

The contribution of pH-dependent mechanisms to fatigue at different intensities in mammalian single muscle fibres

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1. The contribution of intracellular pH (pH_i) to the failure of Ca^{2+} release and inhibition of contractile proteins observed during fatigue was assessed in single intact mouse muscle fibres at 22 °C. Fatigue was induced by repeated tetani at intensities designed to induce different levels of intracellular acidosis. Force and either intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$; measured using indo-1) or pH_i (measured using SNARF-1) were recorded in fibres fatigued at two different intensities.
2. Intensity was varied by the repetition rate of tetani and quantified by the duty cycle (the fraction of time when the muscle was tetanized). Stimulation at the low intensity (duty cycle ~ 0.1) reduced force to 30% of initial values in 206 ± 21 s (60 ± 7 tetani); at the high intensity (duty cycle ~ 0.3) force was reduced to 30% in 42 ± 7 s (43 ± 7 tetani) ($P < 0.05$; $n = 14$).
3. When force was reduced to 30% of initial values, tetanic $[\text{Ca}^{2+}]_i$ had fallen from 648 ± 87 to 336 ± 64 nM (48% decrease) at the low intensity but had only fallen from 722 ± 84 to 468 ± 60 nM (35% decrease) at the higher intensity ($P < 0.05$ low vs. high intensity; $n = 7$).
4. Fatigue resulted in reductions in Ca^{2+} sensitivity of the contractile proteins which were greater at the high intensity (pre-fatigue $[\text{Ca}^{2+}]_i$ required for 50% of maximum force (Ca_{50}) = 354 ± 23 nM; post-fatigue Ca_{50} = 421 ± 48 nM and 524 ± 43 nM for low and high intensities, respectively). Reductions in maximum Ca^{2+} -activated force (F_{max}) were similar at the two intensities (pre-fatigue F_{max} = 328 ± 22 μN ; post-fatigue F_{max} = 271 ± 20 and 265 ± 19 μN for low and high intensities, respectively).
5. Resting pH_i was 7.15 ± 0.05 . During fatigue at the low intensity, pH_i was reduced by 0.12 ± 0.02 pH units and at the high intensity pH_i was reduced by 0.34 ± 0.07 pH units ($P < 0.05$; $n = 5$).
6. Our results indicate that the more rapid fall in force at a high intensity is due to a reduction in Ca^{2+} sensitivity of the contractile proteins, probably related to the greater acidosis. Our data also indicate that the failure of Ca^{2+} release and reduced maximum Ca^{2+} -activated force observed during fatigue are not due to reductions in intracellular pH.

During repeated tetanic contractions there is a reduction in force output known as muscle fatigue. The decrease in force during fatigue is due to at least three mechanisms: (i) decrease in Ca^{2+} released from the sarcoplasmic reticulum (SR); (ii) reduced Ca^{2+} sensitivity of the myofibrillar proteins; and (iii) reduced maximum Ca^{2+} -activated force (see Fitts, 1994; Allen *et al.* 1995*a* for recent reviews). The alterations in contractile protein function appear to be due primarily to the accumulation of metabolites in the myoplasm. Studies with skinned single fibres have shown that increases in hydrogen ions (H^+) and inorganic phosphate (P_i) can account for the reductions in Ca^{2+} sensitivity and maximum Ca^{2+} -activated force observed during fatigue (Metzger & Moss,

1987; Godt & Nosek, 1989). The underlying cause(s) for the reduction in Ca^{2+} release are not fully understood, but there appear to be both metabolic (Westerblad & Allen, 1992; Owen *et al.* 1996; Allen *et al.* 1997) and non-metabolic (Fryer *et al.* 1995; Chin & Allen, 1996) factors involved. In particular, the role of intracellular acidosis in Ca^{2+} release failure and muscle fatigue is still controversial (Allen *et al.* 1995*b*).

Changes in muscle pH have been suggested to play a critical role in fatigue (Fitts, 1994). Numerous studies show strong correlations between muscle $[\text{H}^+]$ and force during exercise and recovery (Hermansen & Osnes, 1972; Kushmerick & Meyer, 1985; Thompson *et al.* 1992). The close association

between force and $[H^+]$ along with evidence that acidosis inhibits the opening of isolated SR Ca^{2+} release channels and reduces ryanodine binding (Ma *et al.* 1988; Favero *et al.* 1995) make it tempting to postulate a causal relationship. However, experiments with intact single fibres have shown that decreases in force and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) occur in the absence of changes in pH during fatigue (Westerblad & Allen, 1992). Conversely, changes in muscle pH induced by CO_2 exposure reduce force but increase $[Ca^{2+}]_i$ indicating that Ca^{2+} release failure is not the cause of the fall in force during acidosis (Westerblad & Allen, 1993). Furthermore, in skinned fibres with intact t-tubular SR coupling, acidosis has very little effect on depolarization-induced Ca^{2+} release (Lamb *et al.* 1992). The extent to which changes in muscle pH explain the fall of force during fatigue is therefore still unclear. A further complication is the recent demonstration that acidosis has much less effect on the contractile proteins at near physiological temperatures than at room temperature (Pate *et al.* 1995; Westerblad *et al.* 1997).

It is well established that the rate at which muscles fatigue depends on the intensity and duration of contraction and on the fibre type composition of the muscle (Metzger & Moss, 1987; Barclay *et al.* 1995). One hypothesis to explain the controversy regarding the role of pH_i in fatigue is that pH_i -dependent mechanisms are intensity and fibre-type specific. For example, H^+ ions may only accumulate to levels that impair force production when muscles contract at a high intensity where rates of ATP demand exceed ATP supply and when primarily fast glycolytic fibres are recruited. In mixed muscle preparations these mechanisms are difficult to assess due to alterations in the numbers and types of fibres recruited. We have therefore used single fibres from a fast muscle to control for fibre type variability and assessed the role of intracellular pH at two different intensities. Our observations indicate that fatigue at a high compared with a low intensity exhibits a greater acidosis, which is associated with a larger decrease in Ca^{2+} sensitivity and a smaller decrease in tetanic $[Ca^{2+}]_i$. These data suggest that in fast fatiguable fibres at room temperature, pH-dependent alterations in contractile protein function play a greater role in fatigue at high intensities and that impairments in excitation-contraction coupling, which appear to be pH independent, predominate during fatigue at low intensities.

