Effects of creatine phosphate and inorganic phosphate on the sarcoplasmic reticulum of saponin-treated rat heart

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1. Ventricular trabeculae from rat heart were permeabilized by treatment with saponin. In the presence of 150 nM Ca^{2+}, application of 20 mM caffeine released Ca^{2+} from the sarcoplasmic reticulum (SR), resulting in a transient contracture. Ca^{2+} released from the SR was detected using fura-2 fluorescence. The amplitudes of the caffeine-induced Ca^{2+} transients were used to assess SR Ca^{2+} content.

2. In the absence of creatine phosphate (CP), introduction of 5–30 mM inorganic phosphate (P_i) caused a net release of Ca^{2+} from the SR. Subsequent caffeine-induced Ca^{2+} and tension transients were smaller in the presence of P_i. Under these conditions, 30 mM P_i decreased the caffeine-induced Ca^{2+} transients by $45 \pm 3{\cdot}1\%$ (mean ± S.D., $n = 14$). On removal of P_i, the [Ca^{2+}] transiently decreased and the caffeine-induced Ca^{2+} transients returned to control levels over 4–6 min.

3. In the presence of CP (5–15 mM), the Ca^{2+} transients were unaffected by the introduction of P_i (5–30 mM) or slightly increased in amplitude. P_i (30 mM) significantly increased the caffeine-induced Ca^{2+} transients by $7 \pm 8{\cdot}8\%$ (mean ± S.D., $n = 19$, $P < 0{\cdot}05$) in the presence of 15 mM CP. The release of Ca^{2+} on addition of P_i and decrease in [Ca^{2+}] on P_i withdrawal was less pronounced or absent completely in the presence of CP. The inhibitory effects of P_i on caffeine-induced Ca^{2+} release became apparent as the [CP] was decreased from 5 to 0 mM

4. In the presence of the creatine phosphokinase inhibitor dinitro-fluorobenzene (DNFB) the effects of P_i (in the presence of CP) were qualitatively similar to the results obtained in the absence of CP, although the decrease in caffeine-induced Ca^{2+} release was less pronounced.

5. These results suggest that the rise in $[P_i]_i$ during ischaemia or anoxia will have little effect on the regulation of Ca^{2+} by the SR while the $[CP]_i$ remains above 5 mM. However, as the [CP] decreases below 5 mM, the accumulation of P_i within the cytosol will progressively reduce the SR Ca^{2+} content. CP may act in conjunction with endogenous creatine phosphokinase to modify the response of the SR to P_i, and possible mechanisms are considered.

Studies using ^{31}P-nuclear magnetic resonance have shown that changes in the intracellular concentration of phosphate metabolites occur under a variety of conditions in cardiac muscle. In the first few minutes of anoxia or global ischaemia, inhibition of aerobic metabolism is accompanied by a pronounced decrease in contractile force (reviewed by Allen & Orchard, 1987). During this phase, the intracellular concentration of ATP ($[ATP]_i$) remains constant as ADP is rephosphorylated by CP, catalysed by creatine phosphokinase. The $[CP]_i$ decreases rapidly from 15–20 mM to undetectable levels and $[P_i]_i$ increases concomitantly from about 2 to 20 mM. After depletion of CP, ATP synthesis continues via anaerobic glycolysis, resulting in lactic acid production and a decrease in intracellular pH (pH_i). With more prolonged periods of global ischaemia, anaerobic glycolysis ceases and a further increase in $[P_i]_i$ occurs due to the net hydrolysis of ATP. Less marked changes in $[P_i]_i$ and $[CP]_i$ may also occur under physiological conditions in response to increased cardiac work, although the extent and significance of these effects remain controversial (for review see Heinemann & Balaban, 1990). Studies on both isolated and *in vivo* hearts suggest that under steady-state conditions, $[P_i]_i$ and $[CP]_i$ change by less than 2 mM during inotropic interventions such as β-adrenergic stimulation and increases in rate (Hoerter, Miceli, Renlund, Jacobus, Gerstenblith & Lakatta, 1986; Katz, Swain & Portman, 1989; Elliott, Smith & Allen, 1994). However, transient reciprocal changes in $[P_i]$ and [CP] of 2–7 mM have been reported following increases in stimulation rate in Langendorff perfused ferret hearts (e.g. Elliott *et al.* 1994).

Recent studies on 'skinned' cardiac preparations have suggested that the increase in $[P_i]_i$ during anoxia or ischaemia may influence the regulation of Ca^{2+} by the sarcoplasmic reticulum (SR). Zhu & Nosek (1991) reported that solutions mimicking the intracellular milieu during anoxia, decreased caffeine-induced Ca^{2+} release from the SR in saponin-treated ventricular trabeculae. This was predominantly attributed to an inhibitory action of P_i on Ca^{2+} uptake by the SR. In a subsequent study, Ca^{2+} release from the SR was measured directly in saponin-treated fibres, allowing the effects of P_i to be investigated in more detail (Smith & Steele, 1992). It was found that introduction of P_i (10–30 mM) released Ca^{2+} from the SR and abolished spontaneous Ca^{2+} release observed at a high bathing $[Ca^{2+}]$. Furthermore, P_i decreased the amount of Ca^{2+} released on application of caffeine, consistent with a reduction in the Ca^{2+} content of the SR. In the presence of 20–30 mM P_i, caffeine-induced Ca^{2+} transients decreased in amplitude by as much as 40–50%. However, the Ca^{2+} content of the SR was also significantly reduced at lower $[P_i]$ within the range 2–10 mM.

These results suggest that the rise in $[P_i]_i$ during the early stages of ischaemia or anoxia will progressively decrease the amount of Ca^{2+} available for release from the SR during systole. In addition, both resting levels of P_i (1–2 mM) and the transient changes in $[P_i]_i$ (over the range 2–7 mM) that may occur during increases in cardiac work, would be expected to affect SR function. However, in the present study we report that the inhibitory effects of P_i on the SR are reversed by CP. Consequently, the response of the SR to an increase in $[P_i]_i$ may depend critically on the cytosolic [CP]. The mechanism underlying the interaction between P_i and CP and the possible consequences of this effect are considered in relation to the events that occur during ischaemia or anoxia. Preliminary results have been presented to the Physiological Society (Steele, McAinsh & Smith, 1994*b*).

METHODS

Sprague–Dawley rats (200–250 g) were killed by a blow to the head and cervical dislocation. Hearts were removed rapidly and bathed in Tyrode solution (Table 1). Free running trabeculae (80–120 μm diameter and 1–2 mm in length) were dissected from the right ventricle. The preparation was permeabilized by exposure to solution C containing 50 μg ml^{-1} saponin for 30 min. Saponin treatment renders the sarcolemma permeable to small ions and molecules without disrupting SR function. Saponin was then removed by washing the preparation in solution C before proceeding with the experiment. All experiments were carried out at room temperature (22–23 °C).

