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Effects of oxidation and cytosolic redox conditions on excitation–contraction coupling in rat skeletal muscle

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In this study the effects of oxidation and reduction on various steps in the excitation–contraction (E–C) coupling sequence was examined in mammalian skeletal muscle. In mechanically skinned fast-twitch fibres, electric field stimulation was used to generate action potentials in the sealed transverse-tubular (T-) system, thereby eliciting twitch responses, which are a sensitive measure of Ca^{2+} release. Treatment of fibres with the oxidant H_2O_2 (200 μM and 10 mM) for 2–5 min markedly potentiated caffeine-induced Ca^{2+} release and the force response to partial depolarisation of the T-system (by solution substitution). Importantly, such H_2O_2 treatment had no effect at all on any aspect of the twitch response (peak amplitude, rate of rise, decay rate constant and half-width), except in cases where it interfered with the T-system potential or voltage-sensor activation, resulting in a reduction or abolition of the twitch response. Exposure to strong thiol reductants, dithiothreitol (DTT, 10 mM) and reduced glutathione (GSH, 5 mM), did not affect the twitch response over 5 min, nor did varying the glutathione ratio (reduced to oxidised glutathione) from the level present endogenously in the cytosol of a rested fibre (30:1) to the comparatively oxidised level of 3:1. In fibres that had been oxidised by H_2O_2 (10 mM) (or by 2,2´-dithiodipyridine, 100 μM), exposure to GSH (5 mM) caused potentiation of twitch force (by ~20 % for H_2O_2); this effect was due to the increase in the Ca^{2+} sensitivity of the contractile apparatus that occurs under such circumstances and was fully reversed by subsequent exposure to 10 mM DTT. We conclude that: (a) the redox potential across the sarcomplamsic reticulum has no noticeable direct effect on normal E–C coupling in mammalian skeletal muscle, (b) oxidising the Ca^{2+}-release channels and greatly increasing their sensitivity to Ca^{2+}-induced Ca^{2+} release does not alter the amount of Ca^{2+} released by an action potential and (c) oxidation potentiates twitches by a GSH-mediated increase in the Ca^{2+} sensitivity of the contractile apparatus.

There have been many suggestions about the importance and possible role(s) of oxidation in normal skeletal muscle function and muscle fatigue (Barclay & Hansel, 1991; Salama *et al.* 1992; Andrade *et al.* 1998; Feng *et al.* 2000; Reid, 2001; Oba *et al.* 2002), but there is little consensus. This is in large part due to major disparities between findings in isolated systems (e.g. single Ca^{2+}-release channels, sarcoplasmic reticulum (SR) vesicles or contractile apparatus) and those in intact muscle fibres. In isolated systems it is possible to tightly control the redox conditions and examine the effects on particular molecular events, but it is quite uncertain whether such findings can be validly extrapolated to the level of a functioning muscle fibre. For example, many studies have shown that oxidation of isolated ryanodine receptors (RyRs), with H_2O_2, reactive disulphides such as dithiodipyridine (DTDP) or other agents, greatly increases their sensitivity to activation by 'cytoplasmic' Ca^{2+} (Zaidi *et al.* 1989; Favero *et al.* 1995; Eager *et al.* 1997; Oba *et al.* 2002), but it is quite unclear whether

this is directly relevant to normal excitation–contraction (E–C) coupling in skeletal muscle, where Ca^{2+} release from the SR is primarily or exclusively under the control of the voltage sensors in the transverse-tubular (T-) system (Melzer *et al.* 1995). Normally, cytoplasmic Ca^{2+} by itself does not readily activate SR Ca^{2+} release in functioning muscle fibres, particularly mammalian muscle fibres, in the presence of physiological $[Mg^{2+}]$ (~1 mM free) and SR Ca^{2+} loading levels (Endo, 1985; Lamb & Stephenson, 1990; Shirokova *et al.* 1998; Lamb *et al.* 2001), although it may play a role in greatly reinforcing Ca^{2+} release if the voltage sensors are activated (Lamb & Stephenson, 1991, 1994; Lamb, 2000). It is possible that the normal mechanism of controlling Ca^{2+} release is altered by oxidation, as oxidation has been found to reduce the inhibitory effect of cytoplasmic Mg^{2+} in isolated RyRs (Donoso *et al.* 2000) and high concentrations of oxidants have been found to prevent activation of isolated RyRs (Favero *et al.* 1995; Eager *et al.* 1997).

Studies on intact fibres give important information about the effects of oxidation on E–C coupling, although the interpretation is not always straightforward. Reid *et al.* (1993) observed that twitch responses in the diaphragm were abolished by high extracellular concentrations of catalase, a scavenger of H_2O_2, and potentiated by addition of H_2O_2, and proposed that this showed that E–C coupling required some level of oxidation of the cytosol for optimal performance. Oba *et al.* (1996) similarly showed that twitch responses in frog fibres were potentiated by the addition of 1.5 mM H_2O_2, with higher concentrations (6 mM) causing greater potentiation and then inhibition of twitches. Although the twitch potentiation might seem to be explained by oxidation-induced increases in the sensitivity of the Ca^{2+}-release channels (see above), Andrade *et al.* (1998) subsequently showed that the analogous potentiation of submaximal tetanic responses occurring in intact murine fibres with H_2O_2 treatment was not due to an increase in cytoplasmic $[Ca^{2+}]$, but instead to an increase in the Ca^{2+} sensitivity of the contractile apparatus. The lack of substantial effect of the H_2O_2 treatment on Ca^{2+} release in the intact fibres (Andrade *et al.* 1998) might reflect that: (a) the release channels *in situ* for some reason are resistant to being oxidised and sensitised, perhaps because they are coupled to the voltage sensors in the T-system, (b) the oxidant was not present at a sufficiently high concentration or was scavenged before reaching the release channels, or (c) most or all of the release channels were oxidised, but this did not cause them to open spontaneously, nor did it alter their activation by the voltage sensors. Recently, Feng *et al.* (2000) have reported that Ca^{2+}-release channels in lipid bilayers can be activated by altering the redox potential difference across the bilayer, and they proposed that this could be important *in vivo* where the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSH), which influences the redox potential, is normally ~30:1 in the cytosol and ~3:1 in the lumen of the SR. In the study of Andrade *et al.* (1998), the intact fibres were bubbled with 95 % O_2 and the GSH:GSSG ratio in the cytosol before and during the H_2O_2 treatment is not known, nor is it known whether this was in any way important.

Mechanically skinned muscle fibres can be used to bridge the gap between intact fibres and isolated vesicles and channel preparations because it is possible to control and manipulate the intracellular environment and yet still be able to activate Ca^{2+} release via the voltage sensors in the T-system (Lamb & Stephenson, 1994). We have shown previously (Posterino & Lamb, 1996) that the force response elicited by depolarising the T-system by ion substitution over several seconds is not noticeably altered by strong reducing conditions, but is abolished when the skinned fibres are exposed to the specific sulphydryl reagent, 2,2´-dithiodipyridine (DTDP; 100 μM), apparently owing to interference with voltage-sensor activation. Brotto & Nosek (1996) reported that oxidation by H_2O_2 (1 mM) also

inhibited depolarisation-induced Ca^{2+} release in skinned fibres and attributed this to the oxidation abolishing Ca^{2+}-induced Ca^{2+} release (CICR). In contrast to this last conclusion, Plant *et al.* (2002) recently showed that treatment with 1 mM H_2O_2 increased the sensitivity of caffeine-induced Ca^{2+} release in both extensor digitorum longus (EDL; fast-twitch) and soleus (slow-twitch) fibres. Plant *et al.* (2002) further found that this H_2O_2 treatment increased the force response to depolarisation (by ion substitution) in slow-twitch fibres, but not in fast-twitch fibres. However, it is unclear (a) whether there was any increase in Ca^{2+} release in the latter case, because the force was already near maximal before exposure to H_2O_2 and (b) whether the increased force response in the slow-twitch fibres was due to increased Ca^{2+} release or an increase in Ca^{2+} sensitivity of the contractile apparatus (Plant *et al.* 2002).

In the study presented here, we investigated how the intracellular redox conditions affect various steps in the E–C coupling sequence in skeletal muscle, using a mechanically skinned fibre preparation (Posterino *et al.* 2000) in which it is possible to trigger Ca^{2+} release by action potential stimulation. We show, using the twitch response to action-potential stimulation as a sensitive measure of Ca^{2+} release, that the normal E–C coupling mechanism in skeletal muscle works well even under very reduced conditions, and also that it is not significantly modified by even large changes in the GSH:GSSG ratio in the cytosol. Importantly, we further show that H_2O_2 treatment does oxidise the Ca^{2+}-release channels *in situ*, greatly potentiating their sensitivity to caffeine-induced Ca^{2+} release and CICR, but that this does not change the amount of Ca^{2+} release elicited by action-potential stimulation; this key observation has major implications in any consideration of the mechanisms involved in activating and terminating Ca^{2+} release in muscle fibres. Finally, we show that the oxidation-induced twitch potentiation that occurs in intact fibres (Oba *et al.* 1996; Andrade *et al.* 1998) is well explained by a paradoxical effect of GSH on the contractile apparatus of oxidised fibres (Lamb & Posterino, 2003).

METHODS

Skinned-fibre preparation and force recording

Male rats (Long Evans hooded; 4–6 months old) were killed humanely by halothane overdose, as approved by the Animal Ethics Committee of La Trobe University. The EDL muscles were dissected and pinned out in paraffin oil in a Petri dish kept on ice. In some experiments, whole EDL muscles were first immersed for 30 min in a mammalian saline solution that contained (mM): NaCl 140, KCl 4, $CaCl_2$ 2.5, $MgCl_2$ 1, Hepes 10, pH 7.1 and 10 μM tetrodotoxin (TTX); these muscles were then blot dried and immersed in paraffin oil. Single fibres were mechanically skinned, mounted on a force transducer (AME801, SensoNor, Norway, resonance frequency > 2 kHz) and stretched to 120 % of their resting length. The fibre was then placed in the standard potassium hexamethylene-diamine-tetraacetate (K-HDTA) solution (see below) with 50 μM total EGTA for 2 min to equilibrate. Force

responses in the electrical stimulation experiments were amplified with a Bioamp pod (ADI Instruments, Sydney, Australia) attached to a 400-series Powerlab and recorded simultaneously on both chart recorder and computer using Chart software (version 3.14); the slower force responses in the caffeine-activation experiments were recorded on a chart recorder. All experiments were carried out at $23 \pm 2\,°C$.

