High intracellular [Ca^{2+}] alters sarcoplasmic reticulum function in skinned skeletal muscle fibres of the rat

G. D. Lamb and M. A. Cellini

Department of Zoology, La Trobe University, Bundoora, Victoria 3083, Australia

1. The effect on sarcoplasmic reticulum (SR) function of exposure to high intracellular [Ca^{2+}] was studied in mechanically skinned fibres from the extensor digitorum longus muscle of the rat, using caffeine to assay the SR Ca^{2+} content.

2. A 15 s exposure to 50 μM Ca^{2+} irreversibly reduced the ability of the SR to load/retain Ca^{2+} and completely abolished depolarization-induced Ca^{2+} release, whereas a 90 s exposure to 10 μM Ca^{2+} had no detectable effect on either function. The reduction in net SR Ca^{2+} uptake: (a) was near-maximal with treatment at 50 μM Ca^{2+}, (b) was unrelated to voltage-sensor function, and (c) persisted unchanged for > 20 min. The reduction was primarily due to a threefold increase in leakage of Ca^{2+} out of the SR. This increased leakage was not substantially blocked by the presence of 10 mM Mg^{2+} or 2 μM Ruthenium Red.

3. The adverse effect on SR function of exposure to high [Ca^{2+}] could also be observed by the reduction in the ability of the SR to maintain a low [Ca^{2+}] within the skinned fibre in the face of elevated [Ca^{2+}] in the bathing solution. When bathed in a solution with ~1·5 μM Ca^{2+} (0·75 mM CaEGTA–EGTA), skinned fibres produced only low force responses for many minutes, but after high [Ca^{2+}] treatment (15 s exposure to 50 μM Ca^{2+}) they showed large, steady or oscillatory force responses.

4. These findings indicate that, in addition to uncoupling the Ca^{2+} release channels from the voltage sensors, exposure of skinned fibres to high [Ca^{2+}] causes a persistent increase in resting Ca^{2+} efflux from the SR. Such efflux in an intact fibre would alter the distribution of Ca^{2+} between the SR, the cytoplasm and the extracellular solution. These results may be relevant to the basis of low-frequency fatigue and possibly other conditions in muscle.

In skeletal muscle, force development by the contractile apparatus is regulated by the intracellular [Ca^{2+}], which in the short term is predominantly influenced by the release and re-uptake of Ca^{2+} by the intracellular store, the sarcoplasmic reticulum (SR). In a sequence of events known as excitation–contraction (E–C) coupling, action potentials depolarize the transverse-tubular (T-) system, activating specialized voltage-sensor molecules (dihydropyridine receptors, DHPRs), which in turn open the ryanodine receptors/Ca^{2+}-release channels in the adjacent SR membrane, allowing efflux of Ca^{2+} into the cytoplasm (Melzer *et al.* 1995). This E–C coupling process can be studied using mechanically skinned muscle fibres, which retain the normal voltage-sensor control of Ca^{2+} release, and yet allow rapid manipulation of the intracellular conditions (Lamb & Stephenson, 1990, 1991).

Excitation–contraction coupling in mechanically skinned fibres is extremely robust, and numerous large depolarization-induced responses can normally be elicited. However, it was found that if the intracellular [Ca^{2+}] was raised to 100 μM for 10 s, depolarization-induced responses in rat extensor digitorum longus muscle (EDL) fibres were completely and

irreversibly abolished, even though Ca^{2+} release could still be elicited by directly stimulating the release channels with caffeine (Lamb *et al.* 1995). Half-maximal effects occurred at ~25 μM Ca^{2+} in EDL fibres and at ~2- to 3-fold lower [Ca^{2+}] in toad iliofibularis fibres. The uncoupling did not involve proteolysis of the ryanodine receptor/Ca^{2+}-release channel, the α_1-subunit of the DHPR or triadin, an associated protein. Furthermore, confocal microscopy of fluo-3 trapped in the T-system of skinned fibres showed that the T-system remained sealed in its entirety. Importantly, electron microscopy of fibres uncoupled by high [Ca^{2+}] treatment revealed that the triad junction, where the T-system abuts the SR, was commonly distorted or even severed (Lamb *et al.* 1995), and this possibly explained the loss of communication between the DHPR/voltage sensors and the Ca^{2+}-release channels.

Lamb *et al.* (1995) suggested that this Ca^{2+}-dependent uncoupling causes the reduction in Ca^{2+} release that underlies 'low-frequency fatigue', the long-lasting muscle fatigue evident at low stimulus frequencies for up to 24 h after prolonged exercise (Edwards *et al.* 1977; Allen *et al.* 1995; Chin & Allen, 1996). Subsequent experiments with

intact fibres of the mouse showed that procedures designed to give large elevations in intracellular $[Ca^{2+}]$ (e.g. tetanic stimulation in the presence of caffeine or a SR pump blocker) did indeed irreversibly reduce Ca^{2+} release and result in low-frequency fatigue (Chin & Allen, 1996; Chin *et al.* 1997). It was not clear, however, whether the high $[Ca^{2+}]$ exposure reduced Ca^{2+} release solely by disrupting the normal coupling mechanism, or whether it also caused some change in SR function and the distribution of Ca^{2+}. One indication that SR function is indeed altered after induction of low-frequency fatigue is the finding that the resting $[Ca^{2+}]$ remains elevated, possibly owing to reduced Ca^{2+} pumping and increased Ca^{2+} leakage from the SR (Westerblad *et al.* 1993).

Here we directly examine whether exposure to high intracellular $[Ca^{2+}]$ modifies the ability of the SR to take up and retain Ca^{2+}. We use caffeine to assay the Ca^{2+} content of the SR in skinned fibres and show that high $[Ca^{2+}]$ treatment induces a substantial increase in Ca^{2+} leakage from the SR. If this phenomenon occurs *in vivo*, it would ultimately affect the amount and distribution of Ca^{2+} within a muscle fibre and contribute to fatigue.

METHODS

Isolation of skinned fibres

Long-Evans hooded rats (*Rattus norvegicus*) aged 14 to 20 weeks were anaesthetized by halothane (2% v/v) and killed by asphyxiation in accordance with guidelines of the La Trobe University Animal Ethics Committee. As described previously (Lamb & Stephenson, 1994), both extensor digitorum longus muscles were then removed and placed in paraffin oil and kept cool on ice. Single muscle fibres were dissected free at one end from the muscle and mechanically skinned. The skinned fibre segment was mounted onto a force transducer (AME875, SensoNor, Horten, Norway), and stretched to 120% of its resting length. The fibre was then placed into a 2 ml Perspex bath containing a potassium or sodium 1,6-diaminohexane-*N,N,N',N'*-tetraacetic acid (HDTA) solution to equilibrate before being stimulated by rapid substitution of an appropriate solution. All experiments were performed at room temperature (23 ± 2 °C).

Solutions

All chemicals were obtained from Sigma unless stated otherwise. The make-up of the solutions used in the different parts of the experiments is set out below. All solutions had a pH of 7.10 ± 0.01 and an osmolality of 295 ± 5 mosmol kg^{-1}, and unless stated otherwise had 1 mM free Mg^{2+} and 8 mM total ATP. Free $[Ca^{2+}]$ at $\geqslant 0.1$ μM was verified with a Ca^{2+}-sensitive electrode (Orion, Cambridge, MA, USA).

Depolarization-induced Ca^{2+} release

Polarizing (K-HDTA) solution. Composition (mM): K^+, 126; Na^+, 37; $HDTA^{2-}$ (Fluka, Buchs, Switzerland), 50; total ATP, 8.0; total creatine phosphate, 10.0; total magnesium, 8.5; Hepes, 90; NaN_3, 1.0; total EGTA; 0.05, with pCa (i.e. $-\log_{10}[Ca^{2+}]$) 6.8.

Depolarizing (Na-HDTA) solution. Same as polarizing solution with all K^+ replaced with Na^+.

High $[Ca^{2+}]$ treatment

Rigor solution. Composition (mM): K^+, 126; Na^+, 37; $HDTA^{2-}$, 68; total magnesium, 1.5; Hepes, 90; NaN_3, 1.0; total EGTA, 0.1; with pCa 7.2. This is similar to the polarizing K-HDTA solution, but with all ATP and creatine phosphate replaced with HDTA. Used to initially induce rigor.

Ca^{2+} rigor solutions. Similar to above rigor solution, but with calcium added to give a free $[Ca^{2+}]$ of 10, 40, 50 or 130 μM. Applied after inducing rigor in absence of Ca^{2+}.

Caffeine-induced Ca^{2+} release

Pre-equilibration solution. K-HDTA solution similar to polarizing solution but with 0.5 mM total EGTA (or 0.5 mM BAPTA) at pCa 8. Used to ensure Ca^{2+} buffer is present in the fibre space before caffeine application.

Caffeine solution. K-HDTA solution with (mM): caffeine, 30; free Mg^{2+}, 0.015 (total magnesium, 1.1); and EGTA, 0.5 (or BAPTA) at pCa 8, but otherwise similar to pre-equilibration solution. Used to induce release of all SR Ca^{2+}.

Wash solution. Identical to pre-equilibration solution. Used to wash out caffeine.

Load solution. K-HDTA solution with 1 mM total EGTA at pCa 6.7 (or 6.4), but otherwise similar to pre-equilibration solution. Used to reload SR with Ca^{2+}.

Leak solution. K-HDTA solution with 2 mM total EGTA (pCa > 8), but otherwise similar to pre-equilibration solution. Used to allow Ca^{2+} leakage from the SR in the absence of Ca^{2+} uptake.

Additional matching solutions were also made with 10 mM free Mg^{2+} (22.7 mM total magnesium) or with all K^+ replaced with Na^+ (for chronic T-system depolarization).

Contractile apparatus experiments

Relaxing solution. Potassium solution with 50 mM EGTA (pCa > 9), which is similar to the polarizing solution with all HDTA replaced with EGTA (see Stephenson & Williams, 1981).

Maximum-activation solution. Same as Relaxing solution but with 50 mM CaEGTA (pCa 4.5). Referred to as 'Max' in the figures.

Solutions of intermediate $[Ca^{2+}]$ were made by appropriate mixture of these two solutions.