Solution composition

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Corporation (UK). H^+ and Ca^{2+} were buffered with Hepes (Boehringer Mannheim) and EGTA respectively. All solutions used after saponin treatment contained ATP to support the activity of the SR and myofilaments. The basic solution compositions are given in Table 1. Ca^{2+} release from the SR was detected in solutions weakly Ca^{2+}-buffered with EGTA containing 4 μM fura-2 (Calbiochem, UK). Calcium chloride (1 M titration standard, BDH, UK) was added to solution A to obtain $[Ca^{2+}]$ greater than 0·08 μM. The $[Ca^{2+}]$ of bathing solutions was calculated from the fluorescence ratio, measured continuously during experiments. Under the conditions of this study, the apparent affinity constant of fura-2 for Ca^{2+} was 400 ± 20 nM ($n = 4$). This value was calculated by measuring the fluorescence ratio in strongly Ca^{2+}-buffered solutions over a range of $[Ca^{2+}]$. The equilibrium concentrations of metal ions in the calibration solutions were calculated using the affinity constants for H^+, Ca^{2+} and Mg^{2+} for EGTA taken from Miller & Smith (1984). The affinity constants for ATP and ADP are those quoted by Fabiato & Fabiato (1979). Corrections for ionic strength, details of pH measurement, allowance for EGTA purity and the principles of the calculations are given in Miller & Smith (1984). In some experiments, 5 mM azide (BDH) was included in the solutions to inhibit possible mitochondrial activity. However, azide had no apparent influence on the effects of P_i and CP reported in this study.

Simultaneous measurement of SR Ca^{2+} release and tension

The apparatus for simultaneous measurement of tension and SR Ca^{2+} release has been described previously (Smith & Steele, 1992). Briefly, trabeculae were mounted between a tension transducer (Akers AE801, SensoNor, Norway) and a fixed support by means of monofilament snares in stainless steel tubes. A Perspex column (5 mm diameter) was lowered to within 5–10 μm of the upper surface of the muscle to minimize the volume of the solution above the preparation. The volume of solution surrounding the preparation was approximately 6 μl. Throughout the experiment, the muscle was perfused by pumping solution through the central bore of the column at 1 ml min^{-1}. The perfusing solution was switched using a series of valves positioned immediately above the column which allowed the bath volume to be exchanged within 10–15 s. Solutions with caffeine were rapidly injected via ducts close to the base of the column. A solenoid-controlled pneumatic system was used to deliver a constant volume of solution (~100 μl) during each 50 ms injection. Using this method, the caffeine concentration of the solution bathing the muscle was increased to 50% of the concentration injected within 8 ms.

The bath was placed on the stage of a Nikon Diaphot inverted microscope. A regular sarcomere pattern was clearly visible under ×40 magnification, and sarcomere length was set at approximately 2·2 μm. Throughout the rest of the experiment, the muscle was viewed via a ×20 Fluor objective lens (Nikon CF Fluor, NA 0·75). The preparation was alternately illuminated with light of wavelengths 340 and 380 nm at 50 Hz frequency using a spinning wheel spectrophotometer (Cairn Research, Sittingbourne, UK). The average $[Ca^{2+}]$ within the visual field containing the preparation was indicated by the ratio of light intensities emitted at >500 nm. Light emitted from areas of the field not occupied by the image of the muscle was reduced using a variable rectangular window on the side port of the microscope.

Data recording and analysis

The ratio signal, individual wavelength intensities and the tension signals were low-pass filtered (−3 dB at 30 Hz) and recorded on tape for later analysis. Data are presented as mean values ± S.D. Where necessary, statistical significance was determined using Student's t test (MINITAB release 7).

Table 1. Composition of solutions

Solution	$[K^+]$	$[Na^+]$	$[Mg^{2+}]$	$[Ca^{2+}]$	[ATP]	[EGTA]	[Hepes]	$[Cl^-]$	$[HPO_4^-]$	[CP]	[ADP]
A	130	40	6	0·04	5	0·2	25	142	—	—	—
B	130	40	7·2	0·04	5	0·2	25	99·4	30	—	—
C	130	40	6	0·04	5	0·2	25	112	—	15	—
D	130	40	7·2	0·04	5	0·2	25	69·4	30	15	—
E	130	40	1·5	0·04	—	0·2	25	133	—	—	—
F	130	40	1·57	0·04	—	0·2	25	103·1	—	15	0·5
G	130	40	6	0·04	5	0·2	25	112	—	20	—
H	130	40	6·8	0·04	5	0·2	25	114·4	20	—	—
Tyrode	5	120	1·5	2	—	—	25	132	—	—	—

All concentrations are millimolar unless otherwise stated. Free $[Mg^{2+}]$ and $[Ca^{2+}]$ were calculated as 1·5 and 0·15 mM, respectively, in solutions A to F.

RESULTS

Effects of P_i on caffeine-induced Ca^{2+} release in the absence and presence of CP

Figure 1*A* shows the effect of P_i on caffeine-induced Ca^{2+} release in the absence of CP. Throughout this protocol, the saponin-treated trabecula was perfused with weakly Ca^{2+}-buffered solutions (solutions A and B, Table 1) including fura-2 (4 μM). Simultaneous records of the fluorescence ratio (upper panel) and isometric tension (lower panel) are shown. The free $[Ca^{2+}]$ of the perfusing solution was below that required to produce Ca^{2+}-activated force (150 nM). A supramaximal dose of caffeine (20 mM) was rapidly applied for 50 ms duration as indicated (▲). Each application resulted in a transient increase in the fluorescence ratio due to Ca^{2+} release from the SR and a corresponding tension response. A series of control responses was obtained by injection of caffeine at 2 min intervals and the amplitude of each caffeine-induced Ca^{2+} transient was used to assess the Ca^{2+} content of the SR.

Introduction of 20 mM P_i, between applications of caffeine, caused a transient release of Ca^{2+} from the SR. Subsequent caffeine-induced Ca^{2+} transients in the presence of P_i were reduced in amplitude, consistent with a reduction in the Ca^{2+} content of the SR. P_i (20 mM) decreased the size of the caffeine-induced Ca^{2+} transients by $45 \pm 3{\cdot}1\%$ (mean ± S.D., $n = 14$) of control values. Tension transients were also smaller in the presence of P_i. This reflects both the decrease in Ca^{2+} release from the SR and a direct depressive effect of P_i on tension generation by the myofilaments (Kentish, 1986). On removal of P_i, the $[Ca^{2+}]$ transiently decreased, presumably due to a net accumulation of Ca^{2+} by the SR. Thereafter, the caffeine-induced responses returned to control levels over two to three load and release cycles. These effects of P_i in the absence of CP are similar to those reported previously in saponin-treated ventricular trabeculae (Smith & Steele, 1992).

