The activity-induced reduction of myofibrillar Ca^{2+} sensitivity in mouse skeletal muscle is reversed by dithiothreitol

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The aim of this study was to further characterize the reduction of myofibrillar Ca^{2+} sensitivity in mouse muscle which has been observed after fatigue at 37°C. Muscle bundles and single fibres were isolated from mouse flexor digitorum brevis muscle and studied at 37°C. The single fibres were injected with the Ca^{2+} indicator indo-1. Muscle fatigue was produced by 0.4 s tetani repeated at 4 s intervals until force had fallen to less than 50% of initial. Excitation–contraction coupling was assessed by measuring the cytosolic calcium concentration ([Ca^{2+}]$_i$) during tetani, and the maximum Ca^{2+}-activated force and the myofibrillar Ca^{2+} sensitivity were estimated from a series of tetani at different stimulation frequencies. Two main results were found. (i) The reduction of Ca^{2+} sensitivity only occurred when the muscle was intensely stimulated leading to fatigue. When the muscle was rested for 10 min at 37°C there was no significant change in Ca^{2+} sensitivity. (ii) If the membrane-permeant thiol-specific reducing agent dithiothreitol (0.5 mM) was applied to the muscle for 2 min following the fatigue protocol, the reduction in Ca^{2+} sensitivity was reversed. Dithiothreitol had no effect on Ca^{2+} sensitivity in unfatigued preparations. There was no effect of fatigue or dithiothreitol on tetanic [Ca^{2+}]$_i$ or on the maximum Ca^{2+}-activated force. These results suggest that intense activity of skeletal muscle at 37°C causes the production of reactive oxygen species which oxidize a target protein. We propose that critical sulphydryl groups on the target protein(s) are converted to disulphide bonds and this reaction reduces Ca^{2+} sensitivity.

There is increasing evidence that reactive oxygen species (ROS) are involved in the regulation of muscle and can contribute to muscle fatigue and/or muscle damage (for review see Supinski, 1998; Reid, 2001). Many studies have shown that during activity muscles accelerate their production of ROS compared to rest (Davies *et al.* 1982; Reid *et al.* 1992*b*). It is also known that exogenous ROS scavengers can slow the rate of fatigue both in isolated muscles (Reid *et al.* 1992*a*) and intact animals and humans (Reid *et al.* 1994). A possible role for ROS in muscle fatigue is further supported by studies showing that exogenous application of ROS can reproduce some of the consequences of fatigue (Brotto & Nosek, 1996).

While a role for ROS in muscle fatigue is widely accepted, the details of how ROS are produced, which ROS have the most critical role and the cellular pathways of the action of ROS remain uncertain. A useful preliminary step in identification of the cellular pathway in muscle is to determine whether ROS affect (i) excitation–contraction coupling, (ii) the maximum Ca^{2+}-activated force, or

(iii) the Ca^{2+} sensitivity of the myofibrillar proteins. Studies of fatigue in isolated muscle fibres, with a Ca^{2+} indicator present to measure cytosolic calcium concentration ([Ca^{2+}]$_i$), allow the contributions of these three components to be identified (Westerblad & Allen, 1991).

There are a number of studies suggesting that ROS may affect excitation–contraction coupling. For instance Favero *et al.* (1995) showed that H$_2$O$_2$ could stimulate Ca^{2+} release from isolated sarcoplasmic reticulum (SR) vesicles and suggested that critical sulphydryl groups near the release site on the ryanodine receptor contribute to the regulation of Ca^{2+} release. In contrast Brotto & Nosek (1996) found that application of H$_2$O$_2$ to single skinned rat skeletal muscle fibres inhibited depolarization-induced release and suggest that this mechanism might contribute to muscle fatigue. However another study using a similar approach failed to identify any major effect of H$_2$O$_2$ on SR Ca^{2+} release (Posterino *et al.* 2003). A study on intact mouse muscle fibres also found that Ca^{2+} release was

relatively insensitive to exogenous application of H_2O_2 (Andrade *et al.* 1998).

The contractile and regulatory proteins may also be sensitive to ROS. A number of studies on skinned muscle have sought to characterize how the myofibrillar function is affected by a variety of exogenous ROS. For instance, superoxide can reduce the maximum Ca^{2+}-activated force in both cardiac and skeletal muscle (MacFarlane & Miller, 1992; Darnley *et al.* 2001; Callahan *et al.* 2001). However studies with H_2O_2 as the oxidant and DTT as reducing agent showed no effect on maximum force (Callahan *et al.* 2001; Lamb & Posterino, 2003). van der Poel & Stephenson (2002) heated intact skeletal muscle fibres to 43–46°C and subsequently skinned them and showed that maximum Ca^{2+}-activated force was reduced. Importantly this reduction of force was prevented by the ROS scavenger 4,5-dihydroxy-1,3-benzene-disulphonic acid (Tiron) and these authors suggested that superoxide produced at elevated temperatures damaged the ability of the contractile proteins to produce maximal force.