Solutions

All chemicals were obtained from Sigma, unless specified otherwise. The standard K-HDTA solution contained (mM): 126 K^+, 37 Na^+, 50 $HDTA^{2-}$ (Fluka, Buchs, Switzerland), 8 total ATP, 8.6 total Mg^{2+}, 10 creatine phosphate, 0.05 total EGTA, 90 Hepes, $1\,N_3^-$, pH 7.10 ± 0.01, and pCa ($-\log_{10}[Ca^{2+}]$) = 7.0. The free $[Mg^{2+}]$ was 1 mM. The Na-HDTA solution was identical except that all K^+ was replaced with Na^+. Solutions with a free $[Mg^{2+}]$ of 0.01, 0.05 mM and 10 mM were similar to the standard K-HDTA solution except that they contained 1.0, 2.1 and 22.7 mM total Mg^{2+}, respectively. Maximum Ca^{2+}-induced activation of skinned fibres was ascertained using a solution similar to the K-HDTA solution but with all HDTA replaced with 50 mM Ca-EGTA (20 μM free Ca^{2+}) and the total Mg^{2+} reduced to 8.3 mM to maintain 1 mM free Mg^{2+}. 'Rigor' solutions were similar to the standard K-HDTA solution except that all ATP and creatine phosphate were replaced with additional HDTA, the total EGTA was 0.25 mM and the total Mg^{2+} was adjusted to give either 0 or 10 mM free $[Mg^{2+}]$. All solutions had an osmolality of 295 ± 10 mosmol kg^{-1}. Free $[Ca^{2+}]$ (for pCa < 7.3) was determined with a Ca^{2+}-sensitive electrode (Orion Research, Boston, MA, USA).

All reagent stocks were made fresh on the day. Dithiothreitol (DTT) was made as a 1 M stock in a K-HDTA solution and diluted as required. GSH and GSSG were made as 100 mM stocks in a K-HDTA solution and the pH adjusted back to 7.10 using KOH. Ratios of GSH:GSSG were made by adding appropriate volumes of the stock solutions together maintaining a 5 mM total glutathione concentration. A volume of H_2O_2 (Sigma ultra) was added directly from a 35 % w/v stock to solutions (to give a final concentration of 10 mM) and an appropriate volume of this solution used to make up 200 μM where required. The fidelity of the 35 % w/v stock was tested by using a colour-change assay with potassium permanganate to verify the concentration of H_2O_2 in the final solution. The reagent DTDP was made as a 100 mM stock in pure ethanol and diluted 1:1000 (final concentration 100 μM) with equivalent volumes of ethanol added to control solutions.

Caffeine-induced Ca^{2+} release

Following skinning and equilibration for 2 min in a K-HDTA solution containing 50 μM total EGTA (pCa 7.0), fibres were briefly equilibrated in a similar solution containing 0.5 mM EGTA ('wash' solution, pCa ~8) for 10 s, and then the endogenous SR Ca^{2+} content was ascertained by releasing all of the Ca^{2+} in the SR using a K-HDTA solution containing 30 mM caffeine, 0.05 mM free Mg^{2+} and 0.5 mM total EGTA to chelate any released Ca^{2+} ('full release' solution, 1 min exposure). The area of the ensuing force response is indicative of the total amount of Ca^{2+} present in the SR (see Bakker *et al.* 1996; Lamb *et al.* 2001). Fibres were washed for 1 min in the wash solution before the SR was re-loaded with Ca^{2+} in a Ca^{2+}-buffered K-HDTA solution (pCa 6.7, 1 mM total EGTA) for various times (10–30 s). Fibres were then equilibrated again in the wash solution for 10 s and the SR emptied using the full release solution. This load–release cycle was repeated several times to find the load time (~20 s in most fibres) required to restore the SR Ca^{2+} content at close to the level that had been present endogenously. The load–release cycle was then

modified as follows to include a test of the responsiveness to 8 mM caffeine. The SR was first loaded with Ca^{2+} for a set time (to close to the original endogenous level) and then the fibre was equilibrated in the standard K-HDTA solution (50 μM total EGTA, pCa 7.0) for 20 s before being exposed to an identical solution containing 8 mM caffeine (for 15 s), which typically elicited a small force response (e.g. top traces in Fig. 1). The SR was then fully depleted again with the full release solution, the area of this response indicating how much Ca^{2+} had remained in the SR after the response in 8 mM caffeine. The entire protocol was then repeated several times to ensure that the force response elicited by the 8 mM caffeine solution was reproducible. Parameters measured from the 8 mM caffeine response were peak force and the rate of rise of force. Both parameters are expressed relative to the maximum Ca^{2+}-activated force; the rate of rise of force was defined as the force at 80 % of peak height divided by the time taken to reach that level after caffeine was applied (expressed as percentage of maximum Ca^{2+}-activated force ('Max') per second). Prior to exposure to a reagent of interest (e.g. 10 mM H_2O_2), fibres were usually first fully depleted of Ca^{2+} and then exposed to a wash solution (pCa ~8) containing the reagent in question for up to 5 min; very similar results were obtained when treatment was applied to fibres in a solution at pCa 7.0 when loaded. Fibres were then washed and the load–deplete cycles and load–caffeine–deplete cycles repeated again as above.

The effect of varying the GSH:GSSG ratio was also examined. Fibres were exposed to the appropriate solutions (see above) containing a 30:1 ratio of GSH:GSSG (5 mM total) immediately after skinning, in order to maintain the GSH:GSSG ratio at an endogenous level both in the cytoplasm and within the SR (see Feng *et al.* 2000), subjected to several load–deplete cycles and the response to 8 mM caffeine tested at a given SR Ca^{2+} load level. Fibres were then exposed to matching solutions all containing a GSH:GSSG ratio of 3:1, reloaded for the same time and the response to caffeine determined under these conditions on several repetitions over ~15–20 min in total. Fibres were then returned to solutions containing the 30:1 GSH:GSSG ratio and caffeine responses again determined over a further 15–20 min.

Ca^{2+}-induced Ca^{2+} release

To measure CICR, the SR was emptied of all Ca^{2+} with the full release solution and reloaded for 30 s as above, and then either (a) emptied again to measure the SR Ca^{2+} content or (b) first exposed to either a 'control' or 'test' set of rigor solutions for a total of 2 min before being emptied (e.g. Fig. 2). In the 'control' case, the fibre was placed for 1 min in a rigor solution (with 10 mM free Mg^{2+} and 0.25 mM EGTA, pCa > 7.5) to remove all ATP (preventing Ca^{2+} uptake), transferred to a similar solution with zero Mg^{2+} for 30 s and then returned to 10 mM Mg^{2+} rigor solution for 30 s. The fibre was then placed in the standard 'wash' solution with ATP for 10 s to relax before being exposed to the full release solution to assay how much Ca^{2+} remained in the SR after the rigor period (see Fig. 2). The $[Mg^{2+}]$ was raised to 10 mM during most of the rigor period to minimise Ca^{2+} leakage. In the 'test' case the procedure was identical except that the free $[Ca^{2+}]$ in the zero Mg^{2+} rigor solution was set at 2 μM to try to elicit CICR.

T-tubular depolarisation by electrical stimulation and solution substitution

Electrical stimulation. EDL fibres were placed in a small Perspex chamber (~400 μl) containing the standard K-HDTA solution (50 μM EGTA, pCa 7.0) and centred between two platinum wire electrodes placed in parallel, 4 mm apart, which were connected to an in-house stimulator with a current output of up to 300 mA

(Posterino *et al.* 2000). Fibres were initially stimulated with single brief pulses (70 V cm^{-1}, 2 ms) which elicited twitch responses at a rate of 1 every 30 s until they reached a steady level (generally ~2–3 min). Where appropriate, fibres were then transferred to a second electrode bath containing a matching solution with the reagent of interest and stimulated at the same frequency for up to 5 min. Twitch parameters (see below) were measured either in the presence of the reagent or after the reagent was washed from fibres for an additional 30 s. Each parameter is expressed as a percentage of its value in the control response (i.e. in the standard K-HDTA solution) measured immediately before the treatment. Twitch parameters measured were: peak amplitude, rise time (calculated between 20–80 % of the peak amplitude), decay rate constant (determined by fitting a single exponential to the last 50 % of the

Figure 1. H$_2$O$_2$ treatment potentiates caffeine-induced Ca^{2+} release

Force response to 8 mM caffeine (indicated by bar, 2 s time calibration) in a mechanically skinned extensor digitorum longus (EDL) fibre. The fibre was subjected to repeated cycles in which the SR was emptied of Ca^{2+}, reloaded for a set time and then 8 mM caffeine applied (see Methods). Between cycles, the fibre was given various treatments (shown on left). The responses to 8 mM caffeine were obtained in order from left to right and top to bottom. The first pair of force responses was elicited in a freshly skinned fibre (Control). Both responses were relatively small (~2–3 % of the maximum Ca^{2+}-activated force, MAX, bottom right panel) and exhibited a considerable delay (~2 s) before force developed. Treatment with 200 μM H$_2$O$_2$ for 2 min increased the response to 8 mM caffeine approximately twofold and an additional 3 min treatment caused a further increase (~10-fold in total). The effect of H$_2$O$_2$ was fully reversed by treatment with 10 mM DTT. Treatment with 10 mM H$_2$O$_2$ for 5 and 20 min produced more marked effects on the 8 mM caffeine response; the peak size increased to ~65 % and ~85 % of MAX, respectively. Time calibration for MAX: 30 s.

decay phase of the twitch response) and half-width (the duration of the response measured at 50 % of peak height). The last three parameters were determined after normalising the peak amplitude.

Solution substitution. Following the initial 2 min equilibration period in a standard K-HDTA solution, the T-system of fibres could be fully depolarised by substituting the K-HDTA solution with the Na-HDTA solution, eliciting a force response as described previously (Lamb & Stephenson, 1994). To elicit submaximal depolarisation-induced force responses, skinned fibres were dissected from the superficial regions of muscles that had been treated with 10 μM TTX (see earlier) to eliminate T-tubular action potentials. The skinned fibre was then depolarised by substituting the K-HDTA solution with the Na-HDTA solution or with a 9:1 mixture of the Na- and K-HDTA solutions (~12 mM K$^+$; see Fig. 5), and treated with H$_2$O$_2$ as described. The T-system was repolarised in the K-HDTA solution for 45–60 s between successive depolarisations. Only TTX-treated fibres in which full Na$^+$ substitution elicited > 60 % of maximum Ca^{2+}-activated force, and the 12 mM K$^+$ substitution elicited > 20 %, were deemed to be properly depolarised and used in this study; about half of the fibres satisfied these criteria. Parameters determined were the peak size of the force response (expressed as a percentage of the maximum Ca^{2+}-activated force, 'MAX', in each fibre), and the rate of force rise (calculated as the force at 80 % of the peak response divided by the time taken to reach that force following immersion in the Na$^+$/K$^+$ solution; expressed as percentage of Max per second).

Statistical analysis

Data are presented as the mean ± S.E.M. and were analysed statistically using either a one-way analysis of variance with Dunnett's post test or Student's paired *t* test, as appropriate. Values were considered significant if $P < 0.05$.