Figure 1*B* shows responses obtained in the constant presence of 15 mM CP. Again, caffeine was applied at 2 min intervals until reproducible Ca^{2+} and tension transients were obtained and 20 mM P_i was introduced between the third and fourth responses. However, the transient release of Ca^{2+} on introduction of P_i was much reduced or (as in this example) absent completely in solutions containing CP. Furthermore, the Ca^{2+} transients in the presence of P_i were generally slightly increased in amplitude. Despite the increase in caffeine-induced Ca^{2+} release, the associated tension responses were smaller in the presence of P_i due to the decreased responsiveness of the myofilaments to Ca^{2+}. However, the decrease in the tension transients was less pronounced than in the absence of CP (e.g. Fig. 1*A*) when *both* myofilament Ca^{2+} sensitivity and caffeine-induced Ca^{2+} release were markedly reduced.

Cumulated data showing the relationship between $[P_i]$ and the Ca^{2+} content of the SR in the presence and absence of 15 mM CP are given in Fig. 2. The abscissa indicates $[P_i]$ and the ordinate the mean (± S.D.) steady-state amplitude of caffeine-induced Ca^{2+} transients expressed as a percentage of control values. In the absence of CP (●), P_i induced a concentration-dependent decrease in the Ca^{2+} content of the SR. P_i (2 and 30 mM) decreased the Ca^{2+} transients by about 10 and 45%, respectively. In contrast, in the presence of CP (▼), increasing the $[P_i]$ caused a slight increase in the caffeine-induced Ca^{2+} transients which was statistically significant at 30 mM P_i ($P < 0{\cdot}05$).

Effect of CP in the presence of 30 mM P_i

The interaction between CP and P_i was studied in more detail using the protocol shown in Fig. 3*A*. Again, the muscle was initially perfused with solutions lacking CP, and caffeine was applied at 2 min intervals. As shown in Fig. 1*A*, introduction of P_i caused a transient release of Ca^{2+} from the SR. Thereafter, the caffeine-induced Ca^{2+} transients in the presence of P_i were markedly reduced in amplitude. Addition of 1 mM CP increased the amplitude of the Ca^{2+} transients by approximately 30%. A further increase in [CP] to 5 mM returned the Ca^{2+} transients to near control levels. The increase in Ca^{2+} release following addition of 5 mM CP approximately doubled the associated tension responses. In contrast, introduction of CP in the absence of P_i had no significant effect on caffeine-induced Ca^{2+} release (not shown).

Cumulated data obtained using this protocol are shown in Fig. 3*B*. The ordinate indicates the mean (± S.D.) amplitude of caffeine-induced Ca^{2+} (□) and tension (▲) transients in the presence of 30 mM P_i, expressed as a percentage of controls (obtained in the absence of P_i). Increasing the [CP] induced a concentration-dependent increase in the amplitude of the caffeine-induced Ca^{2+} and tension transients. In the presence of 5 mM CP, the caffeine-induced Ca^{2+} transients were not significantly different from controls. However, a further rise in [CP] to 10 and 20 mM consistently increased the Ca^{2+} transients to slightly above control levels.

Reciprocal changes in $[P_i]$ and [CP]

Figures 1, 2 and 3 show that CP modifies the response of the SR to P_i. This is relevant to the events that occur during the early stages of anoxia or ischaemia. In these circumstances, $[P_i]_i$ increases from about 2 to 20 mM and $[CP]_i$ decreases concomitantly from 18–20 mM to undetectable levels. Figure 4 shows the effects of reciprocal changes in $[P_i]$ and [CP] on caffeine-induced Ca^{2+} release. The first three control responses were obtained in the presence of 20 mM CP and in the absence of P_i. Simultaneous stepwise increases in $[P_i]$ to 15 mM and decreases in [CP] to 5 mM had little effect or slightly increased caffeine-induced Ca^{2+}

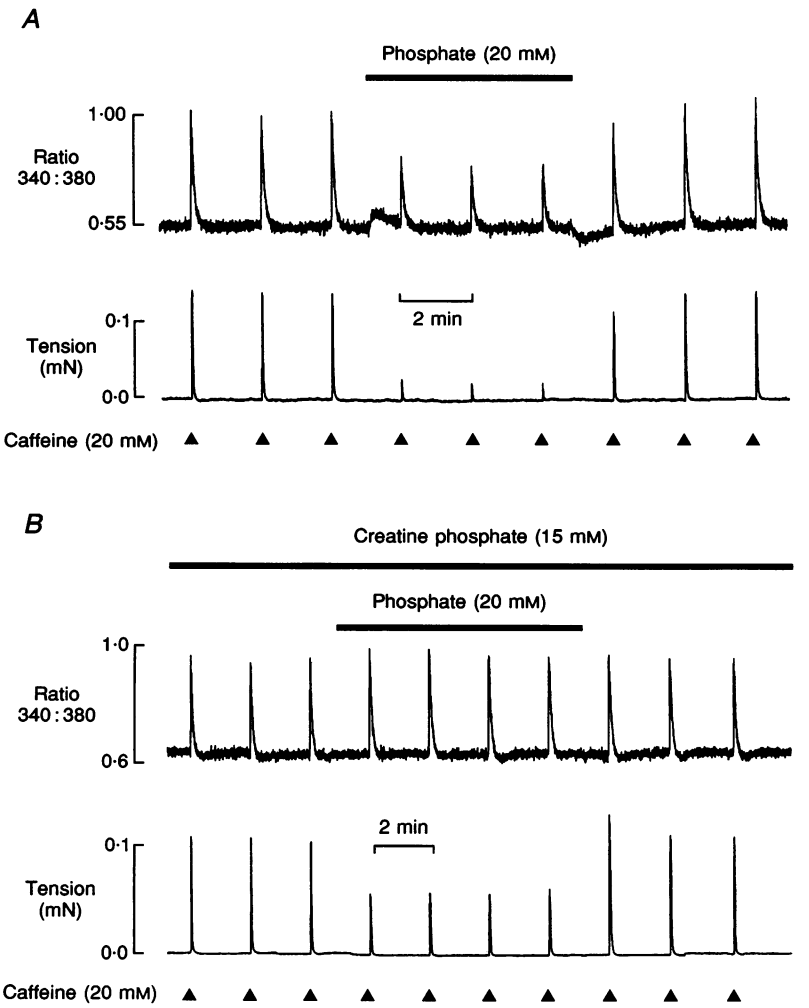

Figure 1. Effects of P_i in the presence and absence of CP

A, effects of 20 mM P_i in the absence of CP. Simultaneous records of fluorescence ratio (upper panel) and isometric tension (lower panel) are shown. The trabecula was initially perfused with solution A. Caffeine (20 mM) was rapidly applied at 2 min intervals (▲) until stable Ca^{2+} and tension transients were obtained. The solution was changed to one with 20 mM P_i (obtained by mixing solutions A and B) as indicated. This resulted in a transient release of Ca^{2+} from the SR, followed by a decrease in the caffeine-induced Ca^{2+} and tension responses. On removal of P_i, the $[Ca^{2+}]$ transiently decreased and the caffeine-induced responses returned to control levels. *B*, typical results obtained in the presence of CP (15 mM). The preparation was initially perfused with solution C. After three control responses, the solution was changed to one containing 20 mM P_i (obtained by mixing solutions C and D). This resulted in a slight increase in the caffeine-induced Ca^{2+} release which was reversible on removal of P_i.

release. However, a further increase in $[P_i]$ to 20 mM and a decrease in [CP] to 0 mM resulted in a marked reduction in the amplitude of the caffeine-induced Ca^{2+} transients to about 50% of control values. Similar results were obtained in three other preparations.

