Mechanisms underlying phosphate-induced failure of Ca^{2+} release in single skinned skeletal muscle fibres of the rat

Giuseppe S. Posterino and Martin W. Fryer

School of Physiology and Pharmacology, The University of New South Wales, Sydney, New South Wales 2052, Australia

(Received 23 February 1998; accepted after revision 11 June 1998)

1. Single mechanically skinned fibres from rat extensor digitorum longus (EDL) muscles were used to investigate the mechanisms underlying inorganic phosphate (P_i) movements between the myoplasm and the sarcoplasmic reticulum (SR). Force transients elicited by caffeine/low Mg^{2+} application were used to assess the rate of P_i-induced inhibition of SR Ca^{2+} release and the subsequent recovery of Ca^{2+} release following removal of myoplasmic P_i.

2. Myoplasmic P_i reduced SR Ca^{2+} release in a concentration- and time-dependent manner. A 10 s exposure to 10, 20 and 50 mM myoplasmic P_i reduced SR Ca^{2+} release by 12 ± 9, 29 ± 5 and 82 ± 5 %, respectively.

3. Removal of myoplasmic ATP at the time of P_i exposure significantly increased the rate and extent of SR Ca^{2+} release inhibition. For example, Ca^{2+} release was reduced by 86 ± 6 % ($n = 6$) after 20 s exposure to 20 mM P_i in the absence of ATP compared with only 47 ± 5 % ($n = 5$) in the presence of ATP.

4. The half and full recovery times for SR Ca^{2+} release following washout of myoplasmic P_i were 35 s and ~7 min, respectively. Recovery of Ca^{2+} release was unaffected by the absence of ATP during washout of P_i but was prevented when fibres were washed in the presence of high myoplasmic P_i (30 mM). Neither the P_i transporter blocker phenylphosphonic acid (PHPA) nor the anion channel blockers anthracene-9-carboxylic acid (9-AC) and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) affected the rate of recovery of SR Ca^{2+} release.

5. These results show that P_i entry and exit from the SR occur primarily through a passive pathway that is insensitive to well-known anion channel blockers. P_i inhibition of SR Ca^{2+} release appears to be a complicated phenomenon influenced by the rate of P_i movement across the SR as well as by the rate, extent and species of Ca^{2+}–P_i precipitate formation in the SR lumen. The more rapid inhibitory effect of P_i in the absence of myoplasmic ATP suggests that P_i may inhibit SR Ca^{2+} release more efficiently during the later stages of fatigue.

Fatigue in skeletal muscle is characterized by a decline in force output as a consequence of intense muscular activity. One of the proposed myoplasmic factors that contributes to the decline of force is inorganic phosphate (P_i), which accumulates to between 30 and 40 mM during fatiguing stimulation (Cady *et al.* 1989; Baker *et al.* 1993). Force reduction by P_i has primarily been attributed to an inhibition of contractile protein function (reviewed by Fitts, 1994). However, P_i has also been shown to reduce force generation in both skinned (Fryer *et al.* 1995) and intact (Westerblad & Allen, 1996) skeletal muscle fibres by decreasing Ca^{2+} release from the sarcoplasmic reticulum (SR). It was concluded in both studies that the mechanism of Ca^{2+} release inhibition involved the movement of P_i from the myoplasm into the SR lumen with subsequent formation of Ca^{2+}–P_i precipitate once the solubility product was exceeded in the SR lumen.

In order to understand these results, and to clarify the role of P_i in interfering with SR Ca^{2+} release during fatigue, it is necessary to determine the rate and magnitude of P_i fluxes between the myoplasm and the SR and to define the pathways through which these fluxes occur. Neither of these are well understood. Previous studies on SR vesicles have suggested that P_i may enter the SR of skeletal muscle via an ATP-dependent P_i transporter that is stimulated by Ca^{2+} and Mg^{2+} (Carley & Racker, 1982) and blocked by phenylphosphonic acid (PHPA; Stefanova *et al.* 1991*a*, *b*). Recently, Fryer *et al.* (1997) estimated that P_i could rapidly enter the SR (30–170 μM s^{-1}) at physiological concentrations of myoplasmic P_i (10–50 mM) in single skinned fibres from

the rat. The partial inhibition (37 %) by PHPA of P_i diffusion into the SR in this study was taken as evidence for a component of P_i entry mediated by the previously described P_i transporter. However, the resistance of most of the P_i entry to PHPA blockade (Fryer *et al.* 1997) along with previous studies in SR vesicles showing inhibition of P_i fluxes by stilbene derivatives (Kasai & Kometani, 1979; Campbell & MacLennan, 1980) suggest that P_i may enter the SR by a passive pathway.

P_i movements between the myoplasm and the SR might occur through one (or several) of the various Cl^- channels (Kourie *et al.* 1996) or voltage-dependent anion channels (VDACs; Lewis *et al.* 1994; Junankar *et al.* 1995; Shoshan-Barmatz *et al.* 1996) which have been found in SR membranes. Many of the former are blocked by 9-AC while VDACs and other anion channels are blocked by stilbene derivatives such as 4,4′-diisothiocyanostilbene-2,2′-di-sulphonic acid (DIDS) and 4-aceto-amido-4′-isothiocyanostilbene-2,2′-disulphonate (SITS) (Kasai & Kometani, 1979; Campbell & MacLennan, 1980).

In this study we assessed the mechanisms underlying P_i entry into and exit from the SR by characterizing the rates of onset and recovery from Ca^{2+}–P_i precipitation-induced failure of SR Ca^{2+} release. The contributions of active and passive P_i movements were assessed by changing myoplasmic [ATP] as well as using known blockers of the P_i transporter and various SR anion channels. The results show that P_i can rapidly equilibrate between the myoplasm and SR and reduce SR Ca^{2+} release via a passive pathway that is actually inhibited by myoplasmic ATP. The time course of recovery of SR Ca^{2+} release appears to be consistent with the gradual resolubilization of Ca^{2+}–P_i precipitate once P_i passively exits the SR. Preliminary results have been reported previously in abstract form (Posterino & Fryer, 1997).

METHODS

Skinned fibre preparation

The skinned fibre preparation was used as described previously (Lamb & Stephenson, 1990; Posterino & Lamb, 1996). Briefly, outbred male Wistar rats (3–6 months old) were anaesthetized with halothane (2 % v/v) in a bell jar and killed by an overdose of the anaesthetic. The extensor digitorum longus (EDL) muscles were removed and subsequently pinned at resting length under paraffin oil (Ajax Chemicals, Sydney) on a bed of Sylgard 184 (Dow Chemicals, Midland, MI, USA). Single muscle fibres were isolated and mechanically skinned with fine forceps, leaving approximately 70–90 % of the fibre bulk. A segment of the skinned fibre was then attached to an isometric force transducer (KG3, Scientific Instruments, Germany), the output of which was sent to a chart recorder. After measuring the resting length of the fibre it was stretched by 20 % to give a sarcomere length of approximately 3·1–3·2 μm (based on previous measurements). Next, the fibre diameter was measured and the preparation moved to a 2 ml Perspex bath containing a potassium hexamethylene-diamine-tetraacetate (HDTA) solution (see below) for 2 min prior to the commencement of the experimental protocol. All experiments were performed at room temperature (23 ± 2 °C).

Solutions

The standard potassium (K-) HDTA solution contained (mM): K^+, 126; Na^+, 37; $HDTA^{2-}$, 50; total ATP, 8; total Mg^{2+}, 8·6; creatine phosphate (CP), 10; total EGTA, 0·05; Hepes, 90; azide, 1; pH 7·10 ± 0·01 and pCa ($-\log_{10}$ [Ca^{2+}]) 7·0–7·1. The free [Mg^{2+}] was 1 mM. All other solutions were variants based on the standard K-HDTA composition. A solution containing lowered free [Mg^{2+}] (0·05 mM) was used to stimulate the SR Ca^{2+}-release channel. This solution contained only 2·15 mM total Mg^{2+}. Maximum Ca^{2+}-activated force was determined in each fibre by exposure to a solution (Max) containing 50 mM Ca-EGTA in place of $HDTA^{2-}$ and a total Mg^{2+} of 8·1 mM. The free calcium ion concentration in this solution (23 μM) was determined by measuring the excess [EGTA] using a titration method previously described (Stephenson & Williams, 1981). Following exposure to the Max solution, fibres were relaxed in a heavily buffered solution in which HDTA was replaced with EGTA (50 mM) and the total Mg^{2+} was 10·3 mM. In some experiments fibres were exposed to solutions that lacked ATP (termed rigor). Rigor solutions lacked ATP and CP and had 1·5 mM total Mg^{2+}. Unless otherwise stated, the free [Mg^{2+}] of all solutions was 1 mM. The osmolality of the above solutions ranged between 280 and 302 mosmol kg^{-1}.