RESULTS

Effect of oxidation on caffeine-induced Ca^{2+} release

We first examined whether oxidation by H$_2$O$_2$ increased the Ca^{2+} sensitivity of the Ca^{2+}-release channels in these skinned fibres by testing their responsiveness to a submaximal caffeine stimulus (see Methods). Exposure to 8 mM caffeine typically triggered a small, slowly rising force response that was quite reproducible on successive test cycles (e.g. top traces in Fig. 1). Treatment with 200 μM H$_2$O$_2$ for 5 min potentiated the response to caffeine in every fibre examined ($n = 7$), increasing the peak force to ~50 % of the maximum Ca^{2+}-activated level and the rate of force development by approximately fourfold (Table 1). Briefer treatment with 200 μM H$_2$O$_2$ (2 min) caused less potentiation (e.g. compare second and third rows in Fig. 1). Treatment with 10 mM DTT for 5 min fully reversed the potentiation in every fibre (e.g. Fig. 1; see Table 1), showing that the potentiation was caused by an oxidation reaction. The increased force response to caffeine was clearly due to increased Ca^{2+} release because (a) the Ca^{2+} sensitivity and maximum force production of the contractile apparatus in the EDL fibres was not significantly altered by 5 min oxidation with 200 μM H$_2$O$_2$ (Lamb & Posterino, 2003) and (b) the SR was depleted of
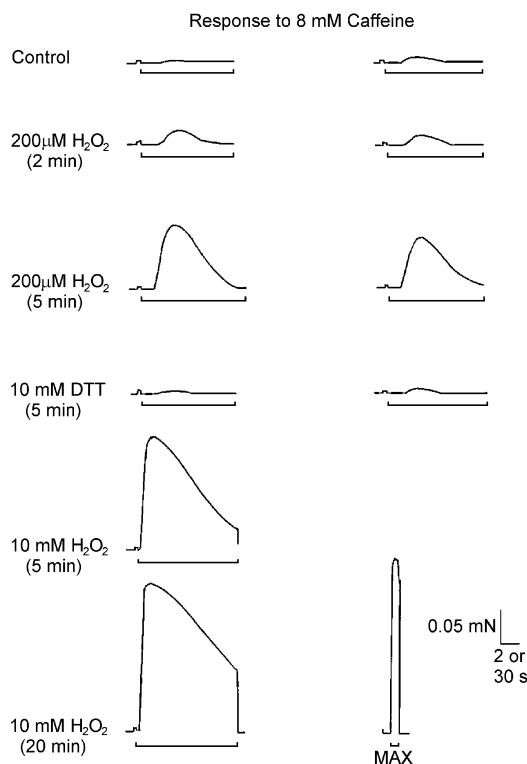
Table 1. Effects of H_2O_2 and DTDP treatments on caffeine-induced force responses

Treatment	Peak of response (% of max force)			Rate of rise of response (% max force s^{-1})		
	Control	Post-treatment	Post-DTT	Control	Post-treatment	Post-DTT
1. 200 μM H_2O_2 (5 min) ($n = 7$)	17 ± 6	51 ± 9	16 ± 6	6 ± 1	23 ± 5	7 ± 3
2. 10 mM H_2O_2 (5 min) ($n = 12$)	18 ± 5	72 ± 6	—	6 ± 1	73 ± 10	—
3. 10 mM H_2O_2 (5 min) (subset of 2; $n = 4$)	25 ± 11	77 ± 15	20 ± 11	7 ± 3	90 ± 22	9 ± 5
4. 10 mM H_2O_2 (20 min) (subset of 2; $n = 3$)	21 ± 9	85 ± 9	—	6 ± 2	87 ± 25	—
5. 100 μM DTDP (5 min) ($n = 3$)	12 ± 5	44 ± 12	27 ± 8	16 ± 10	89 ± 31	19 ± 7

Means ($\pm$ s.e.m.) of peak force and rate of rise of force in skinned EDL fibres upon stimulation with 8 mM caffeine (see Fig. 1). n is the number of fibres. In each fibre the response to caffeine was examined before (control) and after each treatment indicated, and again after exposure to 10 mM DTT for 10 min where indicated. The peak of the response was expressed as a percentage of the maximum Ca^{2+}-activated force (max force) in the same fibre. The rate of rise of force was calculated from the time for the caffeine response to reach 80% of its peak. In every fibre examined the caffeine response increased after the indicated treatment and decreased again after exposure to DTT. The increase in peak and rate of rise were significant ($P < 0.05$) in every case except those for DTDP treatment; the latter was significantly different ($P < 0.05$) when expressed as a relative change on a logarithmic scale. In every case the response after DTT exposure was not significantly different from initial control response.

Ca^{2+} to a greater extent following the potentiated response to 8 mM caffeine, as indicated by the smaller force response found when subsequently emptying the SR of its remaining Ca^{2+} before starting the next cycle. This response indicated that before H_2O_2 treatment 92 ± 4 % of the Ca^{2+} remained in the SR after the 8 mM exposure, whereas following H_2O_2 treatment only 75 ± 4 % remained after the 8 mM exposure ($P < 0.05$, paired t test).

Oxidising the fibres with a higher concentration of H_2O_2 (10 mM) for 5 min had a more marked effect than 200 μM H_2O_2 (e.g. Fig. 1), invariably making the fibre extremely responsive to the 8 mM caffeine stimulus, with the force on average increasing ~12 times faster than in the control case and reaching ~70–80 % of the maximum Ca^{2+}-activated force in that fibre (Table 1). The fibres were also far more responsive than normal to other stimuli, such as to lowering [Mg^{2+}] below 1 mM (not shown). Again these effects were fully reversible with DTT (Table 1). Increasing the total duration of the 10 mM H_2O_2 treatment from 5 to 20 min caused a further small increase in the responsiveness to caffeine in all three fibres examined. Two fibres treated with 10 mM H_2O_2 and then exposed to GSH (5 mM) for 2 min showed potentiation of caffeine-induced Ca^{2+} release that was indistinguishable from the effect of H_2O_2 treatment alone described above. The specific sulphydryl reagent DTDP (100 μM, 5 min) also increased the peak force and the rate of rise of the force response to caffeine in all three EDL fibres examined, with the effects all partially or fully reversed by treatment with DTT for 5 min (see Table 1).

It was also apparent that net Ca^{2+} uptake into the SR under the standard loading conditions (pCa 6.7) was decreased in fibres that had been oxidised by 5 (or 20) min treatment with 10 mM H_2O_2. This was evident from the relatively reduced area (i.e. time integral) of the force response when

a fibre was loaded and then immediately emptied of all Ca^{2+}. This effect, which was reversed by DTT treatment, was seen in every fibre treated with 10 mM H_2O_2 ($n = 15$), with the reduction in area of the response upon full release ranging from ~10–30 %; this can also be seen in Fig. 2 from the extra loading time needed after H_2O_2 treatment to reach approximately the same final SR Ca^{2+} content. Milder oxidising treatment with 200 μM H_2O_2 had no such effect. The reduction in net Ca^{2+} uptake after treatment with 10 mM H_2O_2 could be due both to a reduction in the rate of Ca^{2+} pumping into the SR and to an increase in the rate of efflux of Ca^{2+} from the SR during uptake (Andrade *et al.* 1998).

Increased CICR in oxidised fibres

We examined directly the effect of H_2O_2 treatment on CICR by measuring the amount of Ca^{2+} lost from the SR during a period of Ca^{2+} exposure when all uptake was prevented by having the skinned fibre in 'rigor' solutions with no ATP (Fig. 2). Before oxidative treatment, the SR lost the same amount of Ca^{2+} from the SR during a 2 min rigor period irrespective of whether or not it included a 30 s exposure to 2 μM free Ca^{2+}, indicating that there was no measurable CICR at that free [Ca^{2+}]. This is not surprising as there was no ATP present to stimulate the Ca^{2+}-release channels (Endo, 1985; Lamb, 2000) and the free [Mg^{2+}] was unlikely to have dropped completely to zero from 10 mM during the Ca^{2+} exposure period (see Methods). After treatment with 10 mM H_2O_2, there was substantial CICR (Fig. 2). Similar results were observed in every fibre examined ($n = 6$); before H_2O_2 treatment, the amount of Ca^{2+} in the SR after the rigor-Ca^{2+} period was not significantly different from that found with bracketing rigor-only periods (rigor only: 38 ± 5 %, rigor-Ca^{2+}: 40 ± 5 %; rigor only: 37 ± 6 %; expressed relative to the response obtained without exposure to rigor solutions), whereas after H_2O_2 treatment exposure to Ca^{2+} reduced the

response relative to bracketing rigor-only responses in every fibre ($30 \pm 7\%$, $9 \pm 3\%$ and $20 \pm 6\%$, in same order; middle value significantly smaller than bracketing values, $P < 0.05$). Thus, the H_2O_2 treatment substantially potentiated CICR. This was also the case when stimulating release with $30~\mu M$ Ca^{2+} (with $1~mM$ free Mg^{2+} present). In those experiments (three fibres), there was substantial CICR even before oxidative treatment, and this was increased by the treatment. However, as described previously (Lamb & Cellini, 1999), the 30 s exposure at such a high $[Ca^{2+}]$ caused an increase in Ca^{2+} leakage from the SR, which made it difficult to assay accurately the amount of CICR because the test of CICR deleteriously affected the subsequent control responses.

Effect of the GSH:GSSG ratio on caffeine-induced responses

We also examined the effect on caffeine-induced Ca^{2+} release of altering the ratio of GSH:GSSG in the bathing solution from a relatively 'reduced' level (30:1) to an 'oxidised' level (3:1; see Feng *et al.* 2000). The responsiveness to caffeine was tested with the GSH:GSSG ratio first at 30:1, then 3:1 and then again at 30:1 (see Methods). Such changes have no significant effect on the Ca^{2+} sensitivity of

the contractile apparatus in these fibres (Lamb & Posterino, 2003). In the four fibres examined, the peak size and rate of rise of the caffeine response with a 3:1 GSH:GSSG ratio were $120 \pm 28\%$ and $130 \pm 41\%$, respectively, of the values obtained during the bracketing periods with a 30:1 ratio, thus indicating no significant difference between treatments (paired *t* test, $P > 0.05$). Alteration of the GSH:GSSG ratio from 30:1 to 3:1 also had no significant effect on net Ca^{2+} uptake, as assayed by the area of the response when subsequently emptying the SR with the full release solution (mean area for the 3:1 case being $108 \pm 4\%$ ($n = 4$) of the bracketing 30:1 cases; $P > 0.05$, paired *t* test). Thus, varying the GSH:GSSG ratio in the cytoplasm from 30:1 to 3:1 did not enhance CICR and caffeine-induced Ca^{2+} release in these skinned fibres, which contrasts markedly with the effect of specific oxidative treatments with H_2O_2 or DTDP (see above).