Studies of the Ca^{2+} sensitivity of myofibrillar proteins have often suggested that exposure of skinned fibres to ROS has minimal effect on Ca^{2+} sensitivity (MacFarlane & Miller, 1992; Darnley *et al.* 2001; Callahan *et al.* 2001; van der Poel & Stephenson, 2002). One exception was a study of skinned rat muscle in which, after an initial increase in Ca^{2+} sensitivity, there was a slowly developing reduction of Ca^{2+} sensitivity, which was not reversed by dithiothreitol (Lamb & Posterino, 2003). However in intact cardiac muscle there is good evidence that the post-ischaemia–reperfusion contractile weakness (stunning) is partly due to reduced Ca^{2+} sensitivity as a consequence of oxidative damage to regulatory proteins (for review see Bolli & Marban, 1999). Studies of intact mouse muscle showed that prolonged application of H_2O_2 produced a fall in Ca^{2+} sensitivity which was reversed by dithiothreitol (Andrade *et al.* 1998). Thus it seems that ROS may affect Ca^{2+} sensitivity by pathways which are lost in skinned fibres.

A characteristic finding of oxidative damage is that it can be prevented by pre-application of ROS scavengers and reversed by post-application of reducing agents. In this context dithiothreitol (DTT) is a membrane-permeant thiol-specific reducing agent which is capable of converting disulphide bridges in proteins to sulphydryl groups. Thus it is of particular interest that DTT was shown to improve recovery from fatigue in isolated diaphragm muscle at 37°C (Diaz *et al.* 1998).

We recently studied the fatiguability of isolated mouse skeletal muscle at both 22 and 37°C (Moopanar & Allen, 2005). We confirmed that mouse muscles fatigued more rapidly at 37°C and established that pre-application of the ROS scavenger, Tiron, slowed the rate of fatigue at 37°C but had no effect at 22°C. By measuring $[Ca^{2+}]_i$, we showed that the principal mechanism involved in the rapid onset of fatigue in single fibres at 37°C was a decline in Ca^{2+} sensitivity of the myofibrils. In the present study we have extended this work in two ways. We compare the decline in Ca^{2+} sensitivity at 37°C in fibres with minimal activity to fibres subjected to repeated stimulation leading to fatigue and show that intense muscle activity is necessary for the decline of Ca^{2+} sensitivity. We also show that the decline of Ca^{2+} sensitivity can be at least temporarily reversed by DTT. These results suggest that a specific protein in muscle can have sulphydryl groups converted to disulphide bridges in the oxidizing environment associated with muscle activity and that this change leads to a loss of Ca^{2+} sensitivity.

Methods

Male mice (Balb-C strain) were killed by cervical dislocation and small bundles or single fibres were dissected from the flexor digitorum brevis muscle of the hind limb (Lännergren & Westerblad, 1987). These experiments were approved by the Animal Ethics Committee of the University of Sydney. The flexor digitorum brevis contains about 75% Type IIX fibres with the remaining being Type IIA and Type I; we assume most of our dissected fibres are Type IIX (Allen *et al.* 1993). The isolated fibres had aluminium foil clips attached to the tendons and were mounted between a force transducer and an adjustable holder. Fibre length was adjusted to that producing the maximum tetanic force. The experimental preparation was superfused initially at room temperature (22°C) and thereafter at 37°C with a solution containing (mM): NaCl, 121; KCl, 5.0; $CaCl_2$, 1.8; $MgCl_2$, 0.5; NaH_2PO_4, 0.4; probenecid, 1.0; and glucose, 5.5. The solution was equilibrated with 95% O_2 and 5% CO_2, which gave a pH of 7.3. Probenecid was added to reduce the loss of indo-1 from the cell which was particularly prominent at 37°C. Dithiothreitol (DTT) and caffeine were prepared daily as concentrated stock solution and added to the perfusing solution as required.

Experimental protocol

Preliminary experiments were performed on small bundles without indo-1 injections to determine conditions under which recovery from fatigue would occur. Single fibre preparations were microinjected with indo-1 and allowed to rest at room temperature for 40 min. The preparations were stimulated intermittently at room temperature (0.4 s duration, 100 Hz frequency, 0.5 ms pulse duration, 1.2 × threshold) and only stable preparations used for the main experimental protocol.

The temperature in the muscle chamber was then raised to 37°C over a 2–3 min period. Ca^{2+} sensitivity, defined as the tetanic $[Ca^{2+}]_i$ which produced 50% of maximum force (Ca_{50}), and maximum Ca^{2+}-activated force (F_{max})

were assessed using the protocol described below which involved six tetani at 1 min intervals (see Fig. 1). The muscle preparation was then rested for 2 min before being fatigued by 0.4 s 100 Hz tetani repeated every 4 s until force was reduced to 50% of the initial level. Ca_{50} and F_{max} were reassessed post-fatigue after a 1 min rest. The muscle fibre was then rested for 3 min during which 0.5 mM DTT was applied to the preparation for the last 2 min. DTT was removed just prior to the third determination of Ca_{50} and F_{max}.

The basic protocol described above and in Fig. 1 was modified in various ways for control experiments. One control was similar to the basic protocol except that the muscle was not fatigued but instead rested for 10 min. This control is described as 'No fatigue' and was designed to determine whether the changes in muscle properties were a consequence of time at 37°C or the fatiguing stimulation.

A second set of control experiments were used to determine the effect of DTT on muscle properties in preparations which had not been fatigued, described as 'DTT control'. In these experiments, after the first determination of Ca_{50} and F_{max} at 37°C, the muscle was rested for 10 min, DTT (0.5 mM) was applied for 2 min, and then Ca_{50} and F_{max} were redetermined.

