was measured with slack tests in control, with alkalinization and with acidification at all three temperatures in four fibres (same fibres as in Fig. 3). Figure 5 shows results from one experiment performed at 22 °C under control conditions. Original length and force records are displayed on a slow (Fig. 5*A*) and fast (Fig. 5*B*) time scale. Figure 5*B* shows the fitted exponential functions (dotted lines) used to establish the time at which force redevelopment started. It can be seen that the fit was good at all four shortening steps. Figure 5*B* also illustrates that estimates of the time at which force redevelopment started made directly from the force records will be rather uncertain. In Fig. 5*C*, data points from the four releases have been plotted together with their regression line, the slope of which is used to calculate $V_0$. The series elasticity of fibres is reflected by the intercept on the shortening axis. The series elasticity showed little variation between fibres and the mean value was about 30 μm, which is 5% or less of $L_0$; neither $pH_i$ nor temperature had a significant effect on the series elasticity.

Measurements of $V_0$ in the four fibres are summarized in the upper panels of Fig. 6. $V_0$ showed a marked increase with increasing temperature (Fig. 6*A*) and, under control conditions, $Q_{10}$ was 2·73 ± 0·26 between 12 and 22 °C and 1·80 ± 0·21 between 22 and 32 °C. There was no significant difference between $V_0$ in control and with alkalinization at any temperature. Acidification, on the other hand, significantly reduced $V_0$ by 18% at 12 °C and by 10% at 22 °C, whereas no significant change was observed at 32 °C (Fig. 6*B*). Thus, the depressive effect of acidification on $V_0$ declined with increasing temperature.

The exponential fits of the first part of force redevelopment were mainly used to establish the time to take up the slack. However, the rate of force redevelopment after a shortening step ($k_{tr}$) can also be used to assess cross-bridge kinetics (e.g. Brenner, 1988). The lower panels of Fig. 6 show mean data from measurements of $k_{tr}$ after the smallest shortening steps (40–60 μm) at 22 and 32 °C; at 12 °C the curve fit was less good, making measurements of $k_{tr}$ uncertain. $k_{tr}$ showed an increase with temperature similar to that of $V_0$,

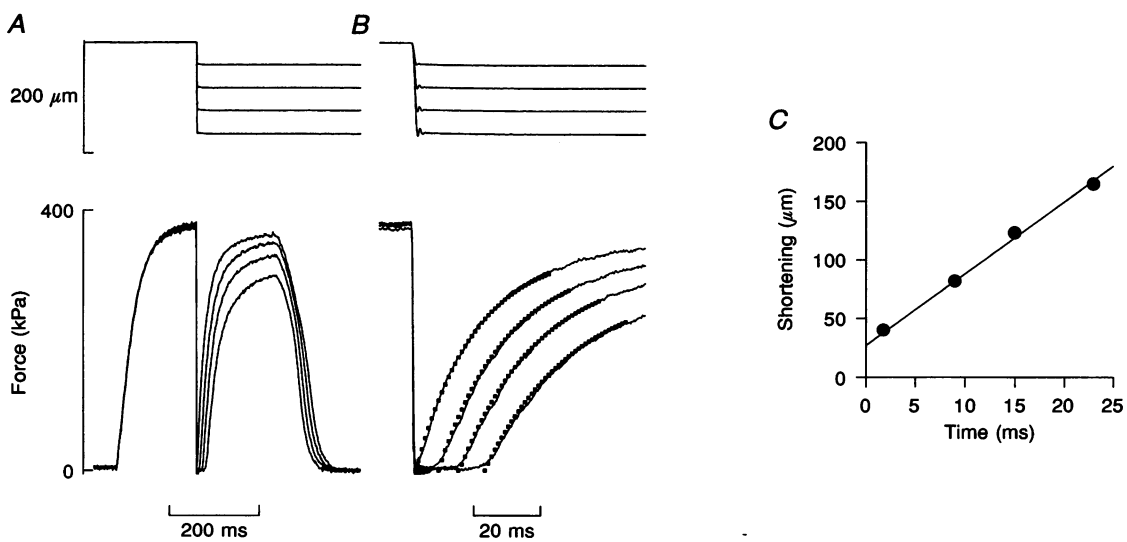


**Figure 5. The shortening velocity at zero load ($V_0$) measured by slack test**

*A*, original records of length changes (upper panel) and force (lower panel) in four consecutive tetani with rapid releases which make the fibre slack; experiment performed at 22 °C. *B*, part of the records in *A* on a faster time scale. The initial 40 ms of force redevelopment was fitted by a single exponential function (dotted lines); the first dot of each fitted curve is at zero force and is used in measurements of the time to take up the slack. *C*, the amplitude of the shortening step is plotted against the time to take up the slack. Data points were fitted by a straight line and the slope of this line gives $V_0$.