METHODS

Mice were killed by cervical dislocation and single muscle fibres dissected from the flexor brevis muscle. Fibres from this muscle are predominantly type II X and II B (Allen *et al.* 1993). Methods for fibre dissection and mounting were similar to those previously used in this laboratory (Westerblad & Allen, 1991). After dissection and transfer to a stimulation chamber fibres were superfused at 22 °C with a solution containing (mM): NaCl, 121; KCl, 5.0; $CaCl_2$, 1.8; $MgCl_2$, 0.5; NaH_2PO_4 , 0.4; $NaHCO_3$, 24; and glucose, 5.5. The solution was bubbled with 95% O_2 and 5% CO_2 to give a pH of 7.3. Approximately 0.2% (v/v) fetal calf serum was added.

The fibres were stimulated with platinum electrodes using pulses of 0.5 ms duration at an intensity of approximately $1.2 \times$ threshold. All tetanic contractions were 0.35 s in duration. Force produced during unfused tetani was assessed by the average output over the final 100 ms.

Intracellular free calcium concentration and pH measurements.

In separate experiments, $[Ca^{2+}]_i$ was measured with the fluorescent dye indo-1 and intracellular pH (pH_i) was measured with SNARF-1. The dyes were introduced into the fibres by micro-injection. For indo-1, fibres were illuminated at 360 nm and the emitted light measured at 400 and 510 nm. For SNARF-1, fibres were illuminated at 540 nm and emitted light collected at 590 and 640 nm. The experimental set-up was similar to that described by Lee *et al.* (1991). Emitted light was measured by two photomultiplier tubes and passed on to an analog divide circuit which allowed continuous monitoring of the 400/510 ($[Ca^{2+}]_i$) or the 590/640 (pH_i) ratio signal. The background fluorescence was subtracted from all measurements. The indo-1 ratio was converted to $[Ca^{2+}]_i$ using the *in vivo* calibration previously described (Westerblad & Allen, 1993) and the steady-state $[Ca^{2+}]_i$ for each tetanus was determined by the average output over the last 100 ms. For pH_i SNARF-1 was calibrated as previously described (Buckler & Vaughan-Jones, 1990; Westerblad & Allen, 1992).

Experimental protocols. The aim of this study was to examine the intensity dependence of pH_i changes and to identify pH-dependent mechanisms of fatigue. We hypothesized that pH_i would change to a greater extent at a high compared with a low work intensity and that Ca^{2+} release failure and alterations in contractile protein function would be exacerbated at the higher intensity. To test these hypotheses we used single muscle fibres in a repeated measures design to control for fibre type differences. Changes in pH_i and $[Ca^{2+}]_i$ were compared during series of tetani at two different intensities. Intensity was varied by altering the rest interval between tetani. For the low intensity, fatigue was induced using a previously characterized protocol (Allen *et al.* 1989; Westerblad & Allen, 1991) with 100 Hz tetani repeated every 4 s for 2 min, then the inter-tetanus interval decreased to 3 s for 2 min, then to 2.5 s for 2 min etc., until force reached 30% of initial values. In a typical fatigue run lasting 3–4 min, the average rate of tetanic contractions was one every 3.5 s. In previous experiments this fatigue protocol resulted in an acidosis of only 0.06 pH units (Westerblad & Allen, 1992). At the higher intensity, fatigue was induced using 100 Hz tetani of the same duration (350 ms) with tetani repeated every 1 s. For both protocols tetani were continued until force was reduced to 30% of initial values. For experiments measuring $[Ca^{2+}]_i$, both protocols were applied to the same fibre with 1 h of recovery between fatigue bouts. For experiments measuring pH_i , fibres were fatigued using both protocols but recovery time was reduced to 20 min to minimize the reduction of SNARF-1 signal over time. In a few experiments pH_i was measured at the two intensities in separate fibres. In experiments where both protocols were used the order was randomized with an equal number of fibres given the high and low intensity first.

Assessment of contractile protein function

In order to determine the Ca^{2+} sensitivity and maximum Ca^{2+} -activated force of the contractile proteins we used a protocol described previously (Westerblad & Allen, 1991). Briefly, before each fatigue bout fibres were activated at a range of frequencies (30, 50, 70, 100 Hz and 100 Hz in the presence of 5 or 10 mM caffeine) at 1 min intervals. Force and $[Ca^{2+}]_i$ at each frequency were used to construct force- Ca^{2+} curves and to determine the Ca^{2+} sensitivity

and maximum Ca^{2+} -activated force of a rested fibre. Force– Ca^{2+} curves were fitted using a Hill equation:

$$F = F_{\max} [\text{Ca}^{2+}]_i^N / (\text{Ca}_{50}^N + [\text{Ca}^{2+}]_i^N),$$

where F is force, F_{\max} is the force at saturating $[\text{Ca}^{2+}]_i$, N is a constant describing the steepness of the relation and Ca_{50} represents the $[\text{Ca}^{2+}]_i$ required for 50% of maximum force. To assess the changes in Ca^{2+} sensitivity during fatigue, force and $[\text{Ca}^{2+}]_i$ from the final phase of fatigue, when both are declining, were used (see Westerblad & Allen, 1991). Force and $[\text{Ca}^{2+}]_i$ from a 100 Hz tetanus in the presence of caffeine, representing maximum Ca^{2+} -activated force, were measured at the end of the fatigue protocol and added to the force– Ca^{2+} plot, which could then be fitted by a Hill equation as described above. Initially maximum Ca^{2+} -activated force was assessed in the presence of 5 or 10 mM caffeine delivered during the fatigue run ($n = 5$). To ensure that the measurement of maximum Ca^{2+} -activated force was not compromised by a depletion of SR Ca^{2+} during fatigue, in some

experiments caffeine was added at the end of the fatigue run and the 100 Hz + caffeine data point obtained after a 15 s pause ($n = 6$). The results of the force– Ca^{2+} curve analyses for the two sets of data were similar so the results were combined.