Solutions containing inorganic phosphate (P_i) were similar in composition to either the K-HDTA or rigor solutions described above except that (i) CP was completely removed and (ii) HDTA was isosmotically replaced with P_i in a ratio of 1 : 1·3. These changes mimic fatigue-like conditions and have been previously shown to have little effect on the ionic strength and osmolality of the solutions (Fryer *et al.* 1995). The osmolality of P_i solutions ranged between 255 and 315 mosmol kg^{-1} (e.g. rigor solutions with 30 mM P_i had an osmolality of 315 mosmol kg^{-1} compared with 302 mosmol kg^{-1} for the standard rigor solution). In all P_i solutions the Hepes concentration was increased to 100 mM in order to make up some of the difference in osmolality due to the removal of CP. Matching solutions in the presence and absence of P_i never varied in osmolality by more than 25 mosmol kg^{-1}. Such differences in osmolality and ionic strength have previously been shown to have negligible effects on Ca^{2+} release and force production (Lamb *et al.* 1993).

Experimental protocols

Estimation of releasable SR Ca^{2+}. The amount of releasable Ca^{2+} within the SR of a single skinned fibre was assessed by exposing the fibre to a standard K-HDTA solution containing 0·05 mM free Mg^{2+}, 30 mM caffeine and 1 mM total EGTA (termed the caffeine solution). The peak force response evoked by the caffeine solution was used as a qualitative estimate of the amount of releasable (free) Ca^{2+} within the SR. Each experiment began with depletion of the SR Ca^{2+} using the above solution in which the released Ca^{2+} is effectively chelated by the EGTA present. Fibres were then washed for 1 min in a standard K-HDTA solution containing 1 mM total EGTA. The SR of fibres was subsequently reloaded with Ca^{2+} to different levels by exposure (for 0 s to 60 s) to a standard K-HDTA solution that contained equal amounts of the Max and relaxing solutions (pCa ~6·7; termed the load solution). Loading of the SR was rapidly stopped by subsequent exposure to a K-HDTA solution containing 1 mM total EGTA for 6 s. The peak force response elicited upon subsequent exposure to the caffeine solution was then compared with the load time. A linear relationship was observed for load times between 0 and 30 s with no additional loading occurring at longer load times (data not shown). Thus the peak force response was found to provide an accurate qualitative estimate of the amount of releasable Ca^{2+}.

Time course of P_i inhibition of SR Ca^{2+} release. The time course of P_i diffusion into the SR and its associated effect on Ca^{2+} release were determined using the following protocol. Single skinned fibres were initially exposed to the caffeine solution for 1 min to both induce maximum Ca^{2+} release and to deplete the SR of all releasable Ca^{2+}. Fibres were then washed in a K-HDTA solution with 1 mM total EGTA for 1 min to wash away the caffeine and to prevent any Ca^{2+} uptake during this time and then exposed to the load solution (pCa 6·7) for 30 s. Ca^{2+} loading was subsequently quenched by exposure to a K-HDTA solution with 1 mM total EGTA for 6 s. Fibres were then exposed to a similar solution (equilibration solution) for between 5 and 30 s, briefly washed in a weakly buffered K-HDTA solution (150 μM total EGTA) for 6 s and then exposed to the caffeine solution again to release all the remaining Ca^{2+} in the SR. The protocol was repeated twice to ensure that the response to the caffeine solution remained constant (fibres were excluded if the peak height of the caffeine response was not stable within a range of 10%). The protocol was repeated with the exception that the equilibration solution was replaced with an appropriate solution containing 10, 20 or 50 mM P_i for between 5 and 30 s. Following a brief wash (6 s) in standard K-HDTA solution containing 150 μM total EGTA, Ca^{2+} release was elicited with the caffeine solution. The brief wash (6 s) prior to exposure to the caffeine solution was found to be sufficient to ensure that myoplasmic P_i was largely washed away from the fibre to remove any direct effect of P_i on the contractile apparatus and to minimize the loss of P_i from the SR.

The presence of 1 mM EGTA in both the equilibration and P_i solutions prevented Ca^{2+} uptake into the SR, as the free [Ca^{2+}] in these solutions was less than 10^{-8} M. However, due to a constant Ca^{2+} leak (~10 μM Ca^{2+} s^{-1}; Bakker *et al.* 1996; Posterino & Lamb, 1996), and the absence of any Ca^{2+} uptake, the SR of fibres was gradually depleted of Ca^{2+} with approximately 30% of the Ca^{2+} content being lost after 30 s. Therefore, in order to equilibrate fibres in P_i solutions for periods > 30 s whilst ensuring the SR still had a measurable Ca^{2+} content, some fibres were subsequently transferred from the equilibration solution (or comparable P_i solutions) after 30 s exposure to paraffin oil for 30 s to 2·5 min. This limited the volume of fluid bathing the fibre, thus preventing any leaked Ca^{2+} from simply diffusing away into the bulk solution. Despite these precautions, a net Ca^{2+} leak from the SR still occurred, albeit at a much slower rate, suggesting that most of the leaked Ca^{2+} was chelated by the 1 mM EGTA, but that some of the leaked Ca^{2+} was also resequestered by the SR of the fibre under oil.

After obtaining a response to caffeine following P_i exposure, the protocol was repeated with the exception that the fibre was washed for 7 min in a P_i-free solution to ensure that most of the P_i was removed from the SR (see Fig. 4). In every fibre the peak force response to caffeine immediately following washout was equivalent to the response before exposure to P_i (± 5%; cf. Fig. 1). Subsequently, the peak height of the caffeine response following P_i exposure was normalized to the average of the caffeine responses before and after P_i exposure.

Rigor solution protocols. The role of passive diffusion of P_i into the SR was determined using the protocol described above with the exception that fibres were equilibrated for 10–30 s in a solution that lacked ATP (rigor solution) in the presence or absence of 10 and 20 mM P_i. Rigor solutions were heavily buffered with 1 mM total EGTA even though SR Ca^{2+} uptake would be eliminated by the absence of ATP. Fibres were also pre-exposed to a P_i-free rigor solution prior to equilibration for 6 s to wash away ATP bound to the fibre. Following equilibration, fibres were washed in a standard K-HDTA solution (with 125 μM total EGTA) for 6 s to restore ATP to the fibre.

Time course of recovery from P_i-inhibited SR Ca^{2+} release. The protocol used was similar to that described above with the exception that after the SR was reloaded with Ca^{2+}, skinned fibres were exposed to either a standard K-HDTA solution or a 50 mM P_i solution (both containing 125 μM total EGTA (pCa < 7·4)) for 20 s and then equilibrated in paraffin oil for a further 2 min. The concentration of P_i used (50 mM) was chosen to maximize the rate of P_i entry into the SR whilst the length of time in oil ensured that P_i had completely equilibrated to a steady-state level in the SR. Fibres were then washed for 6 s to 7 min in a standard K-HDTA solution that was weakly buffered with 125 μM total EGTA. This concentration of EGTA ensured that net Ca^{2+} leak from the SR was small over several minutes of washout and prevented a net accumulation of Ca^{2+}. Fibres were always washed for a minimum of 6 s to remove the bulk of the myoplasmic P_i from the contractile proteins before Ca^{2+} release was evoked with the caffeine solution. Thus, the minimum wash time (6 s) was designated as the 0 s time point (see Fig. 4). All other time points took into account the initial 6 s washout period.

Effects of ATP removal on the recovery of SR Ca^{2+} release. The washout protocol described above was used with the exception that following equilibration in oil (2 min) with or without previous P_i exposure, fibres were initially washed in a rigor solution for 6 s to remove the bulk of myoplasmic ATP from the fibre, and then exposed to an identical rigor solution for an additional 24, 54 or 114 s (30 s, 1 min or 2 min total washout taking into account the 6 s pre-rigor wash). During this time fibres produced rigor-force due to the absence of ATP. Furthermore, ATP-dependent Ca^{2+} uptake or any other ATP-dependent process was eliminated and net Ca^{2+} leak from the SR was also somewhat depressed (previously estimated at 6 μM Ca^{2+} s^{-1} in mechanically skinned EDL fibres; G. S. Posterino, unpublished observations). Fibres were then briefly exposed to a K-HDTA solution containing 125 μM total EGTA (6 s) to restore ATP and relax the fibre before Ca^{2+} release was elicited by exposure to the caffeine solution.

