Effects of creatine phosphate and P_i on Ca^{2+} movements and tension development in rat skinned skeletal muscle fibres

Martin W. Fryer*, Virginia J. Owen, Graham D. Lamb and D. George Stephenson

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

1. Mechanically skinned fast-twitch (FT) and slow-twitch (ST) muscle fibres of the rat were used to investigate the effects of fatigue-like changes in creatine phosphate (CP) and inorganic phosphate (P_i) concentration on Ca^{2+}-activation properties of the myofilaments as well as Ca^{2+} movements into and out of the sarcoplasmic reticulum (SR).

2. Decreasing CP from 50 mM to zero in FT fibres increased maximum Ca^{2+}-activated tension (T_{max}) by 16 ± 2% and shifted the mid-point of the tension–pCa relation (pCa_{50}) to the left by 0·28 ± 0·03 pCa units. In ST fibres, a decrease of CP from 25 mM to zero increased T_{max} by 9 ± 1% and increased the pCa_{50} by 0·16 ± 0·01 pCa units. The effect of CP on T_{max} was suppressed in both fibre types by prior treatment with 0·3 mM FDNB (1-fluoro-2,4-dinitrobenzene), suggesting that these effects may occur via changes in creatine kinase activity.

3. Increases of P_i in the range 0–50 mM reduced the pCa_{50} and T_{max} in both fibre types. These effects were more pronounced in ST fibres than in FT fibres in absolute terms. However, normalization of the results to resting P_i levels appropriate to both fibre types (1 mM for FT and 5 mM for ST fibres) revealed similar decreases in T_{max} (~39% at 25 mM P_i and ~48% at 50 mM P_i) and pCa_{50} (0·25 pCa units at 25–50 mM P_i). The depressant action of P_i on both parameters was considerably reduced when the rise in P_i was accompanied by an equivalent reduction in [CP].

4. Tension development in the presence of complex, fatigue-like milieu changes (40 mM P_i for FT; 20 mM P_i for ST) was decreased by 35–40% at a constant myoplasmic $[Ca^{2+}]$ of 6 μM in both fibre types.

5. SR Ca^{2+} loading at a myoplasmic $[Ca^{2+}]$ of 100 nM was found to increase abruptly when the $[P_i]$ during loading was increased to near 9 mM. At a myoplasmic $[Ca^{2+}]$ of 300 nM, the threshold P_i for this effect dropped to approximately 3 mM.

6. Tension responses evoked by caffeine in the absence of P_i were smaller and slower to peak if fibres were exposed to P_i in a restricted myoplasmic Ca^{2+} pool after SR Ca^{2+} loading. This indicated that myoplasmic P_i can decrease and prolong the rate of Ca^{2+} release from the SR.

7. The apparently opposing effects of P_i on SR Ca^{2+} uptake and release can be reconciled with a simple model in which calcium phosphate precipitation within the SR lumen reduces both the magnitude and the rate of Ca^{2+} release from the SR during a brief period of activation.

The dominant metabolic change that occurs during fatigue in skeletal muscle involves the breakdown of creatine phosphate (CP) to creatine and inorganic phosphate (P_i) via the creatine kinase (CK) and myokinase reactions (Carlson & Wilkie, 1974). Relative changes in the concentrations of these metabolites during fatigue could conceivably decrease tension generation by affecting the contractile proteins and/or the sarcoplasmic reticulum (SR).

* Present address: School of Physiology and Pharmacology, University of New South Wales, PO Box 1, Kensington, NSW 2052, Australia.

Phenotypic differences in myofibrillar ATPase activity and resting metabolite contents between fast-twitch (FT) and slow-twitch (ST) skeletal muscle fibres are probably responsible for the difference in susceptibility to fatigue. A number of studies have shown that resting ST fibres contain less CP (15–25 mM *vs.* 30–50 mM) but more P_i (5–10 mM *vs.* 1–3 mM) than FT fibres (Edström, Hultman, Sahlin & Sjoholm, 1982; Meyer, Brown & Kushmerick, 1985; Kushmerick, Moerland & Wiseman, 1992). As the CP store approaches depletion during fatiguing stimulation the $[P_i]$ in ST and FT fibres (Dawson, Gadian & Wilkie, 1980) could approach 25 and 50 mM, respectively. Some previous studies have looked at comparative effects of P_i on contractile protein function in ST and FT skeletal muscle fibres (Altringham & Johnson, 1985; Nosek, Leal-Cardoso, McLaughlin & Godt, 1990; Millar & Homsher, 1992). However, none of these studies systematically investigated the effects of P_i and CP on both maximum Ca^{2+}-activated tension (T_{max}) and myofilament Ca^{2+} sensitivity over the entire putative physiological range. In addition, these studies failed to relate the reported inhibitory effects of P_i to the differences in resting P_i described above.

A second important component of tension reduction during fatigue is the diminished release of Ca^{2+} from the sarcoplasmic reticulum (SR) during tetanic stimulation (Westerblad & Allen, 1991). One suggested explanation for this effect is a decrease in SR Ca^{2+} content mediated by a metabolite-induced reduction in SR Ca^{2+} pump effectiveness (Westerblad, Lee, Lännergren & Allen, 1991). Here we consider an alternative explanation: that during fatigue the $[P_i]$ in the SR lumen increases to a point such that calcium phosphate precipitate is formed (Hasselbach, 1964), thus reducing the free $[Ca^{2+}]$ in the SR lumen available for release (Owen, Lamb & Stephenson, 1992*a*, *b*).

In the present study we have used mechanically skinned fibres from rat FT and ST skeletal muscles to answer two questions relevant to muscle fatigue. (1) Do the contractile proteins of FT and ST fibres have different sensitivities to CP and P_i changes? (2) Are the effects of P_i on SR Ca^{2+} movements consistent with the decreased SR Ca^{2+} release seen during fatigue?

METHODS

Skinned fibres

Details of the experimental apparatus and procedures have been previously described (Stephenson & Williams, 1981; Fink, Stephenson & Williams, 1986). Briefly, 12- to 15-week-old male Long–Evans hooded rats (*Rattus norvegicus*) were killed by diethyl ether overdose. The soleus and extensor digitorum longus (EDL) muscles were rapidly excised, thoroughly blotted and placed under paraffin oil on a bed of Sylgard 184 (Dow Chemicals, Midland, MI, USA). Single fibres from each muscle were mechanically skinned and then attached to a piezo-resistive tension transducer (Akjeselkapet AE 875, Horten, Norway) for the measurement of isometric tension, which was displayed on a chart recorder. The average sarcomere length was adjusted with a micromanipulator to 2·67 μm as judged by the first-order diffraction maxima produced by a He–Ne laser beam (Spectra Physics 136-04, Melbourne, Australia) crossing the preparation.

In all experiments the skinned muscle fibre was allowed to equilibrate for at least 5 min in a relaxing solution (type A1, Table 1) before any Ca^{2+} activations were performed. In some experiments the fibres were treated with 3% Triton X-100 (v/v) in relaxing solution for 10 min in order to destroy SR function. There was no significant difference in activation properties between treated and non-treated fibres except in solutions containing concentrations of P_i exceeding 10–15 mM, which increase SR Ca^{2+} loading by precipitating Ca^{2+} in the SR lumen (see Results).

Solutions

Details of solution preparation have been presented elsewhere (Fink *et al.* 1986). Three main types of solutions were used (Table 1): high relaxing (type 1), activating (type 2) and low relaxing (type 3). For EGTA-based solutions (A1–D2), type 1 and type 2 solutions were mixed to obtain $pCa \geqslant 4{\cdot}7$ ($pCa = -\log[Ca^{2+}]$). For $pCa \leqslant 4{\cdot}7$ small amounts of $CaCl_2$ ($< 0{\cdot}5$ mM) were added to type 2 solution. The Ca^{2+}-binding ability of all potential ligands was taken into account when calculating the appropriate mixture to achieve a given pCa. Nevertheless, to ensure the accuracy of the Ca^{2+} concentrations in the solutions, the amount of excess EGTA was titrated with $CaCl_2$ using the method of Moisescu & Thieleczek (1978). The pCa of some solutions was also checked using a Ca^{2+}-sensitive electrode. For NTA-based solutions (E1–F2), type 1 and 2 solutions were used to obtain $pCa \geqslant 4$. The apparent affinity constants (K_{app}) of the various cations to ligands in solution were obtained from Sillèn & Martell (1964) and Smith & Martell (1976), or have been previously measured in our laboratory (K_{app} Ca^{2+} to EGTA; K_{app} Mg^{2+} to EGTA and ATP (Fink *et al.* 1986)).

Solutions of [CP] and $[P_i]$ between 0 and 50 mM were obtained by mixing A1–3 solutions with C1–3 and D1–2 solutions, respectively. HDTA was substituted for both CP (in the ratio 1 : 1) and P_i (in the ratio 1 : 1·3). Taking into consideration all species of P_i in our solutions, 50 mM P_i will contribute to the concentration of ionic equivalents, I, and to the osmolality of the solutions by the same amount as 36 mM HDTA and 40·5 mM HDTA, respectively. Therefore, substitution of HDTA for P_i in a ratio of 1 : 1·3 would cause minimal changes to either I or osmolality. Caffeine (30 mM) dissolved in a mixture of B3 and B1 (yielding 0·125–0·50 mM EGTA) was used to evoke SR Ca^{2+} release in some experiments.

In CP solutions Na_2CP was used instead of the more labile K_2CP. Consequently, the control $[Na^+]$ routinely used in our solutions (A, C–F) was 132 mM (100 mM added as Na_2CP and 32 mM as Na_2ATP). These solutions increased the steepness of the tension–pCa relation in soleus fibres and shifted the relation to the right in both muscle types when compared with solutions in which only 32 mM Na^+ and 137 mM K^+ were present (B1–3; see Results). In a number of experiments, exogenous creatine phosphokinase (CK; 15 U ml^{-1}) was added to the solutions. Both the maximum Ca^{2+}-activated tension (T_{max}) and the myofilament Ca^{2+} sensitivity were unchanged by CK addition over the whole range of [CP] used ($P > 0{\cdot}1$, paired t test; 6 FT and 6 ST fibres), indicating that endogenous

Table 1. Composition of solutions (mM)

Solution	CaEGTA	EGTA	CaNTA	NTA	P_i*	CP	HDTA	Na^+
A1	—	10	—	—	—	—	50	132
A2	9·9	0·1	—	—	—	—	50	132
A3	—	0·05	—	—	—	—	60	132
B1	—	10	—	—	—	—	50	32
B2	9·9	0·1	—	—	—	—	50	32
B3	—	0·05	—	—	—	—	60	32
C1	—	10	—	—	—	50	—	132
C2	9·9	0·1	—	—	—	50	—	132
C3	—	0·05	—	—	—	50	10	132
D1	—	10	—	—	50	—	11·5	132
D2	9·9	0·1	—	—	50	—	11·5	132
E1	—	—	—	15	—	—	50	132
E2	—	—	4·5	10·5	—	—	50	132
F1	—	—	—	15	50	—	11·5	132
F2	—	—	4·8	10·2	50	—	11·5	132

All solutions were adjusted to pH 7·10 at 23 ± 1 °C and contained (mM): MgATP, 13·8; Mg^{2+} (free), 1; Hepes, 60; NaN_3, 1; $Na^+ + K^+$ (total), 170. *Solutions had no added phosphate unless otherwise stated. Contaminating [P_i] in CP-based solutions (C1–3) was estimated to be approximately 0·2 mM (see calculation in text). NTA, nitrilotriacetate.

CK activity did not need supplementation. In some experiments FDNB (1-fluoro-2,4-dinitrobenzene) was added to the relaxing solution (0·3 mM) using dimethylsulphoxide (DMSO) as the vehicle.