Assessment of low frequency fatigue

We have previously shown that fatigue induced by repeated short tetani using our standard fatigue protocol (i.e. low intensity) results in a prolonged reduction of Ca^{2+} release and changes in force characteristic of low frequency fatigue. Low frequency fatigue was first defined by Edwards *et al.* (1977) and refers to a reduction of force at low stimulation frequencies (i.e. 30–50 Hz) which persists 1 h after activity with no reduction at high stimulation frequencies (100 Hz) or in maximum force (100 Hz + caffeine). We have also shown that low frequency fatigue is dependent on the elevation of $[\text{Ca}^{2+}]_i$ and the duration of this period of elevated $[\text{Ca}^{2+}]_i$ (i.e. the cumulative $[\text{Ca}^{2+}]_i$ -time integral) (Chin & Allen, 1996). We were interested in whether fatigue at a higher intensity, which would be shortened in duration, would elevate the cumulative $[\text{Ca}^{2+}]_i$ -time

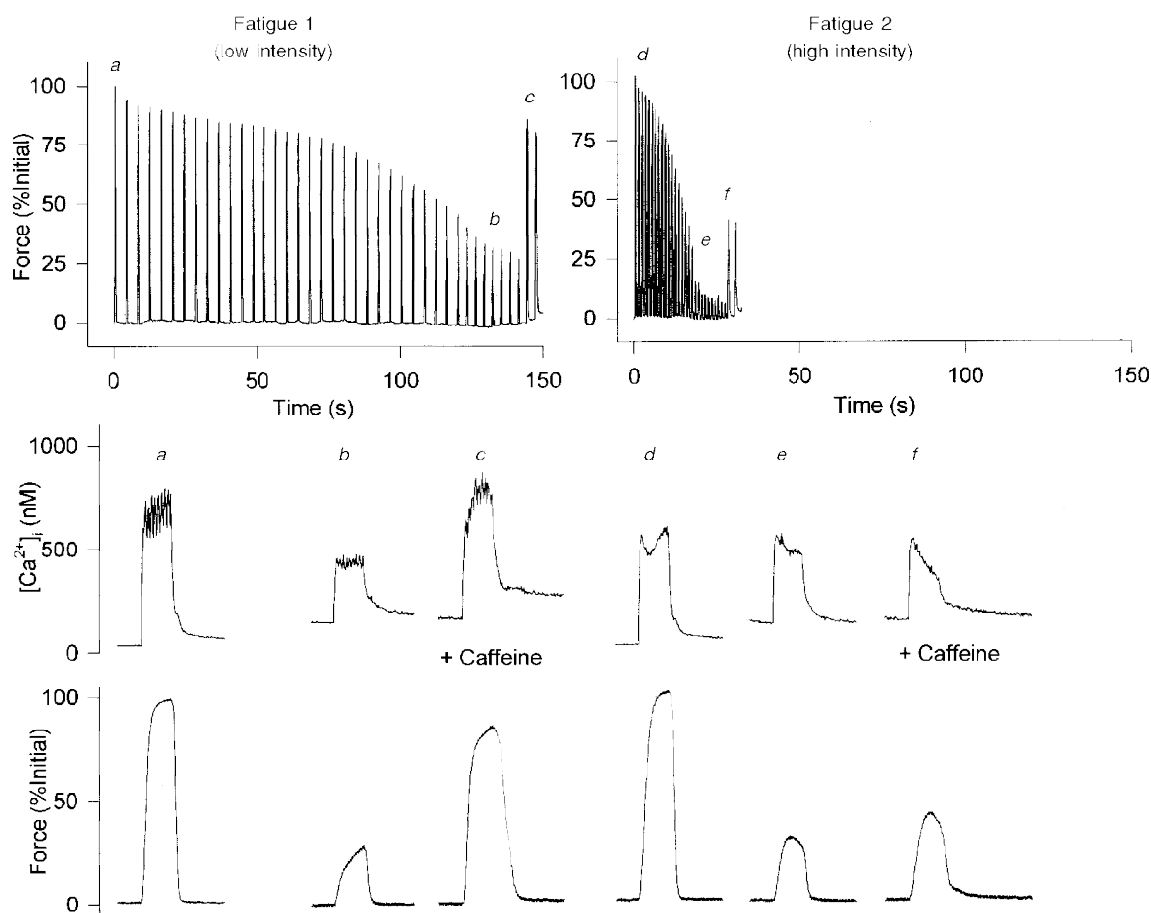


Figure 1. Force and $[\text{Ca}^{2+}]_i$ traces from a single fibre fatigued at low and high intensities

Continuous force records (upper panel) and $[\text{Ca}^{2+}]_i$ and force transients on an expanded time scale (middle and bottom panels, respectively) are shown during fatigue at low and high intensities. Fatigue 1 was induced by tetani repeated initially every 4 s and increased to a rate of 1/3 s after 2 min (low intensity). Following 60 min of recovery Fatigue 2 was induced by tetani repeated every 1 s (high intensity). Tetani were continued until force reached 30% of initial values. The fibre was perfused with a 5 mM caffeine solution towards the end of fatigue to assess E–C coupling failure. For Fatigue 1, force and $[\text{Ca}^{2+}]_i$ transients are shown for the first tetanus (*a*), the tetanus at 30% (*b*) and a tetanus after exposure to caffeine (*c*). For Fatigue 2 force and $[\text{Ca}^{2+}]_i$ transients represent the first tetanus (*d*), the tetanus at 30% (*e*) and a tetanus after exposure to caffeine (*f*).

integral to a level sufficient to induce low frequency fatigue. To assess low frequency fatigue, force and $[Ca^{2+}]_i$ were measured at a range of stimulation frequencies (30, 50, 70, 100 Hz and 100 Hz in the presence of 5 or 10 mM caffeine) 5 min before and 60 min after both fatigue protocols ($n = 9$). Low frequency fatigue was assessed only at the end of the first fatigue run for each fibre.

Statistical analyses

All values are expressed as means \pm s.e.m. Student's paired t tests were used to test for statistical significance when measurements were made in the same fibre (pre vs. post fatigue; $[Ca^{2+}]_i$ for high vs. low intensity). Unpaired t tests were used to test for significance in pH_i experiments. Significance was accepted at $P < 0.05$.

RESULTS

Changes in muscle force and $[Ca^{2+}]_i$ during fatigue at high and low intensities

The underlying mechanisms of fatigue were assessed at two different intensities; at the low intensity tetani were repeated initially every 4 s and at the high intensity tetani were repeated every 1 s. At both intensities tetani were continued until force reached 30% of initial values. Although the relative decrease in force was similar for both intensities, the time course was dramatically different. Figure 1 shows the time course of fatigue and the force and Ca^{2+} transients at the beginning and end of fatigue for a representative fibre at both intensities. During both fatigue runs there was a small initial fall in force; thereafter force declined slowly for 1 to 2 min at the low intensity (Fatigue 1) or declined rapidly at the high intensity (Fatigue 2). The average time to fatigue (30% initial force) was significantly

longer for the low compared with the high intensity (206 ± 21 vs. 42 ± 7 s, $P < 0.05$; $n = 14$). This represented 60 ± 7 and 43 ± 7 tetani ($P < 0.05$) for the low and high intensities, respectively.