with a $Q_{10}$, measured under control conditions, of 1·98 ± 0·33 (Fig. 6*C*). $k_{tr}$ declined when the length of the shortening step was increased; for instance, $k_{tr}$ was reduced by about 20% at 22 °C and 25% at 32 °C after 100–120 μm releases compared with 40–60 μm releases. Changes in $pH_i$ had no significant effect on $k_{tr}$ (Fig. 6*D*).

## DISCUSSION

### Measurements of $pH_i$

The change in $pH_i$ due to alterations of the bath $CO_2$ has been measured previously at 22 °C with another fluorescent pH indicator, BCECF (2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein), in fibres from the same muscle and under the same experimental conditions (Westerblad & Allen, 1992). Under control conditions (5% $CO_2$), Westerblad & Allen reported a mean $pH_i$ of 7·33, similar to the mean value of 7·38 obtained with carboxy SNARF-1 in the present study. $pH_i$ with alkalinization (0% $CO_2$) and acidification (30% $CO_2$) are also similar in the two studies: 7·9 and 6·8 in the study of Westerblad & Allen (1992) and 7·8 and 6·8 in the present study. Thus, BCECF and carboxy SNARF-1 give very similar values of $pH_i$ under the present experimental conditions.

The present values of $pH_i$ agree closely with those obtained with pH-sensitive microelectrodes in mouse soleus muscles bathed in bicarbonate-buffered solution bubbled with 5% $CO_2$ (Aickin & Thomas, 1977). These authors also observed a decline in $pH_i$ with increased temperature following the neutrality of water at different temperatures. At 37 °C Aickin & Thomas reported a mean value of $pH_i$ of 7·07, which is similar to the value we obtained by extrapolation taking the mean value at 32 °C (7·17) and a decline in $pH_i$ of about 0·15 pH units per 10 °C (see Fig. 1).

### Effects of $pH_i$ on tetanic force and relaxation at different temperatures

Tetanic force is the only parameter which showed a monotonic dependence on $pH_i$ (i.e. increase with alkalinization and decrease with acidification) at all three temperatures. The effect of altered $pH_i$ was markedly reduced as the temperature was increased, which agrees with the findings