Caffeine-induced Ca^{2+} release experiments

The skinned fibre was initially equilibrated for 2 min in the polarizing (K-HDTA) solution before commencement of repeated cycles of Ca^{2+} release and reloading. The Ca^{2+} content of the SR was assayed by pre-equilibrating the skinned fibre in 0.5 mM EGTA (pCa 8) (pre-equilibration solution) for 10 s, and then triggering rapid Ca^{2+} release in the 30 mM caffeine–low $[Mg^{2+}]$ solution with 0.5 mM EGTA (caffeine solution). In specified experiments, the Ca^{2+} buffering was altered in both the pre-equilibration and caffeine solutions (to 1.5 mM EGTA or 0.5 mM BAPTA). As shown in Results, the area (i.e. time integral) of the resulting force response is indicative of the amount of Ca^{2+} in the SR. The fibre was left in the caffeine solution for 2 min to ensure complete Ca^{2+} depletion (Fryer & Stephenson, 1996), washed for 30 s in the wash solution and then reloaded for set times in the load solution. The fibre was then either immediately fully depleted again with the caffeine solution or left to 'leak' Ca^{2+} for a specified time in the leak solution before being fully depleted. Fibres in which the area of the caffeine response altered by more than 10% over three successive control load–release cycles were not further examined (< 20% of

fibres tested). When investigating the effects of 10 mM Mg^{2+} or 2 μM Ruthenium Red (RR) on Ca^{2+} leakage, these agents were present in the wash, load and leak solutions, but were not present in the pre-equilibration and caffeine solutions.

High [Ca²⁺] treatment

Skinned fibres were treated with high $[Ca^{2+}]$ in the absence of ATP, whilst they were in rigor. This was achieved by first placing the fibre in a low $[Ca^{2+}]$ solution with no ATP (rigor solution) for 1 min, over which time the fibre developed a steady level of rigor force (Fig. 3). The fibre was then transferred to a similar solution with elevated $[Ca^{2+}]$ (Ca^{2+} rigor solutions – see above) for either 90 s (10 μM Ca^{2+}) or 15 s (for higher $[Ca^{2+}]$), and then moved back to the rigor solution without Ca^{2+} for a further 15 s to wash out the Ca^{2+}. Finally, the fibre was returned to the K-HDTA solution with ATP and it relaxed again. To determine the effect of rigor treatment alone, fibres were kept in the rigor solution (i.e. low $[Ca^{2+}]$) for a total of 2 min without any exposure to high $[Ca^{2+}]$ (e.g. first rigor episode in Fig. 3). When a fibre was transferred between different rigor solutions, the force level often decreased to a lower level due to the slight stretching at the air–water interface and the inability of the fibre to recover its force level whilst in rigor (Fig. 3). Throughout the text, unless specified otherwise, the term 'high $[Ca^{2+}]$ treatment' refers to a 15 s exposure to 50 μM Ca^{2+} during rigor.

RESULTS

Abolition of depolarization-induced force responses

As described previously (Lamb *et al.* 1995), raising the intracellular $[Ca^{2+}]$ to very high levels abolishes depolarization-induced force responses (Fig. 1). In the experiments here, the high $[Ca^{2+}]$ was applied in the absence of ATP (see Methods) in order to prevent Ca^{2+} uptake by the SR from modifying

the $[Ca^{2+}]$ within the fibre. It was shown previously that high intracellular $[Ca^{2+}]$ causes such uncoupling irrespective of whether ATP was present or not (Lamb *et al.* 1995). The effect is evidently steeply dependent on the applied $[Ca^{2+}]$ because a 90 s exposure to 10 μM Ca^{2+} had no effect on the size of the response to depolarization (98 ± 2 % of control level) in the three EDL fibres examined, whereas a subsequent 15 s exposure to 50 μM Ca^{2+} caused complete abolition of depolarization-induced responses in the same three fibres (Fig. 1), as well as in every fibre examined without a preceding exposure to 10 μM Ca^{2+} (seven fibres from five different rats). This effect was shown to occur in tandem with the alteration in the caffeine response described below, using the same solutions and fibres from the same muscles. Following exposure to 50 μM Ca^{2+}, no depolarization-induced responses could be elicited whatsoever for the remainder of the experiment, which could be 30 min or more. In contrast, substantial Ca^{2+} release from the SR could still be elicited by directly stimulating the Ca^{2+} release channels with caffeine (Fig. 1) or with low free $[Mg^{2+}]$ (not shown, see Lamb *et al.* 1995). Furthermore, depolarization-induced responses could not be restored by loading the SR with Ca^{2+} to even above its endogenous level. Thus, it is apparent that the abolition of the depolarization-induced responses is not simply due to depletion of releasable Ca^{2+} and must involve disruption of the normal E–C coupling mechanism.

Caffeine-induced responses

It is nonetheless unclear whether high $[Ca^{2+}]$ exposure, in addition to disrupting the coupling mechanism, also alters
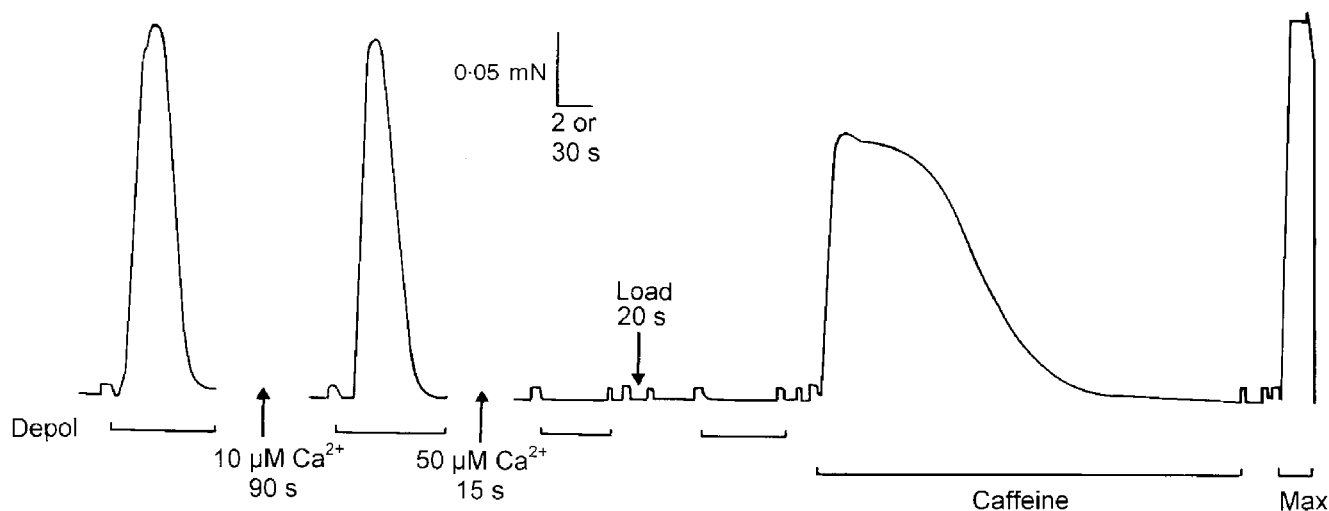

Figure 1. Exposure to high cytoplasmic [Ca²⁺] abolishes E–C coupling in skinned fibres

In a mechanically skinned EDL fibre of the rat, Na^+ substitution depolarizes the sealed T-system (Depol) eliciting Ca^{2+} release from the SR and a large force response. Exposure of the fibre to 10 μM Ca^{2+} for 90 s did not affect the coupling, but exposure to 50 μM Ca^{2+} for 15 s caused complete abolition of depolarization-induced responses, even though caffeine (30 mM caffeine with low $[Mg^{2+}]$ and 0·5 mM EGTA) could still directly elicit Ca^{2+} release. Where indicated, the SR was additionally loaded for 20 s at pCa 6·7 (1 mM total EGTA) to ensure there was ample Ca^{2+} in the SR. The exposures to 10 and 50 μM Ca^{2+} were performed in the absence of ATP (with the fibre in rigor) so as to prevent SR Ca^{2+} uptake from locally altering the applied $[Ca^{2+}]$. Maximum force was determined by exposure to a 50 mM CaEGTA solution at pCa 4·5 (Max). Time scale: 2 s during depolarizations and caffeine exposure and 30 s elsewhere.

the Ca^{2+} handling properties of the SR in some way. This was investigated by using caffeine-induced responses as an assay of the ability of the SR to load and retain Ca^{2+} (Endo, 1985). Rat EDL fibres can be fully depleted of releasable Ca^{2+} by exposure to a solution with 30 mM caffeine and low free $[Mg^{2+}]$ (Fryer & Stephenson, 1996; Owen *et al.* 1997), the latter being necessary in mammalian fibres to ensure potent stimulation of the Ca^{2+}-release channels. Each fibre can be subjected to repeated load–release cycles in which the SR is reloaded with Ca^{2+} by exposure to a load solution (see Methods) for a set period and then depleted again with the caffeine solution, eliciting a highly reproducible force response (Fig. 2*A*). In each fibre, the 'area' (i.e. time integral) of the force response increases progressively with increasing duration of the loading period (Fig. 2), until reaching saturation with long loads (e.g. after ∼60 s at pCa 6·7, 1 mM EGTA) (not shown). This relationship between load time and caffeine-response area (Fig. 2*B*) indicates that the area of the force response is indicative of the amount of Ca^{2+} loaded into the SR. The exact relationship depends on the Ca^{2+} buffering conditions used in the caffeine release solution. Throughout this study, except where specified, there was 0·5 mM EGTA (pCa 8) present in both the pre-

equilibration and caffeine release solutions (see Methods), with these conditions chosen so that the caffeine stimulus elicited a measurable, non-saturated response when the SR was loaded at, or somewhat below, the normal endogenous level. The advantage of using fibres skinned under (paraffin) oil is that the force response to the first caffeine exposure is indicative of the amount of Ca^{2+} present in the SR endogenously. Here it was found that fibres required reloading for between 20 and 40 s (in the standard load solution: pCa 6·7, 1 mM total EGTA) in order to elicit a caffeine response similar to that when releasing the endogenous Ca^{2+} (Fig. 2*A*). For the 12 fibres shown in Fig. 2*B*, the mean area (± S.E.M.) of the caffeine response with the endogenous Ca^{2+} load was 84·6 ± 10·7 % of the response with 40 s loading (i.e. close to the level for 30 s loading).