Solutions mimicking resting and fatigued myoplasm

In some experiments, the solution constituents were altered to mimic the myoplasmic composition of resting and fatigued FT and ST muscle fibres (Table 2). Values for resting concentrations of CP, P_i, creatine and ATP were derived from the literature describing these levels in FT and ST mammalian skeletal muscle fibres (Edström *et al.* 1982; Kushmerick *et al.* 1992). In fatigue solutions CP was completely replaced in equimolar amounts by both P_i and creatine. Polyvinylpyrrolidone (PVP; 4%) was present in resting solutions to maintain the same filament lattice as in intact fibres (Matsubara, Goldman & Simmons, 1984). In fatigue solutions no PVP was present because it is known that the volume of fatigued fibres is about 80% greater than at rest (Gonzales-Serratos, Somlyo, McClellan, Shuman, Borrero & Somlyo, 1978). Fibre diameter in fatigue solutions relative to that in rest solutions was increased by 28 ± 2% in FT fibres ($n = 4$) and 24 ± 2% in ST fibres ($n = 5$). These values correspond to increases in cross-sectional area (volume) of 64 ± 4 and 54 ± 4%, respectively. The observed changes in the cross-sectional area are not likely to cause a significant change in T_{max} since a similar expansion of the fibre in solutions of similar composition to that mimicking fatigued myoplasm did not lead to a significant change in T_{max} (Lamb, Stephenson & Stienen, 1993). However, the expansion of the fibre volume in the above range caused a small reduction in the sensitivity to Ca^{2+} (pCa_{50}) was shifted to the right by 0·13 pCa units; Lamb *et al.* 1993).

Table 2. Composition of solutions mimicking resting and fatigued myoplasm (mM)

	Fast-twitch						Slow-twitch					
	Rest			Fatigue			Rest			Fatigue		
	I	II	III	I	II	III	I	II	III	I	II	III
EGTA	25	25	—	25	25	—	25	25	—	25	25	—
CaEGTA	—	24	—	—	24	—	—	24	—	—	24	—
HDTA	25	25	50	25	25	50	25	25	50	25	25	50
CP	40	40	40	—	—	—	20	20	20	—	—	—
Creatine	19	19	19	59	59	59	16	16	16	36	36	36
P_i*	1	1	1	40	40	40	5	5	5	20	20	20
ATP	9	9	9	9	9	9	6	6	6	6	6	6
PVP	4%	4%	4%	—	—	—	4%	4%	4%	—	—	—

Type I solutions, high relaxing (pCa > 9); Type II solutions, activating; Type III, low relaxing. All solutions contained (mM): Mg^{2+} (free), 1; ADP, 1; Hepes, 60; Na^+, 100; and K^+, 67. The pH of all solutions was adjusted to 7·10. * P_i added in the Na_2HPO_4 form. PVP, polyvinylpyrrolidone (MW, 40000).

Measurement of P_i

Solutions with no added P_i were tested for P_i contamination as a result of CP and ATP hydrolysis prior to or during the experiment. P_i was measured using a modification of the molybdate technique described by Chifflet, Torriglia, Chiesa & Tolosa (1988). The 10 μl sample volume (with or without added P_i standard) was diluted to 100 μl with double-distilled water. The molybdate reaction was initiated by addition of 100 μl of a 1:1 (v/v) solution of freshly prepared 1% ammonium molybdate–6% ascorbic acid in 1 M HCl. After 5 min at 23 °C the reaction was quenched with 150 μl of 2% sodium citrate, 2% sodium meta-arsenite and 2% acetic acid in double-distilled water. The reaction mixture was left at 23 °C for a further 30 min, after which 2 ml of double-distilled water was added and the absorbance at 850 nm read in a spectrophotometer. In solutions containing 16 mM ATP but no added CP, this method yielded a $[P_i] \approx 0{\cdot}1$ mM. For CP-containing solutions, the highly acidic reaction conditions necessary for the colour reaction caused rapid hydrolysis of the CP, causing the release of P_i. For these solutions the molybdate reaction was quenched after 8, 14 and 20 s and the P_i determined at each time interval relative to internal standards. The time course of P_i release over the 20 s period was well described by a single exponential function ($r^2 > 0{\cdot}99$). Back extrapolation to time zero yielded $[P_i]$ of 0·15–0·20 mM (fresh solutions) and 0·20–0·30 mM (at end of an experiment) on solutions containing 50 mM CP.

A final source of P_i to consider is the local increase as a result of myofibrillar ATPase activity. Using the maximum myofibrillar ATPase rates of 0·13 and 0·52 mM s^{-1} at optimum filament overlap for soleus and EDL fibres under similar conditions (Stewart, Stephenson & Wilson, 1987) and a diffusion coefficient for P_i in frog muscle of $3{\cdot}3 \times 10^6$ cm^2 s^{-1} (Yoshizaki, Seo, Nishikawa & Morimoto, 1982) we estimate that the average $[P_i]$ increases by 0·05 and 0·2 mM, respectively, in a 60 μm diameter fibre maximally activated at 23 °C.

Based on the calculations and measurements presented above we estimate the average $[P_i]$ in ST and FT fibres at maximal activation to be ~0·15 and 0·30 mM, respectively, for solutions containing 16 mM ATP and no added P_i and ~0·25 and 0·4 mM for solutions containing an additional 50 mM CP.

Data collection and analysis

Tension–pCa curves were determined for each fibre by sequential activation in a series of solutions of increasing $[Ca^{2+}]$. The steady-state tension developed in each solution was expressed as a percentage of the maximum tension developed in the sequence. The data were corrected for any small ($ca < 10\%$ from start to finish), use-dependent decline in the control level of maximum tension. Fibres exhibiting a steeper decline in maximum tension were discarded unless otherwise noted. Tension–pCa data were fitted by a form of the Hill equation using NFIT software (Island Products, University of Texas, Galveston, TX, USA):

$$\text{Relative tension (\%)} = 100/(1 + ([Ca_{50}]/[Ca^{2+}])^n), \quad (1)$$

where n is the Hill coefficient for Ca^{2+} and $[Ca_{50}]$ is the calcium concentration required for half-maximal tension activation. Fits were chosen by the least-squares method. Changes in calcium sensitivity between test runs and controls were always evaluated within the same fibre.

All soleus fibres used displayed tension oscillations (~0·2 Hz) of myofibrillar origin during submaximal activation in solutions of type A, B and C and gave near maximal tension responses in a type B1 solution of pSr 5·5 ($-\log[Sr^{2+}]$), which are typical characteristics of ST muscle fibres (Fink *et al.* 1986).

All results are expressed as means ± S.E.M. of n observations. Statistical significance was determined at the 95% confidence level using Student's t test for either paired or unpaired samples.

RESULTS

Effects of creatine phosphate on maximum Ca^{2+}-activated tension

Maximum Ca^{2+}-activated tension (T_{max}) was measured in solutions containing CP ranging from 0 to 50 mM. Results of these experiments are shown in Fig. 1*A*. Decreasing CP from 50 mM to zero in FT fibres (simulating complete depletion of the myoplasmic CP store) significantly increased T_{max} (16 ± 2%, $n = 6$). Since ST fibres contain about half as much total CP as FT fibres (Edström *et al.* 1982) the [CP] can only vary between about 25 mM and zero. Decreasing CP in this range significantly increased T_{max} (9 ± 1%, $n = 7$) in ST fibres.

Higher [CP] could have conceivably decreased T_{max} via P_i contamination. However, the P_i content of the CP batches used here was measured as 0·4%, which amounts to 0·2 mM P_i in 50 mM CP, a concentration that has little effect on T_{max} in both muscle types (see Fig. 3).

Another possibility is that changes in CP affect T_{max} by modifying the ability of creatine kinase (CK) to buffer myofibrillar ADP. This idea was tested by comparing CP effects on T_{max} before and after inhibition of CK by 0·3 mM 1-fluoro-2,4-dinitrobenzene (FDNB; Kentish, 1986). Representative results from single FT and ST fibres are shown in Fig. 1*B* and *C*, respectively. Prior to FDNB exposure, the T_{max} ratio (0:50 mM CP) was significantly greater than 1 (FT: 1·13 ± 0·01, $n = 3$; ST: 1·25 ± 0·05, $n = 4$; $P < 0{\cdot}01$) indicating the depressant action of CP on T_{max}. The most dramatic effect of exposure to FDNB (0·3 mM) was a rapid decrease in the T_{max} recorded in zero CP solution to around 60% of its original level in FT fibres and to around 80% in ST fibres. After 10 min of FDNB the T_{max} ratio (0:50 mM CP) was very close to 1 in FT fibres (0·98 ± 0·01, $n = 3$) and slightly less than 1 in ST fibres (0·89 ± 0·04, $n = 4$), indicating the abolition or even slight reversal of CP effects on T_{max}. Control experiments using the DMSO vehicle alone (0·1%) had no effect on T_{max}.

Creatine phosphate effects on the tension–pCa relation

Changes of [CP] in the physiological range were also found to alter the Ca^{2+} sensitivity of the contractile proteins in both FT and ST fibres. Representative results from a single

fibre of each type are shown in Fig. 2 while grouped results are summarized in Table 3. Increasing CP from 0 to 25 mM caused a relatively large and significant shift of the tension–pCa relation to the right, indicating a decrease in the Ca^{2+} sensitivity of tension generation. In six FT and six ST fibres the pCa_{50} shifts between 0, 25 and 50 mM CP were determined within the same fibre. An increase in CP from 0 to 25 mM decreased the pCa_{50} by $0{\cdot}18 \pm 0{\cdot}03$ and $0{\cdot}14 \pm 0{\cdot}01$ pCa units for FT and ST fibres, respectively. A doubling of the CP from 25 to 50 mM had a smaller effect, decreasing the pCa_{50} by a further 0·07 and 0·08 pCa units respectively. These CP-induced changes in Ca^{2+} sensitivity were not significantly different in the two muscle types and were similar in Triton X-100-treated and -untreated preparations. P_i contamination of the CP batch (0·4%) could account for only 0·03 units shift in pCa_{50} in ST fibres at 50 mM CP and would have had no effect on FT fibres (see P_i results in Table 4).

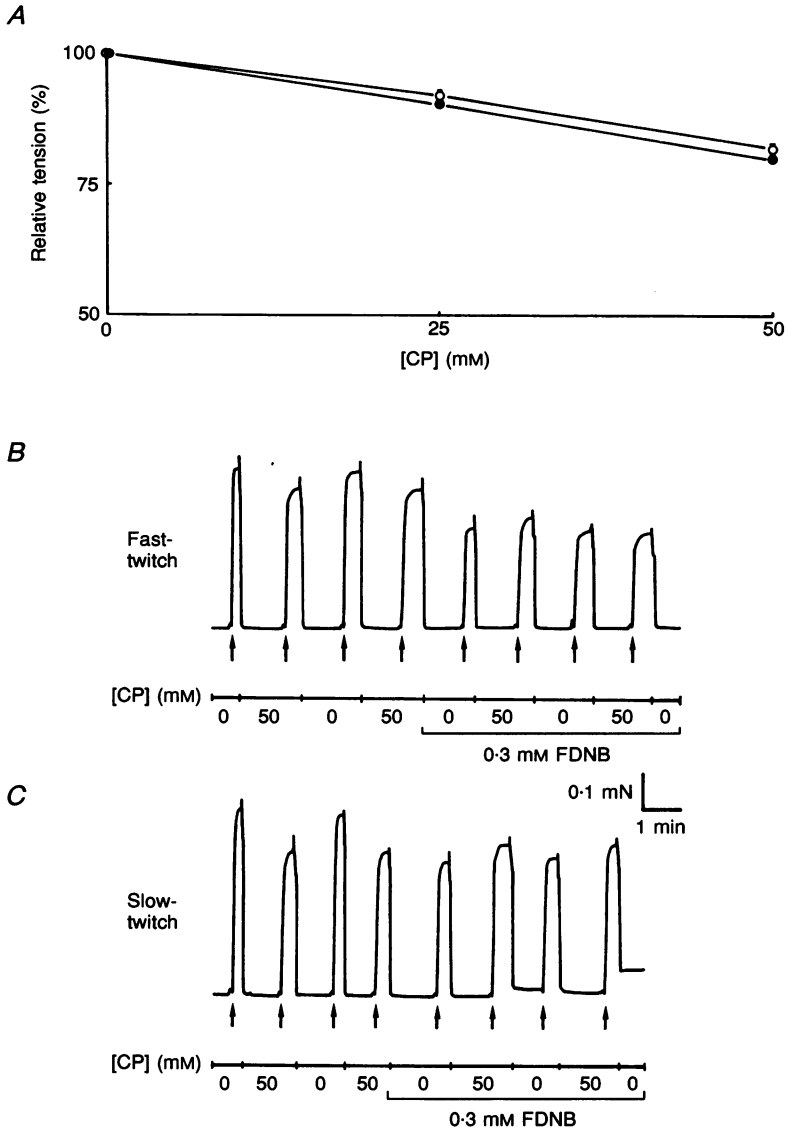

Figure 1. Effects of creatine phosphate on maximum Ca^{2+}-activated tension (T_{max}) in skinned FT and ST skeletal muscle fibres

○, FT fibres; ●, ST fibres. Points in *A* represent the means ± S.E.M. from 6–7 fibres. Error bars are omitted when smaller than the symbol size. Data are normalized to the T_{max} recorded in the absence of CP. *B* and *C*, abolition of CP effects on T_{max} by FDNB in a single FT fibre (*B*) and a single ST fibre (*C*). Fibres were equilibrated in relaxing solution containing either 0 or 50 mM CP prior to exposure to maximum activation solutions (arrows). Fibre dimensions (length, diameter): *B*, 2·2 mm, 50 μm; *C*, 2·1 mm, 43 μm.