Twitch responses in mechanically skinned fibres

Electric field stimulation was used to elicit twitch responses in the skinned EDL fibres, as described previously (Posterino *et al.* 2000; see Methods). The twitch response peaked at ~60% of the maximum Ca^{2+}-activated force (mean $56 \pm 2\%$, in 67 fibres) and lasted ~150–200 ms.

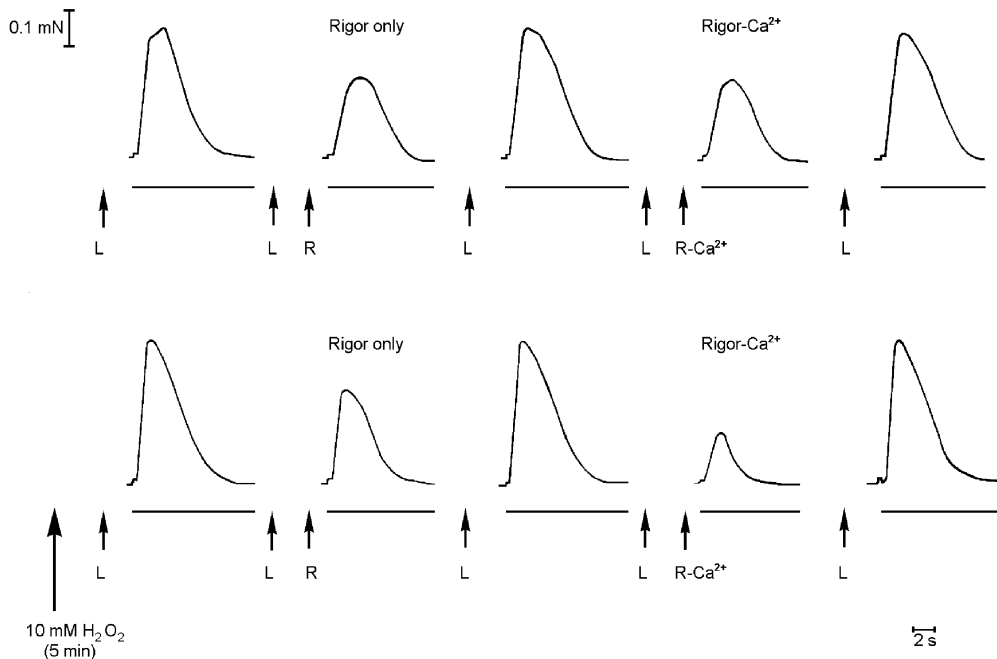**Figure 2. H_2O_2 treatment potentiates Ca^{2+}-induced Ca^{2+} release (CICR)**

Force responses in a fibre when repeatedly emptying the SR of all Ca^{2+} (full release solution, see Methods). In the top traces, before H_2O_2 treatment, the SR was loaded (L) for a set period of 30 s and either emptied straightaway or exposed to a set of rigor solutions (R, no ATP) for 2 min and then emptied. The relative decrease in the response in the latter case ('Rigor only') indicates the amount of Ca^{2+} lost from the SR during the period in the rigor solutions. Exposure to 2 μM free Ca^{2+} for 30 s during the rigor period did not increase the amount of Ca^{2+} lost from the SR (compare traces labelled 'Rigor only' and 'Rigor-Ca^{2+}'), showing there was no detectable CICR under those conditions. After H_2O_2 treatment (lower traces) there was considerable CICR, as shown by the relatively small response for the 'Rigor-Ca^{2+}' case. The 'Rigor only' protocol was repeated after the 'Rigor-Ca^{2+}' protocol in each case (not shown). The loading period (L) had to be increased to 40 s after H_2O_2 treatment to reach the same SR Ca^{2+} content (see text).

The exact size of the response depends on the amount and type of Ca^{2+} buffering in the skinned fibre (i.e. in the bathing solution), with the above values obtained with 50 μM total EGTA (pCa ~7.0); the various effects described below were not qualitatively different between fibres with different size twitches or with different levels of Ca^{2+} buffering (not shown). In any given fibre, twitch responses elicited every 30 s over a 5 min period were very similar (e.g. left panel in Fig. 3*A*), having similar peak amplitudes, with the only discernible difference being a small increase in the duration of the response (indicated by ~11 % increase in half width; see Table 2).

Because the twitch response remains both stable and submaximal, it should be a sensitive measure of any change in Ca^{2+} release from the SR. To demonstrate this, a small additional increment of Ca^{2+} was released from the SR by applying a second (identical) electrical stimulus shortly after the first (e.g. see Fig. 3*B*). When the second stimulus was applied within ~4 ms after the first, the force response was no different from that for a single stimulus; this is consistent with the refractory behaviour of action potentials, as the second stimulus would not be expected to elicit a second action potential. When the second stimulus was applied ~6–20 ms after the first, it elicited a larger force response than the single pulse, typically reaching ~80 % of maximum Ca^{2+}-activated force (e.g.

Fig. 3*B*), with the peak amplitude being virtually identical, irrespective of the exact separation of the two pulses (e.g. compare cases D8 and D10 in Fig. 3*B*). Similar results were observed in every fibre examined. In every case, the peak of the force response to two closely spaced pulses (10 ms apart) was clearly larger (~25 %) than the twitch response to a single stimulus, but smaller than the peak tetanic force reached with 50 Hz stimulation (which typically was the same as maximum Ca^{2+}-activated force measured in the same fibre).

Effect of strong reductants and modifying the GSH:GSSG ratio on twitch responses

In the above fibres, the cytoplasmic factors normally influencing the redox state of the cytoplasm and SR, in particular GSH and GSSH (Feng *et al.* 2000), would have been lost from the fibre soon after skinning, and it might be argued that the fibres subsequently became 'oxidised'. Consequently, we examined the effect on the twitch response of treating the fibres for 5 min in the presence of 5 mM GSH or 10 mM DTT (e.g. right panel in Fig. 3*A*). Neither treatment had any significant effect on the twitch parameters (Table 2). Similarly, restoring the GSH:GSSG ratio to the level present in the cytoplasm *in vivo* (30:1) had no significant effect on the twitch response (Table 2), with the parameters being assessed both in the presence of the redox reagents and immediately after washout, so as to
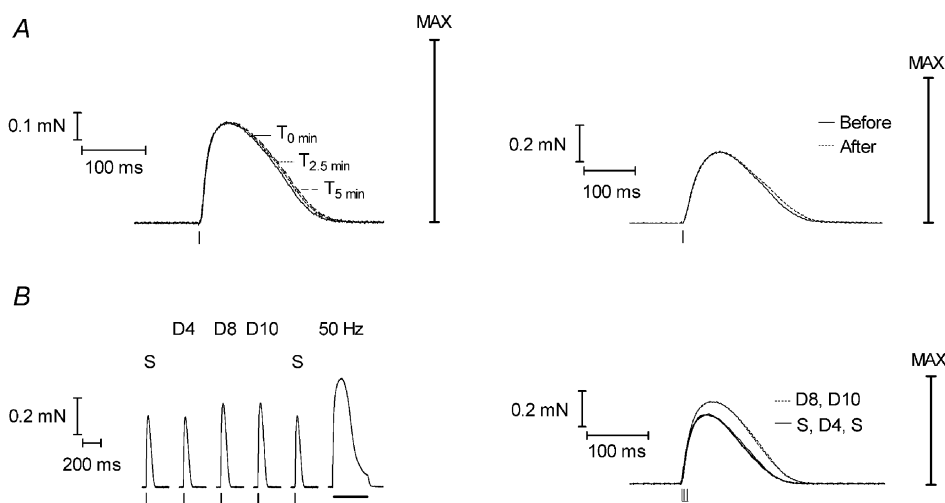

Figure 3. Twitch responses in skinned fibres in control and reducing conditions

A, twitch responses in a mechanically skinned EDL fibre with no redox agents present (left panel). Responses at three different times (*T*) are shown superimposed. The response was virtually unchanged over 5 min, except for a small increase in the half-width. The maximum Ca^{2+}-activated force (MAX) is indicated. The right panel shows twitch responses in another fibre before and immediately after a 5 min treatment with 10 mM DTT; the twitch response was little changed, showing only a slight increase in half-width, as occurs with time alone (left panel). *B*, responses in a fibre stimulated with either a single electric field pulse (S) or a pair of pulses 4–10 ms apart (double pulse, D). Responses are shown in sequence in the left panel and superimposed in the right panel. Stimulating with two pulses 4 ms apart (D4) gave a virtually identical response to stimulating with a single pulse. Increasing the interval between the two pulses to 8 ms elicited a substantially larger response, with no further change occurring when increasing the interval between the pulses to 10 ms (D10). The tetanic response to 50 Hz stimulation (horizontal bar) reached maximum Ca^{2+}-activated force (MAX). Small vertical ticks beneath twitch responses show the time of applied stimuli.

Table 2. Twitch response after 5 min repeated activation under various conditions

		Normalised peak amplitude	Normalised rise time$_{20-80}$	Normalised decay rate constant	Normalised half-width
1. Control		100 ± 2% (5)	98 ± 2%	84 ± 7%	111 ± 3%*
2. 10 mM DTT	(W)	98 ± 4% (6)	94 ± 5%	86 ± 8%	100 ± 3%
3. 5 mM GSH	(W)	105 ± 3% (6)	101 ± 5%	88 ± 7%	112 ± 7%
4. 30:1 GSH:GSSG	(P)	103 ± 6% (3)	108 ± 7%	106 ± 11%	102 ± 11%
	(W)	108 ± 11%	105 ± 3%	101 ± 14%	102 ± 11%
5. 3:1 GSH:GSSG	(P)	101 ± 3% (4)	96 ± 2%	100 ± 5%	94 ± 4%
	(W)	98 ± 6%	98 ± 1%	100 ± 3%	103 ± 4%
6. 2.5 mM GSSG	(P)	97 ± 2% (3)	101 ± 2%	93 ± 3%	104 ± 4%
	(W)	93 ± 3%	99 ± 8%	97 ± 4%	95 ± 4%
7. 200 μM H$_2$O$_2$	(P)	100 ± 3% (10)	96 ± 4%	109 ± 8%	96 ± 3%
8. 10 mM H$_2$O$_2$	(P)	97 ± 3% (8)	108 ± 3%	80 ± 6%	106 ± 5%
9. 10 mM H$_2$O$_2$ Double-pulse	(P)	96 ± 2% (4)	113 ± 7%	110 ± 11	83 ± 15%

Mean values (± S.E.M.) of twitch parameters in skinned EDL fibres measured after 5 min either in the presence (P) of a given reagent (e.g. 10 mM H$_2$O$_2$) or 30 s after the reagent was washed (W) from the fibre. Values were normalised to the response before treatment in each fibre. Fibres were stimulated with a single pulse once every 30 s under all conditions; for row 9 the response to a pair of pulses 10 ms apart was measured before and after the treatment in 4 of the 8 fibres in row 8. In treatment 1, fibres were stimulated in the standard K-HDTA solution (Control) and a paired *t* test used to determine whether there was any significant change in the measured twitch parameter after 5 min (* $P < 0.05$) (e.g. see Fig. 3*A*). For all other treatments, parameter values after 5 min were compared to the corresponding control data with a one-way analysis of variance using Dunnett's multiple comparison post test; no values were significantly different at $P < 0.05$.

identify and distinguish redox effects from any possible direct effects of the reagents or conditions. We also examined the effect of varying the ratio of GSH:GSSG from 30:1 to the relatively oxidised level of 3:1. In these experiments, immediately after skinning the fibre was placed in a solution with a GSH:GSSG ratio of 30:1, so as to keep the ratio constant at its *in vivo* level (Feng *et al.* 2000). Subsequently altering the ratio to 3:1 had no significant effect on the twitch response (Table 2). Furthermore, when other fibres were placed in 5 mM GSH immediately after skinning and then treated in a solution with 2.5 mM GSSG and no GSH for 5 min, there was again no significant change in the twitch response (Table 2).