High $[Ca^{2+}]$ treatment reduces subsequent caffeine responses

Exposure to 50 μM Ca^{2+} for 15 s in the absence of ATP (referred to as 'high $[Ca^{2+}]$ treatment') caused a reduction in the area of the subsequent caffeine response (with 0·5 mM EGTA) in every fibre examined (Fig. 3), with the area on
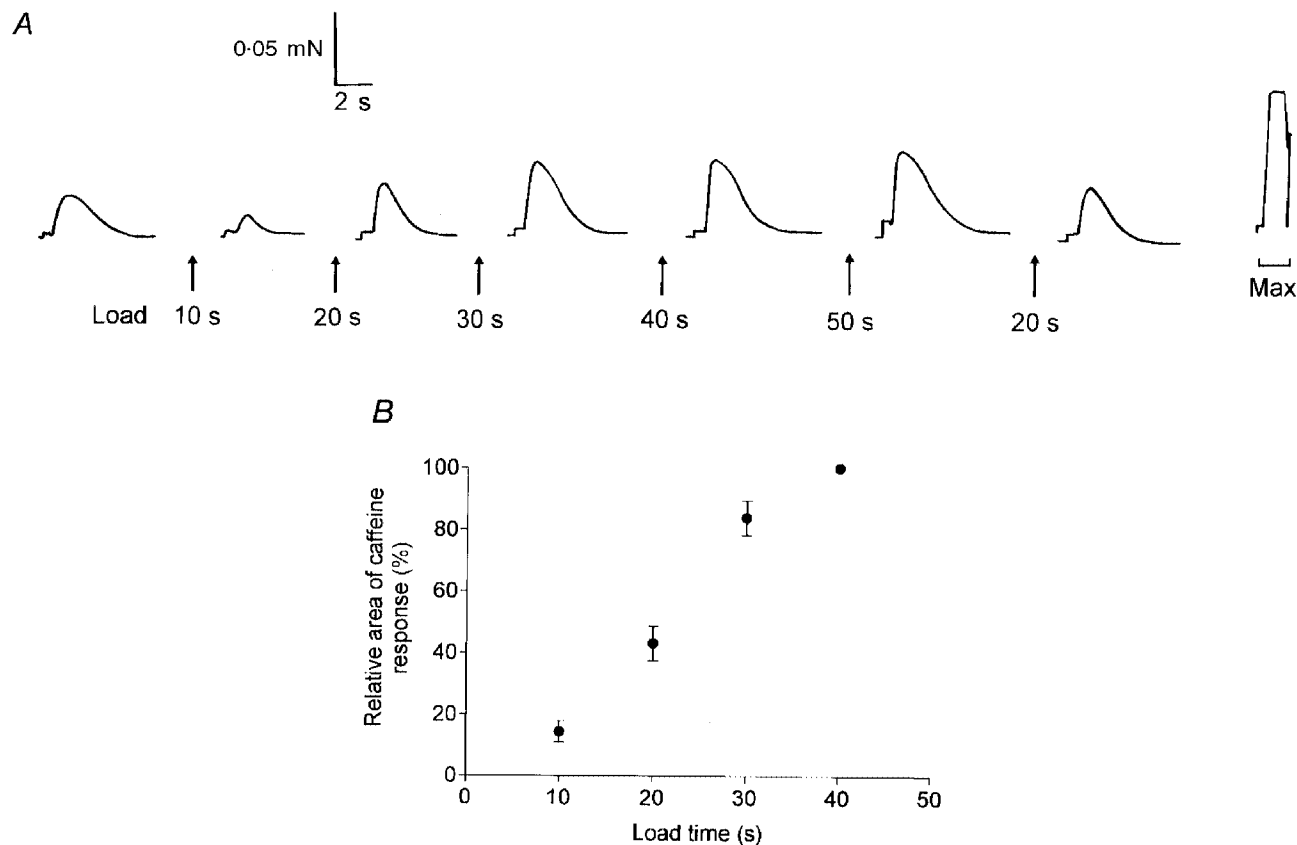

Figure 2. Relationship between Ca^{2+} load time and the size of the caffeine response

A, force responses of an EDL fibre to the caffeine stimulus (i.e. 30 mM caffeine, 0·05 mM Mg^{2+}, 0·5 mM EGTA, pCa 8) after loading the SR with Ca^{2+} to various degrees by exposure to a pCa 6·7 solution (1 mM total EGTA) for the indicated times. The first caffeine response was elicited soon after skinning the fibre, whilst the SR contained the endogenous level of Ca^{2+}. *B*, means ± S.E.M. of the relative area of the force response *versus* loading time in 12 EDL fibres. The responses in each fibre were expressed as a percentage of that for 40 s loading.

Table 1. Mean (± s.e.m.) relative area and duration of the caffeine response after various treatments

Treatment	No. of fibres	Area (%)	Duration (%)
Rigor only	6	$101\cdot7 \pm 7\cdot8$	$108\cdot0 \pm 3\cdot7$
50 μM Ca^{2+} (same fibres)		$59\cdot7 \pm 6\cdot4*$	$83\cdot5 \pm 3\cdot5*$
10 μM Ca^{2+}	9	$104\cdot9 \pm 8\cdot6$	$103\cdot3 \pm 3\cdot4$
50 μM Ca^{2+} (same fibres)		$55\cdot8 \pm 4\cdot7*$	$80\cdot0 \pm 2\cdot8*$
40 μM Ca^{2+} (all fibres)	6	$68\cdot7 \pm 9\cdot5*$	$78\cdot8 \pm 6\cdot3*$
50 μM Ca^{2+} (all fibres)	25	$61\cdot2 \pm 3\cdot3*$	$79\cdot8 \pm 2\cdot3*$
130 μM Ca^{2+} (all fibres)	6	$53\cdot7 \pm 6\cdot4*$	$76\cdot8 \pm 5\cdot2*$

The area (i.e. time integral) and duration of the caffeine response (in 0·5 mM EGTA) were measured after the indicated treatment and expressed as a percentage of the respective value before treatment in the same fibre (see Fig. 3). Duration was measured at 10% of the peak response before treatment. Measurements were made in a total of 25 fibres given a 15 s exposure to 50 μM Ca^{2+}. In 9 of these cases, the fibre was given a 90 s exposure to 10 μM Ca^{2+} before the 50 μM Ca^{2+} treatment. In 6 of these latter cases, the fibre was consecutively given the rigor control treatment, the 10 μM Ca^{2+} treatment and finally the 50 μM Ca^{2+} treatment. Data are also shown for treatment with 40 and 130 μM Ca^{2+}. * value was significantly different after the indicated treatment ($P < 0\cdot05$, Student's paired t test). A sigmoidal fit to the data indicated half-maximal reduction in response area with ~32 μM Ca^{2+}.

average declining to $61\cdot2 \pm 3\cdot3\%$ (mean ± s.e.m., here and following) of that before the high $[Ca^{2+}]$ treatment ($n = 25$, $P < 0\cdot05$) (Table 1). The duration of the caffeine response was also significantly reduced to $79\cdot8 \pm 2\cdot3\%$ of the pre-treatment value (initially $4\cdot2 \pm 0\cdot3$ s, measured at 10% of peak response). In contrast, neither a 90 s exposure to 10 μM Ca^{2+} (not shown) nor rigor treatment alone (Fig. 3), had any significant effect on either the area or the duration of the caffeine response, even though subsequent treatment of the same fibres with 50 μM Ca^{2+} caused a marked reduction in both parameters (Table 1).

The reduction in the caffeine response caused by high $[Ca^{2+}]$ treatment persisted unchanged over a period of at least 10 to 20 min (i.e. three to six repetitions of the load–release cycle), as illustrated by the mean data in Fig. 4A. Individual fibres displayed a similar constancy in the post-treatment response; for example the mean difference between the first and second response after high $[Ca^{2+}]$ treatment was $0\cdot0 \pm 1\cdot9\%$ ($n = 10$). Thus, the spread indicated by the

magnitude of the s.e.m. values following Ca^{2+} treatment in Fig. 4A (~6%) predominantly reflects variability in the extent of the reduction between different fibres, rather than variability between successive responses in the same fibre. In the one fibre examined over six consecutive responses following a single treatment, the area of the caffeine response changed by only 4% (of the initial control) between the first and last repetition. Thus, the area of the caffeine response showed no recovery over a considerable period following the Ca^{2+} treatment. If a fibre was given a second exposure to 50 μM Ca^{2+}, there was no further reduction in the size of the caffeine response (Fig. 4B). In other experiments, fibres were first treated with either 40 μM Ca^{2+} (two fibres) or 50 μM Ca^{2+} (three fibres) and then with 130 μM Ca^{2+}, which resulted in a small, but significant, further reduction in area of the caffeine response in every case (means relative to initial control response: $63\cdot4 \pm 7\cdot1\%$ and $49\cdot3 \pm 5\cdot6\%$, before and after 130 μM Ca^{2+}, respectively, $P < 0\cdot05$, Student's paired t test). These latter data show
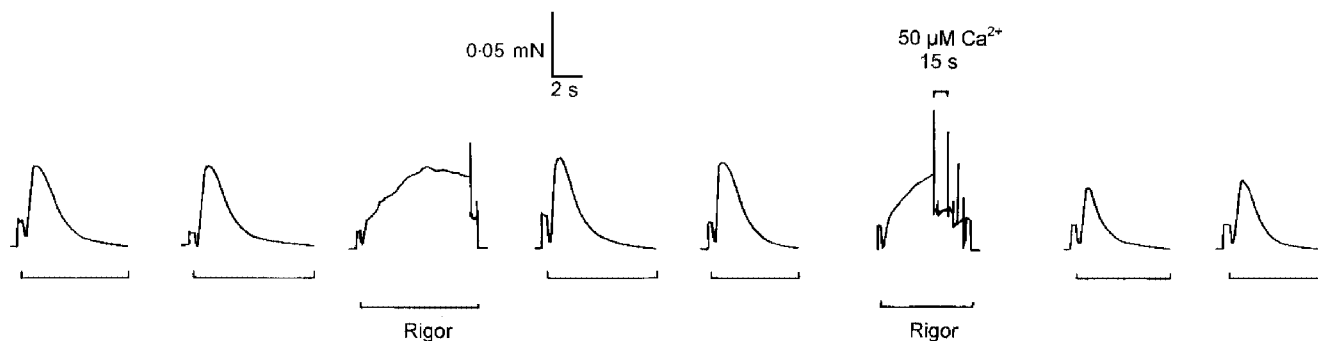

Figure 3. Exposure to high $[Ca^{2+}]$ reduces the size of subsequent caffeine responses

The area of the force response to the caffeine stimulus was reduced substantially after exposure to 50 μM Ca^{2+} for 15 s (with ATP absent and the fibre in rigor), but not after similar treatment without raised $[Ca^{2+}]$. Time scale: 2 s during caffeine stimulus and 30 s elsewhere.

that the reduction in caffeine response does not occur in an all-or-nothing manner in individual fibres, but is instead graded by the [Ca^{2+}] used. The mean values of the relative area and duration of the caffeine response after treatment at different [Ca^{2+}] are shown in Table 1. A plot of mean area of the caffeine response *versus* treatment [Ca^{2+}] was well fitted by a sigmoidal function with half-maximal effect at $\sim$32 μM Ca^{2+}; such analysis indicated that 50 μM Ca^{2+} treatment induced $\sim$80–90 % of the maximum effect.

