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INORGANIC PHOSPHATE DECREASES THE Ca^{2+} CONTENT OF THE SARCOPLASMIC RETICULUM IN SAPONIN-TREATED RAT CARDIAC TRABECULAE

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SUMMARY

1. Measurements of $[Ca^{2+}]$ were made in saponin-permeabilized rat ventricular trabeculae using the fluorescent indicator Indo-1. Application of caffeine (20 mM) caused a transient rise in $[Ca^{2+}]$ within the preparation as a result of Ca^{2+} release from the sarcoplasmic reticulum (SR). The size of the caffeine-induced Ca^{2+} transient was related to the amount of Ca^{2+} accumulated by the SR prior to addition of caffeine. Caffeine-induced Ca^{2+} release was abolished by ryanodine (10 μM), an inhibitor of SR Ca^{2+} release.

2. At a bathing $[Ca^{2+}]$ of 0·2 μM, the amount of Ca^{2+} released from the SR on addition of caffeine was sufficient to generate a tension transient. Ca^{2+} and tension responses were stabilized by application of caffeine at regular intervals (2 min). Addition of 10 mM inorganic phosphate (P_i) induced a transient increase in $[Ca^{2+}]$ within the preparation due to a net release of Ca^{2+} from the SR. The amplitude of subsequent caffeine-induced Ca^{2+} transients were reduced to $65 \pm 7{\cdot}5\%$ (mean $\pm$ S.D., $n = 13$) of control. In addition, the accompanying tension transient fell to $45 \pm 6{\cdot}9\%$ of control. Removal of P_i caused a transient decrease in the $[Ca^{2+}]$ within the preparation consistent with a net increase in Ca^{2+} uptake by the SR. Subsequent caffeine-induced Ca^{2+} and tension transients returned to control levels.

3. Inclusion of P_i (2–30 mM) in the perfusing solution decreased the size of caffeine-induced Ca^{2+} and tension transients in a dose-dependent manner.

4. Addition of 10 mM ADP caused a transient increase in $[Ca^{2+}]$ and depressed subsequent caffeine-induced Ca^{2+} transients to a greater extent than 10 mM P_i. Despite the reduction in Ca^{2+} release from the SR, tension responses were larger in the presence of 10 mM ADP than under control conditions. This is a consequence of an increase in Ca^{2+}-activated force by ADP.

5. A decrease in the amplitude of caffeine-induced Ca^{2+} transients also occurred on changing from a solution containing 1 mM ADP and 10 mM P_i to a solution with 10 mM ADP and 1 mM P_i. This confirms the previous observation that ADP is more effective than P_i at reducing caffeine-induced Ca^{2+} released from the SR.

6. Spontaneous oscillations of $[Ca^{2+}]$ and tension occurred in the presence of 0·5 μM Ca^{2+}. These oscillations result from the cyclic release and uptake of Ca^{2+} from the SR. Under these conditions, introduction of P_i (10 mM) caused a slow transient release of Ca^{2+} from the SR followed by complete abolition of spontaneous activity.

MS 1182

7. The results suggest that P_i (2–30 mM) decreases the Ca^{2+} content of the SR. This could occur either by stimulating SR Ca^{2+} release or by partially inhibiting Ca^{2+} uptake. Based on these results, the rise in intracellular $[P_i]$ that occurs during ischaemia and anoxia in cardiac muscle would be expected to lower the SR Ca^{2+} content and hence reduce the amount of Ca^{2+} released into the cytosol during systole.

INTRODUCTION

In mammalian cardiac muscle, contractile force decreases rapidly during the first few minutes of global ischaemia as a result of the inhibition of aerobic metabolism (review by Allen & Orchard, 1987). Over the same period, intracellular inorganic phosphate concentration ($[P_i]_i$) increases from about 2 to 20 mM. This is accompanied by accumulation of anaerobically produced lactic acid and a marked fall of intracellular pH (pH_i) (Bailey, Williams, Radda & Gadian, 1981). Experiments on permeabilized cardiac preparations have shown that both P_i and low pH depress force production by a direct action on the myofilaments (Fabiato & Fabiato, 1978; Kentish, 1986).

A decrease in Ca^{2+} release from the SR could also contribute to the fall in contractile force during ischaemia. However, it is not clear from work involving intracellular $[Ca^{2+}]$ measurements in intact tissue whether SR function is affected by changes in $[P_i]_i$ and pH_i that occur in early ischaemia. Under normal conditions, approximately 95% of Ca^{2+} released into the cytosol during systole is bound, mainly to troponin-C (Bers, 1991). Under conditions of low pH, the affinity of troponin-C for Ca^{2+} is reduced (Blanchard & Solaro, 1984; Kentish & Palmer, 1989) and more of the Ca^{2+} released from the SR will remain free in the cytosol. This mechanism was proposed to explain the increase in free systolic and diastolic $[Ca^{2+}]_i$ that occurs during ischaemia (Lee, Mohabir, Smith, Franz & Clusin, 1988; Allen, Lee & Smith, 1989). However, since the Ca^{2+} buffering capacity of the cell is reduced, it is not possible to assess whether the amount of Ca^{2+} released from the SR during systole is altered during ischaemia.