Table 3. Effect of creatine phosphate (CP) on Ca^{2+} sensitivity (pCa_{50})

[CP] (mM)	Fast-twitch	Slow-twitch
0	5·89 ± 0·04 (10)	6·11 ± 0·02 (9)
25	5·65 ± 0·03* (6)	5·95 ± 0·01* (6)
50	5·61 ± 0·03* (14)	5·88 ± 0·02* (13)

Means ± S.E.M. (number of fibres). * Significantly different from 0 mM CP ($P < 0·001$).

It should be noted that the results above were obtained in solutions containing high Na^+ concentrations (132 mM) in order to balance the Na^+ contained in the Na_2CP. The main effect of this higher Na^+ was a decrease in Ca^{2+} sensitivity, as was previously reported (Fink *et al.* 1986). Thus, in more conventional K^+-based solutions (containing 32 mM Na^+) the control pCa_{50} values for FT and ST fibres were significantly higher (5·99 ± 0·02 ($n = 6$) and 6·32 ± 0·02 ($n = 6$), respectively).

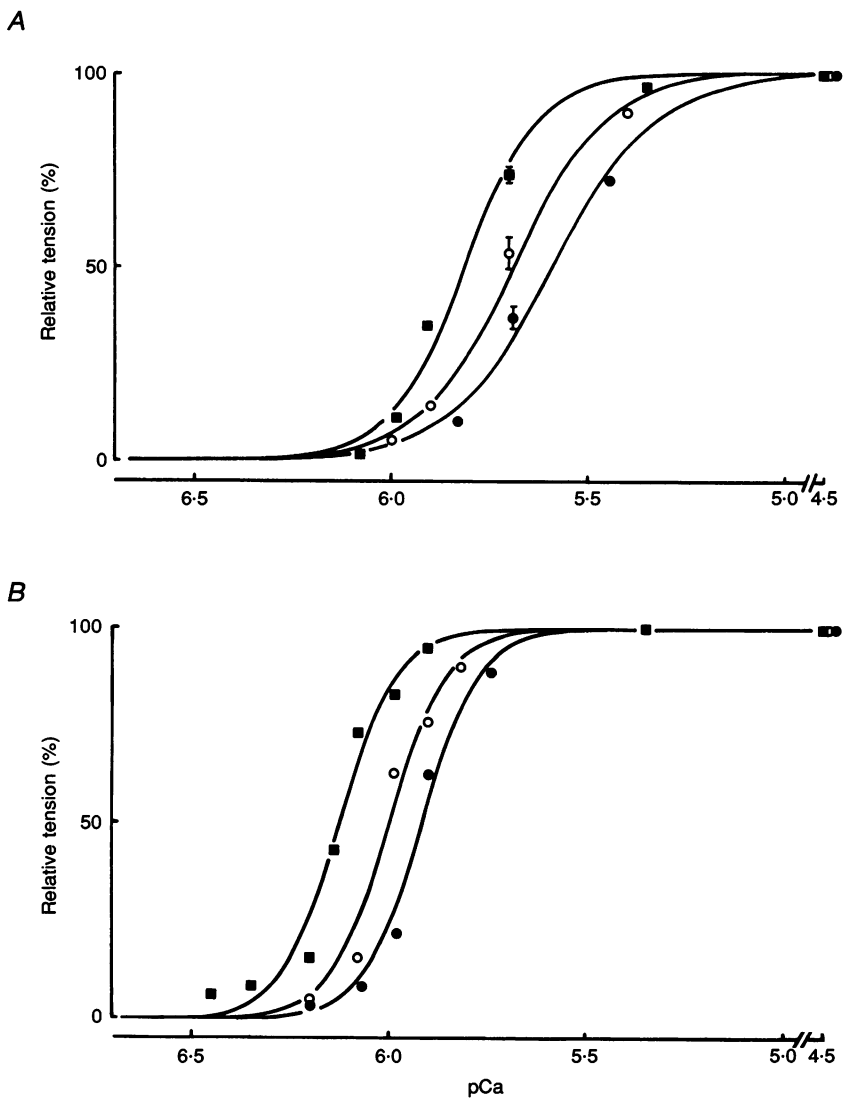

Figure 2. Effects of creatine phosphate on the steady-state tension–pCa relationship of a single FT (*A*) and a single ST (*B*) fibre

■, 0 mM CP; ○, 25 mM CP; ●, 50 mM CP. Solution types used, A and C. Sigmoidal curves represent the least-squares fit of the data to eqn (1) in the Methods. Vertical bars show the range of results when two to three activations were performed. Fibre dimensions (length, diameter): *A*, 2 mm, 50 μm; *B*, 1 mm, 35 μm. Sarcomere length, 2·67 μm.

Table 4. Effects of P_i on Ca^{2+} sensitivity (pCa_{50})

$[P_i]$ (mM)	Fast-twitch	Slow-twitch
0·15–0·30†	5·89 ± 0·04 (10)	6·11 ± 0·02 (9)
1	5·88 ± 0·02 (11)	6·03 ± 0·01* (6)
5	5·82 ± 0·01* (5)	5·88 ± 0·01* (6)
5 (NTA)	5·82 ± 0·01* (2)	5·86 ± 0·01* (3)
25	5·61 ± 0·01* (3)	5·57 ± 0·04* (4)
25 (NTA)	5·65 ± 0·02* (2)	5·65 ± 0·01* (3)
50	5·58 ± 0·08* (3)	5·54 ± 0·11* (3)
50 (NTA)	5·61 ± 0·01* (3)	5·61 ± 0·01* (5)

Means ± S.E.M. (number of fibres). $[Ca^{2+}]$ was buffered with EGTA unless otherwise stated. All fibres were pretreated with Triton X-100 to destroy intracellular compartments. †Calculated upper limit for mean $[P_i]$ in fibre volume during a maximum activation (fast-twitch, 0·30 mM; slow-twitch, 0·15 mM; based on ATPase rates – see Methods). *Significantly different from 0·15–0·30 mM P_i ($P < 0·05$).

Effects of P_i on maximum Ca^{2+}-activated tension

Figure 3 shows that when P_i was increased from control conditions (0·15 mM for ST fibres; 0·30 mM for FT fibres) to as little as 1 mM there were already significant decreases in T_{max} for both muscle types. As $[P_i]$ further increased there was a concentration-dependent decrease in T_{max} that was much greater than that seen with CP.

The T_{max} of ST fibres appeared to be more sensitive to P_i than that of FT fibres at all concentrations tested. However, if the mean T_{max} results from Fig. 3 were normalized to the resting P_i levels appropriate for each muscle type (i.e. 1 mM for FT and 5 mM for ST), then both muscles displayed similar percentage decreases in T_{max} (25 mM P_i: FT, 39·1%; ST, 38·2%; 50 mM P_i: FT, 48·8%; ST, 48·2%).

Effects of P_i on the tension–pCa relation

In preliminary experiments it was found that P_i had profoundly different effects on the tension–pCa relation if the SR was kept intact instead of being destroyed. Thus, when the SR was intact (Fig. 4*B*) a FT fibre was unable to generate any tension for over 2 min while immersed in a

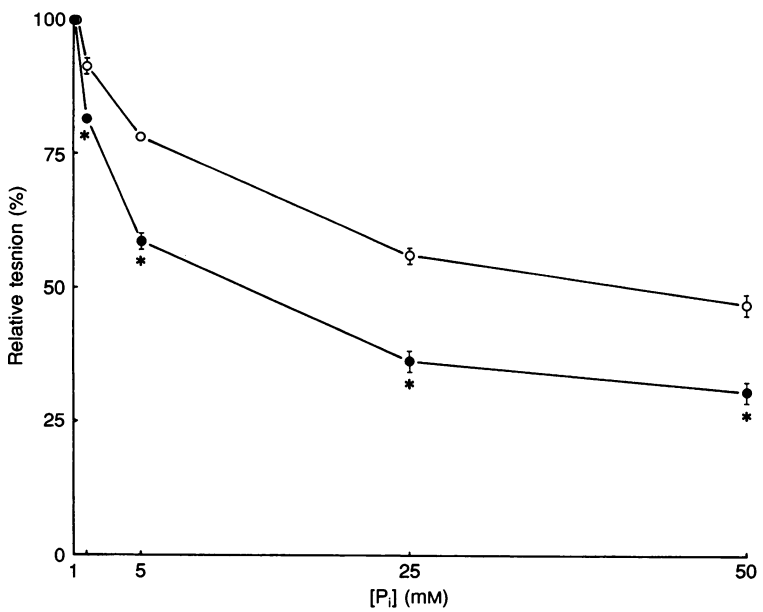

Figure 3. Effects of P_i on maximum Ca^{2+}-activated tension (T_{max}) in skinned FT and ST skeletal muscle fibres

○, FT fibres; ●, ST fibres. Points represent the means ± S.E.M. from 3–10 fibres. Solution types used A and D. Error bars are omitted when smaller than the symbol size. Data are normalized to the T_{max} recorded in the absence of added P_i. *Significant difference between FT and ST fibres ($P < 0·01$).

50 mM P_i solution at pCa 5·5. The absence of tension development was not due to the effect of P_i on the myofilaments because the same fibre rapidly developed a near maximal tension response when placed in the same solution after SR destruction (Fig. 4*D*). SR-dependent differences in tension development were not evident in solutions containing zero added P_i (Fig. 4*A* and *C*) or 1 mM P_i (Fig. 4*B* and *D*), suggesting that the effect required elevated levels of myoplasmic P_i.

The dramatic influence of the SR on the tension response at high P_i meant that data had to be obtained from Triton-treated fibres in order to isolate the effects of P_i on the Ca^{2+} sensitivity of the myofilaments. Typical tension–pCa relations from a single FT and ST fibre in the presence of increasing $[P_i]$ (0–50 mM) are illustrated in Fig. 5 while grouped results are summarized in Table 4.

There was a progressive decrease in pCa_{50} with increasing $[P_i]$ in both muscle types, indicating a decrease in the Ca^{2+} sensitivity of the myofilaments (Fig. 5, Table 4). This effect was significant at P_i concentrations $\geqslant 1$ mM in ST fibres but required $\geqslant 5$ mM P_i in FT fibres (Table 4). There was no further decrease in myofilament Ca^{2+} sensitivity over the 25–50 mM $[P_i]$ range in either muscle type (Fig. 5, Table 4.)