The reduction in the area and duration of the caffeine response following the high [Ca^{2+}] treatment was not due to a change in the Ca^{2+} sensitivity of the contractile apparatus. Experiments in which the contractile apparatus was directly activated by heavily buffered Ca^{2+} solutions (50 mM CaEGTA–EGTA, see Methods) showed no change in the force response to submaximal Ca^{2+} stimuli following 50 μM Ca^{2+} treatment in any of the three fibres examined (mean change in pCa giving half-maximal force: $-0\cdot004 \pm 0\cdot004$). This is in agreement with the previous finding of no change in Ca^{2+} sensitivity or maximum force following similar high [Ca^{2+}] treatment (100 μM Ca^{2+} for 10 s during rigor; Lamb *et al.* 1995). Thus, the reduction in the size of the caffeine response indicates some change in Ca^{2+} handling by the SR.

Effect of different Ca^{2+} buffering in the caffeine solution

In order to investigate what changes in SR function underlie the reduction in the caffeine response, experiments were also performed using 0·5 mM BAPTA instead of EGTA in the caffeine and pre-equilibration solutions. The Ca^{2+} affinity of BAPTA is similar to that of EGTA (at pH 7·1; Harrison &

Bers, 1987) but BAPTA is much faster at chelating Ca^{2+}. The use of such a fast Ca^{2+} buffer permits estimation of the minimum amount and rate of Ca^{2+} release underlying the force response (see Discussion). The force response was smaller and slower to rise when BAPTA was present in the caffeine solution instead of EGTA (Fig. 5). In the five fibres examined with 0·5 mM BAPTA, the peak force and duration of the caffeine response were 55 ± 16 and 83 ± 24 % of the respective values with 0·5 mM EGTA at the same level of SR loading. In the two fibres examined both before and after treatment with 50 μM Ca^{2+}, the area, peak force and duration of the caffeine responses in BAPTA were all reduced after treatment (to 53 and 59 %; to 82 and 84 %; and to 72 and 77 % of the pre-treatment values in the two fibres, respectively), and these reductions were in fact slightly greater than those occurring with EGTA (to 78 and 71 %; to 88 and 94 %; and to 84 and 81 %, respectively). Clearly, high [Ca^{2+}] treatment caused a reduction in the size of the caffeine response, irrespective of the type of Ca^{2+} buffering used in the caffeine solution.

As discussed later, the substantial force responses observed in the presence of the fast buffer, BAPTA, show that the caffeine stimulus induces the rapid efflux of most or all of the SR Ca^{2+} within $\sim$1 s, with the rate of subsequent force decline apparently being determined primarily by the diffusion of Ca^{2+}–buffer complex out of the skinned fibre. It can thus be concluded (see Discussion) that the reduction in the size of the caffeine response after high [Ca^{2+}] treatment must be the result of a reduction in the total amount of releasable Ca^{2+} in the SR, rather than simply a reduction in the rate of release. The size and time course of the force
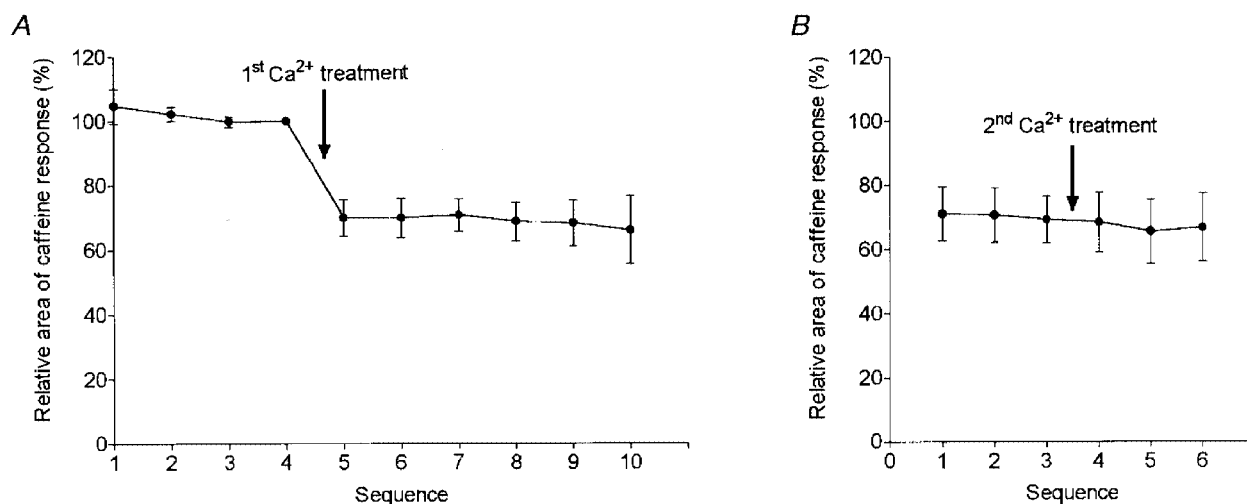

Figure 4. Effects on the caffeine response of two successive Ca^{2+} treatments

A, means $\pm$ S.E.M. of the relative area of the caffeine response before and after a first exposure to 50 μM Ca^{2+} in 10 fibres. The responses in each fibre were expressed as a percentage of that immediately prior to the treatment (i.e. the 4th control response). At least five caffeine responses were recorded following the first Ca^{2+} treatment, with the fibres being subjected to a further (identical) Ca^{2+} treatment following the third such response (6 fibres) or following the second ($n = 1$), fourth ($n = 1$) or fifth ($n = 1$) response or not at all ($n = 1$). *B*, realignment of the responses for the seven fibres from *A* in which three caffeine responses were obtained both prior to and following the second Ca^{2+} treatment. The data in *A* and *B* show that the first Ca^{2+} treatment caused a persistent reduction in the area of the caffeine response, and that a second treatment did not cause any further reduction.

Table 2. Mean (± s.e.m.) Ca²⁺ leakage rates from the SR recorded under various conditions, before and after high [Ca²⁺] treatment

| Treatment	Leakage condition	No. of fibres	Rate of decrease in area of caffeine response during leakage period			
			Before (% s⁻¹)	After (% s⁻¹)	Absolute change (% s⁻¹)	Relative change
Rigor only	Standard	3	1·2 ± 0·1	1·4 ± 0·4	0·2 ± 0·4	1·2 ± 0·4
50 μM Ca²⁺	Standard	4	1·6 ± 0·4	4·6 ± 0·9	3·1 ± 0·6*	3·0 ± 0·2*
50 μM Ca²⁺	10 mM Mg²⁺	3	1·3 ± 0·3	3·0 ± 0·4	1·7 ± 0·2*	2·9 ± 0·3*
50 μM Ca²⁺	2 μM RR	3	1·8 ± 0·2	3·6 ± 0·2	1·8 ± 0·4*	2·1 ± 0·4
50 μM Ca²⁺	Na⁺	3	1·2 ± 0·3	3·4 ± 0·6	2·4 ± 0·1*	3·1 ± 0·4*

The rate of Ca²⁺ leakage out of the SR (both before and after the indicated treatment) was ascertained from the decrease in the area of the caffeine response after various different leakage periods in the indicated conditions, as illustrated in Fig. 6. The rate was determined for each fibre by a linear fit to a graph of caffeine response area *versus* leak duration. The change in the leakage rate after Ca²⁺ treatment (or rigor only) is expressed both in absolute and relative terms, and for the latter a value of 1·0 indicates no change. * Leak rate was significantly higher after the treatment ($P < 0.05$, Student's paired t test). Where indicated, 10 mM Mg²⁺ or 2 μM RR was present before and during loading and during the subsequent leakage period, but was not present in the pre-equilibration (10 s) and caffeine release solutions. 'Na⁺' indicates that the solutions were Na⁺ based, with no K⁺, to ensure the T-system was chronically depolarized and hence the voltage sensors inactivated.

responses before and after high [Ca²⁺] treatment are also consistent with this. In general, the caffeine responses obtained after Ca²⁺ treatment closely resembled those obtained with a shorter loading period before Ca²⁺ treatment. For example in Fig. 5, the characteristics of the caffeine response with EGTA and 20 s loading after Ca²⁺ treatment appear intermediate between those with 14 and 20 s loading before treatment. Similarly, the effect of the Ca²⁺ treatment on the caffeine response appeared to be reversed simply by loading the SR for longer (e.g. 40 s loading instead of the 20 s loading recorded in Fig. 5).

Increase in SR Ca²⁺ leakage following high [Ca²⁺] treatment

The above findings suggested that the ability of the SR to load and/or retain Ca²⁺ was reduced after high [Ca²⁺]

treatment. It is difficult to study Ca²⁺ loading by the SR in isolation, because the SR simultaneously leaks Ca²⁺ whilst loading. In contrast, the rate of Ca²⁺ leakage from the SR can be separately studied simply by loading the SR to some extent and then measuring the rate at which the SR loses Ca²⁺ in a solution where there should be no Ca²⁺ uptake (e.g. pCa > 8·5, 2 mM EGTA). As illustrated in Fig. 6, the area of the caffeine response was used to assay the Ca²⁺ content of the SR with and without various periods for Ca²⁺ leakage (in standard conditions with 1 mM free Mg²⁺ etc.). The area of the caffeine response progressively declined with increasing duration of the leakage period, and in general this was better described by a linear rather than an exponential relationship. The mean rate of decline of the caffeine response area was 1·4 ± 0·2% s⁻¹ (expressed per second of leakage period) in the seven fibres studied with
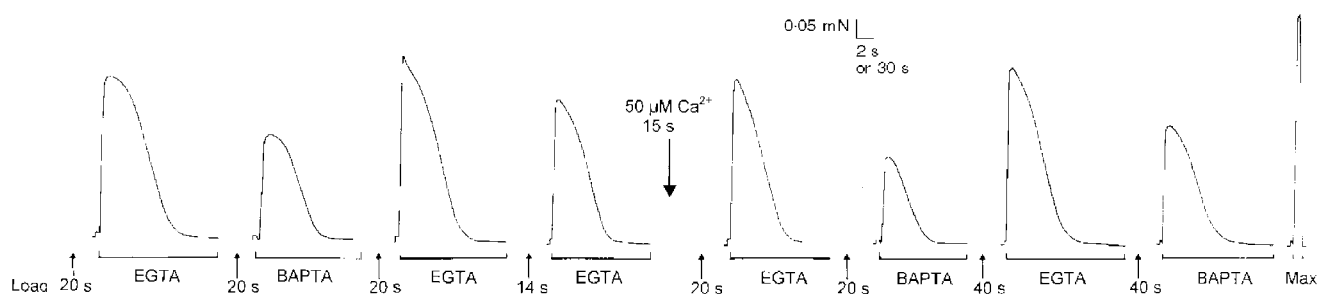
Figure 5. Effect of the fast Ca²⁺ buffer BAPTA

When 0·5 mM BAPTA was used instead of 0·5 mM EGTA in the caffeine and pre-equilibration solutions, the peak size and rate of rise of the caffeine response were reduced for a given level of loading. Ca²⁺ treatment caused a reduction in the area of the responses irrespective of the Ca²⁺ buffer used, with the loading time in both cases having to be increased to about 40 s in order to produce a response similar to that found with 20 s loading before treatment. All loading performed at pCa 6·7 (1 mM EGTA). Time scale: 2 s during caffeine responses, vertical scale 0·05 mN.

the standard conditions (i.e. 1 mM Mg^{2+}). Following Ca^{2+} treatment, the area of the caffeine response (for the same loading period) was reduced, as expected (Fig. 6). The SR was then loaded for a longer period at the same or higher $[Ca^{2+}]$ (i.e. pCa 6·7 or 6·4) in order to restore the area of the response close to that observed before Ca^{2+} treatment (range 70–110%), and the effect of a Ca^{2+}-leakage period was examined (Fig. 6). These experiments showed that the rate of Ca^{2+} leakage out of the SR was increased ~3-fold after high $[Ca^{2+}]$ treatment, but was not significantly changed by rigor treatment alone (Table 2).