Complete withdrawal of CP was frequently associated with a small maintained increase in the resting tension as reported in previous studies on fibres skinned with Triton X-100 (Ventura-Clapier & Vassort, 1985). It is unlikely that this is due to formation of rigor cross-bridges because the response was not inhibited by raising the bathing [ATP] to 10–15 mM or enhanced by decreasing the [ATP] to 1·0 mM (not shown). The increase in resting tension probably results from local accumulation of ADP within the fibre which has been shown to increase myofilament Ca^{2+} sensitivity and maximum Ca^{2+}-activated force by prolonging cross-bridge attachment (Hoar, Mahoney & Kerrick, 1987; Smith & Steele, 1992).

Evidence for endogenous creatine phosphokinase activity in saponin-treated trabeculae

Under physiological conditions, CP rephosphorylates ADP to ATP, catalysed by creatine phosphokinase. Therefore, CP may act via creatine phosphokinase to modify the response of the SR to P_i. However, in this study creatine phosphokinase was not included in the perfusing solutions and it is unclear from previous work to what extent the activity of endogenous enzymes persist after saponin treatment.

The protocol shown in Fig. 5 provides evidence that creatine phosphokinase activity is present after saponin treatment. The preparation was initially perfused with a solution containing 5 mM ATP to support the activity of the SR and myofilaments. The bathing solution was then changed to one lacking ATP which resulted in development of a maintained rigor contracture (lower panel). ATP withdrawal was also associated with a small transient release of Ca^{2+} from the SR (upper panel) similar to that reported previously (Steele & Smith, 1993). Caffeine (20 mM) was then applied briefly (50 ms duration) to release Ca^{2+} accumulated by the SR during exposure to the previous ATP-containing solution. As the myofilaments were in rigor, the transient increase in $[Ca^{2+}]$ did not produce a corresponding Ca^{2+}-activated tension response. The brief upward deflections in the tension record in the absence of ATP are artifacts of rapid caffeine application. After the $[Ca^{2+}]$ returned to control levels, re-addition of caffeine failed to release Ca^{2+} from the SR confirming that (i) the caffeine-accessible pool of Ca^{2+} within the SR was depleted and (ii) the perfusing solution contained insufficient ATP to support the reaccumulation of Ca^{2+} by the SR. The solution was then changed to one with 15 mM CP and 0·5 mM ADP. This produced a rapid relaxation of tension and a transient decrease in $[Ca^{2+}]$ within the trabecula, consistent with a net accumulation of Ca^{2+} by the SR. Thereafter, application of caffeine at 2 min intervals induced transient Ca^{2+} and tension responses similar to those obtained under control conditions in the presence of 5 mM ATP (e.g. Fig. 1*A*). Introduction of 0·5 mM ADP alone (without CP) induces relaxation of the myofilaments or supports SR Ca^{2+} uptake (not shown). Similarly, ADP and CP were ineffective in the presence of the creatine phosphokinase inhibitor dinitro-fluorobenzene (DNFB). This confirms that ATP production by alternative pathways (e.g. myokinase) is not significant. Furthermore,

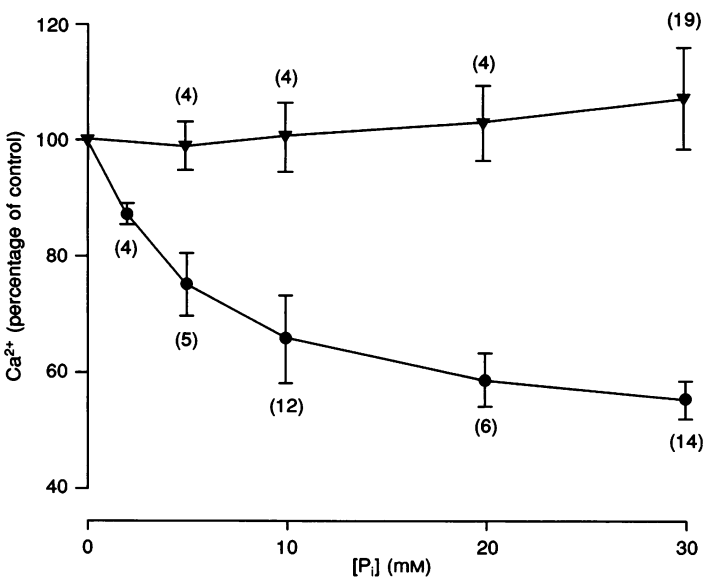

Figure 2. Cumulated data showing the effects of increasing $[P_i]$ on the amplitude of caffeine-induced Ca^{2+} transients in the presence (▼) and the absence (●) of 15 mM CP

Each point represents the mean (± S.D.) expressed as a percentage of the control response. Number of preparations in each case is indicated in parentheses.