In addition to changes in the rate of fatigue, there were differences in the extent to which $[Ca^{2+}]_i$ fell during fatigue at the two intensities. When force was reduced to 30% of initial values, tetanic $[Ca^{2+}]_i$ was higher at the high intensity (Fig. 1*b* vs. *e*). For this fibre, $[Ca^{2+}]_i$ was reduced at the beginning of Fatigue 2 due to a prolonged reduction of Ca^{2+} release induced during Fatigue 1 (see last paragraph of Results). The relative reduction in $[Ca^{2+}]_i$ for Fatigue 2 was therefore even smaller. The lower starting $[Ca^{2+}]_i$ did not alter the overall results since the order of the fatigue protocols was reversed in four of seven fibres. Figure 2 shows the pooled data for seven fibres. When force was reduced to 30% at the low intensity, $[Ca^{2+}]_i$ was reduced from a mean level of 648 ± 87 to 336 ± 64 nM, a decrease of 48%. At the high intensity $[Ca^{2+}]_i$ was reduced from an average of 722 ± 84 to 468 ± 60 nM, a decrease of 35%. The difference between the low and high intensity (48% vs. 35%) was significant. These data suggest that the more rapid fall in force at the higher intensity was due to mechanisms other than Ca^{2+} release failure.

The impairment of excitation-contraction coupling (E-C coupling) was assessed by adding caffeine, a Ca^{2+} release channel agonist, to the perfusing solution at the end of fatigue. Since the effect of caffeine on force and $[Ca^{2+}]_i$ was not immediate, the extent of recovery was determined by

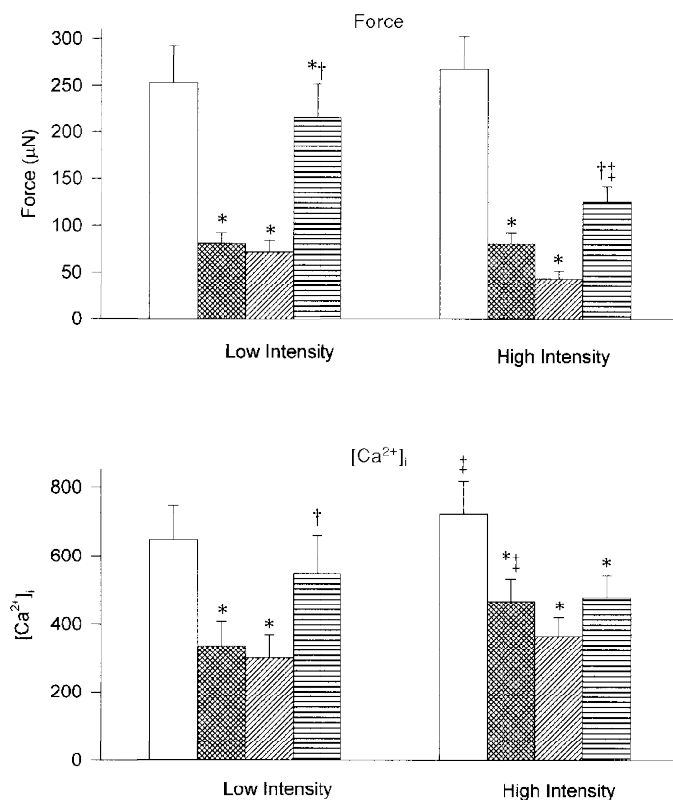


Figure 2. Average force and $[Ca^{2+}]_i$ in response to fatigue at low and high intensities

Force (upper panel) and $[Ca^{2+}]_i$ (lower panel) for the first tetanus (pre-fatigue; □), when force reached 30% (fatigue; ▨), minimum force (▩) and after 5 mM caffeine exposure at the end of fatigue (▧). Minimum force was defined as the force immediately before a response to caffeine was observed. Changes in force and $[Ca^{2+}]_i$ during fatigue at both the low intensity (1/4 s, etc.) and high intensity (1/1 s) are shown. Values represent mean \pm s.e.m. ($n = 7$). * $P < 0.05$ vs. pre-fatigue; † $P < 0.05$ vs. fatigue; ‡ $P < 0.05$ vs. low intensity.

comparing the maximum force and $[Ca^{2+}]_i$ after addition of caffeine to the level which immediately preceded the caffeine response (defined as the minimum level). During stimulation at the low intensity, caffeine was able to restore force and $[Ca^{2+}]_i$ back to 75–85% of starting levels (Fig. 1*c*). In contrast, 5 mM caffeine had only a small effect on force and $[Ca^{2+}]_i$ during stimulation at the high intensity (Fig. 1*f*). As illustrated in Fig. 2, in the presence of caffeine force recovered to $70 \pm 8\%$ at the low intensity and to $51 \pm 4\%$ at the high intensity, a difference which was significant. At the low intensity, this recovery of force was associated with a recovery of tetanic $[Ca^{2+}]_i$ (from a minimum of $46 \pm 11\%$ to $84 \pm 8\%$ of initial values, $P < 0.05$). At the high intensity, however, the recovery of $[Ca^{2+}]_i$ with caffeine was small and not significant (from a minimum of $50 \pm 8\%$ to $67 \pm 9\%$). The decrease in caffeine-activated Ca^{2+} release was not due to a decrease in caffeine sensitivity since similar results were obtained using 10 mM caffeine. These observations suggest there was a decrease in caffeine-activated Ca^{2+} release as well as E–C coupling failure at the high intensity. The decrease in caffeine-activated Ca^{2+} release may be due to insufficient re-filling of the SR Ca^{2+} pool by the SR Ca^{2+} pumps. To test this interpretation, in five additional experiments, fibres were given a 15 s pause between the last tetanus of fatigue and the tetanus in the presence of caffeine. This resulted in partial recovery of the caffeine-evoked force and $[Ca^{2+}]_i$ at the high intensity (force = $72 \pm 1\%$ of initial values and $[Ca^{2+}]_i$ = $78 \pm 17\%$

of initial values; data not shown). This rapid reversal of Ca^{2+} release is consistent with the idea that the impairment of caffeine-activated Ca^{2+} release is related to refilling of the SR Ca^{2+} pool. These data are also consistent with previous reports that SR Ca^{2+} loading but not depolarization-induced Ca^{2+} release is impaired at an acidic pH_i (Lamb *et al.* 1992) (see Discussion).