Effects of 10 mM Mg^{2+} and Ruthenium Red

We then examined whether the SR Ca^{2+} leakage was blocked by factors known to greatly reduce the opening of the ryanodine receptor/Ca^{2+} release channels in mammalian skeletal muscle (RyR1), namely 10 mM Mg^{2+} and 2 μM Ruthenium Red (RR) (Meissner *et al.* 1986; Lamb & Stephenson, 1991; Soler *et al.* 1992; Laver *et al.* 1997). When fibres were pre-equilibrated and loaded in the presence of 10 mM Mg^{2+} (pCa 6·7, 1 mM EGTA), and then Ca^{2+} release stimulated under the standard (1 mM Mg^{2+}) conditions, the area of the caffeine response was $75 \pm 5\%$ of that found after comparable loading at 1 mM Mg^{2+} in the same fibres ($n = 7$), with the relationship between load time and response area not obviously changed. This reduction in net Ca^{2+} uptake at 10 mM Mg^{2+} is similar to that reported previously with skinned EDL fibres (Kabbara & Stephenson, 1994) and probably indicates that the presence of 10 mM Mg^{2+} reduces Ca^{2+} pumping at pCa 6·7. In fibres loaded in 10 mM Mg^{2+}, high $[Ca^{2+}]$ treatment reduced the caffeine response to $46 \pm 9\%$ ($n = 11$) of that before treatment. This indicates that despite the presence of 10 mM Mg^{2+}, there was a reduction in net Ca^{2+} uptake following Ca^{2+} treatment. This must have been caused by either reduced Ca^{2+} pumping into the SR and/or increased leakage of Ca^{2+} during the Ca^{2+} pumping and the subsequent 10 s equilibration period before

the caffeine stimulus was applied. Leakage experiments (e.g. like that in Fig. 6, but with 10 mM Mg^{2+} present during both the uptake and the leakage periods) indicated that: (a) before high $[Ca^{2+}]$ treatment, the Ca^{2+} leakage rate in 10 mM Mg^{2+} was quite similar to that in 1 mM Mg^{2+}, and (b) the Ca^{2+} leakage rate in 10 mM Mg^{2+} was increased significantly after high $[Ca^{2+}]$ treatment (Table 2). Furthermore, the increase in the Ca^{2+} leakage rate following high $[Ca^{2+}]$ treatment was not significantly different between the 1 and 10 mM Mg^{2+} cases (Table 2), but this finding should be viewed with caution because it is not based on paired measurements in the same fibres.

Similar experiments were conducted using Ruthenium Red (RR). We have previously shown that 2 μM RR almost completely blocked (> 95%) the response of EDL fibres to 30 mM caffeine at 1 mM Mg^{2+} (Lamb & Stephenson, 1991). We found here that the response to 30 mM caffeine/low $[Mg^{2+}]$ was also markedly inhibited by the presence of 2 μM RR, but that a substantial response could be elicited if the RR was washed out before transfer to the pre-equilibration and caffeine/low $[Mg^{2+}]$ solutions. In these experiments the SR was loaded in a solution at pCa 6·3 (1 mM total EGTA), because 2 μM RR apparently also inhibited net Ca^{2+} uptake at pCa 6·7, as indicated by the small size or complete absence of a response upon subsequent permeabilization of the SR with Triton X-100 (not shown). This is consistent with the finding that RR decreased the Ca^{2+} sensitivity of uptake in cardiac SR vesicles (Kargacin *et al.* 1998). Even with loading at pCa 6·3 and washout of RR before release, the response to successive repetitions typically declined by 10% or more, making these experiments less reliable. Nevertheless by interpolating between bracketing control responses, it appeared that the area of the caffeine response was approximately linear with the loading duration and could thus be used as a measure of SR Ca^{2+} content. Further experiments with fibres loaded in the presence of 2 μM RR
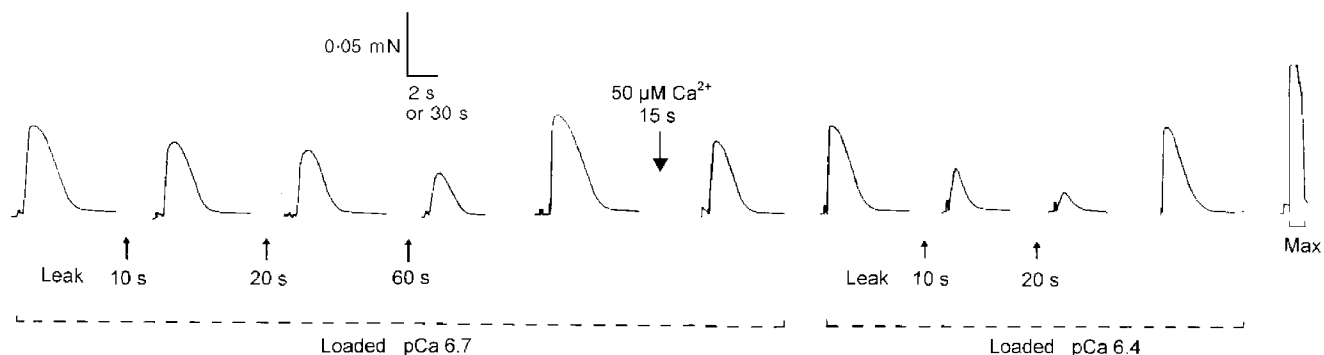

Figure 6. High $[Ca^{2+}]$ treatment causes an increase in the rate of Ca^{2+} leakage from the SR

The caffeine response in this EDL fibre was measured either immediately after loading for 30 s at pCa 6·7 (1 mM EGTA) (first and fifth responses) or after loading and then allowing Ca^{2+} leakage out of the SR for various times (in a solution with 2 mM EGTA, pCa > 8·5) (second to fourth responses). High $[Ca^{2+}]$ treatment caused the typical reduction in the control caffeine response, and so the fibre was subsequently loaded in a solution with higher $[Ca^{2+}]$ (30 s in pCa 6·4, 1 mM EGTA) in order to restore the caffeine response to closer to the pre-treatment level, and then the effect of Ca^{2+} leakage duration was examined. After Ca^{2+} treatment, a 20 s leakage period caused a greater reduction in the caffeine response than did a 60 s leakage period before Ca^{2+} treatment. Time scale, 2 s during caffeine responses.

showed that high $[Ca^{2+}]$ treatment still reduced the area of caffeine response to $40 \pm 7\%$ of the pre-treatment level ($n = 6$), which is comparable to that found in the absence of RR ($61\cdot2 \pm 3\cdot3\%$ with 1 mM Mg^{2+}, Table 1; $46 \pm 9\%$ with 10 mM Mg^{2+}). Leakage experiments indicated that: (a) before high $[Ca^{2+}]$ treatment, the Ca^{2+} leakage rate was similar in the absence and presence of 2 μM RR, and (b) the Ca^{2+} leakage rate in 2 μM RR was significantly increased in absolute terms (but not relative terms) after high $[Ca^{2+}]$ treatment (Table 2). Thus, neither 2 μM RR nor 10 mM Mg^{2+} could prevent the increased SR leakage occurring after high $[Ca^{2+}]$ treatment.

Effect of voltage-sensor inactivation

We also examined whether the increase in Ca^{2+} leakage following high $[Ca^{2+}]$ treatment was the result of increased voltage-sensor stimulation of the Ca^{2+} release channels, which theoretically could arise from some direct effect on the voltage sensors or from depolarization of the T-system. In the following experiments, the T-system was kept chronically depolarized, and hence the voltage sensors inactivated, by the replacement of all K^+ with Na^+ in the bathing solutions (see Methods). In the three fibres examined, the caffeine stimulus elicited little or no response after 10 s loading