These results indicated a greater effect of P_i on ST than FT fibres. However, normalization of the mean pCa_{50} shifts in Table 4 to the appropriate resting $[P_i]$ for each fibre type brought the pCa shifts into roughly similar ranges (FT: 1–25 mM P_i, 0·25 pCa units; ST: 5–25 mM P_i, 0·26 pCa units).

Ca^{2+} sensitivity shifts at high $[P_i]$ moved the tension–pCa relation into a range where total EGTA and total Ca^{2+} were close to equimolar. Since EGTA is a relatively poor buffer in

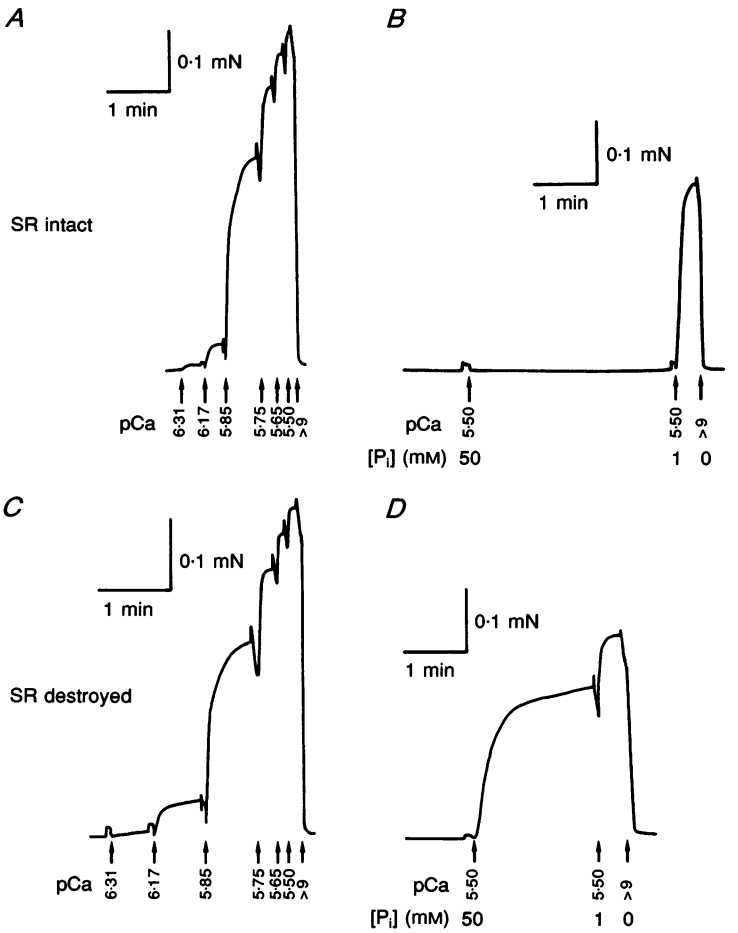

Figure 4. SR-dependent inhibition of Ca^{2+}-activated tension at high concentrations of P_i

A and *C*, Ca^{2+}-activation properties of a single FT fibre (length, 2·4 mm; diameter, 43 μm) in solutions containing no added P_i. Traces were recorded before (*A*) and after (*C*) destruction of the SR by 10 min treatment with Triton X-100. *B* and *D*, tension traces from a different FT fibre (length, 2·0 mm; diameter, 38 μm) at high $[P_i]$ in the presence (*B*) and absence (*D*) of functional SR. Note the absence of tension development in 50 mM P_i at pCa 5·5 in the presence of intact SR (*B*).

this range, a lower affinity Ca^{2+} buffer (NTA) was used in some experiments to confirm the validity of the sensitivity shifts. Tension–pCa relations were constructed using the two different Ca^{2+} buffers, the pCa of each solution being measured with a Ca^{2+}-sensitive electrode. There was no significant difference in results obtained with the two different Ca^{2+} buffers (Table 4).

One final observation was that $[P_i] \geqslant 5$ mM abolished the tension oscillations (~0·2 Hz at 23 °C) of myofibrillar origin that are normally indicative of partially activated ST fibres.

Effects of simple CP–P_i replacement on T_{max}

From Fig. 1*A* it is apparent that CP depletion will cause modest increases in T_{max} while Fig. 3 shows that equivalent P_i accumulation will decrease T_{max} somewhat more. In a highly simplified model of metabolic fatigue, P_i replaces CP in equimolar amounts. The net effect of total conversion of CP to P_i on T_{max} is illustrated in Fig. 6. Transfer of FT fibres from a maximally activating solution mimicking resting conditions (49 mM CP, 1 mM P_i) to one mimicking fatigue (0 mM CP, 50 mM P_i) decreased T_{max} by $27 \pm 4\%$ ($n = 3$). Interestingly, when ST fibres were subjected to a similarly appropriate transition (resting: 20 mM CP, 5 mM P_i, fatigue: 0 mM CP, 25 mM P_i) the T_{max} was suppressed by a similar amount ($28 \pm 2\%$; $n = 3$).

Effects of fatigue-like milieu changes on tension development at constant $[Ca^{2+}]$

Tension development in the presence of complex, fatigue-like milieu changes was studied in solutions of constant $[Ca^{2+}]$. Fibre cross-sectional area and the concentrations of CP, P_i, creatine and ATP were varied as described in the Methods. Resting and fatigue solutions of a given pCa were mixed in varying ratios to provide intermediate points in the transition from rest to fatigue. In both FT and ST fibres, the tension response was normalized to that obtained in the appropriate resting solutions and was plotted against the $[P_i]$ range spanning between rest and fatigued solution mixtures. The effect of these different mixtures on tension development at tetanic-like $[Ca^{2+}]$ (0·85–6·0 μM) is shown in Fig. 7.

ST fibres (Fig. 7*B*) displayed a shallower decline in tension than FT fibres (Fig. 7*A*) when the dynamic range of metabolite changes from rest to fatigue was taken into account. Nevertheless, the results for the two fibre types were similar in a number of respects. (1) At a stage when all the available CP had been converted to P_i and creatine (pure

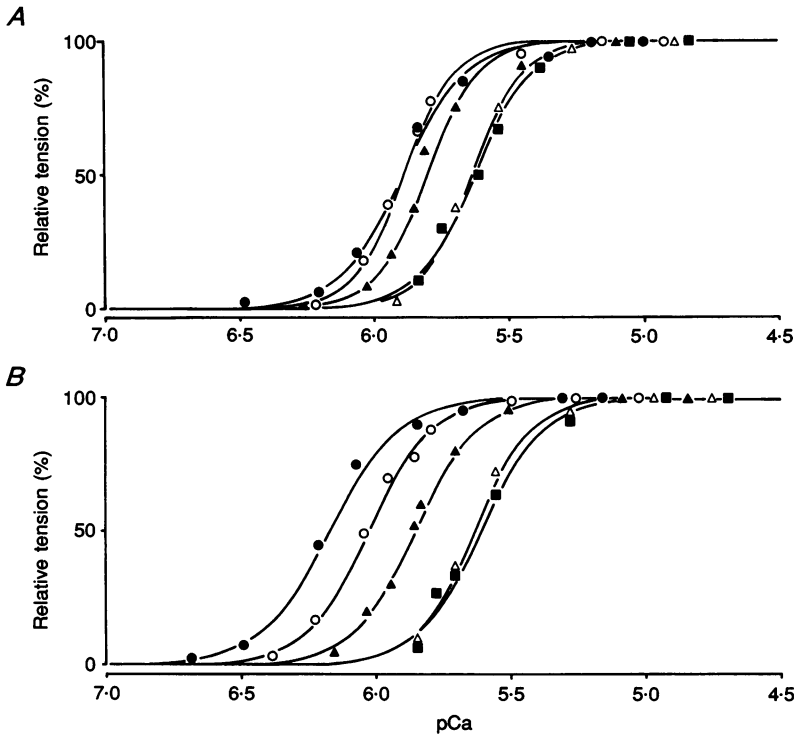

Figure 5. Effects of P_i on the steady-state tension–pCa relationships of single skinned FT (*A*) and ST (*B*) skeletal muscle fibres

P_i concentrations: ●, zero added P_i; ○, 1 mM; ▲, 5 mM; △, 25 mM; ■, 50 mM. Sigmoidal curves represent the least-squares fit of the data to eqn (1) in the Methods. Fibre dimensions (length, diameter): *A*, 1·5 mm, 45 μm; *B*, 1·9 mm, 50 μm. Sarcomere length, 2·67 μm.

fatigue solution; FT, 40 mM P_i; ST, 20 mM P_i), tension was inhibited in both fibre types by 40–50%. (2) Fatigue milieu effects were always greater at the low end of the tetanic Ca^{2+} range (0·85–1·4 μM) than at the higher end (6·0 μM).

P_i effects on SR Ca^{2+} loading in an infinite Ca^{2+} pool

Figure 4 illustrated a marked influence of the SR on tension development that occurred at high P_i (50 mM) but not at low P_i (0–1 mM). One possible explanation was that SR Ca^{2+} loading was markedly stimulated by P_i. This idea was tested in fibres with intact SR by subjecting them to the following protocol. First, the caffeine-sensitive Ca^{2+} store was depleted by exposure to 30 mM caffeine + 0·5 mM EGTA (solutions B3 : B1, 21 : 1) for 4 min. The fibre was then immersed for 5 min in a loading solution of constant [Ca^{2+}] but varying in [P_i]. The loading period was followed by exposure for 1 min to a 0·5 mM EGTA–P_i-free solution. Finally, the cycle was completed by re-exposing the fibre to the original 30 mM caffeine–0·5 mM EGTA solution. The integral of the tension transient obtained in this last step was considered to be a relative indicator of the degree of Ca^{2+} loading of the caffeine-sensitive store. A number of cycles such as this could be performed on the same fibre with only a small degree of run-down (around 5%).

Figure 8 shows the results of one such experiment, in which the [P_i] in the loading solution was varied between 0 and 15 mM, while [Ca^{2+}] was kept constant at a typical resting myoplasmic level (i.e. 100 nM). When the loading solution lacked P_i (Fig. 8*A–C*, left traces), the subsequent caffeine-induced Ca^{2+} release was below the tension threshold in 0·5 mM EGTA. In contrast, the presence of 15 mM P_i in the loading cycle yielded a large, transient tension response to caffeine (Fig. 8*A–C*, right traces). At a loading P_i of 10 mM, however, the response to caffeine was barely above threshold (Fig. 8*B*). Similar results were obtained with caffeine-induced Ca^{2+} releases in 0·5 mM EGTA in two other FT fibres and one ST fibre, highlighting the sharp increase in SR Ca^{2+} loading between 10 and 15 mM P_i at pCa 7·0.