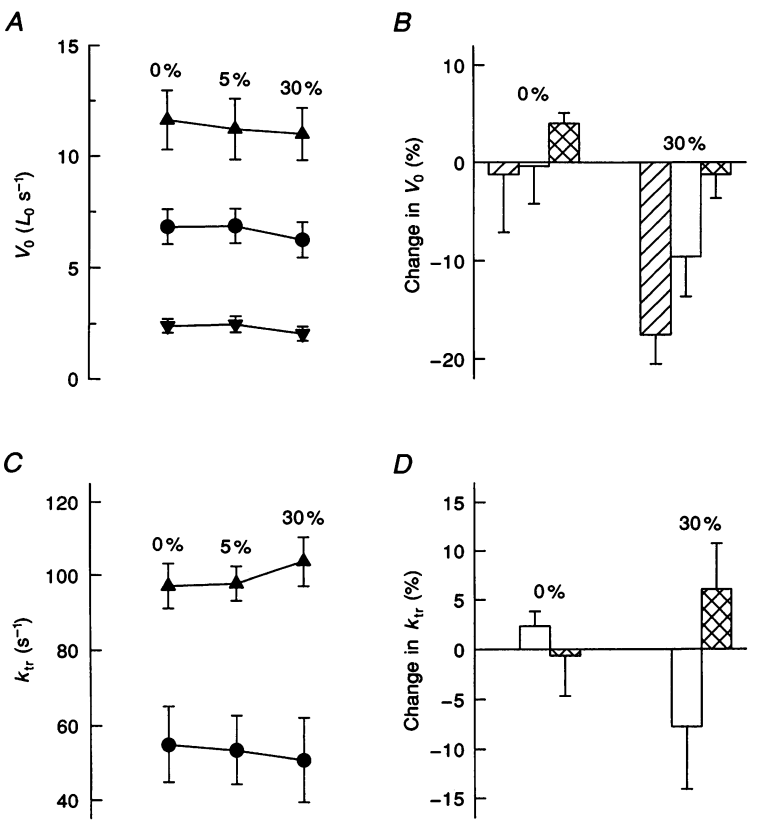


**Figure 6. The effect of $pH_i$ on maximum shortening velocity and rate of force redevelopment**

Mean values (± S.E.M.) of the maximum shortening velocity obtained with slack tests ($V_0$) and the rate of force redevelopment after rapid releases ($k_{tr}$) obtained in the same four fibres as in Fig. 3. *A*, $V_0$ at 12 °C (▼), 22 °C (●) and 32 °C (▲) with alkalinization (0% $CO_2$), in control (5% $CO_2$) and with acidification (30% $CO_2$). *B*, the relative change in $V_0$ in each fibre with alkalinization (0% $CO_2$) and acidification (30% $CO_2$) compared with control. ▨, 12 °C; □, 22 °C; ▩, 32 °C. *C*, $k_{tr}$ at different temperatures and $pH_i$; format as in *A*. *D*, relative change in $k_{tr}$; format as in *B*. Note that no data for $k_{tr}$ are given at 12 °C.

of Pate *et al.* (1995) in skinned fibres. It also agrees with previous results from whole mammalian muscles where an acidification due to increased $CO_2$ resulted in a marked decline in tetanic force at 19 °C (Sahlin, Edström & Sjöholm, 1983) but no decline at 37 °C (Adams, Fisher & Meyer, 1991). The mechanism by which the pH effect on isometric force is reduced with increased temperature is unclear but Pate *et al.* (1995) suggested that it may involve the equilibrium between the myosin–ATP and the myosin–ADP–$P_i$ (inorganic phosphate) states of the cross-bridge cycle.

The rate of relaxation depends on several processes including the rate of $Ca^{2+}$ removal from the myoplasm, the myofibrillar $Ca^{2+}$ sensitivity, and the rate of cross-bridge detachment when the free $[Ca^{2+}]$ in the myoplasm ($[Ca^{2+}]_i$) is low (see Westerblad & Allen, 1993). For instance, a reduction of $pH_i$ will tend to reduce the relaxation speed by reducing the rate of $Ca^{2+}$ removal from the myoplasm (Westerblad & Allen, 1993; Baker, Brandes & Weiner, 1995) and possibly the rate of cross-bridge detachment (see below). On the other hand, a reduction of $pH_i$ decreases the myofibrillar $Ca^{2+}$ sensitivity (e.g. Donaldson & Hermansen, 1978; Metzger & Moss, 1990; Westerblad & Allen, 1993), which will tend to increase the relaxation speed. This relatively complex situation may explain why the relaxation rate was reduced both with alkalinization and acidification (Fig. 3).

At 22 °C alkalinization has previously been shown to slow relaxation in fibres from the same muscle as that used in the present study and this slowing was attributed to an increased myofibrillar $Ca^{2+}$ sensitivity (Westerblad & Allen, 1993). Although we have no data on how temperature affects the rate of $Ca^{2+}$ removal from the myoplasm or the myofibrillar $Ca^{2+}$ sensitivity with alkalinization, it seems likely that the tendency of a reduced relaxation speed with alkalinization at 12 and 32 °C has the same underlying mechanism as that at 22 °C.

Acidification significantly reduced the rate of relaxation at all temperatures but the effect became smaller with increasing temperature (Fig. 3*D*). The finding that relaxation is still significantly slowed at 32 °C is in agreement with the study of Adams *et al.* (1991) where tetanic contractions were produced in intact cat muscle at 37 °C and acidification was found to markedly reduce the rate of relaxation, albeit having no effect on tetanic force.