(pCa $6\cdot7$) and the area of the response increased approximately linearly with longer loading times, with approximately 30 s loading required to elicit a response similar to that with endogenous level of Ca^{2+} loading (not shown). This probably means that the fibre loads Ca^{2+} similarly in the Na^+-based and K^+-based solutions, but that the size of the force response is disproportionately reduced at short load times, possibly due to lower Ca^{2+} sensitivity of the contractile apparatus in Na^+ compared with K^+ (Fink *et al.* 1986). In the fibres kept depolarized in Na^+, the SR Ca^{2+} leakage rate (as indicated by the reduction in the caffeine response with 30 or 40 s loading and various leakage periods), was comparable to that found in (polarized) fibres under the standard conditions with K^+ (Table 2). Following high $[Ca^{2+}]$ treatment, the area of the caffeine response (with 30 or 40 s loading at pCa $6\cdot7$) was reduced to $20 \pm 10\%$ of the pre-treatment value ($n = 3$). The area of the response was restored to $80-95\%$ of the original level by loading (for 20–60 s) at pCa $6\cdot4$, and the Ca^{2+} leakage rate was then found to be significantly higher (about 3-fold) than before Ca^{2+} treatment (Table 2). Thus, high $[Ca^{2+}]$ treatment increases SR Ca^{2+} leakage even if voltage sensors are kept chronically inactivated, indicating that the treatment directly affects the properties of the SR.
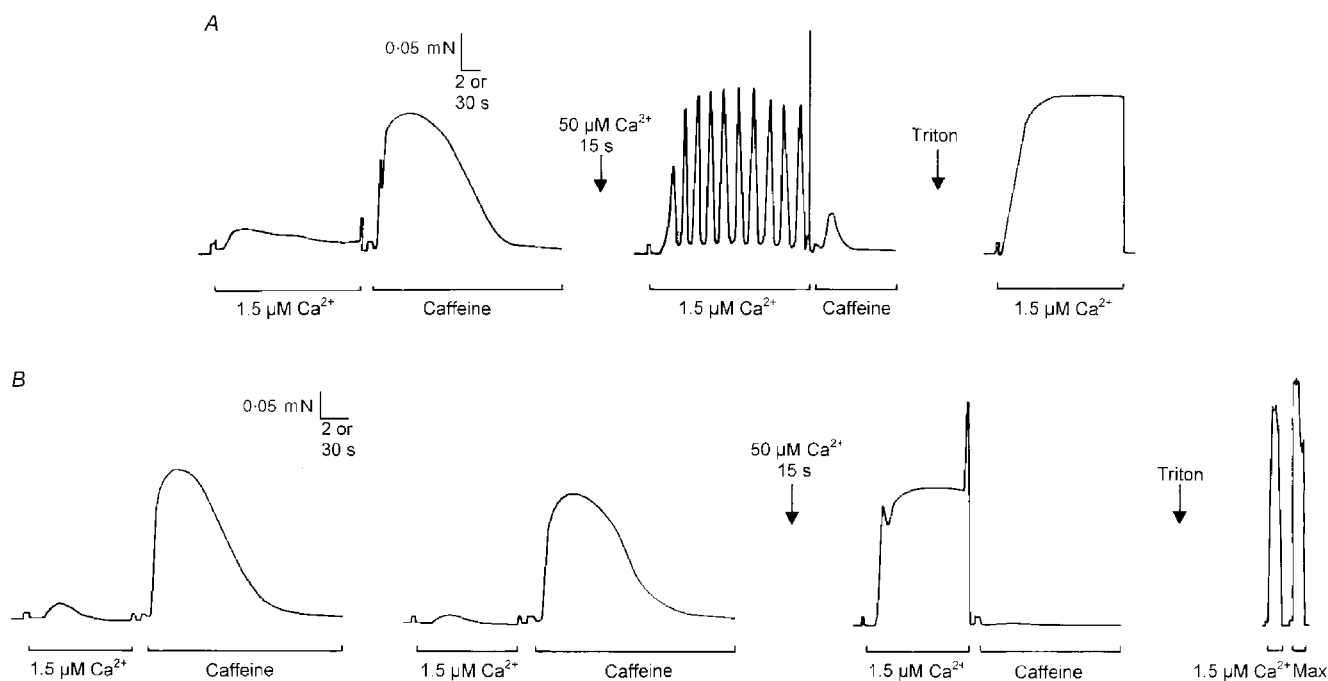

Figure 7. Ca^{2+} treatment reduces the ability of the SR to keep the $[Ca^{2+}]$ low within the fibre

A, the SR was first fully depleted of releasable Ca^{2+} with caffeine (not shown). Subsequent exposure to a solution moderately buffered ($0\cdot75$ mM total EGTA) at $1\cdot5$ μM free Ca^{2+} (i.e. pCa $5\cdot85$) induced only a small force response over a prolonged exposure (3 min), but after Ca^{2+} treatment the same solution caused a large, oscillatory force response (displayed at slow time scale). The caffeine response (fast time scale: caffeine solution with $1\cdot5$ mM EGTA) following exposure to the $1\cdot5$ μM Ca^{2+} solution was much smaller after Ca^{2+} treatment than before, indicating reduced Ca^{2+} uptake by the SR. After the SR was destroyed by treatment with Triton X-100 (2% v/v for 2 min), exposure to the same $1\cdot5$ μM free Ca^{2+} solution caused a large, relatively rapid force response, showing that, before the high $[Ca^{2+}]$ and Triton treatments, the SR had maintained the $[Ca^{2+}]$ near the contractile apparatus at a much lower level than in the bathing solution. *B*, example of a fibre exposed twice to the $1\cdot5$ μM Ca^{2+} bathing solution before high $[Ca^{2+}]$ treatment. Time scale in *A* and *B*: 30 s during exposures to $1\cdot5$ μM Ca^{2+} solution and 2 s during caffeine responses.

Direct evidence of reduced SR Ca²⁺ accumulation

Finally, we wished to see if we could observe the alteration in SR properties more directly, that is without using the caffeine response as an assay of SR Ca^{2+} content. In these experiments, a skinned fibre was initially depleted of releasable Ca^{2+} (with caffeine) and then bathed in a solution moderately buffered (0·75 mM total EGTA) to 1·5 μM free Ca^{2+} (i.e. pCa 5·85) for 2 min or more. If the $[Ca^{2+}]$ near the contractile apparatus reached a level similar to that in the bathing solution, it would induce a large force response. Following the destruction of the SR by treatment with Triton X-100 (2% v/v for 2 min and then washout), this was indeed seen to be the case, with exposure to the 1·5 μM Ca^{2+} solution inducing between 60 and 95% of maximum Ca^{2+}-activated force within 30 s in every fibre examined ($n = 21$) (Fig. 7). However, when the fibres were first exposed to the 1·5 μM Ca^{2+} solution before destruction of the SR with Triton X-100, little or no force was elicited over the whole exposure period (mean peak force 10 ± 3% of force measured after SR destruction; $n = 21$) (Fig. 7). This change in the force response was not due to some effect of the Triton treatment on the contractile apparatus, because solutions in which the $[Ca^{2+}]$ was very heavily buffered (i.e. 50 mM total EGTA) to a similar level induced approximately the same large force response both before and after Triton treatment (not shown). Clearly, the SR, due to its ability to translocate Ca^{2+} and perhaps also its physical structure, kept the $[Ca^{2+}]$ near the contractile apparatus much lower (i.e. < 0·7 μM or pCa 6·15) than in the bathing solution, despite the Ca^{2+} buffering capacity of the EGTA present. (In the few fibres examined, the SR was observed to do this even with exposure to the solution for 10 min or more.) At the end of the exposure period, the SR was evidently very heavily loaded with Ca^{2+}, because caffeine invariably induced a large force response despite the presence of a high free [EGTA] (1·5 or 2 mM, pCa > 8) in the pre-equilibration and caffeine solutions (Fig. 7).

If a fibre was then given the standard high $[Ca^{2+}]$ treatment (15 s exposure to 50 μM Ca^{2+} during rigor), exposure to the same 1·5 μM Ca^{2+} solution invariably induced a large force response, with the peak force reaching 69 ± 6% ($n = 8$) of that elicited after Triton treatment (and with the associated caffeine response in the presence of 1·5 mM EGTA always being greatly reduced, see Fig. 7A). In three of these cases (e.g. Fig. 7A), the force response showed major oscillations, whereas in the other five cases (e.g. Fig. 7B) the force rapidly reached a high steady level after one or two small oscillations (cf. Szentes *et al.* 1998). This altered response to the 1·5 μM Ca^{2+} solution was not due simply to changes occurring during the first exposure (or the associated caffeine response), though in certain cases that first exposure did appear to alter the subsequent properties. These conclusions were made by distinguishing between fibres that gave little or no force response (< 5% of the post-Triton level) upon the first exposure to the 1·5 μM Ca^{2+} solution, and those that gave a more substantial response (> 5%). In the former

group of fibres, those given a second exposure to the 1·5 μM Ca^{2+} solution without any preceding high $[Ca^{2+}]$ treatment, showed no significant increase in the force response (1 ± 1% on first exposure, 3 ± 2% on second, $n = 6$; $P > 0·05$), whereas those fibres given a second exposure after high $[Ca^{2+}]$ treatment (Fig. 7A) showed a large, significant increase (2 ± 1% on first exposure, 60 ± 3% on second, $n = 4$; $P < 0·05$). This shows that the high $[Ca^{2+}]$ treatment deleteriously affected the ability of the SR to maintain the $[Ca^{2+}]$ near the contractile apparatus at a relatively low level, which is consistent with the increased SR leakage detailed above. Interestingly, it was also found that in the fibres in which the first exposure to the 1·5 μM Ca^{2+} solution induced a substantial force response (15 ± 3%, $n = 7$), a second exposure caused a much larger response (51 ± 14%, $P < 0·05$), even though those fibres were not given the high $[Ca^{2+}]$ treatment in between the two exposures. (Not surprisingly, the comparable fibres given the high $[Ca^{2+}]$ treatment between the first and second exposures also showed this significant increase: 23 ± 7 to 79 ± 9%, $n = 4$; $P < 0·05$.) These data suggest that first exposure to the 1·5 μM Ca^{2+} solution (or the associated caffeine response) itself adversely affected the ability of the SR to keep the $[Ca^{2+}]$ relatively low near to the contractile apparatus. This probably indicates that during the exposure to the 1·5 μM Ca^{2+} solution, the $[Ca^{2+}]$ near the release channels increased to such an extent that it induced a similar effect as the high $[Ca^{2+}]$ treatment (see Discussion).

DISCUSSION

Caffeine assay of Ca²⁺ content

In this section we consider the Ca^{2+} release profile underlying the caffeine response, and show that the reduction in the area of the caffeine response after high $[Ca^{2+}]$ treatment is indeed indicative of reduced SR Ca^{2+} content. The amount of releasable Ca^{2+} in the SR was assayed by stimulating rapid and complete release of Ca^{2+} by simultaneously adding caffeine and lowering the free $[Mg^{2+}]$ (Fryer & Stephenson, 1996). This induced a small to large force response, depending on the level to which the SR was loaded with Ca^{2+} and the amount and type of Ca^{2+} buffering present in the cytoplasm (Figs 2 and 5). A lower limit for the amount and rate of Ca^{2+} release can be made as follows, based on the experiments using BAPTA, because this buffer binds Ca^{2+} so rapidly that it should reach equilibrium with the cytoplasmic Ca^{2+} in a matter of milliseconds. When loaded at the endogenous level, the SR of EDL fibres contains ~1 mM of releasable Ca^{2+} (expressed as mmol per litre total fibre volume, as if the Ca^{2+} were released into the whole volume of an intact fibre) (Fryer & Stephenson, 1996; Owen *et al.* 1997). In the EDL fibres here, which were loaded to ~75% of the endogenous level (i.e. with 20 s loading at pCa 6·7, see Fig. 2 and Results), the caffeine stimulus in the presence of 0·5 mM free BAPTA induced a peak force of ~30–40% of maximum, reaching 90% of that level within ~0·5 s of the caffeine addition (Fig. 5). Thus, given: (a) an apparent

calcium association constant for BAPTA of 5×10^6 M^{-1} (Harrison & Bers, 1987), and (b) a force–[Ca^{2+}] relationship with half-maximal force at $\sim$pCa 6·1 in the caffeine solution (Wendt & Stephenson, 1983), this indicates that approximately 75% of the total BAPTA in the fibre space ($\sim$1·06 $\times$ 0·5 mM; see Owen *et al.* 1997), i.e. $\sim$0·4 mM, had Ca^{2+} bound at the peak of the force response. As another $\sim$0·1 mM Ca^{2+} must be bound to troponin C at such force levels (Fuchs, 1985) and additional Ca^{2+} may be bound to other fixed binding sites (Owen *et al.* 1997), the caffeine stimulus must have released at least 0·5 mM Ca^{2+}, which is > 60–70% of the total releasable Ca^{2+}, within $\sim$0·5 s. This estimate of the Ca^{2+} release rate ($\sim$1 mM s^{-1}) is one tenth of the maximum release rate in voltage-clamped EDL fibres at this temperature (10 mM Ca^{2+} s^{-1}; Shirokova *et al.* 1996) and should be considered a lower limit because it ignores the diffusional delays in applying the stimulus and activating the contractile apparatus.