Force– $[Ca^{2+}]_i$ relationship during fatigue at high and low intensities

The decrease in $[Ca^{2+}]_i$ may explain part of the decrease in force during fatigue but another component of force loss is attributable to changes in contractile protein function. We have assessed changes in Ca^{2+} sensitivity of the contractile proteins and changes in maximum Ca^{2+} -activated force by plotting the force– Ca^{2+} relationship before and during fatigue. Figure 3 shows force– Ca^{2+} curves from a fibre that was fatigued at both the high and low intensity. During fatigue at the low intensity there was a rightward shift of the force– Ca^{2+} curve and a reduction in maximum force (F_{max}). During fatigue at the high intensity there was an even greater rightward shift of the force– Ca^{2+} curve and a similar reduction in F_{max} . The mean results for eleven fibres are shown in Table 1. Neither fatigue protocol altered N , suggesting that co-operativity of Ca^{2+} binding was not altered. Ca^{2+} sensitivity of the contractile proteins was reduced during low intensity stimulation resulting in a 19% increase in Ca_{50} . Stimulation at the high intensity reduced

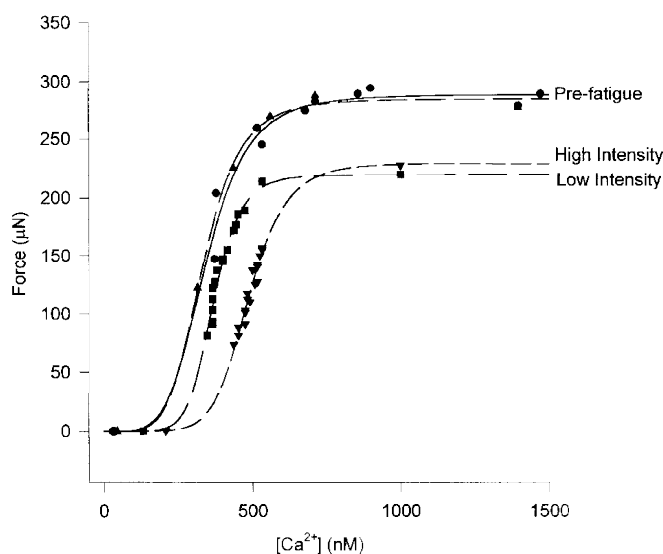


Figure 3. Force– Ca^{2+} relationship in response to fatigue at low and high intensities

Pre-fatigue force and $[Ca^{2+}]_i$ are plotted at 30, 50, 70 and 100 Hz and maximum force obtained at 100 Hz in the presence of 5 mM caffeine. Force and $[Ca^{2+}]_i$ are plotted for pre-fatigue (Fatigue 1, ●; Fatigue 2, ▲) and during the final phase of fatigue, when force and Ca^{2+} are decreasing, for the low intensity (■) and high intensity (▼). Data points representing force and $[Ca^{2+}]_i$ after 5 mM caffeine exposure at the end of fatigue are included. All points are fitted to a Hill equation (see Methods). The rightward shift of the force– Ca^{2+} curve, representing a decrease in Ca^{2+} sensitivity, is greater at the high *vs.* low intensity. The plateau, representing maximum Ca^{2+} -activated force, is reduced during fatigue but to a similar extent at the two intensities.

Table 1. Force–Ca²⁺ relationship during fatigue with different rest intervals

	<i>N</i>	Ca ₅₀ (nM)	<i>F</i> _{max} (μN)
Pre-fatigue	6.076 ± 1.17	354 ± 23	328 ± 22
Low intensity	6.133 ± 0.57	421 ± 48*	271 ± 20
High intensity	6.426 ± 0.43	524 ± 43*†	265 ± 19*

Force and [Ca²⁺]_i were measured at 30, 50, 70, 100 Hz and with 100 Hz in the presence of 5 mM (*n* = 3) or 10 mM (*n* = 8) caffeine. Values were used to construct force–Ca²⁺ curves which were fitted using a Hill equation (see Methods). From this, values were obtained for *N*, which is a constant describing the steepness of the relation, Ca₅₀ which represents the [Ca²⁺]_i required for 50% of maximum force and *F*_{max}, which is the force at saturating [Ca²⁺]_i. The values represent mean ± s.e.m. (*n* = 11). * *P* < 0.05 vs. Pre-fatigue; † *P* < 0.05 vs. 1/4 s.

Ca²⁺ sensitivity to a greater extent (48% increase in Ca₅₀) indicating that contractile protein inhibition was greater with higher intensity stimulation. During stimulation at both high and low intensities, *F*_{max} was reduced to a similar extent (by 17% and 19% for low and high intensity, respectively) suggesting that maximum Ca²⁺-activated force of the contractile proteins was not different between the two intensities.

Changes in pH_i during fatigue at high and low intensities

To assess the importance of pH_i during fatigue at different work intensities, pH_i was measured in fibres fatigued at the high and low intensities. The resting pH_i measured by SNARF-1 was 7.15 ± 0.05. Figure 4 shows the changes in pH_i in a representative fibre in response to stimulation at the two intensities and in response to a brief exposure to 30% CO₂. Fatigue at the low intensity resulted in a small and gradual fall in pH_i. In five fibres the average change in pH_i was 0.12 ± 0.02 pH units, which is similar to previous observations (Westerblad & Allen, 1992). In contrast, fatigue at the high intensity resulted in a large and rapid decrease in pH_i which appeared to coincide with the fall in force. In five fibres, pH_i was reduced by 0.34 ± 0.07 pH units. The increased rate of acidosis can be explained by increased anaerobic glycolysis and inhibition of the lactate–H⁺ co-transporter. At the low intensity the pH change was 0.12 pH units (3.43 min)⁻¹ or 0.03 pH units min⁻¹. At the higher intensity the pH change was 0.34 pH units (0.7 min)⁻¹ or 0.49 pH units min⁻¹. At the low intensity energy is initially supplied by phosphocreatine hydrolysis and some anaerobic glycolysis while oxidative phosphorylation is activated. Our data suggest that at this intensity ATP demand is matched by ATP supply without any excess H⁺ production. As the duty cycle increases, glycolysis is activated to a greater extent but remains at a rate where lactate production can be matched by extrusion