Effects of raised myoplasmic [P_i] on the recovery of SR Ca^{2+} release. The protocol used was identical to the one described above with the exception that following the initial equilibration period in P_i and paraffin oil (2 min), fibres were initially exposed to a rigor solution containing either 5 or 30 mM P_i (6 s) to wash ATP from the myoplasm and then exposed to an identical solution for an additional 24, 54 or 114 s, respectively. During this time rigor force developed although it was depressed (compared with controls) due to the presence of P_i in the solution. Fibres were then briefly washed for 6 s in a standard K-HDTA solution with 125 μM total EGTA to restore ATP, relax the fibre and to wash away the myoplasmic P_i and Ca^{2+} release was again elicited with the caffeine solution.

Chemicals and data analysis

All chemicals were obtained from Sigma-Aldrich (Australia) except for HDTA (Fluka, Buchs, Switzerland). Stock solutions of anthracene-9-carboxylic acid (9-AC), DIDS and phenylphosphonic acid (PHPA) were prepared by dissolving the compounds in double distilled water at concentrations of 100, 10 and 500 mM, respectively. Aliquots of these stock solutions were then added directly to the skinned fibre solutions. None of the blockers altered the pH or osmolality of the solutions.

All results are expressed as the mean ± S.E.M. of n observations. Statistical significance between the results was determined using the Student's t test for paired or unpaired observations where appropriate. Results were considered significantly different if $P < 0{\cdot}05$.

RESULTS

Time course of failure of SR Ca^{2+} release

When P_i enters the SR it may precipitate with Ca^{2+} once the Ca^{2+}–P_i solubility product is exceeded in the SR lumen (Beil *et al.* 1977). If this occurs, the free [Ca^{2+}] within the SR declines leading to a reduction in the amount of Ca^{2+} that can be released upon stimulation (Fryer *et al.* 1995; Westerblad & Allen, 1996). In the first set of experiments the time course of P_i inhibition of SR Ca^{2+} release was estimated by measuring the reduction in the peak force response elicited by the caffeine solution after exposure to different P_i concentrations for different lengths of time.

Figure 1 illustrates the time-dependent effect of P_i exposure on Ca^{2+} release from the SR in a single EDL fibre. Equilibrating the fibre in 20 mM myoplasmic P_i for just 10 s reduced the force response elicited by the caffeine solution by 25% (see 2nd response). Longer exposure to P_i (20 s) caused a further reduction in the force response (by 42%, see 5th response). This effect was completely reversed by subsequently washing the fibre in a P_i-free equilibration solution for 7 min between P_i exposures. The results of such experiments are summarized in Fig. 2*A* which shows both the concentration and time dependence of P_i inhibition of SR Ca^{2+} release. It can be seen that the rate of inhibition of Ca^{2+} release initially increases linearly with increasing myoplasmic [P_i] in the 10–50 mM range. In contrast, the total degree of inhibition of SR Ca^{2+} release varied in a more complex manner with both time and [P_i]. None of the [P_i] completely suppressed Ca^{2+} release. Ca^{2+} release was only reduced by 27 ± 9% ($n = 5$) at 10 mM P_i after 20 s and remained at this level for longer exposure times. At higher myoplasmic [P_i] (20 and 50 mM) the response to caffeine was reduced by a maximum of 78 ± 4% ($n = 5$) and 82 ± 5% ($n = 4$), respectively, which were not significantly different ($P > 0{\cdot}2$) indicating that Ca^{2+}–P_i precipitation mechanisms are saturated at [P_i] > 20 mM P_i. The inhibitory effects of exposure to 50 mM [P_i] appeared to partially reverse after a period of 20–90 s (Fig. 2*A*), but this effect was not statistically significant at the 0·05 level. The cause of the variability of P_i effects in this range is not known. One possibility is that it reflects complicated time-dependent changes in the species of Ca^{2+}–P_i precipitate formed (Walton *et al.* 1967) within the SR lumen.

The complex time dependence of P_i effects on SR Ca^{2+} release is unlikely to represent direct effects of P_i on the contractile proteins. Nevertheless, the time course of P_i washout on the maximum Ca^{2+}-activated force was examined in three fibres (not shown). These fibres were initially exposed to a 50 mM P_i solution containing 1 mM EGTA for 2 min and were then either immediately exposed to a P_i-free maximum activating solution (pCa 4·5) or washed for 6 or 30 s in a P_i-free solution before maximum force was elicited. There was no difference in the peak force response obtained at either washout time compared with controls, demonstrating that the effects of myoplasmic P_i can be rapidly washed away from a skinned fibre in less than 1 s.

Time course of failure of SR Ca^{2+} release in the absence of myoplasmic ATP

It has previously been suggested that P_i enters the SR via an ATP-dependent P_i transporter (Carley & Racker, 1982;

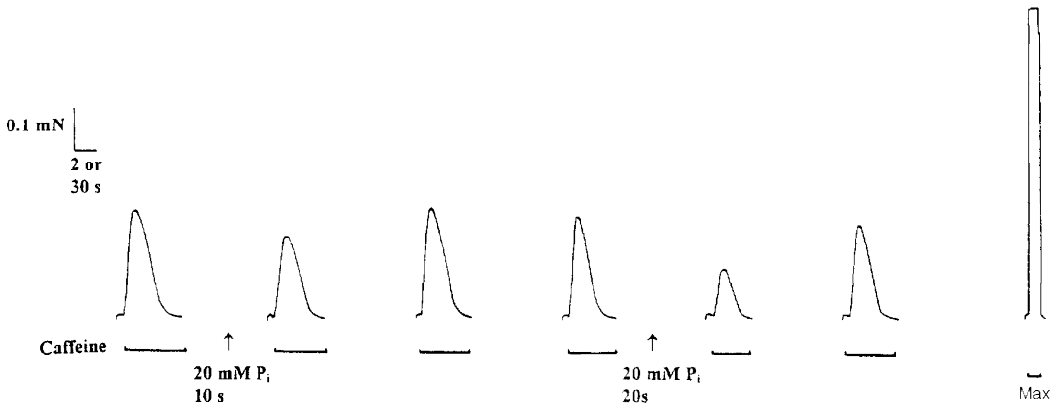

Figure 1. Effect of 20 mM P_i exposure on caffeine-induced Ca^{2+} release from the SR

The SR of a single mechanically skinned fibre was first completely depleted of all Ca^{2+} by exposure for 2 min to a K-HDTA solution containing 30 mM caffeine, 0·05 mM free Mg^{2+} and 1 mM total EGTA (termed caffeine solution: see Methods). The fibre was then reloaded (at pCa 6·7, 1 mM total EGTA) for 30 s, quenched for 10 s in a solution containing 1 mM total EGTA, and then once again depleted of Ca^{2+} in the caffeine solution. The protocol was repeated with the exception that the fibre was equilibrated for 10 s in 20 mM P_i. The subsequent response to caffeine was reduced by 25% compared with controls (2nd response). A repeat of this protocol after the fibre had been washed for 7 min completely restored the response to caffeine (3rd response). The experiment was then repeated with a different equilibration period (20 s) in the presence or absence of 20 mM P_i. The response to caffeine following 20 s equilibration in P_i was reduced by 42% of the interpolated control (5th response). Maximum Ca^{2+}-activated force was determined with a solution of pCa < 4·5 (termed Max). Time scale: 2 s for caffeine responses, 30 s for response to Max.

Stefanova *et al.* 1991*a*,*b*). However, P_i has also been shown to accumulate in SR vesicles in the apparent absence of ATP (Kasai & Kometani, 1979) indicating the additional presence of a passive diffusion mechanism. The role of passive diffusion of P_i into the SR was assessed in the present experiments by exposing fibres to 10 and 20 mM myoplasmic P_i in the absence of ATP (rigor solutions). A surprising result was that the rate of P_i inhibition of Ca^{2+} release was significantly increased in the absence of ATP when compared with P_i exposure in the presence of ATP (Fig. 2*B*). For example, the caffeine response was suppressed by $47 \pm 5\%$ ($n = 5$) after 20 s exposure to 20 mM P_i in the presence of ATP but was inhibited by $86 \pm 6\%$ ($n = 6$) when exposed to the same solution in the absence of ATP. These results indicate that the rate of onset of precipitation-induced Ca^{2+} release inhibition is much faster when myoplasmic ATP is absent. This effect cannot be explained by direct inhibitory effects of the rigor solution on the contractile proteins as prior exposure of skinned fibres to rigor solutions temporarily increased the maximum Ca^{2+}-activated force by approximately 5–10% (examined in three fibres; not shown).