Thus, the force response found using either EGTA or BAPTA apparently results from the rapid 'bolus' release of most of the Ca^{2+} in the SR, followed by the slow diffusional loss of the Ca^{2+}–buffer complex from the fibre. During the latter phase, some of the Ca^{2+} initially released is presumably resequestered by the SR and subsequently released again, though at any time the majority of the Ca^{2+} must be bound to BAPTA/EGTA, troponin C and other fixed buffers in the cytoplasmic space. The peak of the force response with 0·5 mM EGTA was higher than with 0·5 mM BAPTA, presumably because BAPTA chelated a greater proportion of the Ca^{2+} before it reached the troponin C. In contrast, the decline in the force response from a given level was quite similar with either 0·5 mM BAPTA or 0·5 mM EGTA (Fig. 5), because the diffusional loss of Ca^{2+} from the fibre depended on the amount rather than the type of Ca^{2+} buffer present. Typically it took $\sim$3 s for the force to decline to 10% of the peak, with the change in the force indicating that the free [Ca^{2+}] dropped from about pCa 6·15 to pCa 6·4, and hence that the total amount of Ca^{2+} on the BAPTA and troponin C dropped from $\sim$0·5 mM to 0·3 mM, indicating an average diffusional loss of < 0·07 mM Ca^{2+} s^{-1}, which is > 15 times slower than the Ca^{2+} release rate.

The above considerations indicate that most of the Ca^{2+} is rapidly released as a bolus and then the force declines relatively slowly as Ca^{2+}–buffer complex transports Ca^{2+} out of the skinned fibre. As the size and rate of development of the force response in BAPTA indicated that the initial Ca^{2+} release rate was still at least 1 mM s^{-1} after high [Ca^{2+}] treatment (Fig. 5), it is apparent that the reduction in the area of the caffeine response following the treatment cannot be due to a decrease in the rate of Ca^{2+} release, as the rate of Ca^{2+} release still greatly exceeded the rate of diffusional loss. Instead, the reduction in the area of the caffeine response indicates that the amount of releasable Ca^{2+} in the SR was reduced following the high [Ca^{2+}] treatment, which in turn implies a reduction in the ability of the SR to load and/or hold Ca^{2+}.

Ca^{2+} leakage from the SR before and after high [Ca^{2+}] treatment

Even before any high [Ca^{2+}] treatment, the SR of mechanically skinned fibres leaks Ca^{2+}, albeit at a relatively low rate in the presence of a low cytoplasmic [Ca^{2+}] (pCa > 8) and physiological levels of free Mg^{2+} (1 mM), ATP (8 mM total), pH (7·1) (Fig. 6). With the SR loaded at close to the endogenous level ($\sim$1 mM Ca^{2+}; see above), the EDL fibres placed in a solution with 2 mM free EGTA (pCa > 8) lost $\sim$1·4% of the SR Ca^{2+} s^{-1} (Table 2), or $\sim$14 μM s^{-1}, which is similar to that found previously with skinned EDL fibres placed in 5 mM BAPTA (Bakker *et al.* 1996). The Ca^{2+} leakage rate was not very different in the presence of 10 mM Mg^{2+} or 2 μM RR (1·3 and 1·8% s^{-1}, respectively; see Table 2). It is possible that this Ca^{2+} leakage occurs through the normal ryanodine receptor/Ca^{2+}-release channel, and that neither 10 mM Mg^{2+} nor 2 μM RR are able to reduce the channel opening probability below the base level (note: the leakage rate implies a mean opening probability of $\sim$0·001 or lower, because peak release can reach $\sim$10 mM Ca^{2+} s^{-1}; Shirokova *et al.* 1996). Alternatively, the leakage could occur through a modified type of ryanodine receptor, given that it has been reported that Ca^{2+} leaks out of mammalian skeletal muscle SR through a pathway that is insensitive to both ryanodine and RR and that this pathway can be converted back to the normal ryanodine-sensitive state by the sponge extract, bastadin-5 (Pessah *et al.* 1997). A third possibility is that the leak occurs via some quite different pathway, such as through the SR CaATPase; such Ca^{2+} leakage has been observed in frog SR vesicles and was not blocked by Mg^{2+}, ryanodine or RR (Du *et al.* 1996; 1998).

Importantly, the Ca^{2+} leakage rate was increased approximately 3-fold after high [Ca^{2+}] treatment (Fig. 6 and Table 2). This increased leak meant that the SR could only be reloaded to its original, endogenous level by loading for longer at pCa 6·7 (Fig. 5), or if this was still insufficient, by loading at a higher [Ca^{2+}] (Fig. 6). The increased Ca^{2+} leakage was not noticeably blocked by the presence of either 10 mM Mg^{2+} or 2 μM RR (Table 2). It is not known, however, whether this increase in Ca^{2+} leakage is through the same pathway(s) as responsible for Ca^{2+} leakage in untreated fibres or through some additional pathway. As the increased leakage was not blocked by 10 mM Mg^{2+} or 2 μM RR, we were unable to separately examine whether the rate of Ca^{2+} pumping into the SR was also reduced, as we could not prevent Ca^{2+} leakage occurring simultaneously during the Ca^{2+} pumping. Nevertheless, noting that the net Ca^{2+} uptake rate before Ca^{2+} treatment is on average $\sim$33 μM s^{-1} (i.e. about 30 s to reload the SR from empty to $\sim$1 mM) despite simultaneous leakage at $\sim$14 μM s^{-1}, it is apparent that a 3-fold increase in leakage by itself would seemingly fully account for the 40–50% decrease in net Ca^{2+} uptake found after high [Ca^{2+}] treatment. However, in view of the approximations and assumptions in such calculations, we do not rule out that high [Ca^{2+}] treatment might also reduce the SR Ca^{2+} pumping rate to some extent.

In our original study showing that high intracellular $[Ca^{2+}]$ abolishes depolarization-induced responses (Lamb *et al.* 1995), we did not observe any reduction in net Ca^{2+} uptake in the few fibres examined. This is because the fibres were loaded in a very weakly buffered pCa 5·7 solution (50 μM total EGTA) and pre-equilibrated in a pCa 7 solution with only 50 μM EGTA (cf. this study: loading in 1 mM total EGTA at pCa 6·7 and pre-equilibrating in 0·5 mM EGTA at pCa 8), and so most of the extra Ca^{2+} leaking into the fibre space during these periods would have been recovered by the SR rather than chelated by the free EGTA (0·5 mM) present.

$[Ca^{2+}]$ gradients established by the SR

We also obtained further evidence that exposure to high $[Ca^{2+}]$ causes a change in SR Ca^{2+} handling, by examining the ability of the SR to keep the $[Ca^{2+}]$ relatively low near the contractile apparatus in the presence of elevated $[Ca^{2+}]$ in the bathing solution (Fig. 7). Before high $[Ca^{2+}]$ treatment, the SR was evidently able to establish a steady-state $[Ca^{2+}]$ gradient between the weakly buffered bathing solution (0·75 mM total EGTA, 1·5 μM free Ca^{2+}, i.e. pCa 5·85) and the contractile apparatus. The steady-state level of force ($\sim$5 to 10 % of maximum Ca^{2+}-activated force) implied that the $[Ca^{2+}]$ at the contractile apparatus was about 0·8 μM (i.e. pCa 6·1) (see Fink *et al.* 1986; no caffeine) and thus given an apparent Ca^{2+}-affinity constant for EGTA of 5×10^6 M^{-1} (Fink *et al.* 1986), it seems that the ratio of CaEGTA : EGTA was 4 : 1 (i.e. 0·6 : 0·15 mM) near the contractile apparatus compared with 7 : 1 (i.e. 0·66 : 0·09 mM) in the bathing solution. Thus, to establish such a gradient the SR had to continuously translocate only a relatively small amount of Ca^{2+} (0·66 − 0·6 = 0·06 mM) to balance the diffusional influx occurring with 0·66 mM CaEGTA outside and 0·6 mM CaEGTA near the contractile apparatus. It is not clear whether the SR establishes this Ca^{2+} gradient by translocating the Ca^{2+} from the deeper to the more superficial regions of the skinned fibre, or by taking up Ca^{2+} all along the length of each myofibril and releasing it in the triadic regions. Irrespective of this, it was found that after high $[Ca^{2+}]$ treatment, fibres were less able to prevent the Ca^{2+} in the bathing solution from reaching and activating the contractile apparatus (Fig. 7), which is consistent with a reduction in net Ca^{2+} uptake by the SR. In some cases following high $[Ca^{2+}]$ treatment, the force response showed large oscillations (Fig. 7A), similar to those observed in *Xenopus* skinned fibres bathed in comparable solutions (0·5 mM EGTA, pCa 6–5·3; Szentesi *et al.* 1998). These oscillations evidently depend on using weakly buffered high $[Ca^{2+}]$ solutions such that the SR is initially able to sequester sufficient Ca^{2+} to prevent appreciable force development before it eventually becomes so full that it suddenly releases some of the stored Ca^{2+} (perhaps involving a Ca^{2+}-induced Ca^{2+} release mechanism) and then subsequently starts to sequester the Ca^{2+} again.

Interestingly, we also found that if the force rose appreciably during the exposure to the weakly buffered 1·5 μM Ca^{2+}

solution, the force response upon subsequent exposure was greatly increased, just as if the fibre had been given the high $[Ca^{2+}]$ treatment (see Results). This is most easily explained by the $[Ca^{2+}]$ locally near the release channels being considerably higher than in the bathing solution (e.g. $>$10 μM *versus* 1·5 μM), owing to the heavy loading of the SR and the continual efflux of Ca^{2+}, with this elevated $[Ca^{2+}]$ eventually causing alterations in the SR handling properties. Thus, it seems quite possible that the $[Ca^{2+}]$ in intact fibres subjected to prolonged, vigorous activity, would locally reach similar high levels and consequently would induce altered Ca^{2+} handling properties like those described here.