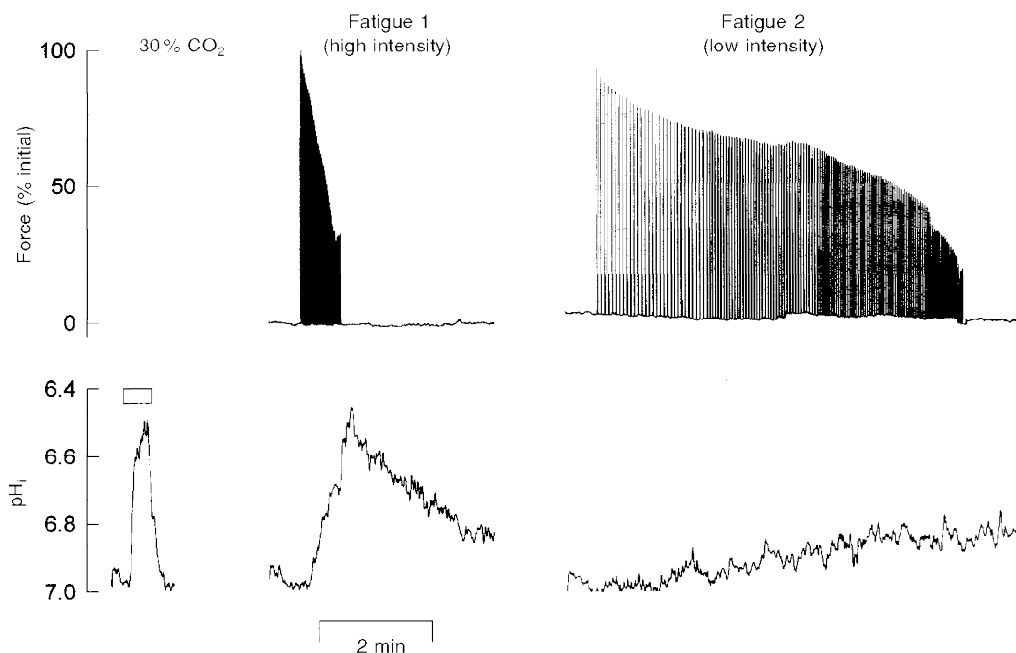


Figure 4. Force and pH_i traces from a single fibre fatigued at low and high intensities

Force (upper panel) and pH_i (lower panel) were recorded simultaneously at both the high and low intensity. pH_i was measured using SNARF-1. Fatigue 1 was induced by tetani repeated every 1 s (high intensity). Following 20 min of recovery, Fatigue 2 was induced by tetani repeated initially every 4 s then increased to 1/3 s, 1/2.5 s, etc. every 2 min (low intensity). Tetani were continued until force reached 30% of initial values. Change in pH_i induced by 30% CO₂ exposure (~0.5 pH unit change) is shown relative to the pH_i changes at high and low intensities.

via the lactate transporter, thus little acidosis results. However, at the high intensity where tetani start at a rate of 1 s^{-1} , anaerobic glycolysis is activated immediately to supply ATP and it becomes the major source of energy. At this rate lactate and H^+ are produced at a faster rate than can be removed by the transporter. The resultant accumulation of H^+ and decrease in pH_i is greater at the higher intensity and indicates that pH_i plays a more important role in fatigue at a higher intensity.

Low frequency fatigue following tetani at high and low intensities

We have previously shown that fatigue induced by stimulation at a low intensity results in a prolonged decrease in Ca^{2+} release and a selective reduction in force at low frequencies known as low frequency fatigue (Westerblad *et al.* 1993; Chin & Allen, 1996). In the present study we were able to assess whether low frequency fatigue was induced during a shorter fatigue run when force fell more rapidly. Figure 5 shows the changes in force and $[\text{Ca}^{2+}]_i$ before and 60 min after fatigue at the low ($n = 6$) and high ($n = 7$) intensities. Similar to previous observations, force at low frequencies (30 and 50 Hz) was still reduced 60 min after fatigue at the low intensity ($55 \pm 13\%$ and $76 \pm 11\%$ initial force for 30 and 50 Hz, respectively). Force was also reduced at 70 Hz ($86 \pm 8\%$ of initial values) but had fully recovered at 100 Hz. At the high intensity, force was depressed at all frequencies after 60 min of recovery (reduced to $30 \pm 8\%$, $50 \pm 8\%$, $74 \pm 7\%$ and $89 \pm 4\%$ of initial values for 30, 50, 70 and 100 Hz, respectively). The differences between intensities were not significant. Furthermore, maximum Ca^{2+} -activated force (100 Hz + Caff) had

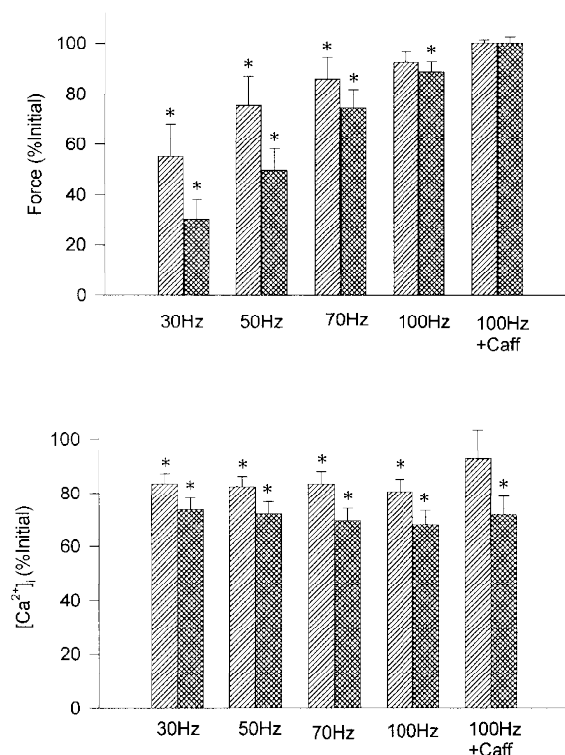
fully recovered for both intensities, indicating that contractile protein function was not impaired and that the fibre preparations remained healthy. The reduction of force can be explained by the prolonged reduction of tetanic $[\text{Ca}^{2+}]_i$ which was still depressed after 60 min of recovery. Similar to previous observations, steady state $[\text{Ca}^{2+}]_i$ was reduced to a similar extent at all frequencies. On average, $[\text{Ca}^{2+}]_i$ recovered to $71 \pm 2\%$ and $84 \pm 2\%$ of initial values at the low and high intensities ($P < 0.05$). These data indicate that fatigue at both intensities resulted in prolonged reductions in Ca^{2+} release and that the reductions were more pronounced at the high intensity even though fatigue occurred over a much shorter period of time.