### Force–velocity relationship and its pH dependence

The present study provides the first measurements of the force–velocity relationship in single, intact mammalian muscle fibres. Under control conditions at 22 °C, we measured a $V_{max}$ (the shortening velocity at zero load obtained from force–velocity curves) of 6·73 $L_0$ $s^{-1}$. This value is similar to that obtained in fast-twitch mouse fibre bundles (Barclay, Constable & Gibbs, 1993) and intact muscles (Luff, 1981) (after correction for the difference in temperature assuming a $Q_{10}$ of about 2). However, our fibres show a somewhat more curved force–velocity relationship ($a/P_0$* equals 0·18 *vs.* about 0·3 in the studies of Barclay *et al.* (1993) and Luff (1981)) and in this respect our fibres are more similar to skinned fibres, which generally show a lower $a/P_0$* than intact muscle (e.g. Ferenczi, Goldman & Simmons, 1984; Julian, Rome, Stephenson & Striz, 1986).

The force at zero velocity extrapolated from force–velocity curves ($P_0$*) was 10–15% higher than the measured isometric force (Table 1). This difference can be explained by the fact that we used no data points with forces higher than 80% of the isometric force in the curve fitting and in frog fibres it has been shown that forces at the high-force end of force–velocity curves (> 80% of the isometric force) deviate downwards from a hyperbola (Edman, Mulieri & Scubon-Mulieri, 1976; Julian *et al.* 1986). Thus, when these high-force points are excluded from the curve fitting, the curve extrapolates to a force which is higher than the isometric force.

The curvature of the force–velocity relationship was similar in control and with alkalinization, whereas the relationship was significantly less curved with acidification. A reduced curvature with acidification has also been observed in intact frog fibres (Lännergren, Lindblom & Johansson, 1982; Curtin & Edman, 1994) and can be explained by a simple cross-bridge scheme (see below). A comparison of the values of $V_{max}$ and $V_0$ (the shortening velocity at zero load obtained with slack tests) reveals that, while the two measures are similar in control and with alkalinization, $V_{max}$ is significantly lower than $V_0$ with acidification. This discrepancy can be explained by the less curved force–velocity relationship with acidification and the fact that in our curve fitting we used no data points in the low-force region (less than 5% of the isometric force). Force–velocity data points have been shown to deviate upwards from a hyperbola in the low-force region and a straightforward hyperbolic curve fit which does not take these low-force points into account will tend to give a value of $V_{max}$ that is too low (Julian *et al.* 1986). Thus, in our study this problem will be most apparent with acidification where the force–velocity relationship is the least curved.

Measurements of $V_0$ were performed at 12, 22 and 32 °C (Fig. 6) and these measurements can be used to assess the $Q_{10}$ of this parameter. Under control conditions, $Q_{10}$ was 2·73 between 12 and 22 °C and 1·80 between 22 and 32 °C. These values of $Q_{10}$ agree with previous results from rat extensor digitorum longus muscles which also display a markedly larger temperature dependence at lower temperatures (Ranatunga, 1984).

Measurements of $V_0$ at different $pH_i$ showed that alkalinization had no significant effect at any of the temperatures studied (Fig. 6*B*). Acidification, on the other hand, reduced $V_0$ by 18% at 12 °C and by about 10% at 22 °C, whereas it had no significant effect at 32 °C. This conspicuous reduction of the pH effect on $V_0$ with increased temperature is consistent with the results of Pate *et al.* (1995), but the

underlying mechanism is unclear (for a brief discussion see Pate *et al.* 1995). Thus, our present results and those of Pate *et al.* (1995) are similar, both concerning the reduced pH effect on isometric force and $V_0$ at higher temperatures.