Time course of recovery of SR Ca^{2+} release

If Ca^{2+}–P_i precipitate formation is an important factor underlying the reduction in force and Ca^{2+} release seen during fatigue, then it is important to determine the time course of recovery from precipitation in order to understand the time course of recovery from fatigue.

Figure 3*A* shows the recovery time course of caffeine responses in a single fibre washed for different lengths of time after establishment of Ca^{2+}–P_i-induced precipitation. Longer wash periods in P_i-free solutions (e.g. 30 s on left-hand side of Fig. 3*A*) were clearly associated with a greater recovery of SR Ca^{2+} release than shorter periods (e.g. 6 s on right-hand side of Fig. 3*A*). Summarized data from four to fourteen fibres at each recovery time point are shown in Fig. 4. The time course of recovery of SR Ca^{2+} release was well described ($R^2 = 0{\cdot}97$) by a single exponential curve with a half-time ($T_{1/2}$) of 35 s (Fig. 4). It should be noted that a relatively large amount of Ca^{2+} (~25%) could still be released when fibres were washed for the minimum period (6 s, designated as 0 s in Fig. 4) whereas full recovery required ~7 min of washing in a P_i-free solution (Fig. 4).

Time course of recovery of SR Ca^{2+} release in the absence of myoplasmic ATP

P_i fluxes between the myoplasm and the SR lumen have not been well characterized. In particular, the mechanism by which accumulated P_i is removed from the SR is unknown and could potentially involve an ATP-dependent transporter or ATP-gated channel. In this group of experiments (example shown in Fig. 3*B*) fibres were washed in a rigor solution (no ATP) for 30 s following equilibration in 50 mM P_i in oil for 2 min. The response to caffeine subsequently recovered to 71% of the control responses, a result which was very similar to the response obtained after washout in a standard K-HDTA solution (with ATP) for 30 s in the same

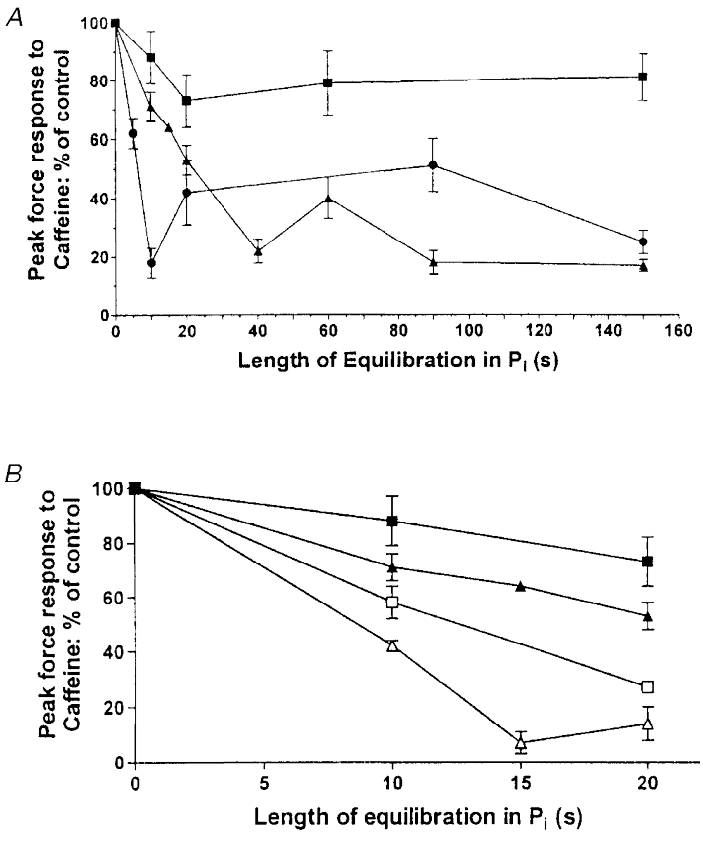

Figure 2. Effect of P_i exposure on caffeine-induced Ca^{2+} release in the presence and absence of myoplasmic ATP

A, concentration and time dependence of caffeine response inhibition in the presence of myoplasmic ATP and varying myoplasmic [P_i]: (■) 10 mM, (▲) 20 mM, (●) 50 mM. *B*, inhibition of caffeine responses by 10 mM P_i (■) and 20 mM P_i (▲) in the presence of myoplasmic ATP compared with the effect of exposure to the same solutions in the absence of myoplasmic ATP (□, △). There was a significant reduction ($P < 0{\cdot}05$) in the peak force response to caffeine following P_i exposure in the absence of ATP compared with the presence of ATP at all times examined. All values are expressed as the mean ± S.E.M. from 3–8 fibres.

fibre (81 % recovery, see Fig. 3*A*). On average, the response to caffeine obtained after a wash period of 30 s to 2 min in the absence of ATP was greater than comparable responses in fibres washed in the presence of ATP (Fig. 5). This effect was only significant at the 1 min time point ($P < 0{\cdot}05$; Fig. 5) and can be explained by a rigor-induced increase in the maximum Ca^{2+}-activated force (5–10 % measured in two fibres; not shown) and to a small increase in the Ca^{2+} sensitivity of the contractile apparatus. The mean response to caffeine following exposure to 50 mM P_i and washout for 30 s under various myoplasmic conditions is also summarized in Table 1. Clearly, the absence of myoplasmic ATP did not inhibit the rate of recovery of SR Ca^{2+} release, suggesting that ATP-dependent processes are not important for the removal of lumenal P_i to the myoplasm.

Time course of recovery of SR Ca^{2+} release in the presence of myoplasmic P_i

The possibility that P_i moves from the SR lumen to the myoplasm via passive diffusion was tested by looking at the time course of recovery of SR Ca^{2+} release in the presence of raised myoplasmic [P_i]. Myoplasmic ATP was omitted in these experiments to rule out any complicating effects resulting from SR Ca^{2+} pump activity or other potential ATP-dependent P_i-extrusion processes.

Figure 3*C* shows an example of the effect of washing a fibre in the absence of ATP and in the presence of 30 mM myoplasmic P_i for 30 s. Compared with washout alone (Fig. 3*A*) or washout in the rigor solution (Fig. 3*B*), washout in the presence of P_i prevented recovery of the force response to caffeine at the same time interval. This can be seen more clearly in the summarized data plotted in Fig. 5 and listed in Table 1. In contrast to rigor wash alone, 1–2 min wash in rigor solution plus 30 mM P_i induced a time-dependent decrease in the caffeine response (Fig. 5), an effect consistent with additional P_i entry from myoplasm to SR lumen during this time. These results support the previous contention that P_i exit from the SR, like its entry (Fig. 3*B*), occurs via a passive diffusion pathway (see Fig. 3*B*) which depends on the P_i concentration gradient across the SR.

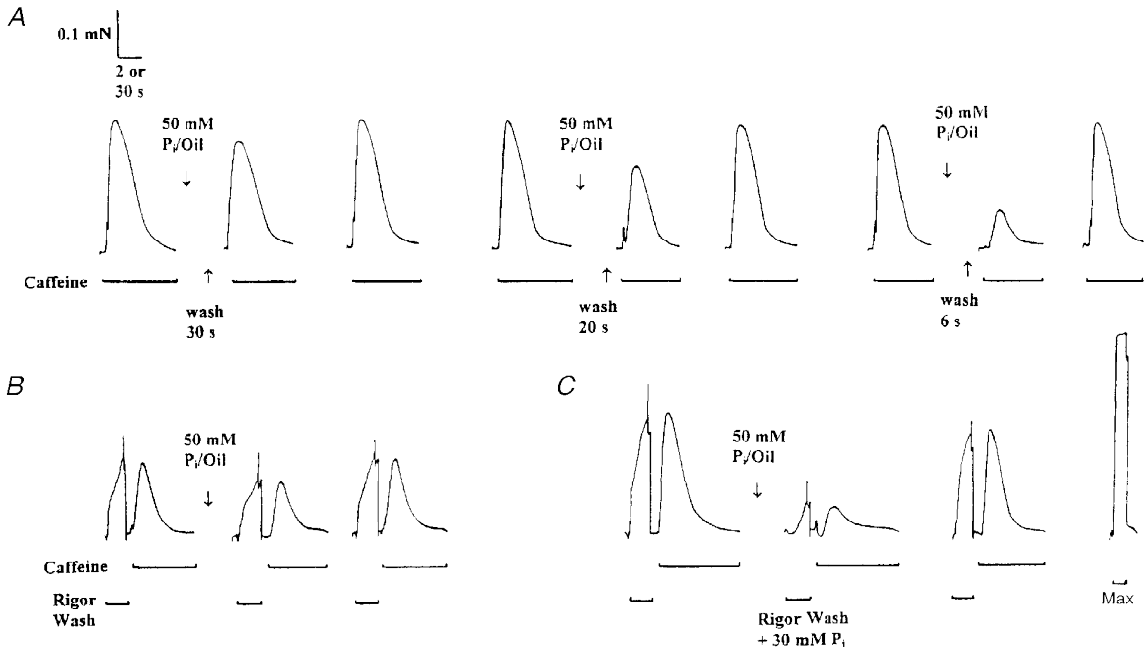

Figure 3. Recovery from P_i-induced Ca^{2+} release inhibition under different myoplasmic conditions