The third control was used to determine how muscle properties changed with time after the fatigue protocol. This protocol was identical to the basic protocol except that DTT was not applied and is described as 'Post-fatigue (ii)'. Only three experiments were completed in this series and there was only one successful caffeine application, so we do not report F_{max} for this series.

Measurement of [Ca²⁺]ᵢ

[Ca²⁺]ᵢ was measured by microinjecting each preparation with the fluorescent Ca²⁺ indicator indo-1 (Westerblad & Allen, 1991). The injected preparation was allowed to rest for 40 min and was thereafter illuminated with monochromatic light at 360 nm wavelength. The light emitted at 405 and 505 nm was measured using two photomultiplier tubes and these signals were then passed to an analog divide circuit which produced a 405/505 nm ratio signal. Background signal was subtracted from each photomultiplier tube (PMT) measurement prior to ratio calculation. The fibre was protected from the exciting illumination by a shutter at all times except during each tetanus. The ratio (R) measurements were later converted to [Ca²⁺]ᵢ using the following equation:

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

where K_D is the apparent dissociation constant of indo-1, β is the ratio of the 505 nm signal at zero and saturating [Ca²⁺]ᵢ, R_{min} is the ratio at very low [Ca²⁺]ᵢ, and R_{max} is the ratio at saturating [Ca²⁺]ᵢ (Grynkiewicz *et al.* 1985). In our present experimental equipment $\beta = 12.6$, $R_{max} = 0.59$, $R_{min} = 0.066$ and K_D was assumed to be 147 nM at 37°C (Moopanar & Allen, 2005).

Determining Ca_{50} and F_{max}

The protocol to determine Ca_{50} and F_{max} involved 0.4 s tetani at a range of frequencies (20, 30, 50, 70, 100 Hz and 100 Hz in the presence of 10 mM caffeine) at 1 min intervals
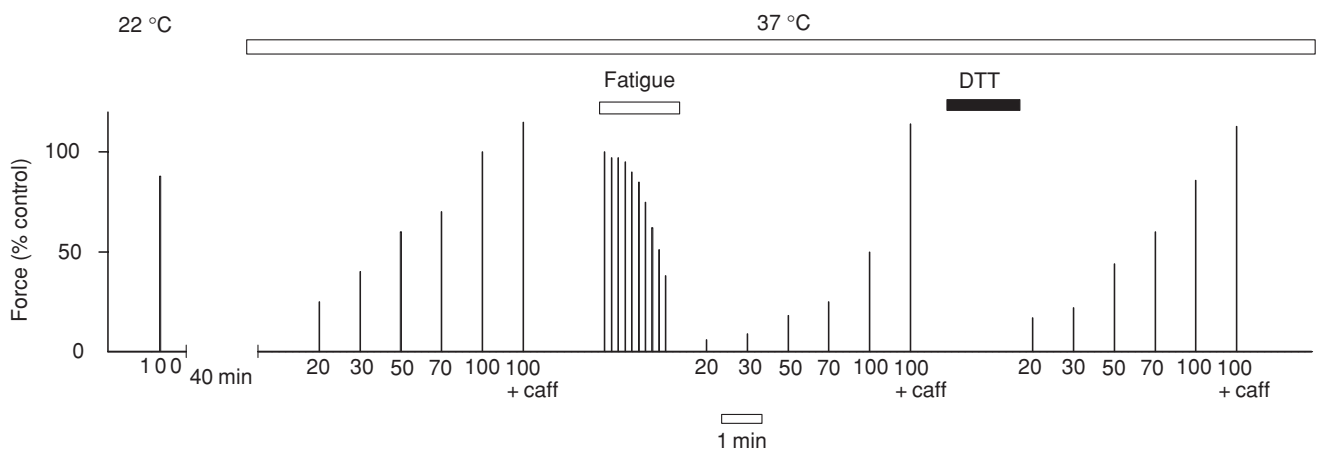

Figure 1. Basic protocol of the experiments
The diagram indicates the timing and amplitude of tetanic contractions; numbers below contractions indicate stimulus frequency (Hz). 100 + caff indicates a 100 Hz tetanus in the presence of 10 mM caffeine. Forces are representative of single fibre experiments and presented as a percentage of the first 100 Hz tetanus at 37°C. This figure is a timing diagram, not an actual experimental record.

(Westerblad & Allen, 1991). Caffeine exposure was kept as brief as possible ($\sim$20 s) since the muscle fibres appeared to be more sensitive to caffeine at 37°C and some fibres developed an irreversible contracture in caffeine. $[Ca^{2+}]_i$ and force during the tetani were estimated by taking an average of the last 200 ms of the tetanic record, by which time the force and $[Ca^{2+}]_i$ appeared to have reached a steady state relationship.