Effect of H$_2$O$_2$ on twitch force responses

Exposure to 200 μM H$_2$O$_2$ had no effect on the size, duration or other twitch parameters over 5 min in 10 of the 11 fibres examined (Table 2), even though it invariably potentiated the response to caffeine (Table 1). In the remaining fibre, the twitch response declined to 69 % of the control level by the end of the 5 min period.

Fibres treated with a much higher concentration of H$_2$O$_2$ (10 mM) for 5 min also displayed responses falling into two distinct categories. In half the cases (8/16) the twitch response fell by at least 20 % before the end of the 5 min treatment period (e.g. Fig. 4*B*), with the mean twitch size after 5 min in these eight fibres being 40 ± 7 % of the initial level. In four of these cases the responses were examined

for a further 2 min after washout of the H$_2$O$_2$, during which time the twitch response declined further or ceased altogether, with there being no recovery after a subsequent 5 min treatment in 10 mM DTT in any case (e.g. Fig. 4*B*). When these fibres were subsequently exposed to a solution with low free [Mg^{2+}] (0.01 mM; see Lamb & Stephenson, 1994), it triggered Ca^{2+} release and a large force response in every case, showing that the SR was still loaded with Ca^{2+} and that the Ca^{2+}-release channels could still be activated directly. This indicates that the 10 mM H$_2$O$_2$ treatment deleteriously affected a more proximal step in the E–C coupling sequence (see Discussion).

In the other eight fibres exposed to 10 mM H$_2$O$_2$ (e.g. Fig. 4*A*), there was no noticeable change at all in the size or other characteristics of the twitch response over the entire 5 min period (Fig. 4*C* and Table 2). In five of the eight cases, the twitch responses were also measured after washout of H$_2$O$_2$ for 2 min and after a further 5 min treatment with 10 mM DTT, neither of which significantly affected the responses (relative twitch size: 96 ± 3 % (washout) and 92 ± 4 % (post-DTT); both $P > 0.05$, paired *t* test). The force response to double-pulse stimuli (10 ms apart) also remained unchanged during the 5 min exposure to 10 mM H$_2$O$_2$ (Table 2; e.g. Fig. 4*A*). These results from fibres where E–C coupling was not interrupted, provide clear evidence that a stringent oxidising treatment sufficient to cause profound potentiation of caffeine-induced Ca^{2+} release and CICR in every fibre (Table 1)

causes no detectable change in the twitch response and Ca^{2+} release following action-potential stimulation.

Effect of H_2O_2 on depolarisation-induced Ca^{2+} release by solution substitution

As the H_2O_2 treatment potentiates caffeine-induced Ca^{2+} release and CICR from the SR but does not alter action-potential-induced Ca^{2+} release, the question arises as to whether this difference is due to the different methods of activating the release channels (i.e. directly or via the voltage sensors) or to some other difference, such as the effectiveness of the stimulus in activating rapid Ca^{2+} release (the action potential triggered Ca^{2+} release more than 100 times faster than did caffeine). To test this, we examined the effect of H_2O_2 treatment on the Ca^{2+} release elicited by submaximal activation of the voltage sensors. This was achieved using the ion-substitution method to depolarise the T-system (Lamb & Stephenson, 1994) in skinned fibres in which the T-system action potential had been blocked with TTX (see Methods). As seen in Fig. 5, substituting the high $[K^+]$ bathing solution with a matching zero $[K^+]$ solution elicited a large force response, although the onset of the response was delayed by about

~0.5–1 s after the solution substitution (mean peak size and rate of rise of force response for the four fibres discussed below: $80 \pm 4\%$ of maximum Ca^{2+}-activated force ('Max') and $51 \pm 8\%$ Max s^{-1}, respectively, measured as described in Methods). The marked delay in the onset of the force response was the result of abolishing the T-system action potential, which meant that the time course of T-system depolarisation (and voltage-sensor activation) was determined primarily by the decline of the $[K^+]$ within the fibre after the solution substitution (time constant < 0.3 s, based on ion size and fibre diameter; Moisescu & Thieleczek, 1978). Submaximal depolarisation could be produced by substituting a solution with 12 mM K^+, rather than no K^+, which elicited a smaller force response that was reasonably reproducible on successive repetitions (Fig. 5). In the four fibres examined, depolarisation by 12 mM K^+ substitution elicited a force response with a mean peak amplitude of $43 \pm 8\%$ of 'Max' and mean rate of force development of $25 \pm 8\%$ Max s^{-1}. One minute after addition of 10 mM H_2O_2, the same depolarising stimulus elicited a substantially larger and faster-rising force response in all four fibres (mean peak and rate of rise: $66 \pm 4\%$ of Max and $46 \pm 11\%$ Max s^{-1}; both $P < 0.05$,

Figure 4. Effect of H_2O_2 treatment on twitch force responses

A, responses in a skinned fibre to stimulation with either a single action potential (S) or two action potentials 10 ms apart (D). The twitch response to a single action potential remained virtually unchanged over a 5 min exposure to 10 mM H_2O_2 and the response elicited by a pair of action potentials afterwards was only slightly smaller than its initial control level. *B*, example of exposure to 10 mM H_2O_2 causing abolition of twitch responses in an EDL fibre; lowering $[Mg^{2+}]$ from 1 to 0.01 mM still induced sarcoplasmic reticulum (SR) Ca^{2+} release and contraction. *C*, mean ($\pm$ S.E.M.) of the peak amplitude of the twitch response during 5 min exposure to 10 mM H_2O_2 in EDL fibres where excitation–contraction coupling was not interrupted (circles; eight fibres) and in fibres maintained in control conditions for a similar period (squares; five fibres). The error bars are smaller than the symbols. Twitch responses in each fibre were normalised to the response obtained under control conditions immediately before the respective 5 min test period. There was no significant difference between the groups in the size of control response as a percentage of maximum Ca^{2+}-activated force ($67 \pm 7\%$, $n = 5$; $58 \pm 6\%$, $n = 8$; $P > 0.05$).
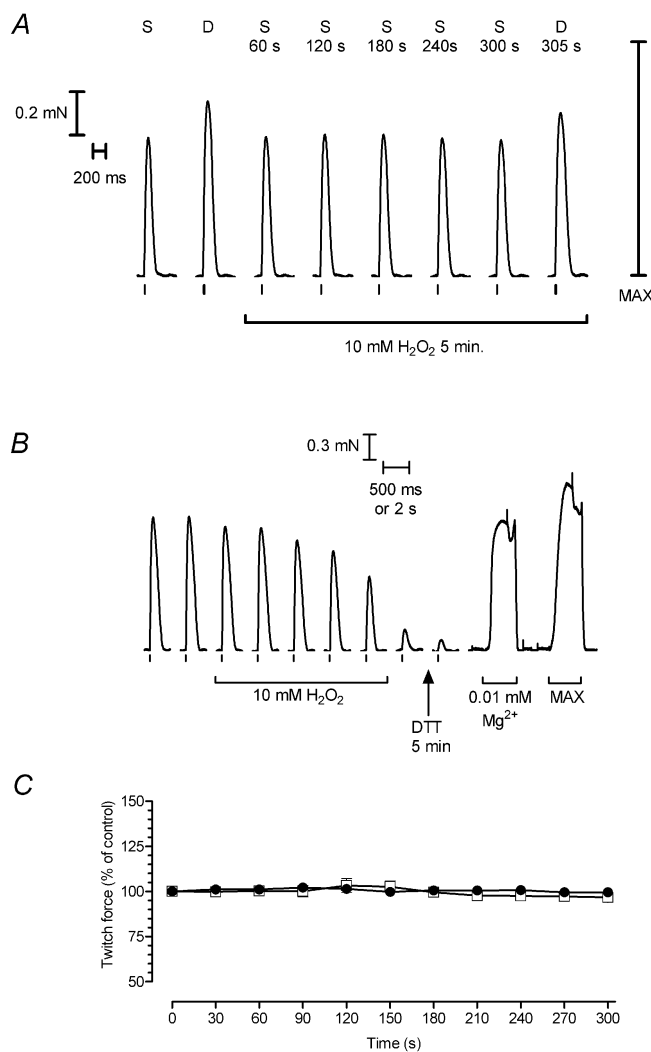
Table 3. Effect of GSH and DTT treatments on twitch response in DTDP-oxidised fibres

		Post-DTDP	Post-GSH	Post-DTT
1.	Peak size	34 ± 11 (4)*	—	53 ± 18 (4)*
	Half-width	73 ± 4 (4)*	—	112 ± 9 (4)
2.	Peak size	39 ± 7 (8)*	90 ± 7(8)	77 ± 4 (3)*
	Half-width	66 ± 5 (8)*	139 ± 13 (8) †	105 ± 12 (3)

Values are the mean (± S.E.M.) of the relative peak size and half-width of the twitch response in skinned EDL fibres after the indicated treatments (e.g. as in Fig. 7). All values are expressed relative to the control twitch before any treatment. In the upper data set, 4 fibres were sequentially treated with DTDP (100 μM, 20 s) and then DTT (10 mM, 2 min). In the lower data set, 8 fibres were sequentially treated with DTDP (100 μM, 20 s) and GSH (5 mM, 2.5 min), and in three cases with DTT (10 mM, 2 min). * and † indicate that the mean value is significantly smaller or greater, respectively, than the original value before any treatment (Student's one-tailed paired *t* test, $P < 0.05$).

paired *t* test; e.g. Fig. 5), and after a further minute in H_2O_2 the same stimulus gave a similar or even larger response (mean: 70 ± 5 % Max and 66 ± 13 % Max s^{-1}). Thus, the treatment in 10 mM H_2O_2 potentiated the peak of the force response to submaximal depolarisation by ~1.6-fold and the rate of force rise by ~2–2.5-fold, producing a response similar to, and even faster than, the response to full depolarisation (i.e. to zero [K$^+$]; see Fig. 5).