The time course of P_i removal from the SR under different myoplasmic conditions was examined in a single mechanically skinned fibre. *A*, following a Ca^{2+}-deplete/Ca^{2+}-load cycle the fibre was exposed to a solution containing 50 mM P_i for 30 s and then equilibrated in paraffin oil for a further 2 min (see Methods for details). The fibre was then washed in a standard (P_i-free) K-HDTA solution for 30, 20 and 6 s before a force response to caffeine was elicited (see 2nd, 5th and 8th response, respectively). Note the reduction in the recovery of the response to caffeine with shorter washout periods. *B*, a similar protocol was employed with the exception that the same fibre was washed in the absence of myoplasmic ATP (rigor solution) for 30 s which resulted in the production of rigor force. Following the brief reapplication of ATP (6 s), Ca^{2+} release was evoked with the caffeine solution. The recovery of the caffeine response was unaffected for the same washout period. *C*, however, when the same fibre was washed for 30 s in rigor solution containing 30 mM P_i, the response to caffeine did not recover. Maximum Ca^{2+}-activated force was induced with a heavily buffered high Ca^{2+} solution (Max). Time scale: 2 s for caffeine responses and 30 s for responses to the rigor solutions and Max.

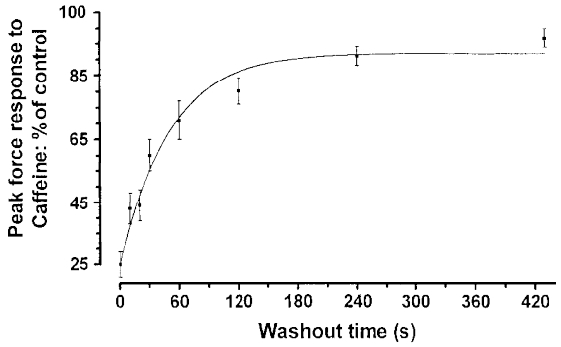

Figure 4. Time course of recovery of SR Ca^{2+} release following removal of myoplasmic P_i

The recovery of the peak force response to caffeine following exposure to 50 mM P_i (cf. Fig. 3*A*) was plotted against the length of time that fibres were washed in P_i -free solution. The curve was fitted with a single exponential ($R^2 = 0{\cdot}97$). The half-time to recovery was 35 s. Points on the graph indicate the mean ± S.E.M. from 4–14 fibres.

The effect of anion channel and transporter blockers on the recovery of Ca^{2+} release

We next tested whether the pathway for P_i exit from the SR is either (i) a DIDS- or 9-AC-sensitive anion channel or (ii) some type of pore associated with the SR P_i transporter protein. The protocol used to determine the rate of P_i exit from the SR was modified such that fibres were washed for 30 s in a standard K-HDTA solution containing either 100 μM 9-AC or 100 μM DIDS. For experiments with PHPA, fibres were washed in a rigor solution containing 2 mM PHPA in order to prevent the interference of PHPA binding by ATP (Fryer *et al.* 1997).

Retardation of P_i exit from the SR by any of the drugs should have been manifested as an impairment in the rate of recovery of the caffeine response. None of the tested compounds had this effect (Table 1), In fact, the response to caffeine after washout of P_i in the presence of DIDS was actually greater than the force response obtained after washout under standard myoplasmic conditions ($P < 0{\cdot}01$), an effect which may be due to DIDS ability to increase the open probability of the ryanodine receptor/SR Ca^{2+} release channels (Oba *et al.* 1996). Overall, these results suggest that neither Cl^- channels nor the VDACs are primarily responsible for the passage of P_i from the SR lumen to the myoplasm. However, these data do not rule out the potential role of all types of Cl^- channel (some of which may not be modulated by myoplasmic ATP and/or which are not affected by these blockers) or other non-specific anion channels residing within the SR membrane.

DISCUSSION

The key findings of this study are: (1) myoplasmic P_i inhibits SR Ca^{2+} release in a concentration- and time-dependent manner, (2) the rate of onset of this inhibition is faster in the absence of myoplasmic ATP, and (3) recovery from P_i-induced inhibition has a $T_{½}$ of ~35 s, is ATP independent, and is dependent upon the $[P_i]$ gradient across the SR. These findings provide further insight into the nature of P_i movements across the SR and are relevant to the understanding of the time course of metabolic fatigue and recovery from fatigue in skeletal muscle.

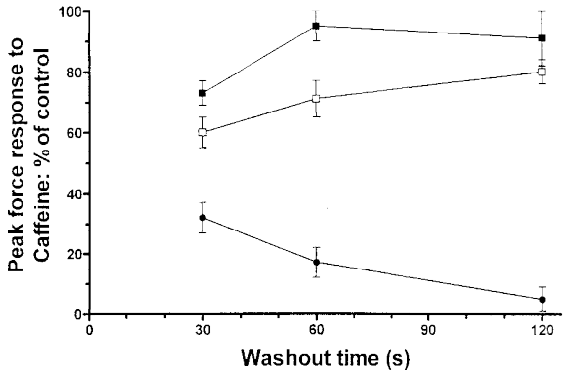

Figure 5. Recovery of SR Ca^{2+} release is not inhibited by myoplasmic ATP removal but is inhibited by the presence of myoplasmic P_i

The recovery of the peak force response to caffeine after prior inhibition by 50 mM P_i was plotted against the length of time fibres were washed (30 s to 2 min) under various myoplasmic conditions: □, presence of myoplasmic ATP, cf. Fig. 3*A*; ■, absence of myoplasmic ATP, cf. Fig. 3*B*; and ●, absence of myoplasmic ATP and presence of 30 mM myoplasmic P_i, cf. Fig. 3*C*. Note that the response to caffeine recovered to a similar extent if fibres were washed in either the presence or absence of ATP, but did not recover when 30 mM P_i was also present during the washout. Points represent mean data ± S.E.M. from 3–14 fibres.

Table 1. Recovery of the response to caffeine after 30 s washout of P_i under various myoplasmic conditions

Washout conditions	Mean response to caffeine (% of controls)
ATP	60 ± 5 (14)
No ATP	73 ± 4 (7)
No ATP + 30 mM P_i	32 ± 5 (4)****
No ATP + 2 mM PHPA	65 ± 3 (3)
ATP + 100 μM DIDS	79 ± 4 (4)***
ATP + 100 μM 9-AC	74 ± 7 (3)

The mean response to caffeine indicates the peak force response to caffeine after washout of P_i for 30 s (as detailed in Fig. 3) as a percentage of the control responses before and after P_i exposure. Washout conditions: ATP refers to a standard K-HDTA solution; No ATP refers to a rigor solution with or without various modifications. All solutions contained 125 μM total EGTA. Values represent the mean response ± S.E.M. with the sample size noted in parentheses. Asterisks represent a significant difference from the response to caffeine following washout under normal myoplasmic conditions (ATP): *** $P < 0{\cdot}01$; **** $P < 0{\cdot}001$.

Inhibition of SR Ca^{2+} release by myoplasmic P_i

A number of mechanisms could potentially account for the inhibitory effects of prior myoplasmic P_i elevation on SR Ca^{2+} release. These include: (i) direct effects of P_i on the myofilaments due to insufficient washout of P_i; (ii) decreased SR Ca^{2+} content due to P_i activation of SR Ca^{2+}-release channels or reduced activity/reversal of the SR Ca^{2+} ATPase pump; or (iii) movement of P_i into the SR with subsequent formation of Ca^{2+}–P_i precipitate.

A residual effect of myoplasmic P_i on the myofilaments is unlikely to account for the decline in caffeine response for several reasons. Firstly, this study has shown, and previous studies have calculated (Fryer *et al.* 1995) that most of the P_i can diffuse out of a 50 μm diameter fibre and into the bulk solution in < 1 s. Such a time course is incompatible with the complex concentration and time dependence seen over many minutes in the present study.