The values of F_{max} and Ca_{50} were determined by plotting peak tetanic force *versus* $[Ca^{2+}]_i$ for each frequency of stimulation including 100 Hz + caffeine and the resting $[Ca^{2+}]_i$ (see Fig. 3). These plots were fitted by a least squares minimization routine to the following Hill curve:

$$F = F_{max}[Ca^{2+}]_i^h / (Ca_{50}^h + [Ca^{2+}]_i^h)$$

where F is the force, F_{max} is the force at saturating $[Ca^{2+}]_i$, h is the Hill coefficient that describes the steepness of the curve, and Ca_{50} is the value of $[Ca^{2+}]_i$ that is required to elicit 50% of F_{max}. The values of h lay between 3.5 and 5.5 and are not reported. In 2 of the 6 main group of experiments, the final post-DTT caffeine tetani were not available. In order to reliably estimate the Ca_{50} for these experiments, we assumed F_{max} was unchanged, based on the fact that in the four experiments for which data were available, F_{max} had not changed significantly. The same approach was used to estimate Ca_{50} for the post-fatigue (ii) group for which only one caffeine result was available.

Statistics

Results are presented as means $\pm$ s.e.m. followed by the n value. Statistical significance was determined using Student's paired t test. Significance was accepted at $P < 0.05$.

Results

The effects of DTT on muscle bundles following fatigue at 37°C

Preliminary studies were performed on small muscle bundles as these were easier to dissect and were more resilient to the stresses of dissection and heat. Muscle bundles (5–10 fibres) were subjected to a similar stimulation protocol as single fibres. After the period of fatigue, the bundles were rested for 3 min before being re-stimulated at 100 Hz. In six experiments, the 100 Hz force after 3 min recovery was $56 \pm 4\%$ of pre-fatigue values.

In a second series of experiments, DTT was applied during the recovery period. In preliminary experiments we found that ≥ 2 mM DTT caused the fibres to become inexcitable. Small concentrations of DTT (≤ 0.25 mM) had only minimal effect on recovery. The optimal recovery was observed with 0.5 mM DTT, which was used for

the main series of experiments. When 0.5 mM DTT was applied in the final 2 min of rest and removed before restimulation, the 3 min recovery force was $102 \pm 6\%$ ($n = 6$) of the initial force. We also experimented with longer duration applications of DTT but the muscle performance rapidly deteriorated if DTT was maintained during the recovery contractions. These results confirm the earlier observations of Diaz *et al.* (1998) but do not reveal the cellular mechanism involved.

Intracellular calcium measurements in single fibres

Single muscle fibres were subjected to the stimulation protocol described in the Methods and required 10 ± 1 tetani ($\sim$40 s) to fatigue to 50% at 37°C. The 100 Hz force post-fatigue was similar to bundle experiments and the application of DTT caused a similar recovery of muscle function. In the following experiments we tested whether this recovery of force was caused by an action of DTT on (i) tetanic $[Ca^{2+}]_i$, (ii) F_{max} or (iii) Ca_{50}. The following experimental data show that the recovery of force triggered by DTT is not caused by changes in tetanic $[Ca^{2+}]_i$.

Figure 2 shows representative results from one experiment and it is clear that the tetanic $[Ca^{2+}]_i$ at 100 Hz is minimally affected following the fatigue protocol and also unaffected by the DTT application. Figure 4A shows averaged data from all experiments and in none of these conditions was tetanic $[Ca^{2+}]_i$ at 100 Hz significantly affected. In the pre-fatigue period, the 100 Hz tetanic $[Ca^{2+}]_i$ was 960 ± 50 nM ($n = 18$). As in our earlier study (Moopanar & Allen, 2005), in the post-fatigue period when force was greatly reduced there was no significant reduction in tetanic $[Ca^{2+}]_i$, which was 870 ± 40 nM ($n = 9$). Finally, following DTT application, tetanic $[Ca^{2+}]_i$ was unaffected (870 ± 60 nM, $n = 6$) even though there was a significant recovery of tetanic force.

When the fatiguing stimulation was omitted, and the muscle was simply rested at 37°C for 10 min, tetanic $[Ca^{2+}]_i$ was not significantly changed at 1080 ± 130 nM ($n = 5$). When DTT was applied to a control (unfatigued) muscle, tetanic $[Ca^{2+}]_i$ was not significantly different at 830 ± 60 nM ($n = 4$). Lastly, when the fatiguing protocol was used without DTT, it was found that tetanic $[Ca^{2+}]_i$ had still not significantly changed after the longer period post-fatigue (920 ± 40 nM, $n = 3$). These results show that neither the decline in force associated with fatigue nor the recovery in force with DTT are associated with changes in tetanic $[Ca^{2+}]_i$.

Maximum Ca²⁺-activated force

F_{max} and Ca_{50} were obtained for each stage of the experiment by fitting Hill curves to the tetanic data at the various frequencies. Figure 3 shows plots of $[Ca^{2+}]_i$ and force for the experimental data shown in Fig. 2 and

also shows the fitted curves from which Ca$_{50}$ and F_{max} were determined. Note that in the initial (pre-fatigue) series the 100 Hz force (rightmost unfilled circle), the 100 Hz + caffeine force (the filled circle), and F_{max} (the value to which the fitted curve approaches asymptotically) are all quite similar in magnitude. This is similar to previous results and suggests that the [Ca^{2+}]$_i$ during a normal 100 Hz tetanus is close to the level which produces maximum Ca^{2+}-activated force (Allen & Westerblad, 1995). In contrast in the post-fatigue data, the 100 Hz force (rightmost open triangle) is greatly reduced and addition of caffeine produces a large increase in force. We have already established that the 100 Hz tetanic [Ca^{2+}]$_i$ is unchanged by fatigue; thus the smaller force could either be because the Ca$_{50}$ is increased or F_{max} is reduced by fatigue. The fitting of the Hill curve clarifies the fact that F_{max} is unchanged by fatigue but Ca$_{50}$ increases substantially. Figure 4B shows average F_{max} values from all experiments under the various conditions studied. The pre-fatigue value for F_{max} was $117 \pm 2\%$ of the initial 100 Hz tetanus at 37°C ($n = 18$). After the muscle was fatigued, there was no significant difference in F_{max} ($116 \pm 6\%$, $n = 6$). Finally, after DTT was applied, it was found that F_{max} remained unchanged at $116 \pm 5\%$ ($n = 4$) of the initial.