Following 2 min exposure to 10 mM H_2O_2, the response to successive depolarisations (by either submaximal or maximal stimuli) progressively declined, irrespective of whether the H_2O_2 remained or was washed out. Nevertheless, it was still always possible to elicit Ca^{2+} release and a large force response by lowering the free [Mg^{2+}] (not shown). Thus, the treatment with 10 mM H_2O_2 evidently interfered (directly or indirectly) with voltage-sensor activation of Ca^{2+} release in these solution substitution experiments, just as seen in the action-potential-stimulation experiments

(e.g. Fig. 4*B*). In two fibres that were exposed to 1 mM H_2O_2, the response to submaximal depolarisation became progressively larger over the first four depolarisations (1 min apart) before stabilising; the response was little changed when the H_2O_2 was washed out and when 10 mM H_2O_2 was subsequently added. Thus, exposure to 1 mM H_2O_2 caused an increase in the response to submaximal depolarisation similar to that with exposure to 10 mM H_2O_2, although it took longer for maximal effect to occur (~4 min *versus* < 1 min).

Effect of GSH exposure after oxidation

Finally, we also examined whether fibres that had been oxidised by H_2O_2 (or DTDP) treatment showed any alteration in twitch response when subsequently exposed to GSH, as this type of treatment has been shown to have a significant effect on the steady-state Ca^{2+} sensitivity of the contractile apparatus (Lamb & Posterino, 2003) and could potentially account for the oxidation-induced increase of
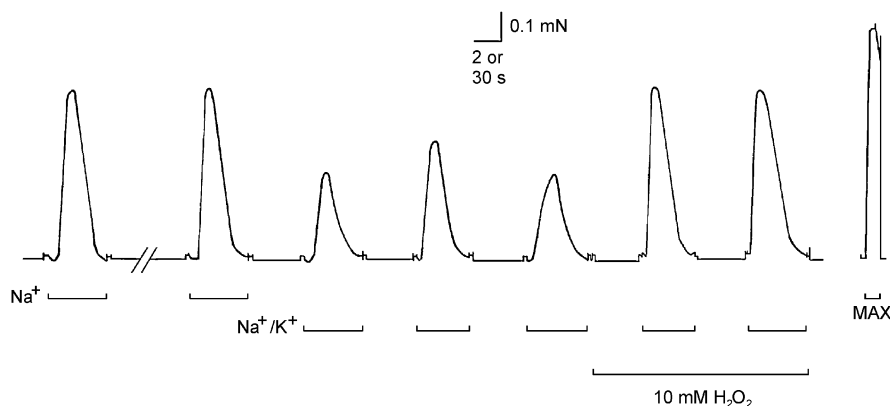

Figure 5. H_2O_2 treatment potentiates the force response to submaximal depolarisation elicited by solution substitution

A skinned EDL fibre (pretreated with TTX) was depolarised by substituting the standard high [K$^+$] solution with a matching Na$^+$ solution with no K$^+$ (see Methods), eliciting Ca^{2+} release and a large force response lasting 2–3 s. When the T-system was depolarised only partially by substituting a Na$^+$/K$^+$ solution containing 12 mM K$^+$, the resulting force response was approximately twofold smaller than the response to full Na$^+$ substitution. Application of 10 mM H_2O_2 greatly increased both the peak and rate of rise of the force response to submaximal depolarisation. Time scale: 2 s during depolarisations (bars) and 30 s between depolarisations and maximum Ca^{2+}-activated force (MAX).

twitch and submaximal tetanic responses in intact fibres (Oba *et al.* 1996; Andrade *et al.* 1998; see Introduction).

Each skinned EDL fibre was exposed to 10 mM H_2O_2 for 2 min, then the H_2O_2 was washed out and the fibre exposed to 5 mM GSH for ~2.5 min. As seen in the example in Fig. 6*A* and the mean data in Fig. 6*B*, the twitch response increased progressively during the exposure to GSH and remained at a similar level after washout of the GSH. A subsequent 2 min treatment with 10 mM DTT reversed the twitch potentiation (Fig. 6). Control fibres, which were tested alternately with the above fibres but not subjected to the H_2O_2 treatment, showed no increase in twitch force when placed in the same GSH solution (Fig. 6*B*). Comparison of the relative change in the twitch response between the control and the H_2O_2-oxidised fibres in Fig. 6*B* indicates that the GSH exposure potentiated the twitch in the H_2O_2-oxidised fibres by ~15–20 %. This corresponds on average to the twitch force increasing from ~60 % to ~70–75 % of maximum Ca^{2+}-activated force.

We also examined the effect of oxidising the fibres with DTDP (100 μM, 20 s) and then exposing them to GSH, as this causes an even more marked increase in the steady-state Ca^{2+} sensitivity of the contractile apparatus (increase in pCa at 50 % maximum force of ~0.27 pCa units; Lamb & Posterino, 2003). The treatment in DTDP substantially reduced the size of the twitch response (e.g. Fig. 7), with the peak decreasing on average to 37 ± 6 % ($n = 12$) and the half-width decreasing to 68 ± 4 % of their original size (Table 3). The reduction in the size of the twitch is almost certainly due in part to a decrease in the Ca^{2+} sensitivity of the contractile apparatus that occurs with such treatment (Lamb & Posterino, 2003), but it is also likely that there was some irreversible oxidation-induced disruption in the voltage-sensor activation of Ca^{2+} release because (a) coupling was irrevocably lost in many fibres if the duration of the DTDP treatment was increased only two- or threefold (compare effect of 10 mM H_2O_2 above; Fig. 4*B*) and (b) a 2 min DTT treatment was only able to partially restore the size of the twitch response (Table 3),

Figure 6. Exposure of H_2O_2-oxidised fibres to reduced glutathione (GSH) causes potentiation of twitch force

A, the twitch response in a skinned EDL fibre was virtually unchanged during a 2 min exposure to 10 mM H_2O_2 (responses elicited every 30 s, last two shown), but increased progressively during a subsequent 2.5 min exposure to GSH (5 mM). The response was unchanged when the GSH was washed out, but decreased to close to the original control level (dashed line) following a 2 min treatment with 10 mM DTT. *B*, mean data ($\pm$ S.E.M.) of relative twitch size in six fibres exposed successively to H_2O_2 and GSH as in *A* (squares) and six other fibres exposed to GSH without a preceding exposure to H_2O_2 (circles). The response following DTT exposure was obtained in only four of the six fibres exposed to H_2O_2 and GSH. Twitch forces in each fibre were normalised to the first of the two 'control' responses obtained preceding GSH exposure.
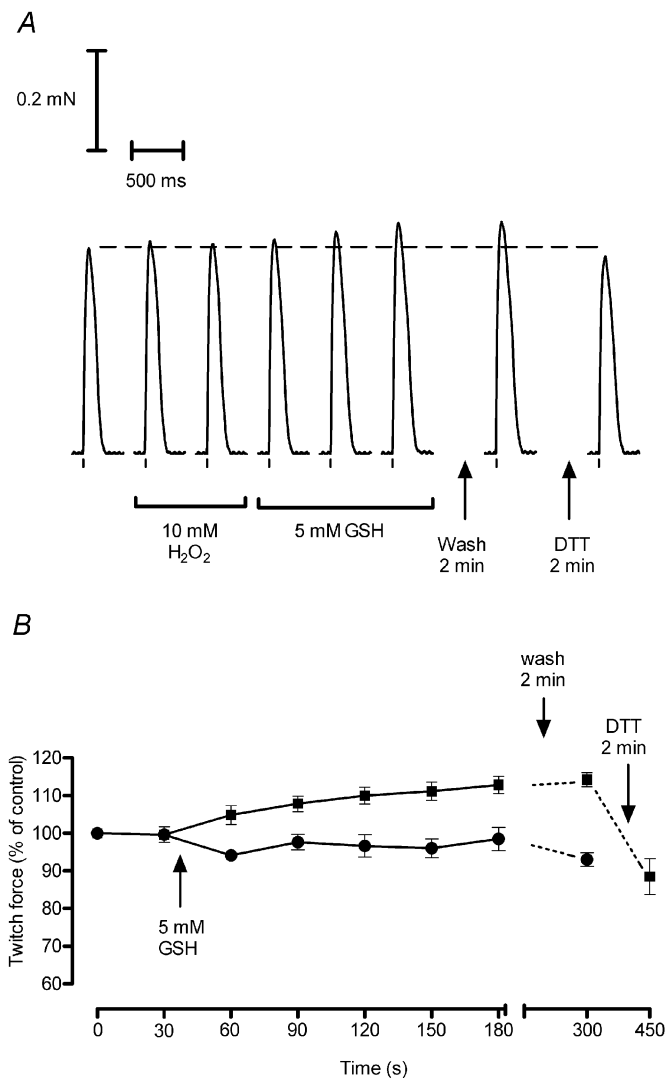
even though the decrease in Ca^{2+} sensitivity of the contractile apparatus is fully reversed by a much briefer exposure to DTT (15 s; Lamb & Posterino, 2003).

When such DTDP-oxidised fibres were exposed to GSH (5 mM), the twitch response noticeably increased within ~10 s and reached a steady level within 1–2 min, remaining unchanged upon washout of the GSH (e.g. Fig. 7). In the eight fibres examined in this way, the peak amplitude and the half-width of the twitch response increased to $292 \pm 66\%$ and $216 \pm 21\%$, respectively, of their values after DTDP treatment ($P < 0.05$ in both cases), that is, to $90 \pm 7\%$ and $139 \pm 3\%$, respectively, of their original values (Table 3). In three of these cases the fibres were then treated with 10 mM DTT (2 min) and the peak amplitude and the half-width of the twitch response decreased to $78 \pm 6\%$ and $65\% \pm 3\%$ of their respective values before the DTT treatment ($P < 0.05$; e.g. see Fig. 7). Thus, in DTDP-oxidised fibres, exposure to GSH caused marked potentiation and prolongation of the twitch response, with the effect being much larger than seen with DTT exposure (top data set in Table 3) and not attributable simply to reversal of the effect of DTDP on the sensitivity of the contractile apparatus. Furthermore, subsequent DTT treatment substantially reversed the effects of the GSH treatment, restoring the twitch parameters to values similar to those seen when treating DTDP-oxidised fibres with DTT without any intervening exposure to GSH (compare upper with lower data set in right-most column of Table 3). The effect of GSH found in DTDP-treated fibres is completely analogous with its effect in H_2O_2-treated fibres described above, and together these data provide strong evidence that GSH potentiates twitch responses in oxidised fibres by increasing the Ca^{2+} sensitivity of the contractile apparatus, in accord with observations on the steady-state properties of the contractile apparatus (Lamb & Posterino, 2003).