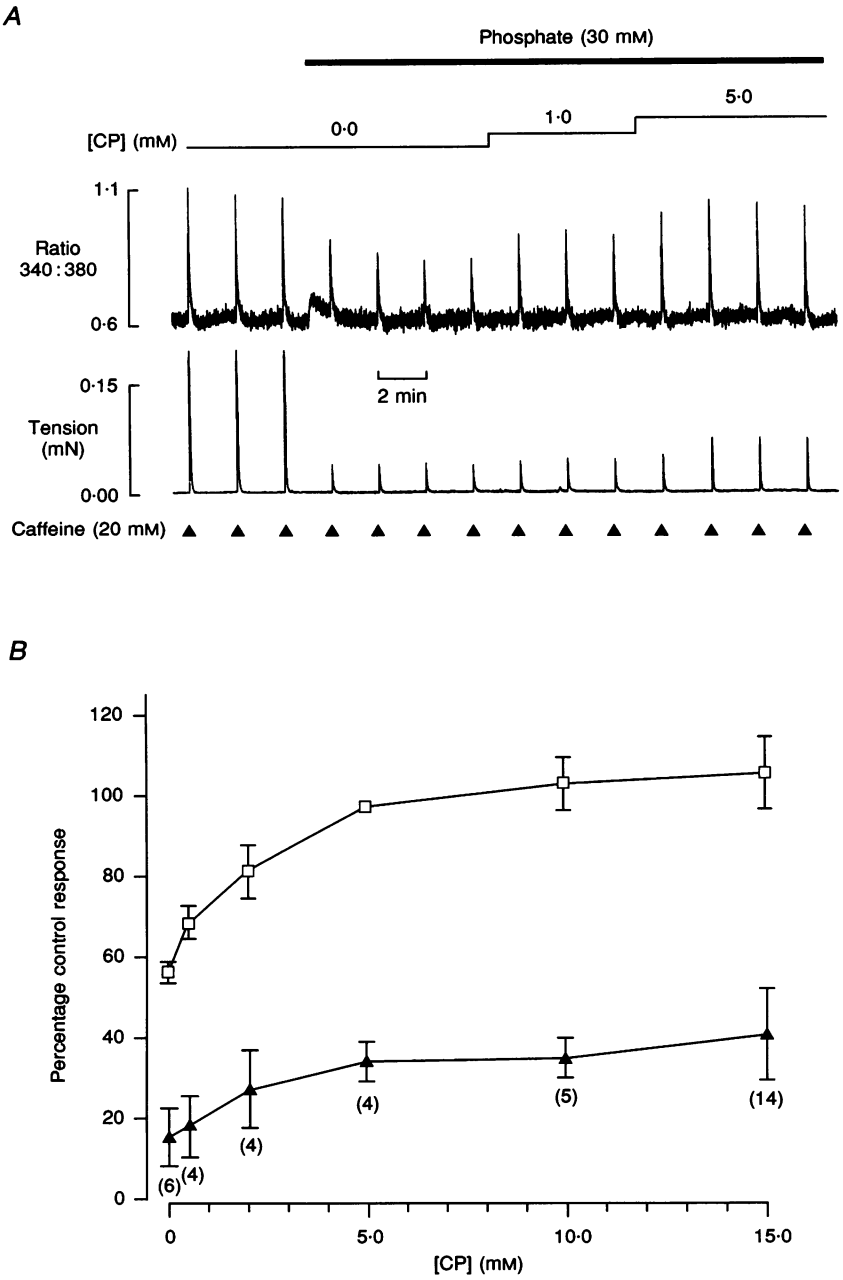

Figure 3. Cumulative addition of CP in the presence of P_i

A, introduction of CP in the presence of 30 mM P_i. The preparation was initially perfused with a solution lacking CP and P_i (solution A) and caffeine was applied at 2 min intervals. Introduction of 30 mM P_i (solution B) induced a release of Ca^{2+} followed by a decrease in caffeine-induced Ca^{2+} release. Once the caffeine-induced Ca^{2+} and tension transients reached a steady state in the presence of P_i, [CP] was increased to 1 and then 5 mM. This produced an increase in the caffeine-induced Ca^{2+} and tension transients. CP concentrations less than 15 mM were obtained by mixing solutions B and D. *B*, cumulated data showing the effect of [CP] on caffeine-induced Ca^{2+} (□) and tension (▲) transients in the presence of P_i (30 mM) obtained using the protocol shown in *A*. Each point represents the mean (± S.D.) expressed as a percentage of the control response. The number below each point is the number of preparations.

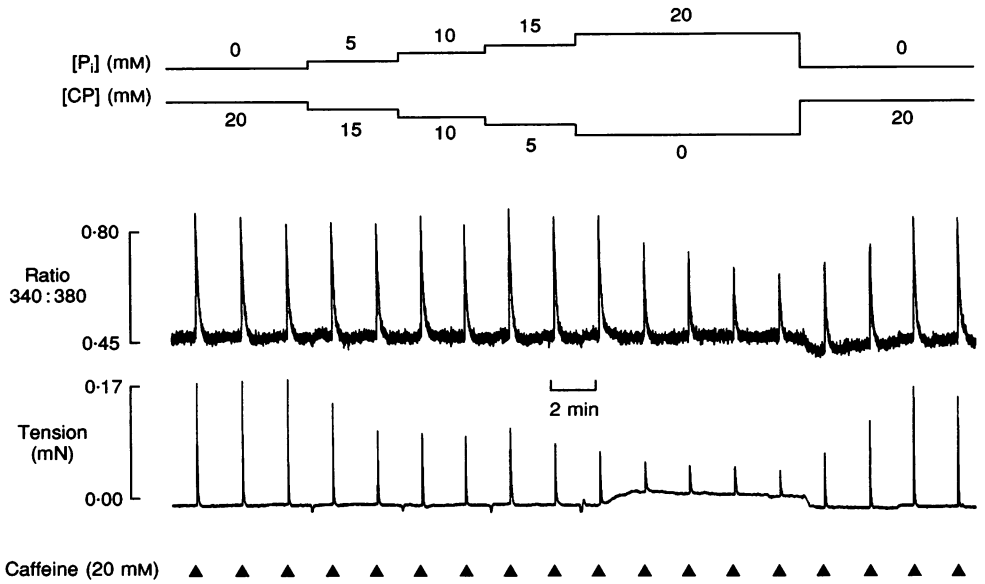

Figure 4. Reciprocal changes in $[P_i]$ and [CP]

Simultaneous records of fluorescence ratio (upper panel) and isometric tension (lower panel) are shown. The trabecula was initially perfused with solution G and caffeine was briefly applied at 2 min intervals. The $[P_i]$ was then increased (0 to 20 mM) and the [CP] simultaneously decreased (20 to 0 mM) in a stepwise manner as indicated. [CP] and $[P_i]$ ratios were obtained by mixing solutions G and H.

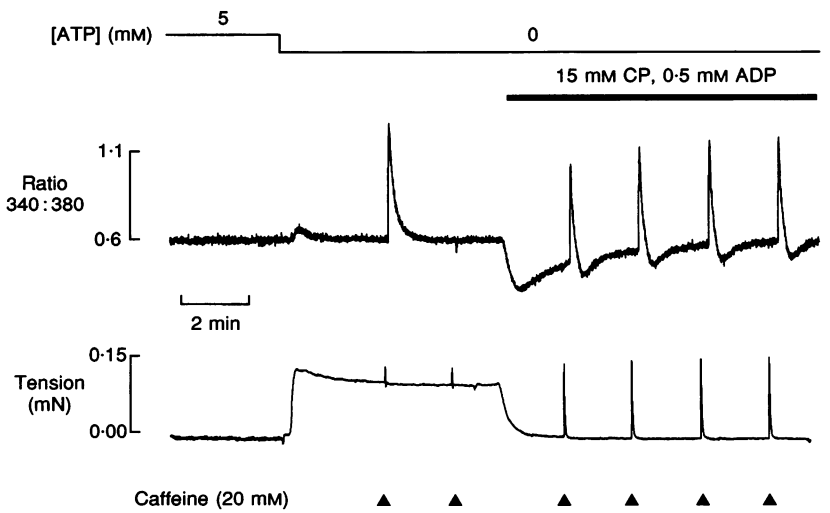

Figure 5. Myofilament and SR activity supported by ATP generated from ADP and CP

Simultaneous records of fluorescence ratio (upper panel) and isometric tension (lower panel) are shown. The preparation was initially perfused with solution A. The perfusing solution was then changed to solution E without ATP, resulting in the development of a rigor contracture (lower panel). Caffeine was rapidly applied as indicated (▲) to release Ca^{2+} from the SR (upper panel). Introduction of solution F containing 0·5 mM ADP and 15 mM CP induced relaxation of the rigor contracture. Thereafter, application of caffeine induced transient Ca^{2+} and tension responses.

the protocol suggests that the activity of the SR and myofilaments can be supported by ATP produced entirely by the rephosphorylation of ADP, catalysed by endogenous creatine phosphokinase. Similar results were obtained in four other preparations.