In control experiments in which fatigue was omitted, the second determination of F_{max} was not significantly different ($110 \pm 2\%$ of control, $n = 5$). When DTT was applied without fatigue, F_{max} was not significantly changed at $117 \pm 2\%$ of control ($n = 4$). The post-fatigue (ii) value is not estimated because the caffeine exposure was only successfully complete in one out of three experiments. These results suggest that the decline in muscle performance at 37°C post-fatigue and the improvement in muscle function associated with DTT application are not due to changes in F_{max}.

Myofibrillar Ca^{2+} sensitivity

Figure 3 illustrates the results of plotting tetanic [Ca^{2+}]$_i$ and force during the various stages of one experiment. The open circles represent force and [Ca^{2+}]$_i$ for the initial stimulation protocol, and the fitted Hill curve (the
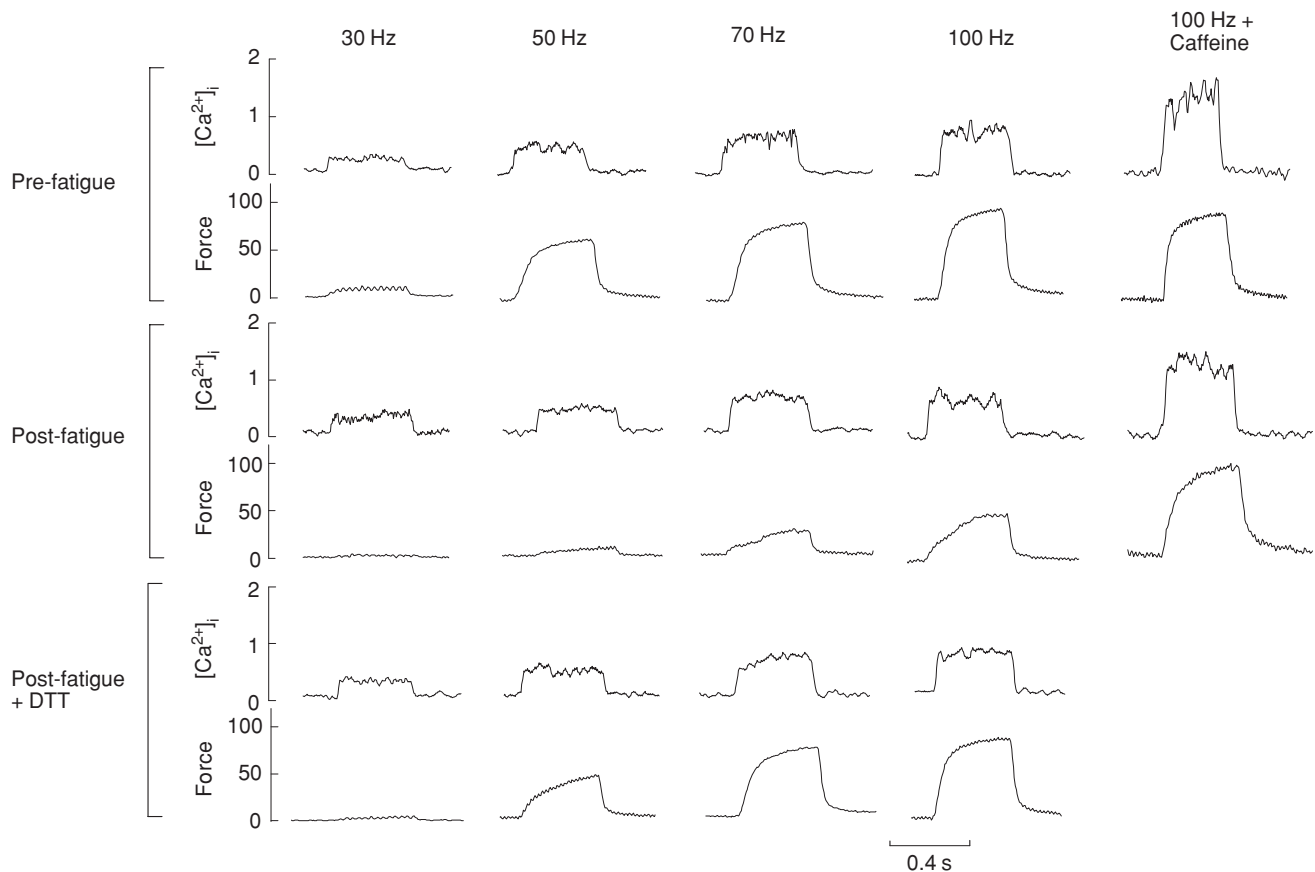