DISCUSSION

This study presents a number of findings that are important for understanding (a) the normal physiological processes controlling Ca^{2+} release and contraction in skeletal muscle fibres and (b) the effects of oxidation on particular steps in the E–C coupling sequence and their possible roles in normal muscle function and in fatigue. By using action-potential stimulation of Ca^{2+} release in skinned fibres it was possible to examine the response of the normal, rapid E–C coupling mechanism whilst controlling the redox conditions in the cytoplasm during Ca^{2+} release, and to examine the response both before and after applying particular oxidation and reduction treatments. Furthermore, it was also possible to determine the parallel effects of these redox conditions and treatments on the responsiveness of the Ca^{2+}-release channels to direct activation by caffeine and Ca^{2+}.

Caffeine-induced Ca^{2+} release and CICR

The present results on the caffeine sensitivity of Ca^{2+} release (e.g. Fig. 1, Table 1) indicate that Ca^{2+}-release channels functionally coupled to voltage sensors are quite responsive *in situ* to oxidation by either H_2O_2 or the specific sulphydryl reagent DTDP, and that the effect is fully reversible by reduction with DTT, in agreement with the recent results of Plant *et al.* (2002). The channels in fibres obtained from rested muscles were evidently in a reduced state, with further reduction having no noticeable effect. This is similar to the conclusions reached about the redox state of the contractile apparatus in such fibres (Lamb & Posterino, 2003).

The conclusion that oxidation increased CICR and caffeine-induced Ca^{2+} release in skinned fibres (this study and Plant *et al.* 2002) is consistent with the effects of oxidation on SR vesicles and single RyRs (Zaidi *et al.* 1989; Favero *et al.* 1995) but conflicts markedly with the skinned fibre study of Brotto & Nosek (1996), which concluded that CICR was completely abolished by oxidation (5 min
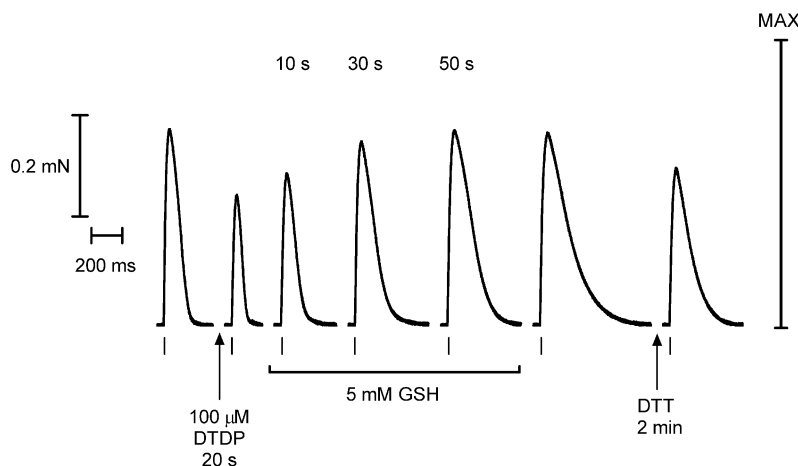

Figure 7. GSH potentiates twitch force in dithiodipyridine (DTDP)-oxidised fibres

The twitch response in an EDL fibre decreased in both peak amplitude and half-width after a brief (20 s) exposure to 100 μM DTDP (in the absence of any stimulation). When the fibre was subsequently exposed to 5 mM GSH and stimulated at ~20 s intervals (time in GSH shown), the twitch response increased progressively in peak amplitude and half-width, with each reaching a steady level within 1–2 min. These changes persisted when GSH was washed out. Subsequent exposure to 10 mM DTT for 2 min partially reversed the potentiation occurring with GSH treatment.

treatment with 1 mM H_2O_2). The difference was not attributable to 'over-oxidation' of the RyRs causing inhibition of CICR, because CICR in skinned fibres was increased, not inhibited, by applying a more severe and prolonged treatment (20 min, 10 mM H_2O_2; Table 1). Instead, it is possible that the apparent abolition of CICR reported by Brotto & Nosek (1996) was actually due to an oxidation-induced increase in CICR that (a) reduced net uptake during the loading period at pCa 6.6 and (b) caused increased loss of SR Ca^{2+} during the equilibration and wash periods necessary for the assay of CICR (see Fig. 2), particularly given that the SR had been made leaky by the saponin treatment used in those experiments (see Launikonis & Stephenson, 1997).

Ca^{2+} release with action-potential stimulation

It was found that the twitch response, which is indicative of the amount of Ca^{2+} released by action-potential stimulation of the voltage sensors, was not appreciably altered by varying the redox condition of the cytoplasm from a highly reduced level (e.g. with 10 mM DTT or 5 mM GSH) to a highly oxidised level (e.g. GSH:GSSG at 3:1 or all GSSG; Table 2). It was further found that oxidising the Ca^{2+}-release channels by H_2O_2 exposure (200 μM or 10 mM), greatly sensitising their activation by cytoplasmic Ca^{2+} and caffeine (Table 1), did not change any of the characteristics of the twitch response (Table 2; Fig. 4). As the pCa_{50} of the force–pCa relationship is quite unaffected by these H_2O_2 treatments (Lamb & Posterino, 2003), this indicates that the amount of Ca^{2+} released by an action potential was unchanged. These results are consistent with the study of Andrade *et al.* (1998) in intact muscle fibres, in which they observed little or no effect of H_2O_2 treatment (100–300 μM) on cytoplasmic $[Ca^{2+}]$ during submaximal tetanic stimulation. The above findings also show that the effects of oxidation and reduction observed in single Ca^{2+}-release channels and SR vesicles (Feng *et al.* 2000; Oba *et al.* 2002) cannot be extrapolated in any simple way to give insight into the effects and role of oxidation in functioning muscle fibres.

The concentration of H_2O_2 required to induce substantial oxidative effects in studies with skinned fibres, isolated SR and single release channels (e.g. 0.2–10 mM), is considerably higher than that in intact fibres. This is probably because H_2O_2 itself is a comparatively unreactive reagent and most likely mediates its effects after conversion to the highly reactive hydroxyl radical. This will occur far more readily in an intact muscle fibre than in perfused preparations where myoglobin has been washed out and Fe^{2+} and similar ions are buffered to an extremely low concentration by the addition of EGTA (see discussion in Lamb & Posterino, 2003). In any case, whatever the reason for this disparity, we show here that the oxidative treatment did greatly upmodulate CICR but did not affect the twitch response in the same preparation.

Oba *et al.* (2002) report that oxidation reversibly converts single RyRs from a low open probability (P_o) state, where they are very poorly responsive to stimulation by Ca^{2+}, caffeine and ATP, to a high P_o state, where they are very responsive to such stimuli. The present finding that oxidation greatly increases the sensitivity of the Ca^{2+} release in skinned fibres to such stimuli could be seen as in some ways consistent with this. However, it is evident that the release channels in the oxidised fibres here, with 8 mM ATP present, could not have had a high P_o all the time, because very little Ca^{2+} 'leaked' out of the SR in the absence of voltage-sensor activation (i.e. P_o was $\ll 0.01$); this was presumably the result of the presence of 1 mM free Mg^{2+} in the experiments here and possibly also because of the presence of associated proteins (e.g. calmodulin and FKBP12) and interactions between adjacent release channels *in situ*. It should also be noted that if the release channels in the fibres did convert from 'low P_o' to 'high P_o' states as reported by Oba *et al.* (2002), this had no apparent consequence for their activation by the voltage sensors, which potently stimulated the channels and elicited the same total amount of Ca^{2+} release irrespective of the particular P_o state.

Activation and termination of Ca^{2+} release

The lack of effect of oxidation on action potential stimulation of Ca^{2+} release is not because voltage-sensor control of the release channels is in some way immune to the effect of oxidising the release channels. On the contrary, oxidative treatment substantially increased the effectiveness of the voltage-sensor activation of Ca^{2+} release when using relatively weak depolarisations that induced only submaximal activation of the voltage sensors (e.g. Fig. 5). After oxidation, the submaximal depolarising stimulus elicited as big a response as the full depolarising stimulus, presumably indicating that less voltage-sensor activation (i.e. fewer voltage sensors activated or activated at any given instant) is needed to open the release channels when they are oxidised. This effect of oxidation is highly analogous to the ability of caffeine to increase the effectiveness of the coupling between the voltage sensors and the Ca^{2+}-release channels; that is, to increase the 'transfer function' (Shirokova & Rios, 1996). This effect could be the result of some overall increase in the general excitability of the release channel, or alternatively it might be due specifically to the increase in sensitivity of the channel to activation by cytoplasmic Ca^{2+}. Given that voltage-sensor activation of Ca^{2+} release is indeed sensitised by oxidation, the most straightforward explanation for the inability of the oxidative treatment to increase action-potential-induced Ca^{2+} release is that all the channels are already fully activated (i.e. $P_o \approx 1$) by the stimulus. Indeed, this is also suggested by calculations comparing the rate of Ca^{2+} efflux from the SR during an action potential with the Ca^{2+} flux through single release channels (Mejia-Alvarez *et*

al. 1999). If $P_o \approx 1$ during the action potential, oxidation could not increase channel activation any further.

The question then arises as to why Ca^{2+} release doesn't continue after the action potential ends, especially given that the oxidative treatment evidently increased the sensitivity of CICR (Fig. 2). During the phase of action-potential-induced Ca^{2+} release, the $[Ca^{2+}]$ in the vicinity of the release channels should be relatively high, and yet the Ca^{2+}-release channels evidently close after releasing approximately the same amount of Ca^{2+}, irrespective of whether or not they have been oxidised. The fact that an additional quantum of Ca^{2+} can be released by another action potential as little as 6 ms after the first, indicates that the termination of Ca^{2+} release is not due to the SR being fully depleted of Ca^{2+}. It is possible, however, that partial or local depletion of SR Ca^{2+} is the reason why CICR fails to stimulate Ca^{2+} efflux after the action potential. We have recently shown that caffeine-induced Ca^{2+} release and CICR are severely inhibited by decreasing the amount of Ca^{2+} in the SR, even though voltage-sensor activation is still able to elicit Ca^{2+} release (Lamb *et al.* 2001); evidently CICR only occurs if the SR is loaded with Ca^{2+} above a certain level (see Endo, 1985). This effect might also be exacerbated by local depletion of Ca^{2+} in the lumen of the SR near the release channels, which could result from the sudden efflux of Ca^{2+} and what may be relatively slow replenishment from other regions of the SR owing to diffusion being limited by Ca^{2+} binding to calsequestrin.