DISCUSSION

Previous studies have shown that muscle fatigue is due to a combination of decreased release of Ca^{2+} from the SR, decreased Ca^{2+} sensitivity of the contractile proteins and reduced maximum Ca^{2+} -activated force (Allen *et al.* 1989; Westerblad & Allen, 1991). We have examined changes in force, $[\text{Ca}^{2+}]_i$ and pH_i during fatigue at two different intensities to try to elucidate the pH dependence of these mechanisms. Our results indicate that although $[\text{Ca}^{2+}]_i$ declined at a faster rate, the decrease in tetanic $[\text{Ca}^{2+}]_i$ that occurred during fatigue was attenuated at a higher intensity when acidosis was more pronounced. Ca^{2+} release failure therefore does not require acidosis nor does it appear to be greater when acidosis is present. Our data also show that Ca^{2+} sensitivity of the contractile proteins was reduced to a greater extent during fatigue where a large acidosis was observed. The reduced Ca^{2+} sensitivity of the contractile

Figure 5. Force and $[\text{Ca}^{2+}]_i$ after 60 min recovery demonstrate low frequency fatigue

Force (top panel) and $[\text{Ca}^{2+}]_i$ (bottom panel) after 60 min of recovery from fatigue at low and high intensities (▨ and ▩, respectively). Reductions in force and $[\text{Ca}^{2+}]_i$, shown as % of initial values, were of similar magnitude at both intensities. Values represent mean \pm s.e.m. ($n = 6-7$). * Significant difference ($P < 0.05$) compared with initial force or $[\text{Ca}^{2+}]_i$ at the same frequency or condition.



proteins is due to both pH-dependent and pH-independent mechanisms and appears to be relatively more important in fatigue at high intensities.

Role of pH_i in Ca^{2+} release failure during fatigue

The contribution of intracellular acidosis to the failure of E–C coupling during muscle fatigue remains controversial. Observations that acidosis inhibits the opening of SR Ca^{2+} release channels and reduces ryanodine binding (Ma *et al.* 1988; Favero *et al.* 1995) have implicated a role for pH_i in the failure of Ca^{2+} release. If acidosis was responsible for the reduction in Ca^{2+} release one would expect a greater reduction in tetanic $[\text{Ca}^{2+}]_i$ during fatigue in which a substantial level of acidosis occurred. However, this was not observed in the present study. Instead $[\text{Ca}^{2+}]_i$ was 13% higher at the same relative force output when fatigued at a high intensity ($\text{pH}_i \sim 6.8$) compared with a low intensity ($\text{pH}_i \sim 7.0$). These observations are consistent with previous findings that acidosis induced by CO_2 exposure in intact single muscle fibres (Allen *et al.* 1989; Westerblad & Allen, 1993) and in whole frog muscles (Baker *et al.* 1995) was associated with a 33–37% increase in tetanic $[\text{Ca}^{2+}]_i$. Thus it appears that Ca^{2+} release evoked by normal E–C coupling mechanisms is not inhibited by a decrease in pH_i and that reductions in pH_i probably do not contribute to the Ca^{2+} release failure observed during fatigue.

It is not clear whether the increased tetanic $[\text{Ca}^{2+}]_i$ during acidosis is caused by increased Ca^{2+} release, by reduced Ca^{2+} buffering or by a reduction in the SR Ca^{2+} pump rate. Studies using isolated SR vesicles indicate that Ca^{2+} release can be augmented by acidosis (Donoso *et al.* 1996), but studies with skinned single fibres show that acidosis has little effect on depolarization-induced Ca^{2+} release (Lamb *et al.* 1992) and no effect on t-tubule charge movement (Fitts & Balog, 1996). Acidosis can also alter intracellular Ca^{2+} regulation by increasing the leak of Ca^{2+} from the SR (Lamb *et al.* 1992) and slowing the SR Ca^{2+} pump (MacLennan, 1970). Under acidotic conditions single fibres show a 50–65% greater rise in resting $[\text{Ca}^{2+}]_i$ (Westerblad & Allen, 1993; present study) and a slower rate of decline of the Ca^{2+} tails (Westerblad & Allen, 1993), which are consistent with a pH-induced leak of Ca^{2+} from the SR and a reduction in Ca^{2+} uptake. The increased Ca^{2+} leak and impaired Ca^{2+} reuptake may be due to direct inhibitory effects of H^+ ions on the Ca^{2+} -ATPase or they may be secondary to reduced maximal Ca^{2+} binding to calsequestrin as pH in the SR lumen is reduced (Donoso *et al.* 1996). Currently there are no data to indicate whether Ca^{2+} buffering is altered by changes in sarcoplasmic pH so it is not known whether this mechanism also contributes to the rise in tetanic $[\text{Ca}^{2+}]_i$ when the muscle becomes acidotic.

An interesting observation from the present study is that reductions in pH_i impaired caffeine-activated Ca^{2+} release. Reductions in caffeine- and Ca^{2+} -activated Ca^{2+} release have also been shown in isolated SR vesicles (Favero *et al.* 1995; Kentish & Xiang, 1997) and in skinned fibres (Lamb *et al.*

1992). There are several possible explanations for the reduction in caffeine-activated Ca^{2+} release. First, there may be insufficient refilling of the SR Ca^{2+} pools because of slowing of the SR Ca^{2+} pump. The rapid recovery of caffeine-activated force and tetanic $[\text{Ca}^{2+}]_i$ by 15 s following fatigue supports this interpretation. However, if there was inadequate Ca^{2+} available for release it would suggest there was a decrease in total SR Ca^{2+} storage and there is reasonable evidence to indicate that this does not happen during fatigue (Gonzalez-Serratos *et al.* 1978). A second possibility is that the gating mechanisms regulating caffeine and Ca^{2+} -induced Ca^{2+} release are pH sensitive even though depolarization-induced Ca^{2+} release is not. There is no direct evidence for differential pH sensitivities of the various gating mechanisms but this may explain the conflicting reports in the literature regarding pH effects on Ca^{2+} release (Ma *et al.* 1988; Lamb *et al.* 1992; Favero *et al.* 1995). A third possible explanation for the decrease in caffeine-activated Ca^{2+} release is that P_i becomes elevated in the SR lumen as well as in the cytoplasm and Ca^{2+} then binds to P_i , forming a Ca^{2+} orthophosphate precipitate. This mechanism is thought to be involved in the impaired depolarization-induced release of Ca^{2+} during fatigue (Fryer *et al.* 1995) and it may also explain the reduced caffeine-activated release.