Figure 2. Tetanic [Ca^{2+}]$_i$ and force records from various stages of the experiment
Data from one experiment showing the tetanic [Ca^{2+}]$_i$ (μM) and force (% initial) records for the series of tetani required to determine F_{max} and Ca$_{50}$. Upper group from before fatigue; middle group from after fatigue; lower group after 2 min application of DTT (0.5 mM) to the fatigued muscle. Note that in this experiment the post-fatigue + DTT 100 Hz + caff tetanus was unsuccessful and is not shown.

continuous line) has a Ca_{50} of 561 nM. The open triangles represent stimulation just after the muscle was fatigued and the fitted line (long dashes) shows that Ca_{50} had increased to 728 nM. It is clear that, as in our earlier study (Moopanar & Allen, 2005), fatiguing stimulation at 37°C causes a substantial fall in Ca^{2+} sensitivity. The squares represent measurements of $[Ca^{2+}]_i$ and force after DTT was applied, and the fitted line (short dashes) shows that the Ca^{2+} sensitivity had largely recovered (Ca_{50} 577 nM).

Figure 4C shows a bar graph of Ca_{50} from all experiments including the various controls. The initial value of Ca_{50} was 650 ± 40 nM ($n = 18$). After fatigue, Ca_{50} was significantly increased to 870 ± 40 nM ($n = 9$) indicating a loss in myofibrillar Ca^{2+} sensitivity. After the DTT application to the fatigued muscle, the Ca_{50} recovered to 680 ± 40 nM ($n = 6$), which was not significantly different from the initial control value. In control experiments in which muscle preparations were stimulated 10 min apart, it was found that there was no significant difference in Ca_{50} (630 ± 110 nM, $n = 5$). Also, the application of DTT in unfatigued preparations had no significant effect on Ca^{2+} sensitivity ($Ca_{50} = 620 \pm 20$, $n = 4$). When the muscle was stimulated a second time after fatigue, Ca_{50} showed a significant further increase to 1050 ± 90 nM ($n = 3$), showing that there was an ongoing decline in Ca^{2+} sensitivity post-fatigue.
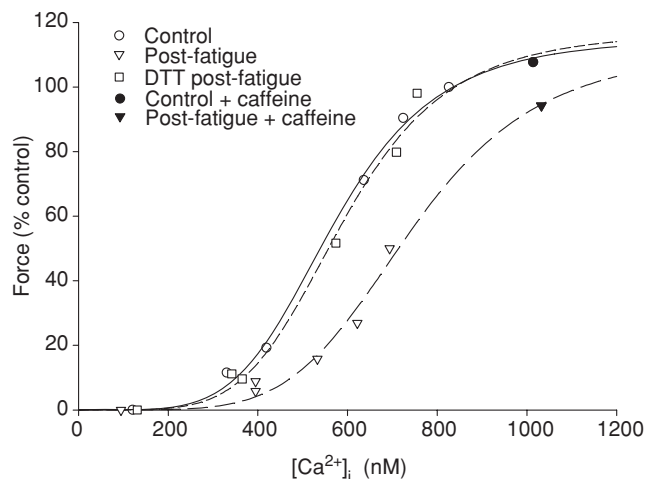

Figure 3. Force/[Ca²⁺]ᵢ plots from various stages of the experiment
Each point is the average $[Ca^{2+}]_i$ and force for the final 200 ms of the tetani shown in Figure 2. Forces are presented as percentage of the first 100 Hz tetanus at 37°C. The left-most points show resting $[Ca^{2+}]_i$. ○, before fatigue; ●, 100 Hz + caffeine before fatigue; ▽, post-fatigue; ▼, 100 Hz + caffeine post-fatigue; □, after DTT in the post-fatigue state. Lines show fits of the Hill equation to the data points; continuous line, pre-fatigue; long dashes, post-fatigue; short dashes, after DTT in the post-fatigue state. The line fitted to the DTT in the post-fatigue state data points has been fitted on the assumption that F_{max} was unchanged (see Methods).

Discussion

In our earlier study (Moopanar & Allen, 2005), we showed that fatigue in isolated single mouse muscle preparations at 37°C has different properties to the fatigue observed at 22°C. Specifically fatigue at 37°C develops more rapidly, but the increased rapidity can be prevented by including a ROS scavenger in the solution before and during the fatigue period. The accelerated fatigue does not involve large changes in F_{max} or tetanic $[Ca^{2+}]_i$ but is associated with a substantial fall in Ca^{2+} sensitivity and shows only a partial recovery with time. In the present study we confirm that fatigue at 37°C is caused by a substantial fall in Ca^{2+} sensitivity (increase in Ca_{50}) using a more rigorous protocol in which the identical sequence of stimulation was repeated after fatigue rather than using the tetani during the fatigue protocol. We also show that if sensitivity is redetermined after a further 9 min (post-fatigue (ii)), then it shows an additional decline. If the fall in Ca^{2+} sensitivity were caused by the effect of metabolites on the contractile proteins, as is thought to occur at room temperature (Allen *et al.* 1995), then one would have predicted that there would be an improvement over this period of rest as metabolites return towards normal rapidly during the resting period (Kushmerick & Meyer, 1985).