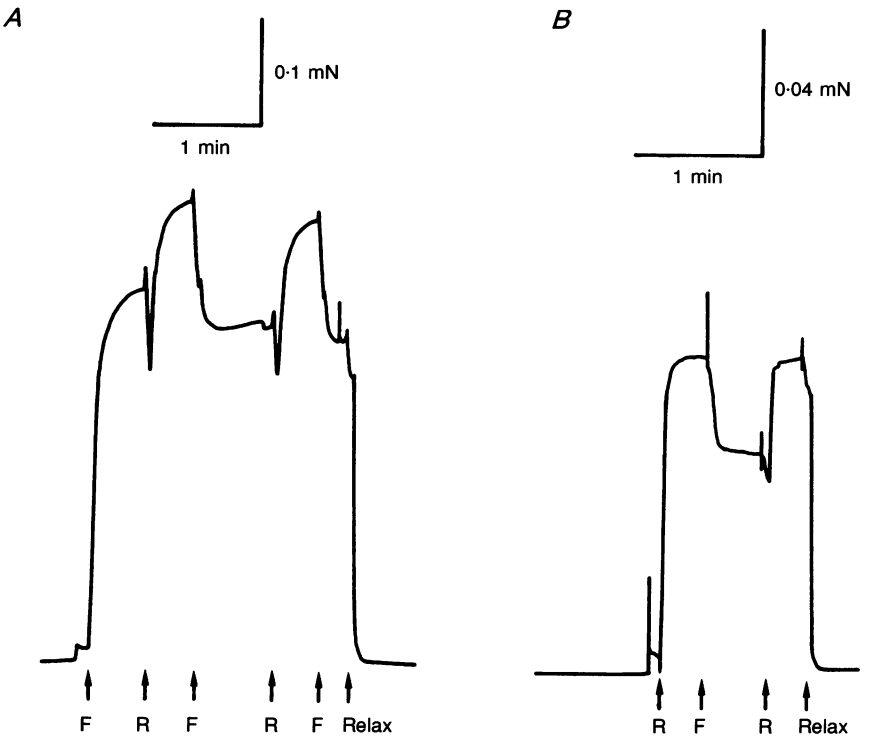

Figure 6. Effect of simultaneous CP and P_i changes on maximum Ca^{2+}-activated tension

A, representative tension results from a single FT fibre (length, 2·4 mm; diameter, 45 μm) transferred between a maximum activation solution mimicking normal resting myoplasmic levels (R: 49 mM CP, 1 mM P_i, pCa 4·5) to one mimicking fatigued myoplasm (F: 0 mM CP, 50 mM P_i, pCa 4·5). In this fibre the use-dependent decline of T_{max} in both F and R solutions over successive applications was greater than 10%. However, the decline occurred in parallel such that the ratio of T_{max} between the two conditions remained constant. *B*, results from a single ST fibre (length, 1·6 mm; diameter, 32 μm). Solution R: 20 mM CP, 5 mM P_i, pCa 4·5; solution F: 0 mM CP, 25 mM P_i, pCa 4·5. Tension recordings start and finish with the fibre in high-relaxing solution (pCa > 9). Both fibres were pre-treated for 10 min with 3% Triton X-100 (v/v).

In a second series of experiments the [EGTA] was lowered to 0·25 mM with the aim of detecting the threshold [P_i] for enhanced Ca^{2+} loading more accurately. Note that under these conditions (Fig. 9) caffeine-induced Ca^{2+} release was sufficient to elicit tension responses under control loading conditions (pCa 6·5 or 7·0, traces marked 0). In this particular fibre the threshold [P_i] for enhanced loading at pCa 7·0 was between 3 and 5 mM, as indicated by the large, reversible increase in the caffeine-induced tension response (Fig. 9, top panel). When the same fibre was loaded with Ca^{2+} at pCa 6·5, the threshold [P_i] for enhanced loading dropped to 2 mM (Fig. 9, lower panel).

When the results from a number of experiments on FT fibres similar to that shown in Fig. 9 were grouped, the threshold [P_i] for enhanced SR Ca^{2+} loading was found to be significantly higher at pCa 7·0 (9·2 ± 2·6 mM, $n = 6$; range, 3–15 mM) than at pCa 6·5 (2·3 ± 0·6 mM, $n = 4$; range, 1–4 mM). Paired results in four of these fibres showed a highly significant ($P < 0·001$) 2·5 ± 0·5-fold decrease in [P_i] threshold between pCa 7·0 and 6·5.

The enhanced Ca^{2+} loading by P_i is most likely to be due to formation of calcium phosphate precipitate in the SR lumen (Hasselbach, 1964). The [P_i] at which this occurs would be critically dependent on the solubility product [Ca^{2+}][P_i]. An upper limit for the [Ca^{2+}][P_i] was estimated by adding a P_i solution (pH 7·1) to a strongly buffered Ca^{2+} solution containing (mM): NTA, 60; $CaCl_2$, 30; Hepes, 50; K^+, 130; pH 7·1; 23 °C. The solutions were stirred thoroughly after each addition and the [P_i] was noted at which light scattering was apparent to the naked eye while observing at 90 deg to the incident light (75 W cold light source, fibre optic) under a light microscope. A clear formation of precipitate was observed at a [Ca^{2+}][P_i] product of $\leqslant 6$ mM^2, which corresponds to a [Ca^{2+}][HPO_4^{2-}] of ~ 3 mM^2. Given that the affinity of H^+ for HPO_4^{2-} is $6·2 \times 10^6$ M^{-1} at 25 °C (0·2 M ionic strength; Smith & Martell, 1976) and that of K^+ for HPO_4^{2-} is 3 M^{-1}, then about 50% of the P_i will be HPO_4^{2-}, 30% will be HPO_4^- and 19% will be $KHPO_4^-$ at pH 7·1 and 130 mM K^+. The published solubility of $CaHPO_4$ at 23 °C is 0·0316 g (100 ml H_2O)$^{-1}$ (Weast, 1977), which is equivalent to a solubility product [HPO_4^{2-}][Ca^{2+}] of about 3 mM^2.

Exposure of the SR to P_i in a finite Ca^{2+} pool

In order to mimic the physiological situation more closely, an experiment was designed in which the SR was exposed to P_i within a restricted myoplasmic Ca^{2+} pool (Fig. 10*A*). This was achieved by first loading the fibre with Ca^{2+} (2 min, pCa 7·0), then briefly exposing it to P_i (50 mM, 20 s) and then placing the fibre into paraffin oil for 3 min. After this period of Ca^{2+} pool restriction the fibre was returned to a P_i-free, low-relaxing solution (0·125 mM EGTA) for 20 s to wash out the myoplasmic P_i and to equilibrate the fibre with EGTA prior to caffeine exposure.

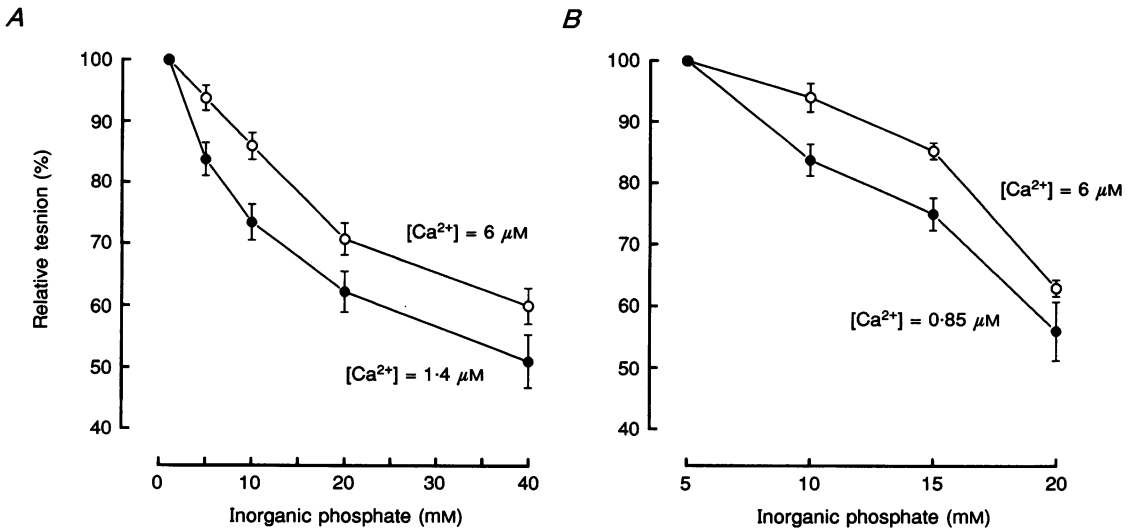

Figure 7. Effects of complex fatigue-like milieu changes on tension development at constant [Ca^{2+}]

A, fast-twitch fibres; *B*, slow-twitch fibres. Solutions from Table 2 were mixed in different proportions to mimic the range of myoplasmic constituent changes progressing from the resting to the fatigued state. Tension is expressed as a percentage of the response obtained in solutions mimicking resting myoplasmic conditions, and is plotted as a function of increasing myoplasmic [P_i]. Points represent the means ± S.E.M. from 5 or 6 Triton-treated fibres in each muscle type.

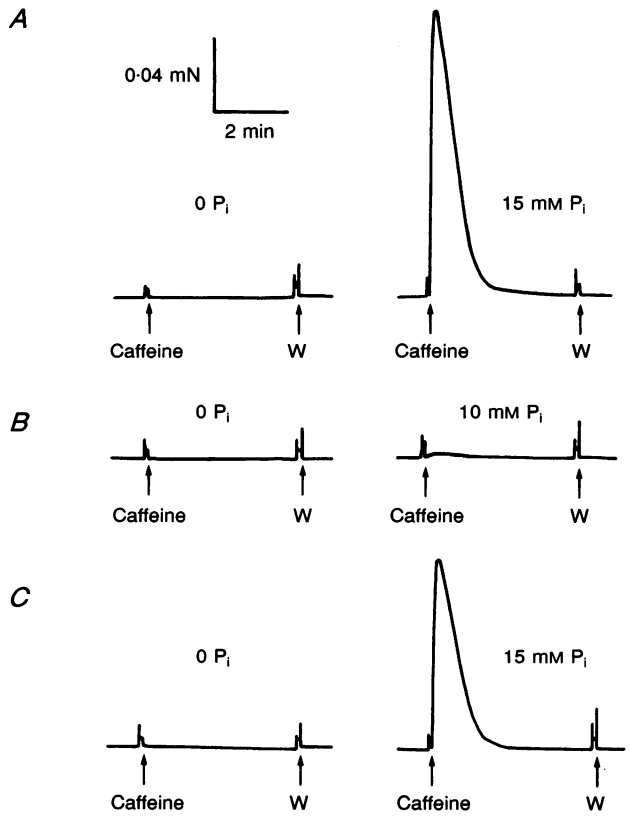

Figure 8. The presence of 10–15 mM P_i markedly enhances SR Ca^{2+} loading

The degree of SR Ca^{2+} loading was assessed by the size of the tension transient recorded from a single FT fibre (length, 1·9 mm; diameter, 48 μm) in response to a low-relaxing solution containing 30 mM caffeine, 0·5 mM EGTA and no added P_i. The fibre was then washed (W) for 5 min in high-relaxing solution (pCa > 9), re-loaded for 5 min in a pCa 7·0 solution containing 0, 10 or 15 mM P_i and then equilibrated for 1 min in a 0·5 mM EGTA, P_i-free solution prior to the next caffeine exposure. Traces were recorded left to right in the sequence *A* to *C*. Numbers above each trace refer to the $[P_i]$ present during the re-loading phase. Note that Ca^{2+} release under control loading conditions (left traces) was insufficient to achieve the tension threshold in the presence of 0·5 mM EGTA.

The middle trace in Fig. 10*A* shows that when the fibre was exposed to P_i in a restricted Ca^{2+} pool, the ensuing caffeine-induced tension response was significantly decreased in amplitude and had a much slower time to peak than the controls. In four experiments of this kind (with a wash-out period prior to caffeine exposure of 20 s) the peak amplitude of the tension response was 19 ± 7% of control while the time to peak tension was increased 5·8 ± 1·4-fold. Suppression of the tension response following P_i exposure was greater if the wash period prior to the

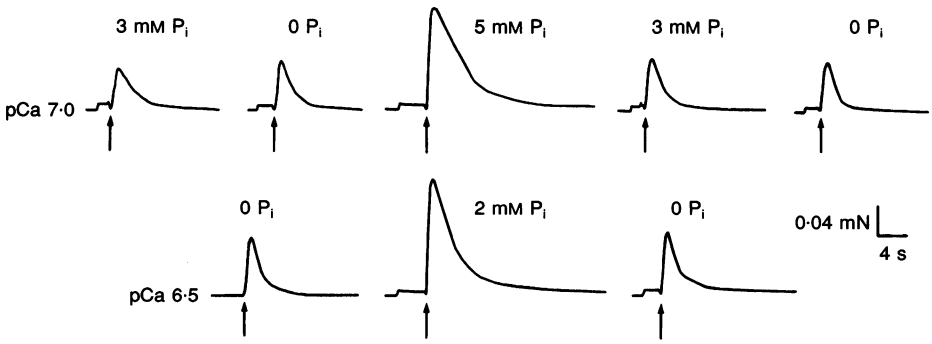

Figure 9. The threshold $[P_i]$ required for enhancing SR Ca^{2+} loading decreases as myoplasmic $[Ca^{2+}]$ increases

Numbers above each trace indicate the $[P_i]$ present during SR Ca^{2+} loading at either pCa 7·0 (upper traces) or pCa 6·5 (lower traces). Tension was elicited at the arrows from a single FT fibre (length, 2·5 mm; diameter, 40 μm) by exposure to a low-relaxing solution containing 30 mM caffeine, 0·25 mM EGTA and no added P_i. The experimental protocol and solutions were similar to that described in Fig. 8 except that the wash period was doubled (to 10 min) and the EGTA was halved in the caffeine and pre-equilibration solutions (0·25 mM). Lowering the [EGTA] allowed caffeine to evoke considerable tension under control loading conditions (traces marked 0), in contrast to Fig. 8.

caffeine response was shorter (2 fibres). This suggested a possible direct effect on myofilament Ca^{2+} sensitivity due to the inadequate wash-out of myoplasmic P_i. However, complete recovery of the tension response after P_i exposure required wash periods of at least 5–10 min, a time course considerably longer than that calculated for P_i wash-out from a 50 μm diameter skinned fibre (99% in < 1 s).