It has been previously shown that 10 mM P_i can increase the open probability of the skeletal muscle ryanodine receptor by ~2-fold (Fruen *et al.* 1994). In addition, it has been suggested that myoplasmic P_i can inhibit or even reverse the SR Ca^{2+}–ATPase pump (Barlogie *et al.* 1971; Zhu & Nosek, 1991; Xiang & Kentish, 1995; Steele *et al.* 1996). As P_i is present in the equilibration solutions in our experimental protocols, it is possible that the above actions of P_i would lead to a reduced SR Ca^{2+} content and hence caffeine response. Recovery of the caffeine response in such a situation would require a period of SR Ca^{2+} re-loading, a process that requires the presence of myoplasmic ATP. However, our results clearly demonstrated that the caffeine response could recover just as quickly in the absence of ATP as it did in its presence (Fig. 5), indicating that the releasable Ca^{2+} freed up during the recovery period was already present within the SR lumen.

Given the arguments above, the most likely mechanism to explain the inhibition of the caffeine response involves the movement of P_i from the myoplasm to the SR lumen with subsequent reduction of SR Ca^{2+} release once the Ca^{2+}–P_i solubility product is exceeded (Fryer *et al.* 1995). In these circumstances the rate of decline of the caffeine response should be limited by either the rate of P_i entry or by the subsequent rate of formation of Ca^{2+}–P_i complexes and precipitates.

P_i entry into the SR and Ca^{2+}–P_i precipitate formation

The results (Figs 1, and 2*A* and *B*) suggested that the process responsible for the decline in releasable Ca^{2+} after P_i exposure has the following properties: (i) an initial rapid phase, the rate of which is almost linearly related to myoplasmic $[P_i]$ in the 10 to 50 mM range; (ii) an even faster initial rate in the absence of myoplasmic ATP; and (iii) a complicated time dependence.

If the process described above is rate limited by P_i entry into the SR, then its properties should be compatible with known P_i channel and/or transport mechanisms. A P_i transporter that is dependent on myoplasmic Ca^{2+}, Mg^{2+} and ATP (Carley & Racker, 1982; Stefanova *et al.* 1991*b*) and largely blocked by phosphonic acid derivatives such as PHPA (Stefanova *et al.* 1991*a*) has been described in SR vesicles. In skinned fibres, Fryer *et al.* (1997) found that P_i entry into the SR could be partially blocked by PHPA (37 %); however, this was only found under conditions where the blocker was applied in the absence of both myoplasmic P_i and ATP, which presumably compete with PHPA at a binding site on the P_i transporter (Stefanova *et al.* 1991*a*). These previous results, plus the present observation showing an apparent stimulation of the rate of P_i entry in the absence of myoplasmic ATP (Fig. 2*B*) suggest that the ATP-dependent P_i transporter is not a main pathway for P_i entry into the SR of skinned fibres, implying that some other pathway is primarily responsible.

This leaves the possibility that P_i enters the SR passively through either ion channels or pores in the SR membrane. It is known that P_i can pass through certain SR Cl^- channels (Tanifuji *et al.* 1987; D. Laver, unpublished observations) as well as through the SR VDAC/porin pathway that has been previously characterized in mitochondria (Hodge & Colombini, 1997). However, given that P_i does not appear to pass through the 87 pS conductance Cl^- channel incorporated into artificial bilayers (Stefanova *et al.* 1991*b*), and that neither 9-AC nor DIDS had much effect on the rate of P_i exit from the SR (Table 1), we can only suggest that P_i fluxes in the present study occur through an as yet unknown pathway that is quite insensitive to DIDS and 9-AC.

One possible explanation for the results shown in Figs 1 and 2 is that the rate and extent of Ca^{2+}–P_i precipitate formation within the SR lumen is rate limiting for the decline in SR releasable Ca^{2+} after myoplasmic P_i exposure. If higher concentrations of myoplasmic P_i lead to faster P_i entry then the Ca^{2+}–P_i solubility product is attained more quickly and Ca^{2+} release is attenuated accordingly (Fig. 2*A*). Even though the rate of decline of SR Ca^{2+} release varied almost linearly with myoplasmic P_i in the 10 to 50 mM range, the maximum extent of inhibition saturated between 20 and 50 mM P_i, consistent with the previous suggestion that the SR [P_i] within mechanically skinned rat EDL fibres saturates at around 30–35 mM (Fryer *et al.* 1997). Saturation of the SR [P_i] places a limit on the total amount of lumenal [Ca^{2+}] that can be precipitated and thus the lowest level at which the releasable [Ca^{2+}] may be clamped (~20 % of control values in these fibres).

In order to more fully understand the complex time-dependent changes in releasable [Ca^{2+}] (Fig. 2*A*) as well as its modulation by myoplasmic [ATP], it is necessary to appreciate that the rate and extent of such a process is not only governed by the Ca^{2+}–P_i solubility product but also by the rates at which various Ca^{2+}–P_i precipitate species are formed and with which they are mutually interconverted (Walton *et al.* 1967; Beil *et al.* 1977). The final effect of precipitate formation on releasable Ca^{2+} will ultimately be determined by the proportion of stable (i.e. relatively insoluble) crystal species to metastable (i.e. relatively soluble) crystal species. Time-dependent transformations between such species may explain the complex time dependence of changes in releasable Ca^{2+} at longer P_i exposure times (Fig. 2*A*). It is also interesting and important to note that the rate of Ca^{2+}–P_i crystal formation is markedly inhibited in the presence of millimolar levels of Mg-ATP (Feher & Lipford, 1985), a phenomenon which may explain the results in Fig. 2*B* and also have implications for precipitate formation during the late stages of fatigue (further discussed in a separate section below). Feher & Lipford (1985) suggested a number of possible mechanisms including (i) prevention of crystal nucleation, (ii) prevention of crystal growth by adsorption to nuclei, or (iii) complexation of Mg^{2+} with P_i and Ca^{2+} with ATP to lower the effective concentration of precipitating anions. As precipitation occurs in the SR lumen, one way ATP may regulate this mechanism would be if the lumenal [ATP] falls as a consequence of ATP removal from the myoplasm, a process which could occur by ATP equilibration through VDACs in the SR membrane (Shoshan-Barmatz *et al.* 1996). Alternatively, the results in Fig. 2*B* could be explained if ATP simply blocked P_i entry into the SR through certain channels. One such channel could be the small Cl^- channel (conductance of 75 pS) found in the SR membrane of rabbit skeletal muscle which is inhibited by high myoplasmic [ATP] (Kourie, 1997). Preliminary experiments on these channels incorporated into bilayers show that they can conduct P_i (D. Laver, unpublished data). Irrespective of the exact mechanism of P_i entry and its possible modulation by ATP, it is known that Ca^{2+}–P_i precipitation is modulated by myoplasmic ATP, perhaps making this the ATP-sensitive step.

Recovery of P_i-inhibited SR Ca^{2+} release

The results suggested that the process responsible for the recovery of releasable Ca^{2+} after P_i exposure: (i) has a $T_{½}$ of ~35 s (Fig. 4), (ii) is active in the absence of myoplasmic ATP (Fig. 3*B*, Table 1), (iii) is inhibited by 30 mM myoplasmic P_i (Fig. 3*C*, Table 1), and (iv) is not blocked by either 9-AC, DIDS or PHPA (Table 1). As previously mentioned, the recovery of releasable Ca^{2+} in the absence of myoplasmic ATP indicates that the process involves a freeing up of calcium that is already available in the SR lumen rather than a re-accumulation of Ca^{2+} that has been lost from the SR. Thus, the recovery process must require solubilization of Ca^{2+}–P_i precipitate that has previously been formed in the SR lumen.