Another process that could contribute to the termination of Ca^{2+} release following an action potential is Ca^{2+}-dependent (or activation-dependent) inactivation of the release channel (Schneider & Simon, 1988; Jong *et al.* 1993; Hollingworth *et al.* 1996; Pizarro *et al.* 1997; Laver & Lamb, 1998). In voltage-clamp experiments in frog fibres, Jong *et al.* (1993) found evidence that the total amount of Ca^{2+} released from the SR at the time of peak rate of release appeared to be independent of SR Ca^{2+} content, 'consistent with the idea that a single activated channel might pass, on average, a fixed number of ions' before inactivating, possibly by a Ca^{2+}-dependent mechanism. This phenomenon, whatever its molecular basis, seems highly comparable with the fixed amount of Ca^{2+} release found here with action-potential stimulation.

The present results do not show whether oxidation reduced the inhibitory effect of Mg^{2+} at the low-affinity Ca^{2+}/Mg^{2+} site on the release channel, as occurs in isolated channels with at least some oxidation procedures (Donoso *et al.* 2000). It is clear, however, that Mg^{2+} still potently inhibited Ca^{2+} release in the oxidised fibres by some mechanism, possibly by its action at the Ca^{2+}-activation site (Meissner *et al.* 1986; Laver *et al.* 1997), because lowering $[Mg^{2+}]$ (from 1 to 0.01 mM) invariably triggered massive Ca^{2+} release (e.g. Fig. 4*B*). The $[Mg^{2+}]$ threshold for this effect was not examined, although it did appear

that the oxidised fibres responded more rapidly to lowering $[Mg^{2+}]$. Thus, even though the present results do not show whether normal E–C coupling involves changes in Mg^{2+} inhibition of the release channel (Lamb & Stephenson, 1991, 1994), they do point to the importance of cytoplasmic Mg^{2+} in inhibiting Ca^{2+} release in general, and in particular in inhibiting spontaneous CICR, which could otherwise be a major problem in oxidised fibres (e.g. Fig. 2).

Effect of GSH in oxidised fibres

Oxidation of intact muscle fibres with H_2O_2 was found to potentiate submaximal tetanic force responses, not by increasing Ca^{2+} release, but by increasing the Ca^{2+} sensitivity of the contractile apparatus (Andrade *et al.* 1998). We showed recently in experiments examining the steady-state Ca^{2+} sensitivity of the contractile apparatus (Lamb & Posterino, 2003) that this effect was not due simply to H_2O_2 oxidising the contractile proteins. Instead, it appeared that oxidation only led to an increase in steady-state Ca^{2+} sensitivity of the contractile apparatus if the fibres were briefly exposed (e.g. $\leqslant 2$ min) to GSH afterwards, apparently because of the formation of a mixed disulphide between GSH and a particular free sulphydryl group on the contractile apparatus. GSH had no such effect in fibres that had not be oxidised, and the sensitivity increase occurring with the oxidation-GSH treatment was reversed by exposure to DTT or much more prolonged exposure (> 20 min) to GSH (Lamb & Posterino, 2003). Thus, we sought here to establish whether or not these distinctive and potentially important effects on the steady-state Ca^{2+} sensitivity were also apparent in twitch responses, where dynamic changes in cytoplasmic Ca^{2+} and force may alter the overall behaviour.

In fibres that had been oxidised by either H_2O_2 or DTDP, and not control fibres, exposure to GSH indeed caused potentiation of the twitch within 1–2 min in a redox-related manner, the effect being reversed by exposure to DTT (Figs 6 and 7). The reduction in peak force and half-width seen with the initial DTDP treatment, and the respective increases with subsequent GSH treatment (e.g. Fig. 7), are the opposite of the changes one would expect if the effects were due to the actions of the reagents on CICR (see Results). In contrast, all the effects are very well explained by the highly distinctive actions of all these agents on the Ca^{2+} sensitivity of the contractile apparatus (Lamb & Posterino, 2003; with DTDP also apparently having a deleterious effect on voltage-sensor activation, see Results). Furthermore, the increase in twitch size in fibres exposed to H_2O_2 and then GSH (Fig. 6), from ~60 % to ~70–75 % of maximum Ca^{2+} force, is very similar to that simplistically 'expected' from the increase in steady-state Ca^{2+} sensitivity (pCa_{50} increasing by ~0.027 pCa units) measured in the contractile apparatus experiments with virtually the same treatment (10 mM H_2O_2, 5 min; 5 mM

GSH, 2 min; Lamb & Posterino, 2003), assuming that the $[Ca^{2+}]$ at the peak of the twitch was unchanged in the two cases. Here, the sizes of the twitch before and after treatment, expressed as percentages of maximum force, are simply mapped on to the respective force–pCa curves describing the steady-state Ca^{2+} sensitivity of the contractile apparatus. The potentiation of the twitch with DTDP-GSH treatment (e.g. Fig. 7), however, is considerably smaller than what would be similarly 'expected' from the increase in steady-state Ca^{2+} sensitivity (~0.27 pCa increase; compare changes in Fig. 7 with the force–pCa curves in Fig. 2 of Lamb & Posterino, 2003). This quite possibly simply reflects the fact that the total amount of Ca^{2+} released during the twitch is unchanged, and that a larger force response can only occur if the increase in the Ca^{2+} sensitivity of troponin-C is sufficiently great that more Ca^{2+} binds to the troponin-C at the expense of Ca^{2+} binding to other sites (e.g. the SR Ca^{2+} pump). Thus, even though it is not possible to make precise quantitative comparisons between the twitch potentiation data reported here and the steady-state force–pCa data obtained in the contractile apparatus experiments (Lamb & Posterino, 2003), there is clearly reasonable agreement between the studies, with both showing exactly the same highly distinctive sets of effects of the various agents. In view of this, we suggest that the potentiation of twitches and submaximal tetanic force observed in intact muscle fibres with H_2O_2 (Oba *et al.* 1996; Andrade *et al.* 1998) could be due to an increase in the Ca^{2+} sensitivity of the contractile apparatus following its oxidation by H_2O_2 (or more likely its redox products) and subsequent reaction with GSH present within the cytoplasm (see Lamb & Posterino, 2003).

Oxidation in normal function and fatigue

Finally, it is worth trying to consider how the various effects of oxidation bear on normal muscle function and muscle fatigue. The Ca^{2+} release channel in a muscle fibre is evidently readily oxidised *in situ* by the various oxidative treatments, such as those used here, in agreement with many previous studies on isolated preparations showing its ready susceptibility to oxidation (e.g. Zaidi *et al.* 1989; Favero *et al.* 1995). Although the sensitivity of the release channels to CICR was greatly enhanced, this did not alter the response to action-potential stimulation, indicating that this effect of oxidation has little, if any direct effect on normal Ca^{2+} release in a rested fibre. The coupling mechanism is evidently normally highly efficacious. This is also shown by the fact that action-potential-induced Ca^{2+} release is not noticeably affected by the presence of 30 mM lactate in the cytoplasm, even though this depresses CICR in the same fibres (Posterino *et al.* 2001). It is possible, nevertheless, that in certain conditions, various inhibitory effects on release channels are so profound that the voltage sensors are not able to elicit the normal amount of Ca^{2+} release. For example, this might happen after vigorous and prolonged stimulation, when there may be major changes

in the cytoplasm (e.g. decrease in [ATP], increase in $[Mg^{2+}]$; Allen *et al.* 1995, 2002), and possibly some level of chronic T-system depolarisation that inactivates some of the Na^+ channels and voltage sensors there (Dulhunty, 1992; Fitts, 1994; Balog & Fitts, 2001). In such a case, oxidation of the release channels, which can improve the efficacy of its coupling with the voltage sensors (Fig. 5), might well help to maintain Ca^{2+} release at its normal level, that is, it may oppose factors that could cause muscle fatigue.

Oxidation of the release channels might also have relatively subtle effects, both in the short and long term. Increasing the sensitivity of CICR could appreciably increase the amount of Ca^{2+} 'leaking' out of the SR at rest (e.g. pCa $\geqslant 7$), and although this is evidently a small flux in absolute terms (never leading to noticeable SR depletion or building up to give a detectable 'spontaneous' force response in the experiments here), it would presumably lead to a small elevation in resting cytoplasmic $[Ca^{2+}]$ in intact fibres (e.g. see Andrade *et al.* 1998). Such a rise in resting $[Ca^{2+}]$ could appreciably alter the distribution of cellular Ca^{2+} both within the cytoplasm and between the cytoplasm and the SR, and in that way indirectly affect the force response to stimulation. It could also affect many cellular signalling pathways, and hence affect the muscle in many other ways. In the extreme, continued Ca^{2+} leakage might trigger substantial reinforcement of CICR and a detectable force response, particularly in amphibian fibres (see Oba *et al.* 1996), where some of the release channels may be more sensitive to CICR than in mammalian muscle.

A second major site for oxidative effects is the contractile apparatus, as covered in detail in our previous study (Lamb & Posterino, 2003). This represents a second way in which oxidation could increase force responses, by increasing the Ca^{2+} sensitivity of the contractile proteins (Andrade *et al.* 1998), apparently by a specific GSH-mediated mechanism as outlined above. However, prolonged or stringent oxidative treatment subsequently depresses the force response (Andrade *et al.* 1998), by decreasing both the Ca^{2+} sensitivity and maximum force production of the contractile apparatus (Lamb & Posterino, 2003). By this latter action, oxidation could also contribute to muscle fatigue.

The third major way oxidation may impact on E–C coupling is by inhibiting activation of the voltage sensors, either by depolarising the T-system in some way (e.g. by opening or blocking particular ion channels in that membrane) or by directly interfering with the voltage sensors. If the T-system is chronically depolarised it can cause inactivation of the Na^+ channels, leading to action potential failure, and inactivation of the voltage sensors (Dulhunty, 1992). We found that treatment with 200 μM–1 mM H_2O_2 (< 5 min) did not cause much interruption to the coupling, but that treatment with 10 mM H_2O_2 reduced or abolished action-potential-

induced responses (e.g. Fig. 4*B*) and responses to depolarisation by ion substitution in a large proportion of fibres within 5 min. DTDP (100 μM) had an even greater deleterious effect. The effect was not due to depletion of SR Ca^{2+} or block of the release channel function, as it remained hypersensitive to direct activation even at 20 min in 10 mM H_2O_2 (Fig. 1; Table 1). The concentration- and time-dependence of the loss of the twitch response are very similar to those occurring with H_2O_2 treatment of intact fibres from frog muscle (Oba *et al.* 1996; see Introduction). Oxidation by H_2O_2 evidently interferes with the coupling, just as does oxidation by DTDP (Posterino & Lamb, 1996). The precise site(s) of this effect is not clear, but the fact that other sulphydryl reagents interfere with activation by voltage steps in voltage-clamped fibres (Caputo *et al.* 1993) indicates that the voltage sensors themselves are directly involved in at least some cases. Thus, this type of effect represents a second major way in which oxidation could deleteriously affect E–C coupling and hence contribute to muscle fatigue.

In conclusion, this study gives important information about the characteristics of the E–C coupling mechanism in skeletal muscle and how various steps are affected by oxidation, and thus how they can contribute to, or counter, impairment of muscle performance.

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