Time course of P_i removal from the SR

In experiments like the one shown in Figs 8 and 9, the fibres were washed for 1 min in a P_i -free solution prior to caffeine exposure. This washing off of the P_i allowed caffeine to elicit a rapid force response. Trace *a* in Fig. 10*B* is another example of such a response recorded on a faster time base. The time taken between immersion in caffeine solution and the attainment of half-maximal tension was about 1 s. If 50 mM P_i was present right up to the moment of caffeine exposure (trace *b*), there was ~3 s latency until force slowly started to develop. The lack of force response for 3 s after exposure to caffeine can only be explained by a delay in the solubilization of the calcium phosphate precipitate.

DISCUSSION

The present results indicate that changes of [CP] and [P_i] in the range expected to occur during fatigue have significant effects on both the Ca^{2+}-activation properties of the myofilaments and SR function in mammalian skeletal muscle fibres. As such, these findings are relevant to understanding the decline in tension development that occurs with metabolic fatigue.

Effects of P_i on SR Ca^{2+} movements

Using different experimental conditions, two major effects of P_i on SR Ca^{2+} movements have been demonstrated in this paper: (1) an increase in SR Ca^{2+} loading (Figs 8 and 9), and (2) a decrease in SR Ca^{2+} release (Fig. 10*A* and *B*). Both of these effects may be explained by a decrease in the free [Ca^{2+}] in the SR lumen ([Ca^{2+}]$_{SR}$) due to formation of calcium phosphate precipitate.

P_i enhances SR Ca^{2+} loading

P_i and oxalate have previously been used at high concentrations (50–100 mM) in isolated SR vesicles to

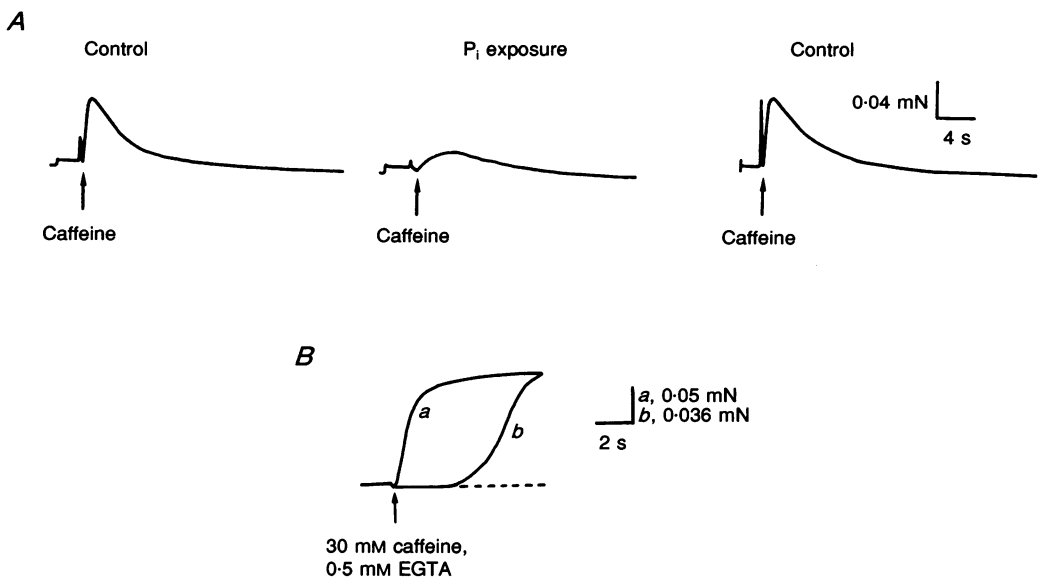

Figure 10. P_i exposure reduces and prolongs SR Ca^{2+} release

A, reduction and prolongation of the rate of SR Ca^{2+} release by exposure to P_i within a restricted myoplasmic Ca^{2+} pool. Tension responses were evoked at the arrows by exposing a single FT fibre (length, 2·5 mm; diameter, 32 μm) to a low-relaxing solution containing 30 mM caffeine and 0·125 mM EGTA. Prior to recording the control traces the fibre was transferred through the following solution sequence: (1) 5 min in relaxing solution (pCa > 9), (2) 2 min load at pCa 7·0, (3) 20 s in low-relaxing solution containing 0·125 mM EGTA, (4) 3 min in paraffin oil, and (5) 20 s equilibration in P_i-free low-relaxing solution containing 0·125 mM EGTA. The middle trace was recorded after an identical protocol except the solution used in step (3) contained an additional 50 mM P_i. *B*, time course of P_i removal from the SR. In traces *a* and *b* the same FT fibre was exposed to a 30 mM caffeine solution containing 0·5 mM EGTA and zero added P_i to evoke SR Ca^{2+} release. Prior to caffeine exposure, the fibre had been loaded for 5 min in a pCa 7·0 solution containing 50 mM P_i and then equilibrated for 1 min in 0·5 mM EGTA. In *a* the P_i was washed off in this 1 min equilibration period while in *b* the [P_i] was maintained at 50 mM up to the point of caffeine application. The peak response in *b* was 1·4 times smaller than *a*, but the traces have been normalized for easier comparison. Fibre dimensions: length, 1·9 mm; diameter, 48 μm.

precipitate Ca^{2+} in the SR lumen (Hasselbach, 1964). Precipitate formation prevents the rapid increase in the $[Ca^{2+}]_{SR}$ that would normally inhibit further SR Ca^{2+} loading, thus prolonging steady-state SR Ca^{2+} uptake (Inesi & De Meis, 1989). An important and novel finding in the present study is that this phenomenon occurs in an experimental preparation with an intact SR at physiological levels of myoplasmic $[Ca^{2+}]$ (pCa, 6·5–7·0) and $[P_i]$ (2–15 mM). Previous skinned-fibre studies may have failed to detect enhanced SR Ca^{2+} uptake at such low $[P_i]$ because the loading times may have been too short to allow equilibration of P_i between the myoplasm and the SR (Nosek & Jain, 1988; Zhu & Nosek, 1991; Stienen, Graas & Elzinga, 1993).

The following evidence is consistent with the interpretation that $CaHPO_4$ precipitation in the SR is responsible for the enhanced loading in the presence of P_i:

(1) Enhancement of SR Ca^{2+} loading was dependent on myoplasmic $[P_i]$ at a constant loading time of 5 min (Figs 8 and 9). Similar results could be obtained with short exposures to high $[P_i]$ (1–5 min at 20–50 mM P_i) or long exposures to low $[P_i]$ (10–30 min at 1–5 mM P_i; 3 FT fibres and 2 ST fibres), suggesting that P_i entry into the SR is rate limiting for precipitate formation.

(2) Enhancement of SR Ca^{2+} uptake occurred at a distinct threshold $[P_i]$ at constant loading time (Figs 8 and 9). This is consistent with a sudden initiation of calcium phosphate precipitation once the $[Ca^{2+}][P_i]$ solubility product is exceeded in the SR lumen.

(3) The threshold $[P_i]$ for enhancing SR Ca^{2+} loading was lower when the myoplasmic $[Ca^{2+}]$ used for loading was higher (Fig. 9). Given a constant period of loading, a rat FT fibre will load more Ca^{2+} at myoplasmic pCa 6·5 than at

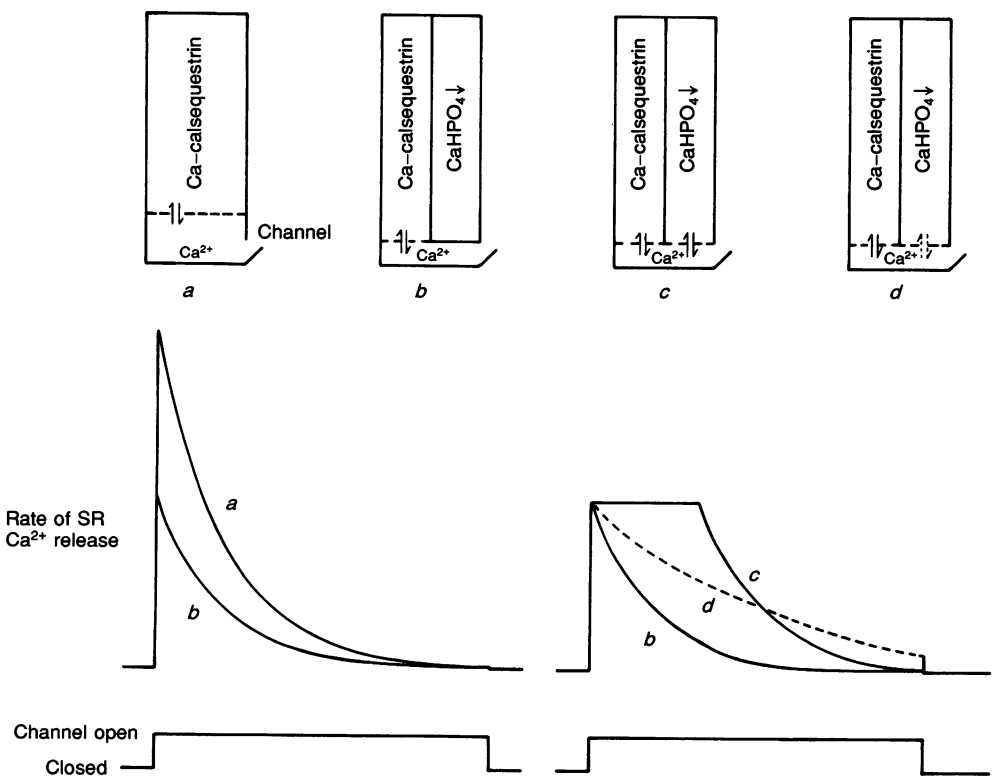

Figure 11. A qualitative model of decreased SR Ca^{2+} release during fatigue

The top 4 panels display the 3 possible states of Ca^{2+} in the SR lumen: free Ca^{2+}, Ca^{2+} bound to calsequestrin, and calcium precipitated as $CaHPO_4$. The upper traces are schematic profiles of SR Ca^{2+} release rates during the period of channel opening indicated beneath them. Trace *a* represents the rate of Ca^{2+} release prior to the $[Ca^{2+}][P_i]$ solubility product being exceeded in the SR lumen. During fatigue, myoplasmic $[P_i]$ and SR luminal $[P_i]$ ($[P_i]_{SR}$) progressively increase until the solubility product is exceeded. $CaHPO_4$ then precipitates in the SR lumen, reducing the free Ca^{2+} available for release (*b*). If the $CaHPO_4$ precipitate can dissolve immediately upon channel opening (*c*) or during the time course of channel opening (*d*) the Ca^{2+} release waveform will be both reduced and prolonged compared with the control (*a*). In this diagram it is assumed that the total SR $[Ca^{2+}]$ remains constant and that $[P_i]_{SR}$ is increased such that 50% of the total SR $[Ca^{2+}]$ has precipitated as $CaHPO_4$ in traces *b*, *c* and *d*. A more detailed description of the model is provided in the text.

pCa 7·0 (Fryer & Stephenson, 1993). If $[Ca^{2+}]_{SR}$ is proportional to the total SR Ca^{2+} content then $[Ca^{2+}]_{SR}$ should have been higher during loading at pCa 6·5 than at pCa 7·0. Thus, at pCa 6·5, a lower $[P_i]$ was required to exceed the calcium phosphate solubility product in the SR lumen (Fig. 9). Using our measured value of $[Ca^{2+}][P_i]$ (6 mM^2) and assuming that calcium phosphate precipitation starts to occur at our measured $[P_i]$ threshold, the calculated $[Ca^{2+}]_{SR}$ is $1·2 \pm 0·4$ mM ($n = 6$) at myoplasmic pCa 7·0 and $3·4 \pm 0·9$ mM at pCa 6·5. These values are likely to underestimate the ionized $[Ca^{2+}]$ in the SR lumen because $[P_i]$ in the SR lumen would be less than the myoplasmic $[P_i]$ after only 5 min equilibration. Indeed, longer exposures of FT fibres to P_i (10–30 min) apparently caused precipitation to occur at lower $[P_i]$ (1–5 mM) at myoplasmic pCa 7·0. From these experiments the $[Ca^{2+}]_{SR}$ of FT fibres at pCa 7·0 was estimated to be about 3 mM. Since the total Ca^{2+} in the SR of intact mammalian fibres under resting conditions is around 10 mM (Lüttgau & Stephenson, 1986) we can conclude that a significant amount of this SR Ca^{2+} is bound to calsequestrin.