Responses obtained following inhibition of creatine phosphokinase

The experiment shown in Fig. 6 suggests that modification of the SR response to P_i may involve the interaction of CP with endogenous creatine phosphokinase. Throughout this protocol the preparation was perfused with a solution including 15 mM CP, which is normally sufficient to completely abolish the inhibitory effects of P_i on the SR (Figs 1*B*, 2 and 4). However, the solutions also contained DNFB to inhibit endogenous creatine phosphokinase activity. In the presence of 0·5 mM DNFB, introduction of 20 mM phosphate consistently decreased caffeine-induced Ca^{2+} transients (by 28 ± 2·4%, mean ± S.D., $n = 4$). Furthermore, introduction of P_i was associated with a transient increase and withdrawal of P_i, and a transient decrease in $[Ca^{2+}]$ similar to that observed in the absence of CP (e.g. Fig. 1*A*). The mean reduction in the caffeine-induced Ca^{2+} transient was less than that in the complete absence of CP, which may result from incomplete inhibition of creatine phosphokinase. In most preparations DNFB also affected the myofilaments, resulting in a gradual increase in resting tension and prolongation of the tension transients (lower panel). This suggests that, in addition to the known inhibitory effects on creatine phosphokinase, DNFB also has a deleterious action on the contractile apparatus.

DISCUSSION

In previous studies it has been suggested that early contractile failure during ischaemia or anoxia results from accumulation of P_i and H^+ in the cytosol (Allen & Orchard, 1987). Both P_i and low pH reduce myofilament Ca^{2+} sensitivity and maximum tension generated at a saturating $[Ca^{2+}]$ (Fabiato & Fabiato, 1978; Kentish, 1986). Acidosis may also decrease the supply of Ca^{2+} to the myofilaments during systole by inhibiting the uptake and release of Ca^{2+} from the SR (Fabiato & Fabiato, 1978). More recently it has been shown that increases in $[P_i]$ within the range 2–30 mM can markedly reduce the Ca^{2+} content of the SR (Zhu & Nosek, 1991; Smith & Steele, 1992). Based on these results, the rise in $[P_i]_i$ during the early stages of ischaemia or anoxia would be expected to contribute to contractile failure by decreasing the amount of Ca^{2+} available for release from the SR during systole. Furthermore, smaller transient changes in $[P_i]_i$ that occur following increases in cardiac work might also affect SR function.

Effects of P_i on caffeine-induced Ca^{2+} release in the presence and absence of CP

Figures 1*A* and 2 confirm previous reports that P_i (2–30 mM) induces a concentration-dependent reduction in the Ca^{2+} content of the SR. However, in this study we have shown that the effects of P_i are modified by CP. In the presence of 15 mM CP, raising the $[P_i]$ over the range 2–30 mM progressively increased the Ca^{2+} content of the SR (Figs 1*B* and 2). The inhibitory effect of 30 mM P_i on Ca^{2+} release was almost completely reversed by addition of 5 mM CP and partial reversal was obtained with 0·5–5 mM CP

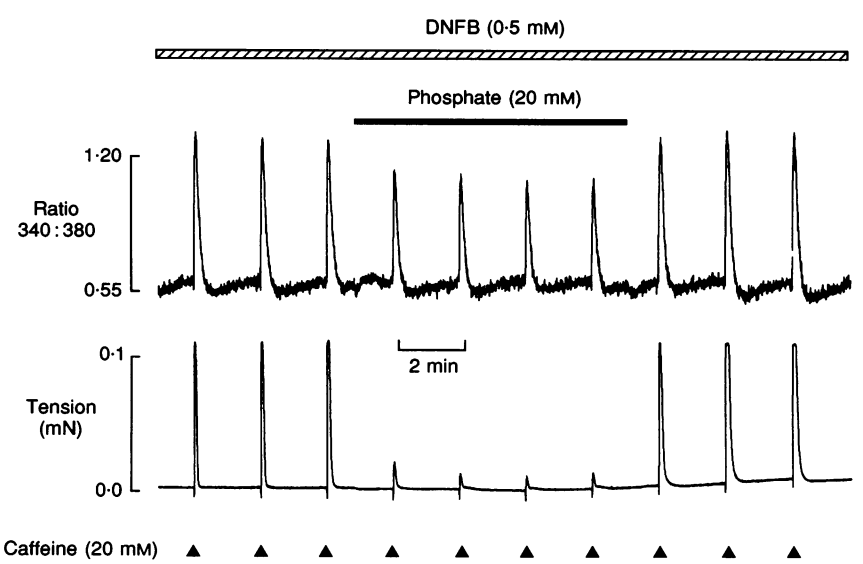

Figure 6. Effects of 20 mM P_i following inhibition of creatine phosphokinase

Simultaneous records of fluorescence ratio (upper panel) and isometric tension (lower panel) are shown. The muscle was initially perfused with solution C containing 15 mM CP and 20 mM caffeine applied at 2 min intervals. P_i (20 mM) was introduced as indicated. All solutions contained 0·5 mM DNFB.

(Fig. 3*A* and *B*). The protocol shown in Fig. 4 was designed to mimic the reciprocal changes in $[P_i]_i$ and $[CP]_i$ that occur during ischaemia or anoxia in cardiac muscle. Stepwise increases in $[P_i]$ over the range 0–15 mM and decreases in [CP] from 20 to 5 mM had no effect or slightly increased caffeine-induced Ca^{2+} release. However, a further rise in the $[P_i]$ from 15 to 20 mM and decrease in [CP] from 5 to 0 mM induced a marked reduction in the Ca^{2+} content of the SR similar to that shown in Fig. 1*A*. These results suggest that P_i will have no effect or may slightly increase the Ca^{2+} content of the SR while the cytosolic [CP] remains above 5 mM. However, if the [CP] decreases below this level, P_i will markedly reduce the amount of Ca^{2+} available for release from the SR.