Ca^{2+}–P_i precipitate solubilization and P_i exit from the SR

Solubilization of Ca^{2+}–P_i precipitate can only occur if either lumenal [Ca^{2+}] or [P_i] drops such that the solubility product for crystal formation is no longer exceeded. The time-dependent recovery of the caffeine response in myoplasmic solutions that are P_i free implies that lumenal [Ca^{2+}] is actually increasing. Therefore, solubilization of Ca^{2+}–P_i precipitate in the present experiments is likely to result from the reduction of lumenal [P_i] due to its movement into the myoplasm. This contention is supported by the close agreement between our measured half-recovery time (35 s) and that seen for the rate of efflux of P_i from passively loaded SR vesicles (~30 s, Campbell & MacLennan, 1980). The present data suggest that P_i passively exits the SR down a concentration gradient because this process was substantially inhibited in the presence of 30 mM myoplasmic P_i (Figs 3*C* and 5, Table 1) and was not inhibited by the absence of myoplasmic ATP (Figs 3*B* and 5, Table 1). In fact, the recovery of releasable Ca^{2+} appeared to be slightly faster in the absence of myoplasmic ATP, a result which would appear to contradict the precipitate-stabilizing effect of low Mg-ATP discussed above. However, Feher & Lipford (1985) found that the re-introduction of Mg-ATP had little effect on Ca^{2+}–P_i crystals once they were already formed. The apparent speeding of recovery in the absence of myoplasmic ATP could potentially be explained by an increase in the maximum Ca^{2+}-activated force (5–10 % measured in two fibres, not shown) and to a small increase in the Ca^{2+} sensitivity of the contractile apparatus following rigor-induced force. Alternatively, the presence of myoplasmic ATP might impede the exit of P_i into the myoplasm after its passage through the SR membrane in the same way it might impede P_i diffusion into the SR.

The lack of effect of PHPA on the recovery time course suggests that the SR P_i transporter characterized by Stefanova *et al.* (1991*a*, *b*) is not responsible for trans-SR P_i

movement. Similarly, both 9-AC (100 μM) and DIDS (100 μM) failed to attenuate the rate of P_i exit, indicating that the pathway involved is not a 9-AC-sensitive Cl^- channel nor is it the VDAC/porin in the SR membrane. The reason for the significant speeding up of recovery in the presence of DIDS (Table 1) is not known, but could involve some type of residual effect of DIDS on either the SR Ca^{2+} release channel (Oba *et al.* 1996) or the contractile proteins.

Relevance to intact skeletal muscle function at rest and during fatigue

This study confirms and extends previous work (Fryer *et al.* 1995, 1997) showing that physiological levels of myoplasmic P_i can equilibrate across the SR and lead to alterations in SR Ca^{2+} handling. Typically, the myoplasmic $[P_i]$ estimated in resting mammalian fibres ranges from $\leqslant 1$ mM in fast-twitch fibres to ~6 mM in slow-twitch fibres (Kushmerick *et al.* 1992). Given that the free Ca^{2+} in the SR lumen is ~1 mM (Gonzalez-Serratos *et al.* 1978), and that the solubility product of Ca^{2+}–P_i is ~ 6 mM^2 (Fryer *et al.* 1995), then it is possible that slow-twitch fibres may already contain calcium in the form of Ca^{2+}–P_i precipitates. This may help explain why the SR Ca^{2+} content of slow-twitch fibres responds very differently to myoplasmic Ca^{2+} loads when compared with fast-twitch fibres (Fryer & Stephenson, 1996).

Myoplasmic $[P_i]$ and the onset of fatigue

Many skinned fibre studies have shown that myoplasmic P_i can depress force generation by inhibiting both the calcium sensitivity and the maximum force-generating capacity of the myofilaments (reviewed by Fitts, 1994). These effects of P_i mean that the changes in force observed during the onset and recovery from fatigue do not necessarily reflect corresponding changes in SR Ca^{2+} release. The validity of the Ca^{2+}–P_i precipitate hypothesis of fatigue can therefore only be discussed with reference to studies in which myoplasmic $[P_i]$ is elevated during the simultaneous measurement of both force and $[Ca^{2+}]_i$. For example, Westerblad & Allen (1996) found that microinjection of P_i to a myoplasmic concentration of 10–20 mM caused a large decrease in SR Ca^{2+} release within 4–12 min. Whilst no detailed analysis of the time course of onset of this effect was presented, they concluded that a Ca^{2+}–P_i precipitation mechanism was the only one compatible with the observed results. Nevertheless, a bolus injection of P_i from a micropipette would be expected to produce a very different $[P_i]$ transient to that seen during prolonged, intermittent muscle activity. During the early and intermediate stages of this type of fatigue the decline in force appears to be at least partly due to P_i effects on the contractile proteins, with SR Ca^{2+} release staying relatively unchanged until late in fatigue (Allen *et al.* 1995). Thus, Westerblad & Allen (1996) point out that Ca^{2+}–P_i precipitation is unlikely to explain the failure of SR Ca^{2+} release in late fatigue because this occurs long after P_i has been substantially elevated, and coincides more readily with the time when myoplasmic Mg^{2+} rises and [ATP] falls (Westerblad & Allen, 1992). However, it is possible to reconcile both observations as follows: in early and intermediate fatigue myoplasmic $[P_i]$ rapidly accumulates to ~10–20 mM and starts to equilibrate across the SR via an unknown pathway. Even though myoplasmic P_i is elevated there are a number of processes operating within intact muscle fibres which delay and inhibit any rapid formation of SR lumenal precipitate at this stage. Firstly, intact fibres contain other known P_i sinks (such as mitochondria) which could compete with the SR for P_i. Secondly, our data suggest that P_i equilibration will introduce a necessary delay between $[P_i]$ changes in the myoplasm and those in the SR, the duration of which will shorten as myoplasmic $[P_i]$ increases. Thirdly, the rate of formation of stable precipitate will be strongly inhibited by normal levels of MgATP (Feher & Lipford, 1985) and could potentially be inhibited by other myoplasmic factors such as phosphocreatine.

We propose that rapid formation of precipitate within the SR is more likely to occur in late fatigue because myoplasmic $[P_i]$ has had sufficient time to equilibrate across the SR. An attractive (though speculative) hypothesis based on our results (Fig. 2*B*) is that the rapid fall in SR Ca^{2+} release seen in late fatigue (Allen *et al.* 1995) coincides with a fall in local [ATP] within the SR lumen that greatly enhances the rate of formation of stable Ca^{2+}–P_i precipitate. Further experiments beyond the scope of the present study are clearly required to validate this proposal.

Myoplasmic $[P_i]$ and the recovery from fatigue

With regard to recovery from fatigue, many previous studies have generally found a good correlation between the rate of decline of myoplasmic $[P_i]$ and the rate of force recovery. Baker *et al.* (1993) noted a good correlation between the recovery of maximal voluntary contractions of human muscles and the decline in myoplasmic $[P_i]$ after short duration exercise, but found that force recovery lagged well behind $[P_i]$ after long duration exercise.

For short duration exercise, our results would predict a transient formation of Ca^{2+}–P_i precipitate, which will re-solubilize at a rate determined by P_i exit from the SR (Fig. 4) which itself depends on the myoplasmic $[P_i]$ (Fig. 5). The drop in myoplasmic P_i in this situation is probably determined by the rate of its incorporation into the organic phosphate pool, and might explain why a portion of recovery from fatigue is sensitive to the rate of glycogen re-synthesis (Chin & Allen, 1997). Thus, after short duration activity, there is generally a good correlation between recovery of $[P_i]$, force and SR Ca^{2+} release.

The dissociation between the time course of recovery of myoplasmic $[P_i]$ and force after longer duration exercise (Baker *et al.* 1993) means that P_i effects on the contractile proteins can be ruled out, suggesting the presence of a prolonged inhibition of SR Ca^{2+} release. This finding can be explained by the formation of greater proportions of more stable (less soluble) forms of Ca^{2+}–P_i crystal species over time (Walton *et al.* 1967). Such species would be expected to re-solubilize extremely slowly in response to a reduction of SR lumenal $[P_i]$, leading to an apparent dissociation between

[P_i] (myoplasmic and lumenal) and the recovery of SR Ca^{2+} release. This mechanism would also explain why the recovery of SR Ca^{2+} release following intracellular P_i injections was extremely slow (~1 h), despite indications that the myoplasmic [P_i] had returned to normal (Westerblad & Allen, 1996). In addition, the formation of highly insoluble Ca^{2+}–P_i species might explain the component of prolonged reduction of SR Ca^{2+} release which was found to be independent of glycogen re-synthesis (Chin & Allen, 1997).

Concluding remarks

This study has provided further insight into the mechanisms of P_i inhibition of SR Ca^{2+} release. We have shown that both P_i entry into and exit from the SR occurs largely by a passive mechanism that is insensitive to well-known anion channel blockers and SR P_i transport inhibitors. Such a mechanism must involve ion channels or pores; however, little is known regarding their identity. From our results we conclude that P_i inhibition of SR Ca^{2+} release is a complicated phenomenon influenced by the rate of P_i movement across the SR and the rate, extent and species of Ca^{2+}–P_i precipitate formation in the SR lumen. Interestingly, the more rapid inhibitory effect of P_i in the absence of myoplasmic ATP suggests that P_i may inhibit SR Ca^{2+} release more efficiently during the later stages of fatigue.