P_i decreases SR Ca^{2+} release

If the rate of Ca^{2+} release from the SR is proportional to $[Ca^{2+}]_{SR}$, then the decrease in $[Ca^{2+}]_{SR}$ by calcium phosphate precipitation should decrease the rate of SR Ca^{2+} release. The results in Figs 8 and 9 appear to contradict this prediction since caffeine-induced Ca^{2+} release was increased after SR Ca^{2+} loading in the presence of P_i. This was initially taken as evidence that P_i could not be responsible for the decrease in SR Ca^{2+} release seen during fatiguing stimulation (Westerblad & Allen, 1991). However, in an intact fibre the SR is exposed to P_i in a finite myoplasmic Ca^{2+} pool, while in skinned-fibre experiments the myoplasmic Ca^{2+} pool is essentially infinite. When the myoplasmic Ca^{2+} pool was kept restricted with P_i present after SR Ca^{2+} loading (Fig. 10*A*) the ensuing caffeine-induced tension response was always smaller and slower to peak than its respective control. This result is consistent with a calcium phosphate precipitation mechanism for a prolonged and decreased rate of SR Ca^{2+} release (see Fig. 10*B*). Thus, it is suggested that the decrease in SR Ca^{2+} release seen in late fatigue (Westerblad & Allen, 1991) is not due to a decrease in total SR Ca^{2+} content (Westerblad *et al.* 1991; Smith & Steele, 1992), but rather to a decrease in the free Ca^{2+} in the SR lumen. With such a mechanism in place, it is not necessary to invoke a role for P_i in either reversing the SR Ca^{2+} pump (Zhu & Nosek, 1991) or impairing pump function by altering the free energy of ATP hydrolysis (Westerblad & Allen, 1991; Westerblad *et al.* 1991; Smith & Steele, 1992).

Decreased SR Ca^{2+} release during fatigue: a simple model

Figure 10 demonstrated that P_i could decrease and prolong the caffeine-induced tension transient in skinned fibres. This result is similar to the smaller and longer intracellular free Ca^{2+} transients measured during tetani in intact mammalian skeletal muscle fibres during late fatigue (phase 3; Westerblad & Allen, 1991). Figure 11 illustrates a simple qualitative model in which $CaHPO_4$ precipitation in the SR lumen can both reduce and prolong SR Ca^{2+} release by reducing $[Ca^{2+}]_{SR}$. For simplicity, we assume in this model that channel gating remains constant.

Under pre-fatigue conditions (trace *a*, Fig. 11), most of the Ca^{2+} in the SR is bound to calsequestrin but is in rapid equilibrium with $[Ca^{2+}]_{SR}$. The Ca^{2+} release profile suddenly rises when release channels open, and then declines exponentially as the releasable Ca^{2+} pool approaches depletion.

As myoplasmic $[P_i]$ increases during fatigue, $[P_i]$ enters the SR lumen and starts to accumulate. Once the $[HPO_4^{2-}][Ca^{2+}]$ solubility product is exceeded in the SR lumen, $[Ca^{2+}]_{SR}$ will proportionally decline with further increases in P_i. If the $CaHPO_4$ precipitate is unavailable for the duration of channel opening (trace *b*, Fig. 11), then Ca^{2+} release will be reduced as a result of the decrease in $[Ca^{2+}]_{SR}$. If, however, the $[Ca^{2+}]_{SR}$ is allowed to be in instantaneous equilibrium with both calsequestrin and the $CaHPO_4$ precipitate (*c*, Fig. 11) then $[Ca^{2+}]_{SR}$ will be buffered more strongly than usual at its new, lower level. The effect of this increased buffering on the subsequent Ca^{2+} release profile is to slow the depletion of the releasable store, and hence clamp release at a lower, steady level until the $CaHPO_4$ precipitate is completely dissociated (*c*). Finally, if the $CaHPO_4$ precipitate can enter solution within the time scale of channel opening (but not instantaneously) (*d*), the Ca^{2+} release waveform will lie somewhere intermediate between conditions *b* and *c*.

From this model it is clear that the time course of precipitate dissociation in the SR lumen will be crucial in determining the rate of recovery of SR Ca^{2+} release. Experiments like that shown in Fig. 10*A* showed that partial recovery of SR Ca^{2+} release (around 20%) occurred within 20 s of P_i wash-out but total recovery required 5–10 min. The fast phase of recovery has a similar time course to the rate of $CaHPO_4$ dissolution measured *in vitro* when $[Ca^{2+}]$ is rapidly dropped with EGTA (half-time of 10 s; Owen, Lamb & Stephenson, 1992*b*). We believe that the slower component represents the slow wash-out of P_i from the SR in the skinned-fibre preparation. At present, it is difficult to assess exactly how fast the precipitate will dissolve in intact skeletal muscle fibres because there is little information available regarding P_i fluxes between the myoplasm and the SR. However, it is conceivable that SR Ca^{2+} release could recover over tens of seconds as a consequence of a rapid drop in either the free $[Ca^{2+}]$ or free $[P_i]$ within the SR lumen. For example, if the calcium phosphate solubility product were only marginally exceeded in the SR lumen, then recovery might require the removal of only a few millimolar P_i from the SR lumen. If

this is the case then the time course of $CaHPO_4$ dissolution may be adequate to explain the marked recovery of SR Ca^{2+} release seen during 10 s rest periods in late fatigue (Westerblad & Allen, 1991).

The model in Fig. 11 can also be used to explain the results obtained when the FT fibres were loaded in the presence of an infinite pool of P_i at pCa 7·0 and 6·5 (Figs 8 and 9). In the absence of precipitate formation the ionized Ca^{2+} within the SR rises rapidly to levels that inhibit further SR Ca^{2+} loading (Inesi & De Meis, 1989). However, when the $[P_i]$ in the SR lumen ($[P_i]_{SR}$) is high enough to exceed the solubility product for $CaHPO_4$ formation (i.e. $[P_i]_{SR} \times [Ca^{2+}]_{SR} = 6$ mM^2), the $[Ca^{2+}]_{SR}$ cannot rise past a certain value which is determined by the solubility product. Therefore, in the presence of an infinite P_i pool the SR loading can continue with the deposition of $CaHPO_4$ precipitate in the SR lumen. If the P_i is washed out from the SR at this point (as was the case in the experiments) the $[Ca^{2+}]_{SR}$ will increase following the solubilization of the $CaHPO_4$ precipitate, as long as the product between $[P_i]_{SR}$ and $[Ca^{2+}]_{SR}$ is smaller than the solubility product for $CaHPO_4$ formation. With this type of mechanism in place, $[Ca^{2+}]_{SR}$ can easily exceed the value which would normally inhibit SR Ca^{2+} loading in the absence of $CaHPO_4$ precipitate formation. Thus, caffeine responses recorded after SR Ca^{2+} loading in the presence of P_i are greater than those recorded under control conditions (Figs 8 and 9). This is analogous to the conditions shown in the model in panel *b* where $[Ca^{2+}]_{SR}$ is lower, and panel *a* where $[Ca^{2+}]_{SR}$ is higher.

Effects of CP and P_i on the contractile proteins

The results indicated that large decreases in [CP] (25–50 to 0 mM) modestly increased T_{max} but markedly enhanced myofilament Ca^{2+} sensitivity in both FT and ST fibres (Figs 1*A* and 2, Table 3). These effects of CP depletion are important because they counteract the inhibitory actions of P_i on contractile protein function. Thus, when the [CP] and $[P_i]$ were varied reciprocally (as should occur *in vivo*) the inhibitory effects of P_i on both T_{max} and Ca^{2+} sensitivity were significantly reduced (Figs 6 and 7).

Godt & Nosek (1989) found that decreasing CP from 15 to 1 mM increased T_{max} by about 10% and increased the myofilament sensitivity (pCa_{50}) by 0·07 pCa units in skinned FT rabbit skeletal muscle fibres. We found similar effects of CP depletion on T_{max} but observed larger increases in Ca^{2+} sensitivity. Extrapolation of Godt & Nosek's results yielded a sensitivity increase of 0·12 pCa units for a decrease in CP from 25 to 1 mM while the results in the present study for FT fibres were twice as great (0·24 pCa units; Table 3).

CP effects on T_{max} were abolished in FDNB-treated fibres from both FT and ST muscles (Fig. 1*B* and *C*). These results are consistent with the previous suggestion (Godt & Nosek, 1989) that decreases in [CP] affect T_{max} by modifying creatine kinase activity. At low [CP], creatine kinase is less efficient at maintaining low [ADP], leading to an elevation of ADP within the preparation and a subsequent increase in T_{max} (Cooke & Pate, 1985).

P_i accumulation decreases T_{max} and Ca^{2+} sensitivity

Two new aspects of P_i effects on the contractile proteins were addressed in the present study: (1) $[P_i]$ was studied over the full range (1–50 mM) that is likely to occur in FT fibres when the CP is almost fully depleted, and (2) the comparative effects of P_i on FT and ST fibres were fully characterized.

P_i was found to decrease T_{max} (Fig. 3) and shift the tension–pCa relation to higher $[Ca^{2+}]$ (Fig. 5, Table 4) in both fibre types. It is believed that P_i depresses T_{max} by affecting the tension-producing step of the cross-bridge cycle, leading to a decrease in the number of attached bridges (Hibberd, Dantzig, Trentham & Goldman, 1985). Changes in myofilament Ca^{2+} sensitivity by P_i have been suggested to be a result of changes in the rate constants of the cross-bridge cycle (Brandt, Cox, Kawai & Robinson, 1982). The decrease in Ca^{2+} sensitivity could also be due to a P_i-induced decrease in the number of attached cross-bridges, since these are known to influence the Ca^{2+} affinity of troponin C (Bremel & Weber, 1972; Guth & Potter, 1987). Consistent with this interpretation is the observation that raising $[P_i]$ (e.g. from 0 to 50 mM) had a greater absolute effect on ST than on FT fibres with regard to both T_{max} and Ca^{2+} sensitivity (Fig. 3, Table 4).