Possible effects of P_i and CP during ischaemia, anoxia or metabolic inhibition

One consequence of these results is that transient increases in $[P_i]_i$ (over the range 2–7 mM) that may occur following changes in cardiac work are unlikely to influence SR function as the $[CP]_i$ constantly remains above 5 mM (Elliott *et al.* 1994). However, the accumulation of P_i within the cytosol might influence SR function during ischaemia, anoxia or metabolic blockade, when the $[CP]_i$ can decrease to less than 0·5 mM. Developed pressure is abolished completely within 5–10 min of global ischaemia and during this period, the $[CP]_i$ falls to about 5 mM (Koretsune & Marban, 1990; Elliott *et al.* 1992). The results shown in Fig. 4 suggest that P_i will have no effect or may slightly increase the Ca^{2+} content of the SR under these conditions. Consequently, a P_i-induced reduction in SR Ca^{2+} release is unlikely to contribute to the initial rapid decline in developed force during global ischaemia. However, with longer periods of ischaemia, P_i may reduce the Ca^{2+} content of the SR as cytosolic CP is further depleted (Figs 1*B* and 4). During anoxia or exposure to cyanide, developed pressure decreases to about one-third of normal but can continue at this level for prolonged periods (> 30 min). The $[CP]_i$ rapidly decreases (Smith, Donoso, Bauer & Eisner, 1993) to undetectable levels (< 0·5 mM) and under these conditions the accumulation of P_i would be expected to progressively reduce the Ca^{2+} content of the SR, contributing to the reduction in force.

Any change in the SR Ca^{2+} content during metabolic inhibition might produce a corresponding change in the systolic Ca^{2+} transient. However, previous studies involving measurement of intracellular $[Ca^{2+}]_i$ in cardiac muscle have produced conflicting results which may reflect the influence of several factors with opposing effects on the systolic Ca^{2+} transient. In particular, a marked intracellular acidosis develops during global ischaemia due to accumulation of anaerobically produced lactic acid. It has been shown that acidosis inhibits the uptake and release of Ca^{2+} from the SR, decreases the affinity of troponin-C for Ca^{2+} and raises diastolic $[Ca^{2+}]_i$ (for review see Orchard & Kentish, 1990). The net result of these actions is an increase in the intracellular Ca^{2+} transient at low pH. Therefore, the acidosis that occurs during ischaemia may dominate other changes in the intracellular environment producing the reported increase in peak systolic $[Ca^{2+}]$ (Allen, Lee & Smith, 1989; Kihara, Grossman & Morgan, 1989; Koretsune & Marban, 1990; Mohabir, Lee, Kurz & Clusin, 1991). However, during anoxia or exposure to cyanide the intracellular acidosis is less pronounced as lactic acid is extruded from cells into the perfusate. Under these conditions, a decrease in the intracellular Ca^{2+} transient has been reported in a variety of cardiac preparations, particularly when anaerobic glycolysis is prevented (Allen & Orchard, 1983; MacKinnon, Gwathemy & Morgan, 1987; Lee & Allen, 1988; Smith & Allen, 1988; Stern *et al.* 1988; Kihara *et al.* 1989; Northover, 1990; Goldhaber, Parker & Weiss, 1991). There is evidence to suggest that a reduction in action potential duration may underlie this effect (Stern *et al.* 1988). However, the present results suggest that accumulation of P_i will markedly reduce the Ca^{2+} content of the SR under conditions where $[CP]_i$ is depleted, which may also contribute to the decrease in systolic $[Ca^{2+}]$.

The inhibitory effects of P_i on SR function

In previous studies, the reduction in the SR Ca^{2+} content in the presence of P_i has been ascribed to inhibition of net Ca^{2+} uptake (Zhu & Nosek, 1991; Smith & Steele, 1992). This has been demonstrated in isolated SR vesicles and in saponin-skinned cardiac preparations where P_i reduced both the rate and steady-state amount of Ca^{2+} accumulated by the SR (Perlitz, Long, Adams & Nosek, 1990; Steele *et al.* 1994*a*). However, a reduction of net Ca^{2+} uptake could result from activation of Ca^{2+} efflux or inhibition of the Ca^{2+} pump and the mechanism of action remains to be established.

One possible explanation for the inhibitory effects of P_i is that Ca^{2+} uptake by the SR is reduced due to a decrease in the free energy of ATP hydrolysis. The energy available from the hydrolysis of ATP (ΔG_{ATP}) and therefore the maximum theoretical $[Ca^{2+}]$ gradient that can be maintained across the SR membrane is dependent upon the [ATP] and products of hydrolysis (for a more complete discussion see Smith & Steele, 1992). Raising the $[P_i]$ decreases ΔG_{ATP} which may in turn reduce the amount of Ca^{2+} available for release by caffeine. Increasing the [ADP] or decreasing the [ATP] should have a similar effect on ΔG_{ATP} and therefore the Ca^{2+} content of the SR. However, ADP decreases the Ca^{2+} content of the SR by more than P_i (Smith & Steele, 1992). Furthermore, the SR Ca^{2+} content was unaffected by increasing the bathing [ATP] from 0·5 to 10 mM while similar changes in $[P_i]$ (and hence ΔG) decreased the caffeine-induced Ca^{2+} transients by approximately 25% (D. S. Steele & A. M. McAinsh, unpublished observations). These results suggest that a simple explanation for the effects of P_i based on free energy is not appropriate.

The present study on saponin-skinned trabeculae appears more consistent with previous reports describing the action

of P_i and ADP on isolated skeletal and cardiac muscle SR vesicles. Addition of ATP to previously unloaded SR vesicles is followed by a rapid phase of net Ca^{2+} accumulation. However, the rates of Ca^{2+} uptake and ATP hydrolysis are progressively inhibited by the rising intravesicular [Ca^{2+}]. A steady state is finally obtained when Ca^{2+} influx equals efflux and the [Ca^{2+}] within the vesicles remains constant. Unidirectional Ca^{2+} flux measurements under steady-state conditions of Ca^{2+} loading have shown that micromolar levels of ADP stimulate a rapid exchange between intra- and extravesicular Ca^{2+} (Wass & Hasselbach, 1981; Feher & Briggs, 1983; Soler, Teruel, Fernandez-Belda & Gomez-Fernandez, 1990). ADP-stimulated Ca^{2+}–Ca^{2+} exchange is present in SR membrane fractions lacking Ca^{2+} release channels and is markedly reduced by specific inhibitors of the SR ATPase (Soler *et al.* 1990). This has led to the suggestion that ADP-activated Ca^{2+}–Ca^{2+} exchange may result from partial reversal of phosphorylated intermediate states of the SR ATPase.