During anoxia, the increase in $[P_i]_i$ is similar to that in ischaemia but pH_i falls only slightly since lactic acid is lost from the cell. Studies of intracellular $[Ca^{2+}]$ during anoxia have provided conflicting results. Allen & Orchard (1987) found no consistent change in the size of the Ca^{2+} transient while MacKinnon, Gwathmey & Morgan (1987) reported a fall in the Ca^{2+} transient during anoxia. Lee & Allen (1988) attributed these different results to variations in lactic acid accumulation in isolated papillary muscle preparations. This complication should not arise in isolated cardiac cells, where anoxia or cyanide have little or no effect on the amplitude of the Ca^{2+} transient (Stern, Silverman, Houser, Josephson, Capogrossi, Nichols, Lederer & Lakatta, 1988; Eisner, Nichols, O'Neill, Smith & Valdeolmillos, 1989). One interpretation of this result is that the rise in $[P_i]_i$ does not affect SR function. However, the metabolic state of isolated cells is uncertain since the few measurements available show that myocytes have high intracellular $[P_i]$ even in the presence of fully oxygenated bathing solution (Wittenberg, Doeller, Gupta & White, 1988).

The effects of pH on the SR have been studied in mechanically skinned cardiac cells. In this preparation, the intracellular environment can be controlled, allowing the effects of pH to be studied independently of the changes in $[Ca^{2+}]$ and $[P_i]$ that

occur during ischaemia in intact cells. Acidosis decreased SR Ca^{2+} uptake and hence reduced the amount of Ca^{2+} available for subsequent release by caffeine or Ca^{2+}-induced Ca^{2+} release (Fabiato & Fabiato, 1978). In addition, the optimum free [Ca^{2+}] for triggering Ca^{2+}-induced Ca^{2+} release was shifted to higher [Ca^{2+}] by a decrease in pH (Fabiato & Fabiato, 1978). These results suggest that the intracellular acidosis associated with ischaemia decreases the amount of Ca^{2+} released from the SR during systole.

Similar experiments have yet to be done to assess the effects of P_i in skinned muscle preparations. Shoshan-Barmatz (1987) reported that P_i stimulated Ca^{2+} efflux from isolated skeletal muscle SR vesicles, an effect ascribed to an action of P_i on ATP-dependent SR Ca^{2+} channels. However, P_i can increase Ca^{2+} uptake in cardiac, skeletal and smooth muscle SR vesicles by an action similar to oxalate (Mermier & Hasselbach, 1975; Feher & Lipford, 1985; Kwan 1985). Interpretation of these results is complicated because the experimental conditions used in these SR vesicle studies are generally quite unlike those of the normal intracellular environment.

In this study, we have examined the effects of P_i on the Ca^{2+} content of SR in saponin-permeabilized cardiac muscle. Ca^{2+} released from the SR was measured directly using Indo-1. The amplitude of the caffeine-induced Ca^{2+} transient was used as an assay of the Ca^{2+} content of the SR. The Ca^{2+} content of the SR was monitored before, during and after exposure to P_i. The range of P_i concentrations used is believed to reflect levels during normal and ischaemic conditions in cardiac muscle. Preliminary results have been presented to the Physiological Society (Smith, Crichton & Steele, 1992).

METHODS

Sprague–Dawley rats (200–250 g) were killed by a blow to the head and cervical dislocation. Their hearts were removed rapidly and bathed in Tyrode solution. Free-running trabeculae 80–120 μm diameter and 1–2 mm in length were dissected from the right ventricle. Experiments were performed at room temperature (22–23 °C).