We also show that if the muscle was left resting for 10 min, instead of the fatiguing protocol, there was no significant change in Ca^{2+} sensitivity. This observation shows that the reduced sensitivity is not simply a consequence of time at 37°C but requires muscle contraction for its development. In fact although the preparation was rested for 10 min, it received 12 contractions spread over 20 min (6 tetani for each sensitivity determination) so that it appears that infrequent tetani do not induce the reduction in Ca^{2+} sensitivity. This strongly suggests that either rapidly repeated tetani, or perhaps fatiguing tetani, are required to induce this effect. This conclusion is consistent with our earlier observation that ROS scavengers prevent the reduced Ca^{2+} sensitivity, since it is known that the rate of production of ROS increases with frequency of activation (Reid *et al.* 1992a). It also fits well with the observation that this mechanism does not appear to operate at room temperature, since it is known that production of ROS is increased steeply by temperature (Zuo *et al.* 2000).

Our experiments also confirm the earlier observation that intermediate concentrations of DTT are capable of reversing the force deficit after fatigue at 37°C (Diaz *et al.* 1998). Diaz *et al.* showed that the beneficial effects of DTT were not apparent at ≤ 0.1 mM or ≥ 5 mM and were not apparent in unfatigued muscle, and we confirm both these observations. Diaz *et al.* applied 0.5–1.0 mM DTT continuously after fatigue and observed an improvement in performance over at least 90 min. However in our experiments the improved performance only lasted

10–20 min after which performance deteriorated (data not shown). We are unsure of the reason for this difference. Single fibres seem to be particularly susceptible to ROS at 37°C, possibly because intense illumination of cells required for $[Ca^{2+}]_i$ measurements is capable of generating additional ROS (King & Oh, 2004). If this is the case, it might be more difficult for DTT to reverse this process in our single fibre preparation.

An important novel finding in our study is that the mechanism of action of dithiothreitol is a reversal of the reduced Ca^{2+} sensitivity which characterizes this type of fatigue. In the post-fatigue (ii) condition Ca_{50} was

1050 ± 90 nm. At the same time in the DTT treated muscle fibres Ca_{50} was 680 ± 40 nm. Diaz *et al.* speculated that 'endogenously produced ROS down-regulate force production during fatigue by oxidizing critical sulphydryl groups on important redox-sensitive proteins'. Our findings strongly suggest that the protein(s) affected are involved in regulating Ca^{2+} sensitivity.

Cellular site of action

Many different protein modifications have been identified as a consequence of oxidative stresses. Methionine residues
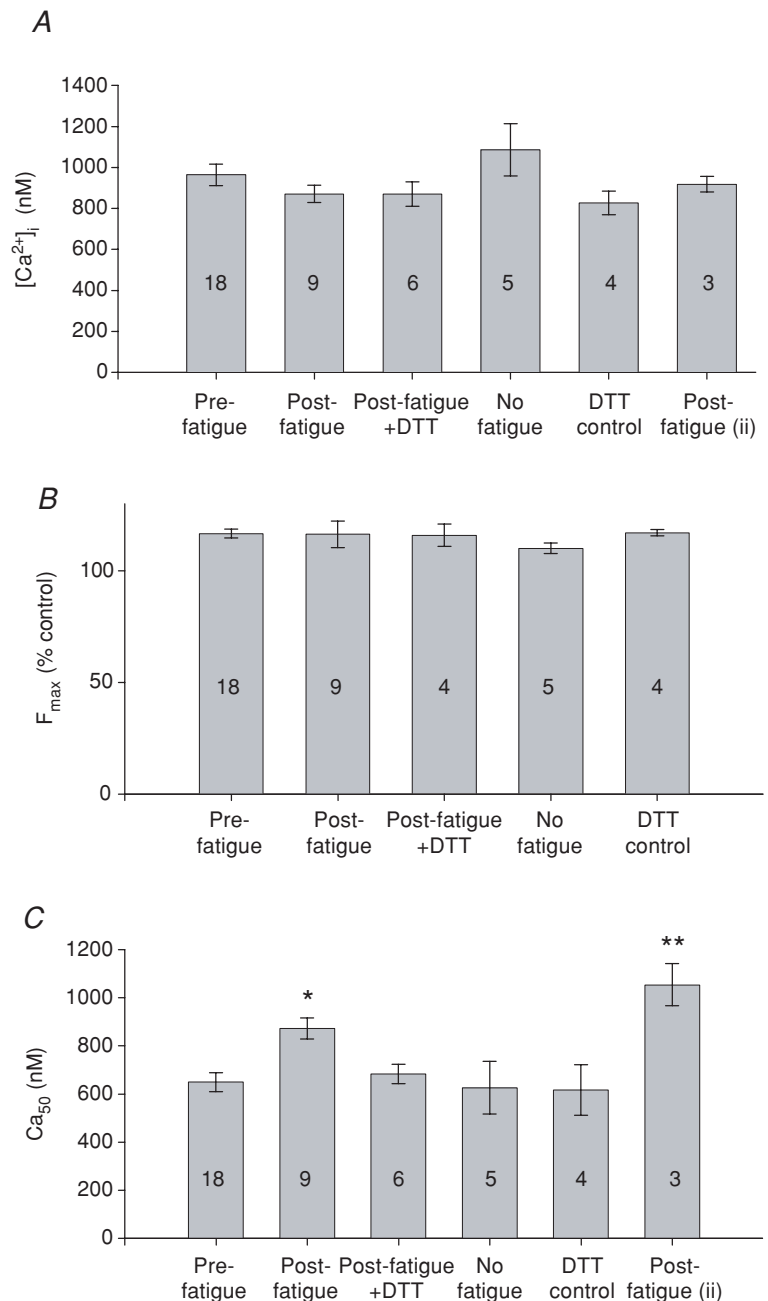