Physiological relevance

Westerblad *et al.* (1993) observed that the resting $[Ca^{2+}]$ in mouse fast-twitch fibres was still elevated by $\sim$40 % more than 30 min after induction of long-lasting 'low-frequency' fatigue. Based on mathematical modelling of the final fall in $[Ca^{2+}]$ after a tetanus, they suggested that there had been a 2-fold decrease in SR pumping rate and a $\sim$2·5-fold increase in the small level of SR Ca^{2+} leakage, though the exact values were quite model dependent. It has also been reported that following extremely prolonged (7 h) exercise in rats, the Ca^{2+} uptake capacity of the SR from both fast- and slow-twitch muscles was decreased by about 20 to 40 %, with no change in SR ATPase activity (Fitts *et al.* 1982). The authors suggested that Ca^{2+} transport by the SR ATPase became uncoupled or that the SR became 'leaky'. The results of both studies might be explained by findings here. Specifically, the high $[Ca^{2+}]$ reached locally during the stimulus periods may have induced a long lasting increase in the rate of Ca^{2+} leakage from the SR, leading to reduced net Ca^{2+} uptake capacity and an increased resting $[Ca^{2+}]$.

If net Ca^{2+} uptake by the SR is reduced at the $[Ca^{2+}]$ close to the resting level (i.e. pCa $\leqslant$ 7·0), be this due entirely to increased Ca^{2+} leakage or also to reduced Ca^{2+} pumping, the amount of releasable Ca^{2+} in the SR of an intact fibre must inevitably be reduced over time. Furthermore, because the $[Ca^{2+}]$ in the cytoplasm is regulated in the long term by Ca^{2+} movements into and out of the muscle fibre, it is to be expected that the elevated intracellular $[Ca^{2+}]$ will ultimately lead to net loss of Ca^{2+} from the fibre as a whole. Thus, if the changes in net SR Ca^{2+} uptake reported here occurred in an intact fibre, it might well lead to substantial depletion of releasable Ca^{2+} in the SR. This effect would compound any decrease in Ca^{2+} release caused by uncoupling of the voltage sensors and Ca^{2+}-release channels. Consequently, the reduction in Ca^{2+} release seen in low frequency fatigue may be due to the high local $[Ca^{2+}]$ occurring during stimulation inducing changes in both the SR properties and the coupling mechanism. It is not certain whether or how these two processes are linked, but one explanation is that both are caused by the physical changes occurring at the triad junction (Lamb *et al.* 1995; see swollen SR and altered triad dimensions). Alternatively, high $[Ca^{2+}]$ treatment might cause some change in the proportion of Ca^{2+} release channels in

the ryanodine-sensitive and ryanodine-insensitive states (Pessah *et al.* 1997) which results both in the uncoupling and increased Ca²⁺ leakage. Whatever the relationship, if any, between these two phenomena, both may be of importance in muscle function in exercise and disease.

ALLEN, D. G., LÄNNERGREN, J. & WESTERBLAD, H. (1995). Muscle cell function during prolonged activity: cellular mechanisms of fatigue. *Experimental Physiology* **80**, 497–527.

BAKKER, A. J., LAMB, G. D. & STEPHENSON, D. G. (1996). The effect of 2,5-di-(tert-butyl)-1,4-hydroquinone on force responses and the contractile apparatus in mechanically skinned muscle fibres of the rat and toad. *Journal of Muscle Research and Cell Motility* **17**, 55–67.

CHIN, E. R. & ALLEN, D. G. (1996). The role of elevations in intracellular [Ca²⁺] in the development of low frequency fatigue in mouse single muscle fibres. *Journal of Physiology* **491**, 813–824.

CHIN, E. R., BALNAVE, C. D. & ALLEN, D. G. (1997). Role of intracellular calcium and metabolites in low-frequency fatigue of mouse skeletal muscle. *American Journal of Physiology* **272**, C550–559.

DU, G., ASHLEY, C. C. & LEA, T. J. (1996). Ca²⁺ effluxes from the sarcoplasmic reticulum vesicles of frog muscle: effects of cyclopiazonic acid and thapsigargin. *Cell Calcium* **20**, 355–359.

DU, G., ASHLEY, C. C. & LEA, T. J. (1998). The cation selectivity of the sarcoball Ca²⁺ channel in frog muscle fibres. *Pflügers Archiv* **436**, 365–370.

EDWARDS, R. H. T., HILL, D. K., JONES, D. A. & MERTON, P. A. (1977). Fatigue of long duration in human skeletal muscle after exercise. *Journal of Physiology* **272**, 769–778.

ENDO, M. (1985). Calcium release from sarcoplasmic reticulum. *Current Topics in Membranes and Transport* **25**, 181–230.

FINK, R. H. A., STEPHENSON, D. G. & WILLIAMS, D. A. (1986). Potassium and ionic strength effects on the isometric force of skinned twitch muscle fibres of the rat and toad. *Journal of Physiology* **370**, 317–337.

FITTS, R. H., COURTRIGHT, J. B., KIM, D. H. & WITZMANN, F. A. (1982). Muscle fatigue with prolonged exercise: contractile and biochemical alterations. *American Journal of Physiology* **242**, C65–73.

FRYER, M. W. & STEPHENSON, D. G. (1996). Total and sarcoplasmic reticulum calcium contents of skinned fibres from rat skeletal muscle. *Journal of Physiology* **493**, 357–370.

FUCHS, F. (1985). The binding of calcium to detergent-extracted rabbit psoas muscle fibres during relaxation and force generation. *Journal of Muscle Research and Cell Motility* **6**, 477–486.

HARRISON, S. M. & BERS, D. M. (1987). The effect of temperature and ionic strength on the apparent Ca-affinity of EGTA and the analogous Ca-chelators BAPTA and dibromo-BAPTA. *Biochimica et Biophysica Acta* **925**, 133–143.

KABBARA, A. A. & STEPHENSON, D. G. (1994). Effects of Mg²⁺ on Ca²⁺ handling by the sarcoplasmic reticulum in skinned skeletal and cardiac muscle fibres. *Pflügers Archiv* **428**, 331–339.

KARGACIN, G. J., ALI, Z. & KARGACIN, M. E. (1998). Ruthenium red reduces the Ca²⁺ sensitivity of Ca²⁺ uptake into cardiac sarcoplasmic reticulum. *Pflügers Archiv* **436**, 338–342.

LAMB, G. D., JUNANKAR, P. R. & STEPHENSON, D. G. (1995). Raised intracellular [Ca²⁺] abolishes excitation–contraction coupling in skeletal muscle fibres of rat and toad. *Journal of Physiology* **489**, 349–362.

LAMB, G. D. & STEPHENSON, D. G. (1990). Calcium release in skinned muscle fibres of the toad by transverse tubule depolarization or by direct stimulation. *Journal of Physiology* **423**, 495–517.

LAMB, G. D. & STEPHENSON, D. G. (1991). Excitation–contraction coupling in skeletal muscle fibres of rat and toad in the presence of GTPγS. *Journal of Physiology* **444**, 65–84.

LAMB, G. D. & STEPHENSON, D. G. (1994). Effects of intracellular pH and [Mg²⁺] on excitation–contraction coupling in skeletal muscle fibres of the rat. *Journal of Physiology* **478**, 331–339.

LAVER, D. R., OWEN, V. J., JUNANKAR, P. R., TASKE, N. L., DULHUNTY, A. F. & LAMB, G. D. (1997). Reduced inhibitory effect of Mg²⁺ on ryanodyine receptor-Ca²⁺ release channels in malignant hyperthermia. *Biophysical Journal* **73**, 1913–1924.

MEISSNER, G., DARLING, E. & EVELETH, J. (1986). Kinetics of rapid Ca²⁺ release by sarcoplasmic reticulum. Effects of Ca²⁺, Mg²⁺, and adenine nucleotides. *Biochemistry* **25**, 236–244.

MELZER, W., HERRMANN-FRANK, A. & LÜTTGAU, H. C. (1995). The role of Ca²⁺ ions in excitation–contraction coupling of skeletal muscle fibres. *Biochimica et Biophysica Acta* **1241**, 59–116.

OWEN, V. J., LAMB, G. D., STEPHENSON, D. G. & FRYER, M. W. (1997). Relationship between depolarization-induced force responses and Ca²⁺ content in skeletal muscle fibres of rat and toad. *Journal of Physiology* **498**, 571–586.

PESSAH, I. N., MOLINSKI, T. F., MELOY, T. D., WONG, P., BUCK, E. D., ALLEN, P. D., MOHR, F. C. & MACK, M. M. (1997). Bastadins relate ryanodine-sensitive and -insensitive Ca²⁺ efflux pathways in skeletal SR and BC₃H1 cells. *American Journal of Physiology* **272**, C601–614.

SHIROKOVA, N., GARCIA, J., PIZARRO, G. & RIOS, E. (1996). Ca²⁺ release from the sarcoplasmic reticulum compared in amphibian and mammalian skeletal muscle. *Journal of Physiology* **107**, 1–18.

SOLER, F., FERNANDEZ-BELDA, F. & GOMEZ-FERNANDEZ, J. C. (1992). The Ca²⁺ release channel in junctional sarcoplasmic reticulum: gating and blockade by cations. *International Journal of Biochemistry* **24**, 903–909.

STEPHENSON, D. G. & WILLIAMS, D. A. (1981). Calcium-activated force responses in fast-and slow-twitch skinned muscle fibres of the rat at different temperatures. *Journal of Physiology* **317**, 281–302.

SZENTESI, P., ZAREMBA, R. & STIENEN, G. J. M. (1998). Calcium handling by the sarcoplasmic reticulum during oscillatory contractions of skinned skeletal muscle fibres. *Journal of Muscle Research and Cell Motility* **19**, 675–687.

WENDT, I. R. & STEPHENSON, D. G. (1983). Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal muscle fibres of the rat. *Journal of Physiology* **398**, 210–216.

WESTERBLAD, H., DUTY, S. & ALLEN, D. G. (1993). Intracellular calcium concentration during low-frequency fatigue in isolated single fibres of mouse skeletal muscle. *Journal of Applied Physiology* **75**, 382–388.

Corresponding author

G. D. Lamb: Department of Zoology, La Trobe University, Bundoora, Victoria, 3083 Australia.

Email: zoogl@zoo.latrobe.edu.au