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

Baker, A. J., Kostov, K. G., Miller, R. G. & Weiner, M. W. (1993). Slow force recovery after long-duration exercise: metabolic and activation factors in muscle fatigue. *Journal of Applied Physiology* **74**, 2294–2300.

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 fibres of the rat and toad. *Journal of Muscle Research and Cell Motility* **17**, 55–68.

Barlogie, B., Hasselbach, W. & Makinose, M. (1971). Activation of calcium efflux by ADP and inorganic phosphate. *FEBS Letters* **12**, 265–268.

Beil, F. U., von Chak, D., Hasselbach, W. & Weber, H. H. (1977). Competition between oxalate and phosphate during active Ca^{2+} accumulation by sarcoplasmic vesicles. *Zeitschrift für Naturforschung* **32**, 281–287.

Cady, E. B., Jones, D. A., Lynn, J. & Newham, D. J. (1989). Changes in force and intracellular metabolites during fatigue of human skeletal muscles. *Journal of Physiology* **418**, 311–325.

Campbell, K. P. & MacLennan, D. H. (1980). DIDS inhibition of sarcoplasmic reticulum anion efflux and calcium transport. *Annals of the New York Academy of Sciences* **358**, 328–331.

Carley, W. W. & Racker, E. (1982). ATP-dependent phosphate transport in sarcoplasmic reticulum and reconstituted proteoliposomes. *Biochimica et Biophysica Acta* **680**, 187–193.

Chin, E. R. & Allen, D. G. (1997). Effects of reduced glycogen concentration on force, Ca^{2+} release and contractile protein function in intact mouse skeletal muscle. *Journal of Physiology* **498**, 17–29.

Feher, J. J. & Lipford, G. B. (1985). Calcium oxalate and calcium phosphate capacities of cardiac sarcoplasmic reticulum. *Biochimica et Biophysica Acta* **818**, 373–385.

Fitts, R. H. (1994). Cellular mechanisms of muscle fatigue. *Physiological Reviews* **74**, 49–93.

Fruen, B. R., Mickelson, J. R., Shomer, N. H., Roghair, T. J. & Louis, C. F. (1994). Regulation of the sarcoplasmic reticulum ryanodine receptor by inorganic phosphate. *Journal of Biological Chemistry* **269**, 192–198.

Fryer, M. W., Owen, V. J., Lamb, G. D. & Stephenson, D. G. (1995). Effects of creatine phosphate and P_i on Ca^{2+} movements and tension development in rat skinned skeletal muscle fibres. *Journal of Physiology* **482**, 123–140.

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.

Fryer, M. W., West, J. M. & Stephenson, D. G. (1997). Phosphate transport into the sarcoplasmic reticulum of skinned fibres from rat skeletal muscle. *Journal of Muscle Research and Cell Motility* **18**, 161–167.

Gonzalez-Serratos, H., Somlyo, A. V., McClellan, G., Shuman, H., Borrero, L. M. & Somlyo, A. P. (1978). Composition of vacuoles and sarcoplasmic reticulum in fatigued muscle: Electron probe analysis. *Proceedings of the National Academy of Sciences of the USA* **75**, 1329–1333.

Hodge, T. & Colombini, M. (1997). Regulation of metabolic flux through voltage-gating of VDAC channels. *Journal of Membrane Biology* **157**, 271–279.

Junankar, P. R., Dulhunty, A. F., Curtis, S. M., Pace, S. M. & Thinnes, F. P. (1995). Porin-Type 1 proteins in plasmalemmal and sarcoplasmic reticulum of striated muscle fibres. *Journal of Muscle Research and Cell Motility* **16**, 595–610.

Kasai, M. & Kometani, T. (1979). Inhibition of anion permeability of sarcoplasmic reticulum vesicles by 4-acetoamido-4′-isothiocyano-stilbene-2,2′-disulfonate. *Biochimica et Biophysica Acta* **557**, 243–247.

Kourie, J. I. (1997). ATP-sensitive voltage- and calcium-dependent chloride channels in sarcoplasmic reticulum vesicles from rabbit skeletal muscle. *Journal of Membrane Biology* **157**, 39–51.

Kourie, J. I., Laver, D. R., Junankar, P. R., Gage, P. W. & Dulhunty, A. F. (1996). Characteristics of two types of chloride channel in sarcoplasmic reticulum vesicles from rabbit skeletal muscle. *Biophysical Journal* **70**, 202–221.

Kushmerick, M. J., Moerland, T. S. & Wiseman, R. W. (1992). Mammalian skeletal muscle fibres distinguished by contents of phosphocreatine, ATP and P_i. *Proceedings of the National Academy of Sciences of the USA* **89**, 7521–7525.

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

Lamb, G. D., Stephenson, D. G. & Stienen, G. J. M. (1993). Effects of osmolality and ionic strength on the mechanism of Ca^{2+} release in skinned skeletal muscle fibres of the toad. *Journal of Physiology* **464**, 629–648.

Lewis, T. M., Roberts, M. L. & Bretag, A. H. (1994). Immunolabelling for VDAC, the mitochondrial voltage-dependent anion channel, on sarcoplasmic reticulum from amphibian skeletal muscle. *Neuroscience Letters* **181**, 83–86.

Oba, T., Koshita, M. & Van Helden, D. F. (1996). Modulation of skeletal muscle Ca^{2+} release channel gating by anion channel blockers. *American Journal of Physiology* **271**, C819–824.

Posterino, G. S. & Fryer, M. W. (1997). Time course of inorganic phosphate (P_i) removal from the sarcoplasmic reticulum of rat fast-twitch skeletal muscle. *Proceedings of the Australian Physiological and Pharmacological Society* **28**, 85P.

Posterino, G. S. & Lamb, G. D. (1996). Effects of reducing agents and oxidants on excitation-contraction coupling in skeletal muscle fibres of rat and toad. *Journal of Physiology* **496**, 809–825.

Shoshan-Barmatz, V., Hadad, N., Feng, W., Shafir, I., Orr, I., Varsanyi, M. & Heilmeyer, L. M. G. (1996). VDAC/porin is present in sarcoplasmic reticulum from skeletal muscle. *FEBS Letters* **386**, 205–210.

Steele, D. S., McAinsh, A. M & Smith, G. L. (1996). Comparative effects of inorganic phosphate and oxalate on uptake and release of Ca^{2+} from the sarcoplasmic reticulum in saponin skinned rat cardiac trabeculae. *Journal of Physiology* **490**, 565–576.

Stefanova, H. I., East, J. M. & Lee, A. G. (1991*a*). Covalent and non-covalent inhibitors of the phosphate transporter of sarcoplasmic reticulum. *Biochimica et Biophysica Acta* **1064**, 321–328.

Stefanova, H. I., Jane, S. T., East, J. M. & Lee, A. G. (1991*b*). Effects of Mg^{2+} and ATP on the phosphate transporter of sarcoplasmic reticulum. *Biochimica et Biophysica Acta* **1064**, 329–334.

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.

Tanifuji, M., Sokabe, M. & Kasai, M. (1987). An anion channel of sarcoplasmic reticulum incorporated into planar lipid bilayers: Single-channel behavior and conductance properties. *Journal of Membrane Biology* **99**, 103–111.

Walton, A. G., Bodin, W. J., Furedi, H. & Schwartz, A. (1967). Nucleation of calcium phosphate from solution. *Canadian Journal of Chemistry* **45**, 2695–2701.

Westerblad, H. & Allen, D. G. (1992). Myoplasmic free Mg^{2+} concentration during repetitive stimulation of single fibres from mouse skeletal muscle. *Journal of Physiology* **453**, 413–434.

Westerblad, H. & Allen, D. G. (1996). The effects of intracellular injections of phosphate on intracellular calcium and force in single fibres of mouse skeletal muscle. *Pflügers Archiv* **431**, 964–970.

Xiang, J.-Z. & Kentish, J. C. (1995). Effects of inorganic phosphate and ADP on calcium handling by the sarcoplasmic reticulum in rat skinned cardiac muscles. *Cardiovascular Research* **29**, 319–400.

Zhu, Y. & Nosek, T. M. (1991). Intracellular milieu changes associated with hypoxia impair sarcoplasmic reticulum Ca^{2+} transport in cardiac muscle. *American Journal of Physiology* **261**, H620–626.

Acknowledgements

This work was supported by the National Health and Medical Research Council of Australia.

Corresponding author

M. W. Fryer: School of Physiology and Pharmacology, University of New South Wales, Sydney 2052, Australia.

Email: m.fryer@unsw.edu.au