Figure 4. Average data for tetanic $[Ca^{2+}]_i$, F_{max} and Ca_{50}
Bars show the mean ± S.E.M.; numbers in the bars are the *n* values. *$P < 0.002$ compared to pre-fatigue; **$P < 0.001$ compared to pre-fatigue (paired *t* test comparing data points with their controls in the pre-fatigue group). The experimental conditions under each bar are defined in Methods.

are particularly susceptible and can be reversibly oxidized to methionine sulphoxide (Vogt, 1995; Davies, 2005). Cysteine residues are also susceptible and may be subject to *S*-glutathiolation, *S*-nitrosolation, as well as disulphide formation both within proteins and between proteins (Hogg, 2003; Davies, 2005). It is also known that oxidative damage makes proteins more susceptible to breakdown both by proteasome and by Ca^{2+}-activated proteases (Nakashima *et al.* 2004). While other possibilities are not excluded, the development of disulphide bonds seems an important possibility because of the reversal by DTT. Disulphide bonds are particularly common in extracellular proteins where they help stabilize proteins but may also be cleaved as part of the regulatory pathway for a particular protein (Hogg, 2003). Under most circumstances intracellular proteins are thought to be protected from disulphide bridge formation by the reducing environment of the cell but it seems that the oxidative stresses of intense muscle activity could be sufficient to allow this reaction to occur (Brennan *et al.* 2004).

The fact that myofibrillar Ca^{2+} sensitivity seems to be a target for oxidative damage suggests that troponin, tropomyosin, actin, myosin or myosin light chains may be possible targets. Cardiac troponin can form both inter- and intramolecular disulphide bonds and this process leads to an increase in Ca^{2+} sensitivity (Putkey *et al.* 1993). However skeletal troponin C does not have cysteines at the same location and it requires a mutant skeletal troponin C to form an intradomain disulphide bond (Grabarek *et al.* 1990). Brotto *et al.* (2000) and de Paula Brotto *et al.* (2001) have shown that hypoxic fatigue in diaphragm muscle at 22°C causes a loss of Ca^{2+} sensitivity which is associated with damage to troponin C and I; it is not clear how this relates to oxidative damage at 37°C but points to troponins as possible targets. Oxidative damage to tropomyosin and actin have been demonstrated in the heart following reperfusion damage (Canton *et al.* 2004). Evidence for disulphide bridges in myosin comes from application of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), an oxidizing agent which produces disulphide links between appropriately positioned cysteines. DTNB applied to skinned skeletal fibres caused force to fall rapidly to zero but force recovery occurred with DTT (Wilson *et al.* 1991). Thus currently, while troponins are the most obvious target, there is little evidence of oxidative damage to skeletal troponins and it remains possible that a variety of proteins might be involved in the loss of Ca^{2+} sensitivity.

Physiological significance

Single fibres are a useful model for identifying mechanisms of fatigue but they obviously differ in many ways from intact muscles. The time course of fatigue in single fibres at 37°C is very fast and they fail to fully recover; both features suggest that some aspect of their performance is unphysiological. It is probable that either endogenous ROS production is increased or ROS scavenging is reduced since addition of the ROS scavenger Tiron improves both these features (Moopanar & Allen, 2005). Thus it is important to stress that although in the single fibre model at 37°C changes in Ca^{2+} sensitivity due to oxidative damage appear to be the principal mechanism of fatigue, we suspect this mechanism may only be one contributor in intact muscles. Furthermore, it is generally accepted that there are many mechanisms of fatigue and the relative importance of a particular mechanism, such as oxidative damage, is likely to vary according to the fatigue protocol.

Persistent muscle weakness following intense exercise, or even relatively normal daily activities, is a common complaint whose cellular basis, if any, is poorly understood. Edwards *et al.* (1977) described a form of weakness in humans following intense muscle activity which could persist for several days. Part of the explanation for this type of weakness may be a persistent reduction in SR Ca^{2+} release (Westerblad *et al.* 1993), possibly triggered by Ca^{2+}-activated proteases acting on the ryanodine receptor (Lamb *et al.* 1995; Chin & Allen, 1996; Verburg *et al.* 2005). It is interesting to speculate whether reduced Ca^{2+} sensitivity secondary to oxidative damage might also contribute to this type of weakness. Westerblad *et al.* measured Ca^{2+} sensitivity 30 min after a fatiguing protocol and by that time Ca^{2+} sensitivity had returned to normal; however, the Westerblad *et al.* study was at room temperature so the kinds of changes seen in the present study would not be expected. We have been unable to find studies in which muscles are fatigued *in vivo* or at least at 37°C, and skinned fibres subsequently studied for changes in Ca^{2+} sensitivity. Thus two important goals for the future are to identify the target proteins for oxidative damage and to assess whether this mechanism operates in intact muscles during fatigue.

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