A hyperbolic relationship was found between the depression of T_{max} and the $[P_i]$ in the 1–50 mM range in both FT and ST fibres (Fig. 3). The degree of T_{max} depression in ST fibres seen in the present study was similar to that seen in skinned cardiac fibres (Kentish, 1986; Nosek *et al.* 1990) but was significantly greater than that observed in FT fibres (Fig. 3). Similar results have been previously reported for chemically skinned FT and ST fibres from fish muscle (Altringham & Johnson, 1985). However, the present results differ from studies on rabbit skeletal muscle, in which little difference was found between FT and ST fibres (Nosek *et al.* 1990), or where FT fibres were more sensitive to P_i than ST ones in the steady state (Millar & Homsher, 1992).

Increases in $[P_i]$ were also shown to have inhibitory effects on myofilament Ca^{2+} sensitivity in both ST and FT fibres (Fig. 5, Table 4). An interesting and novel result was the greater sensitivity of ST fibres to low $[P_i]$ (1–5 mM). This effect could be physiologically important since the measured resting myoplasmic $[P_i]$ in intact ST muscles is around 5 mM (Kushmerick *et al.* 1992). The implication of this high resting $[P_i]$ is that myofilament Ca^{2+} sensitivity in ST fibres is inhibited in the rested state, an effect which would help dampen any further inhibitory effects of P_i accumulation *in vivo*.

$[P_i]$ increases above 5 mM inhibited myofilament Ca^{2+} sensitivity of FT and ST fibres by about the same extent (Table 4), but no further effect was observed in the 25–50 mM range. A change in P_i from 1 to 25 mM in FT fibres increased the pCa_{50} by 0·25 pCa units, corresponding to a 78% increase in the $[Ca^{2+}]$ required to produce 50% of T_{max}. The present result with rat FT fibres falls at the low end of the range previously reported for rabbit FT skeletal muscle fibres in this $[P_i]$ range (0·3–0·5 pCa units; Godt & Nosek, 1989; Millar & Homsher, 1990; Nosek *et al.* 1990).

Effects of milieu changes mimicking fatigue

In a highly simplified version of fatigue, [CP] and $[P_i]$ were varied reciprocally to mimic the extremes of resting and fatigued myoplasm. In these experiments T_{max} was decreased by similar amounts in FT and ST fibres (to around 70–75% of control T_{max}; Fig. 6). Such decreases in T_{max} were considerably less than seen with P_i alone (Fig. 3), indicating that CP depletion considerably aids T_{max} maintenance.

In an experimental model of low-frequency fatigue Westerblad & Allen (1991) showed that the myoplasmic Ca^{2+} transient recorded from single, intact mouse fibres did not decrease significantly until fairly late in fatigue. In this kind of fatigue, gradual changes in myoplasmic [CP] and $[P_i]$ should be superimposed on a relatively constant myoplasmic Ca^{2+} transient. Any decline in tension during this period must reflect a decrease in the maximum tension-generating capacity and/or Ca^{2+} sensitivity of the myofilaments. Our results from experiments mimicking this situation (Fig. 7) demonstrated that the myofilaments in ST fibres were less affected by myoplasmic metabolite changes than those in FT fibres when the dynamic range of metabolic changes between rest and fatigue was taken into account for each fibre type. The pH in these experiments was not varied because the myoplasmic pH changes measured during fatigue in single, intact mammalian fibres are minimal (Westerblad & Allen, 1992). Given a constant tetanic Ca^{2+} transient of around 1 μM, we would predict that tetanic tension would drop by about 40–50% in late fatigue as a consequence of the inhibitory effects of milieu changes on the myofilaments alone. These results compare favourably with those obtained in single, intact mouse fibres, where the relative tetanic tension declined from around 1·0 to approximately 0·6 at a constant myoplasmic pCa of 6·0 during late fatigue (Fig. 3 of Westerblad *et al.* 1991).

Concluding remarks

The comparative results reported in the present study indicate that the resting metabolic profile of a slow-twitch muscle fibre helps preserve its myofilaments from the inhibitory effects of myoplasmic milieu changes during fatigue. This represents another means by which the oxidative capacity of these fibres contributes to their superior fatigue resistance.

Physiological concentrations of P_i (2–20 mM) stimulated SR Ca^{2+} uptake and decreased SR Ca^{2+} release. These two observations can be reconciled by a mechanism in which calcium phosphate precipitates in the SR lumen. We suggest that this is the mechanism responsible for the decrease in SR Ca^{2+} release seen during late fatigue in mammalian skeletal muscle rather than the previous suggestions of SR Ca^{2+} pump inhibition or a decrease in total SR Ca^{2+} content.

Altringham, J. D. & Johnson, I. A. (1985). Effects of phosphate on the contractile properties of fast and slow muscle fibres from an antarctic fish. *Journal of Physiology* **368**, 491–500.

Brandt, P. W., Cox, R. N., Kawai, M. & Robinson, T. (1982). Regulation of tension in skinned muscle fibres: Effect of cross-bridge kinetics on apparent Ca^{2+} sensitivity. *Journal of General Physiology* **79**, 997–1016.

Bremel, R. D. & Weber, A. (1972). Co-operation within actin filament in vertebrate skeletal muscle. *Nature* **238**, 97–101.

Carlson, F. D. & Wilkie, D. D. (1974). *Muscle Physiology.* Prentice Hall, Englewood Cliffs, NJ, USA.

Chifflet, S., Torriglia, A., Chiesa, R. & Tolosa, S. (1988). A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases. *Analytical Biochemistry* **168**, 1–4.

Cooke, R. & Pate, E. (1985). The effects of ADP and phosphate on the contraction of muscle fibres. *Biophysical Journal* **48**, 789–798.

Dawson, M. J., Gadian, D. G. & Wilkie, D. R. (1980). Mechanical relaxation rate and metabolism studied in fatiguing muscle by phosphorus nuclear magnetic resonance. *Journal of Physiology* **299**, 465–484.

Edström, L., Hultman, E., Sahlin, K. & Sjoholm, H. (1982). The contents of high energy phosphates in different fibre types in skeletal muscles from rat, guinea-pig and man. *Journal of Physiology* **332**, 47–58.

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.

Fryer, M. W. & Stephenson, D. G. (1993). Calcium loading characteristics of sarcoplasmic reticulum (SR) in single mammalian skeletal muscle fibres. *XXXII Congress of the International Union of Physiological Sciences* 247.2/P.

Godt, R. E. & Nosek, T. M. (1989). Changes of intracellular milieu with fatigue or hypoxia depress contraction of skinned rabbit skeletal and cardiac muscle. *Journal of Physiology* **412**, 155–180.

Gonzales-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.

Guth, K. & Potter, J. D. (1987). Effect of rigor and cycling cross-bridges on the structure of troponin C and on the Ca^{2+} affinity of the Ca^{2+}-specific regulatory sites in skinned rabbit psoas fibres. *Journal of Biological Chemistry* **262**, 13627–13635.

Hasselbach, W. (1964). Relaxing factor and the relaxation of muscle. *Progress in Biophysics and Biophysical Chemistry* **14**, 167–222.

Hibberd, M. G., Dantzig, D. R., Trentham, D. R. & Goldman, Y. E. (1985). Phosphate release and force generation in skeletal muscle fibres. *Science* **228**, 1317–1319.

Inesi, G. & De Meis, L. (1989). Regulation of steady-state filling in sarcoplasmic reticulum. *Journal of Biological Chemistry* **264**, 5929–5936.

Kentish, J. C. (1986). The effects of inorganic phosphate and creatine phosphate on force production in skinned muscles from rat ventricle. *Journal of Physiology* **370**, 585–604.

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. & 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.

Lüttgau, H. Ch. & Stephenson, D. G. (1986). Ion movements in skeletal muscle in relation to the activation of contraction. In *Physiology of Membrane Disorders*, ed. Andreoli, T. E., Hoffman, Y. F., Fanestil, D. D. & Schultz, S. G. Plenum Press, New York.

Matsubara, I., Goldman, Y. E. & Simmons, R. M. (1984). Changes in the lateral filament spacing of skinned muscle fibres when cross-bridges attach. *Journal of Molecular Biology* **173**, 15–33.

Meyer, R. A., Brown, T. R. & Kushmerick, M. J. (1985). Phosphorus nuclear magnetic resonance of fast- and slow-twitch muscle. *American Journal of Physiology* **248**, C279–287.

Millar, N. C. & Homsher, E. (1990). Kinetics of force generation and phosphate release in skinned rabbit soleus muscle fibres. *American Journal of Physiology* **262**, C1239–1245.

Millar, N. C. & Homsher, E. (1992). The effect of phosphate and calcium on force generation in glycerinated rabbit skeletal muscle fibres. *Journal of Biological Chemistry* **265**, 20234–20240.

Moisescu, D. G. & Thieleczek, R. (1978). Calcium and strontium concentration changes within skinned muscle preparations following a change in external bathing solution. *Journal of Physiology* **275**, 241–262.

Nosek, T. M. & Jain, A. (1988). Inorganic phosphate has a biphasic effect on calcium loading by the sarcoplasmic reticulum of skinned cardiac fibres. *Biophysical Journal* **53**, 607a.

Nosek, T. M., Leal-Cardoso, J. H., McLaughlin, M. & Godt, R. E. (1990). Inhibitory influence of phosphate and arsenate on contraction of skinned skeletal and cardiac muscle. *American Journal of Physiology* **259**, C933–939.

Owen, V. J., Lamb, G. D. & Stephenson, D. G. (1992*a*). Effect of phosphate on excitation–contraction coupling in skeletal muscle of the toad. *Proceedings of The Australian Physiological and Pharmacological Society* **23**, 93P.

Owen, V. J., Lamb, G. D. & Stephenson, D. G. (1992*b*). Effects of IMP and inorganic phosphate on depolarization-induced and caffeine-induced calcium release in skeletal muscle fibres of the toad. *Proceedings of The Australian Physiological and Pharmacological Society* **23**, 148P.

Sillèn, L. G. & Martell, A. E. (1964). *Stability Constants of Metal–Ion Complexes*. Chemical Society, London.

Smith, R. M. & Martell, A. E. (1976). *Critical Stability Constants*, vol. 4, *Inorganic Complexes*. Plenum Press, New York.

Smith, G. L. & Steele, D. S. (1992). Inorganic phosphate decreases the Ca^{2+} content of the sarcoplasmic reticulum in saponin-treated rat cardiac trabeculae. *Journal of Physiology* **458**, 457–473.

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

Stewart, A. W., Stephenson, D. G. & Wilson, G. J. (1987). Simultaneous measurement of Ca^{2+}-dependent force and ATPase activity in fast- and slow-twitch skinned muscle fibres of the rat. *Proceedings of The Australian Physiological and Pharmacological Society* **18**, 46P.

Stienen, G. J, Graas, I. A. & Elzinga, G. (1993). Uptake and caffeine-induced release of calcium in fast muscle fibres of *Xenopus laevis*: effects of MgATP and P_i. *American Journal of Physiology* **265**, C650–657.

Weast, R. C. (1977). *Handbook of Chemistry and Physics*, 58th edn. CRC Press, Boca Raton, FL, USA.

Westerblad, H. & Allen, D. G. (1991). Changes of myoplasmic calcium concentration during fatigue in single mouse muscle fibres. *Journal of General Physiology* **98**, 615–635.

Westerblad, H. & Allen, D. G. (1992). Changes of intracellular pH due to repetitive stimulation of single fibres from mouse skeletal muscle. *Journal of Physiology* **449**, 49–71.

Westerblad, H., Lee, J. A., Länneergren, J. & Allen, D. G. (1991). Cellular mechanisms of fatigue in skeletal muscle. *American Journal of Physiology* **261**, C195–209.

Yoshizaki, K., Seo, Y., Nishikawa, H. & Morimoto, T. (1982). Application of pulsed-gradient ^{31}P NMR on frog muscle to measure the diffusion rates of phosphorus compounds in cells. *Biophysical Journal* **38**, 209–211.

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

We thank Mrs R. Cafarella for expert technical assistance. This work was supported by a NH & MRC C. J. Martin Fellowship (M. W. F.), NH & MRC Project Grant and the ARC.

Received 29 July 1993; accepted 8 June 1994.