### Effects of $pH_i$ on cross-bridge kinetics at different temperatures

A simple two-state cross-bridge scheme (Brenner, 1988) can be used to explain some of the present results. Three rate constants are used in this model: $f_{app}$ and $g_{app}$ are the rate constants for attachment and subsequent detachment of force-generating cross-bridge states, respectively, and $g_2$ is the rate constant for detachment of negatively strained cross-bridges. $g_2$ is proportional to the shortening velocity at zero load and changes in $g_2$ can therefore be assessed from changes in $V_0$ or $V_{max}$. The sum of $f_{app}$ and $g_{app}$ reflects the rate of cross-bridge cycling under isometric conditions and can be estimated from the rate of force redevelopment after a shortening step ($k_{tr}$). The optimal protocol for measuring $k_{tr}$ involves a short period of rapid shortening of a contracting fibre followed by a rapid re-extension to the original length and the rate of force redevelopment after the re-extension is measured while sarcomeres are clamped at a constant length (Brenner & Eisenberg, 1986). Our measurements of $k_{tr}$ were simplified in that no re-extension of the fibres was performed and no sarcomere clamp was used during the period of force redevelopment. The rate of force redevelopment has been shown to be underestimated without sarcomere clamp (Brenner & Eisenberg, 1986), but still our estimates of $k_{tr}$ are similar to those obtained in skinned fibres with sarcomere clamp (Brenner & Eisenberg, 1986; Metzger & Moss, 1990).

The isometric force can be expressed as:

$$nFf_{app}/(f_{app} + g_{app}), \quad (3)$$

where $n$ is the number of cycling cross-bridges per half-sarcomere, $F$ is the mean force produced by a cross-bridge in force-generating states, and $f_{app}/(f_{app} + g_{app})$ represents the fraction of cycling cross-bridges in force-generating states. Since we consistently used maximum tetanic stimulation, it can be assumed that $n$ is constant. The reduction of isometric force with falling $pH_i$ can then be explained by a reduction of the force per cross-bridge ($F$) or a reduced fraction of force-generating cross-bridges. Alteration of $pH_i$ had no effect on $k_{tr}$ in our experiments and this agrees with results from maximally activated skinned fibres (Metzger & Moss, 1990). Thus, since $(f_{app} + g_{app})$ is not affected by $pH_i$, the easiest way to explain the reduction in isometric force with falling $pH_i$ is that it is due to a reduction of $F$. Furthermore, the pH effect on force declines with increasing temperature and hence $F$ has to be less pH sensitive at high temperature.

The curvature of the force–velocity relationship is related to $g_2/(f_{app} + g_{app})$ and an increase in this ratio will give more curved force–velocity curves (see e.g. Woledge *et al.* 1985). While $pH_i$ had no effect on $(f_{app} + g_{app})$ (see above), acidification markedly reduced $g_2$ at 12 and 22 °C (judged from the reduction in $V_0$). Consistent with this we found a markedly less curved force–velocity relationship with acidification than with control and alkalinization.

The rate of relaxation was significantly reduced with acidification at all temperatures. The slowed relaxation with acidification has been ascribed to the cross-bridge component of relaxation (Westerblad & Allen, 1993). This might seem puzzling since the rate of cross-bridge cycling, $(f_{app} + g_{app})$, during tetanic stimulation is not affected by $pH_i$. However, Metzger & Moss (1990) have shown that, while $k_{tr}$ at maximum $Ca^{2+}$ activation is not affected by pH, it is markedly depressed by an acidification at reduced $Ca^{2+}$ activation. During tetanic contraction $[Ca^{2+}]_i$ is close to saturation but $[Ca^{2+}]_i$ falls rapidly during relaxation and hence the rate of cross-bridge cycling during relaxation might be depressed by acidification. The reduced effect of acidification on the relaxation rate with increased temperature might then be explained by the pH effect becoming smaller at high temperatures, which seems likely since we have shown that most other aspects of cross-bridge cycling become less pH sensitive with a rise in temperature.

### Acidosis and fatigue

Acidosis is generally believed to be one of the most important causes of skeletal muscle fatigue. However, this can be questioned on the basis of the present results and those of Adams *et al.* (1991) and Pate *et al.* (1995) since, except for causing a slowed relaxation, an acidification has little effect on the contractile function of mammalian muscle at physiological temperatures. Nevertheless, a reduced $pH_i$ acidosis cannot be excluded from having some effect on skeletal muscle fatigue. For instance, an acidification is probably an important factor in fatigue of cold-blooded animals like frogs and it has been shown that reduced $pH_i$ has a marked effect on the contractile function of frog muscles at about 20 °C (Renaud, Allard & Mainwood, 1986; Stevens & Godt, 1990), which is a physiological temperature for these animals. Furthermore, a reduction of $pH_i$ opens ATP-dependent potassium channels (Standen, Pettit, Davies & Stanfield, 1992), which may lead to impaired action potential activation in fatigue.

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