The relationship between the indo-1 ratio and $[\text{Ca}^{2+}]_i$ is slightly sensitive to pH with a 1 pH unit decrease in pH causing a 25% reduction in the Ca^{2+} sensitivity of indo-1 (Westerblad & Allen, 1993). Thus the peak tetanic $[\text{Ca}^{2+}]_i$ at the end of the low intensity fatigue when an acidosis of 0.12 pH units was present would be underestimated by about 3% ($25\% \times 0.12$) while the peak systolic $[\text{Ca}^{2+}]_i$ at the end of high intensity fatigue would be underestimated by 8.5% ($25\% \times 0.34$). These small corrections would not affect any of our conclusions.

Role of duty cycle in low frequency fatigue

While the pH-mediated increase in tetanic $[\text{Ca}^{2+}]_i$ cannot explain the reduction in force during fatigue, it may contribute to the prolonged reduction in Ca^{2+} release characteristic of low frequency fatigue (Westerblad *et al.* 1993; Chin & Allen, 1996). In the present study, low frequency fatigue was observed even at the higher intensity when force was reduced to 30% in less than a minute. The prolonged impairment in Ca^{2+} release is related to a sustained rise in cytosolic $[\text{Ca}^{2+}]_i$ (Lamb *et al.* 1995; Chin & Allen, 1996), which occurs both because of the rise in resting $[\text{Ca}^{2+}]_i$ and the more frequent tetani. Thus, even when muscles fatigue rapidly the cumulative $[\text{Ca}^{2+}]_i$ -time integral can be elevated above the threshold level required to induce low frequency fatigue (Chin *et al.* 1997) which suggests that both intensity-dependent and Ca^{2+} -dependent components are of importance in determining whether low frequency fatigue will occur.

Role of pH_i in impaired Ca^{2+} sensitivity during fatigue

A key observation from the present study is that the predominant pH-dependent mechanism of fatigue is a

reduction in Ca^{2+} sensitivity of the contractile proteins. During fatigue reductions in Ca^{2+} sensitivity are also attributed to elevations in P_i and reduced cellular ATP concentrations (Godt & Nosek, 1989; Parkhouse, 1992). In addition, these metabolic effects may be partly offset by increases in ADP, which increase Ca^{2+} sensitivity (Godt & Nosek, 1989). The contribution of pH_i to alterations in the force– Ca^{2+} relationship can be predicted from experiments in frog and mouse skeletal muscle (Westerblad & Allen, 1993; Baker *et al.* 1995). It was previously shown that a 0.5 pH unit change increased Ca_{50} from 357 to 532 nM, a 1.5-fold increase (0.17 pCa unit) (Westerblad & Allen, 1993). It is expected that a 0.22 pH unit change (0.34–0.12 pH units at the high and low intensities, respectively) would increase Ca_{50} by $0.22/0.5 \times 0.17$ pCa units = 0.076 pCa units or a factor of 1.19; this agrees with the observed change in Ca_{50} of 1.24 (524 nM/421 nM). Thus, the increased acidosis can explain most of the reduction in Ca^{2+} sensitivity. The molecular mechanism for the pH-induced reduction in Ca^{2+} sensitivity is not known, but one possibility is that the excess H^+ ions compete with Ca^{2+} and reduce their binding to the Ca^{2+} binding sites on troponin C (TnC) (Ball *et al.* 1994). Additionally, there may be changes in interactions between TnC and the other regulatory proteins (troponin I, troponin T and tropomyosin) and/or to altered binding of the troponin complex to the actin filament (Ball *et al.* 1994; Ding *et al.* 1996). The precise molecular interactions disrupted by reductions in pH_i in skeletal muscle are not currently known.

Role of pH_i in impaired maximum Ca^{2+} -activated force

pH-induced decreases in maximum Ca^{2+} -activated force of 10–15% have been shown in single muscle fibre preparations (Godt & Nosek, 1989; Westerblad & Allen, 1993) as well as in intact whole muscles (Baker *et al.* 1995). The decrease in maximum Ca^{2+} -activated force during isometric contractions at an acidic pH_i has been attributed to a decrease in the mean force produced per cross-bridge (see Westerblad *et al.* 1997). This is consistent with *in vitro* studies that show a 50% decrease in myofibrillar ATPase activity when pH_i is reduced from 7.0 to 6.5 (Parkhouse, 1992). While reductions in pH_i can decrease maximum force by impairing myosin ATPase activity, the contribution of this mechanism to the reduction in force during fatigue is still questionable. In the present study we observed a 17–19% decrease in F_{\max} at both intensities despite the differences in pH_i . Also, during repeated tetanic contractions the decline in force during early fatigue (~10%) occurs in the first 10–15 tetani. This initial fall in force is due to a reduction in maximum Ca^{2+} -activated force which is not associated with a decline in pH_i (Westerblad & Allen, 1992, 1993). Thus, decreases in maximum Ca^{2+} -activated force during repeated tetani and fatigue probably do not result from H^+ -induced effects on myosin ATPase activity. We cannot, however, rule out the possibility that in late fatigue pH_i -induced reductions in myosin ATPase occur but are offset by increases in ADP which increase maximum Ca^{2+} -activated force (Pate & Cooke, 1989).

In summary, the present study shows that in fast fatiguable fibres the predominant mechanism of muscle fatigue and the role of pH_i in fatigue are dependent on the work intensity. At lower intensities the predominant mechanism of fatigue is failure of E–C coupling, which is pH independent. In contrast, at higher intensities the reduction in Ca^{2+} sensitivity of the contractile proteins is largely due to the accompanying acidosis and takes on an increasing role in fatigue. The contribution of acidosis to the reduction in force during fatigue, however, would be smaller at more physiological temperatures.

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