In saponin-treated trabeculae, the mean [ADP] may exceed 60 μM in the absence of an ATP-regenerating system (Smith & Steele, 1992), which would be expected to stimulate Ca^{2+}–Ca^{2+} exchange. This may also occur in intact cardiac preparations, under conditions of ischaemia or metabolic inhibition where the cytosolic [ADP] can increase to 20–100 μM following depletion of CP (Allen, Morris, Orchard & Pirolo, 1985). This form of Ca^{2+}–Ca^{2+} exchange may have little effect on the Ca^{2+} content of the SR as Ca^{2+} influx and efflux appear to be increased to a similar extent in the presence of ADP alone (Wass & Hasselbach, 1981; Feher & Briggs, 1983; Soler *et al.* 1990). However, of particular relevance to the present study, Ca^{2+} efflux from the SR can be markedly increased by millimolar levels of P_i (Barlogie, Hasselbach & Makinose, 1971) and this is accompanied by a net synthesis of ATP from ADP (Makinose & Hasselbach, 1971; Deamer & Baskin, 1972). It has been suggested that this form of ADP-dependent Ca^{2+} efflux results from full reversal of the SR Ca^{2+}pump with the energy required for ATP synthesis provided by the [Ca^{2+}] gradient across the SR membrane. Therefore, the inhibitory effects of P_i reported in this study might result from the dual action of P_i and ADP (produced by cellular ATPases) which together induce a net Ca^{2+} efflux via the SR Ca^{2+} pump. In these circumstances, the reduction in the SR Ca^{2+} content would result from activation of Ca^{2+} efflux rather than inhibition of Ca^{2+} uptake *per se*. This is supported by the observation that introduction of P_i (in the absence of CP) consistently induced a rapid release of Ca^{2+} from the SR which was then followed by a reduction in the caffeine-induced Ca^{2+} transients (Fig. 1*A*). It is possible that P_i-induced Ca^{2+} release could result from direct activation of SR Ca^{2+} channels rather than reversal of the Ca^{2+} pump. However, this is unlikely because it has been shown recently that P_i activates skeletal but not cardiac muscle SR Ca^{2+} channels (Fruen, Mickelson, Shomer, Roghair & Louis, 1994).

Possible mechanisms underlying the interaction between P_i and CP

Introduction of CP (0·5–15 mM) had no significant effect on caffeine-induced Ca^{2+} release in the absence of P_i (not shown). This suggests that CP acts by preventing the inhibitory effects of P_i rather than increasing the Ca^{2+} content of the SR by an independent mechanism. Under physiological conditions, CP synthesizes ATP from ADP and CP, catalysed by creatine phosphokinase (the Lohmann reaction). Figure 5 shows that the activity of the SR and myofilaments can be supported entirely by ATP generated from ADP and CP, confirming that endogenous creatine phosphokinase activity persists after saponin treatment. This introduces the possibility that CP may interact with endogenous creatine phosphokinase to influence the response of the SR to P_i. In support of this, P_i reduced the SR Ca^{2+} content in the presence of CP, after treatment with the creatine phosphokinase inhibitor DNFB (Fig. 6). Furthermore, the concentration dependence of the effect of CP (Fig. 3*B*) is consistent with the measured K_m of CP binding to cardiac creatine phosphokinase of 1–1·5 mM CP (Saks, Chernousova, Vetter, Smirnov & Chazov, 1976).

As considered above, millimolar levels of P_i can stimulate Ca^{2+} efflux from the SR via the SR Ca^{2+} pump and this form of Ca^{2+} efflux requires micromolar levels of ADP. In the presence of CP, ADP produced by ATPases including the SR and myofilaments is rephosphorylated to ATP where endogenous creatine phosphokinase is localized within cells (e.g. the SR). Therefore, the results of this study could be explained if CP antagonizes the effects of P_i by reducing ADP levels within the skinned muscle preparations. This interpretation is supported by the reduction or abolition of P_i-induced Ca^{2+} release in the presence of CP (cf. Fig. 1*A* and *B*). Furthermore, experiments on SR vesicles have shown that ADP-dependent Ca^{2+} efflux is greatly reduced in the presence of an ATP regenerating system comprising phosphoenolpyruvate (PEP) and pyruvate kinase, due to lowering of the [ADP] (Feher & Briggs, 1983; Soler *et al.* 1990). In intact preparations, it has been calculated that increasing the [CP] from ~0·3 mM to normal levels will decrease the [ADP] from approximately 20–100 μM (during ischaemia or anoxia) to submicromolar levels (Allen *et al.* 1985). This reduction in [ADP] would be expected to inhibit ADP-dependent Ca^{2+} efflux (Barlogie *et al.* 1971), thereby rendering the SR insensitive to P_i.

We have previously reported that the PEP–pyruvate kinase ATP-regenerating system does not mimic the effects of CP in saponin-skinned fibres (Steele *et al.* 1994*b*) which may appear to contradict earlier studies on isolated vesicles described above. However, in experiments similar to that shown in Fig. 5, we have found that addition of ADP to solutions with PEP and pyruvate kinase (but without ATP) failed to relax rigor tension and only partially supported SR Ca^{2+} uptake (not shown). This is probably because pyruvate kinase added to the bathing solution does not have free access to the cytoplasm in trabeculae permeabilized with saponin. The limited SR

activity may result from the inward diffusion of ATP regenerated outside the permeabilized cells. Consequently, PEP and pyruvate kinase may not reduce the [ADP] within saponin-skinned fibres sufficiently to prevent the effects of P_i.

At higher concentrations, P_i significantly increased the Ca^{2+} content of the SR in the presence of CP (Fig. 2). This is consistent with a previous study on saponin-skinned cardiac muscle in which a P_i-induced increase in Ca^{2+} content of the SR was reported in the presence of CP (Zhu & Nosek, 1991). The mechanism underlying this effect is unclear. In some circumstances, P_i (like oxalate) can precipitate within the SR (as calcium phosphate) although this does not occur under our conditions in the absence of CP (Steele *et al.* 1994*a*). As introduction of CP appears to relieve the inhibitory effects of P_i on the SR (e.g. Fig. 1*B*), the luminal [Ca^{2+}] may reach high enough levels in these circumstances to allow calcium phosphate to precipitate. This reaction is not readily reversible and precipitated calcium phosphate is unlikely to be available for release by caffeine. However, assuming a luminal [Ca^{2+}] of 1–5 mM a significant proportion of the Ca^{2+} within the SR would be expected to be in a form that is exchangeable with P_i and therefore available for release. This may account for the slight increase in the amount of Ca^{2+} available for release by caffeine at higher [P_i] and in the presence of CP (Fig. 2).

Summary

In the absence of CP, introduction of P_i induced a marked concentration-dependent reduction in the Ca^{2+} content of the SR. This inhibitory effect of P_i was reversed by CP within the concentration range 0·5–5 mM. These results suggest that resting levels of P_i (1–2 mM) or increases in cytosolic [P_i] that may occur during changes in cardiac work are unlikely to affect SR function since the [CP] remains above 5 mM. However, under conditions of ischaemia or metabolic inhibition where the [CP] can decrease to undetectable levels, the accumulation of P_i would be expected to markedly reduce the Ca^{2+} content of the SR. The reduction in the Ca^{2+} content of the SR in the presence of P_i may involve stimulation of Ca^{2+} efflux via the Ca^{2+} pump by a mechanism that requires ADP. The ability of CP to prevent the inhibitory effects of P_i may result from removal of ADP from the cytosol by the Lohmann reaction, catalysed by endogenous creatine phosphokinase.

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