Solution composition

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Corporation. H^+ and Ca^{2+} were buffered with HEPES (Boehringer Mannheim) and EGTA respectively. All solutions contained ATP to support the activity of the SR and myofilaments in saponin-permeabilized muscle. Creatine phosphate is normally included to buffer the [ATP] within the preparation during sustained contractions when the ATPase rate is maintained at a high level. However, creatine phosphate can contain significant P_i contamination (Kentish, 1986). Therefore, in experiments to study the effects of P_i on the SR, creatine phosphate was not included in the solutions. ATP buffering is less important because the basal ATPase rate is low and the contractile responses are only transient. Compositions of solutions are given in Table 1. Ca^{2+} release from the SR was detected in solutions weakly Ca^{2+} buffered with EGTA (solution A, Table 1) containing 4 μM Indo-1 (Calbiochem). Calcium chloride (1 M titration standard, BDH) 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 fluorescence ratio displayed an apparent affinity constant of Indo-1 for Ca^{2+} of 705 ± 5 nM ($n = 4$). This value was calculated by measuring the fluorescence ratio in strongly Ca^{2+}-buffered solutions over a range of [Ca^{2+}]. Maximum and minimum values of the ratio were obtained using solutions D and E with 4 μM Indo-1. Solutions with intermediate [Ca^{2+}] were prepared by mixing solutions D and E. 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 Smith & Miller (1984). The affinity constants for ATP and ADP are those quoted by Fabiato & Fabiato (1979).

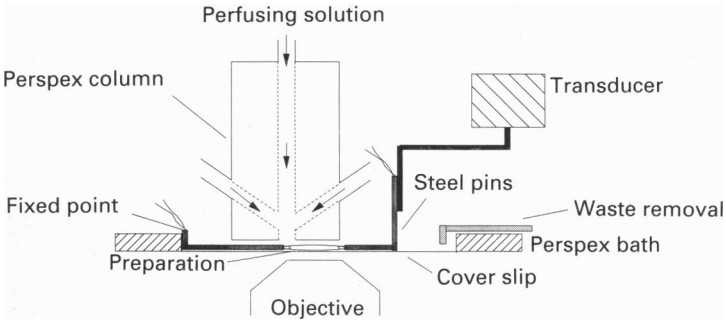

Fig. 1. Diagram of the apparatus used to make simultaneous measurements of $[Ca^{2+}]$ and tension from saponin-treated preparations.

TABLE 1. Composition of solutions (mM except where stated)

Solution	K^+	Na^+	Mg^{2+}	Total Ca^{2+}	$[Ca^{2+}]$ (μM)	ATP	EGTA	HEPES	Cl^-	HPO^{4-}	ADP
A	130	40	6·5	0·02	0·08	5	0·2	25	174	—	—
B	130	40	7·8	0·02	0·08	5	0·2	25	126	30	—
C	130	40	6·8	0·02	0·08	5	0·2	25	149	—	10
D	130	40	6·7	0·02	0·001	5	10·0	25	154	—	—
E	130	40	6·7	10·1	100·0	5	10·0	25	154	—	—
Tyrode	5	120	2	1·0	—	—	—	5	132	—	—

All solutions had a pH (activity) of 7·00 and a free $[Mg^{2+}]$ of 2·0 mM.

Corrections for ionic strength, details of pH measurement, allowance for EGTA purity and the principles of the calculations are given in Smith & Miller (1984). Ionic strength of solutions was maintained by reducing $[Cl^-]$ in solutions containing P_i or ADP.

Chemical skinning procedure

During dissection and mounting, the muscle was bathed in Tyrode solution. The preparation was permeabilized by exposure to solution A containing 50 μg ml^{-1} saponin for 30 min. Saponin treatment renders the sarcolemma permeable to small ions and molecules without disrupting SR function (Endo & Kitazawa, 1978). Saponin was removed by washing the preparation in solution A before proceeding with the experiment.

Apparatus for simultaneous measurement of SR Ca^{2+} release and tension

As shown in Fig. 1, trabeculae were mounted between a tension transducer (Akers 46136, SensoNor a.s. 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 top surface of the muscle to minimize the volume of the solution above the preparation. The perfusing solution with 4 μM Indo-1 was pumped constantly (1 ml min^{-1}) through the central bore of the column and collected at the edge of the column. The volume of solution surrounding the preparation was approximately 4 μl. A solution containing 20 mM caffeine was injected onto the preparation via the manifold at the base of the column. A solenoid-controlled pneumatic system ensured that a uniform volume of solution (approximately 100 μl) was applied to the preparation during a 50 ms injection period. The bath was placed on the stage of a Nikon Diaphot inverted microscope. A regular sarcomere pattern was clearly observable under 400 × magnification, allowing the sarcomere length to be set for these experiments to approximately 2·2 μm. The preparation was illuminated with light of wavelength 360 nm via a 20 × Fluor objective (Nikon CF Fluor, NA 0·75). The average $[Ca^{2+}]$ within the visual field containing the preparation was indicated by the ratio of light

intensities emitted at 405 and 495 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. The ratio of light intensities emitted at the two wavelengths was obtained using an analog divider circuit. The ratio signal, the individual wavelength intensities and the tension signals were low-pass filtered (−3 dB at 30 Hz) and recorded on tape for later analysis.

RESULTS

Measurement of $[Ca^{2+}]$ *and isometric tension in saponin-treated trabeculae*

Figure 2 shows typical fluorescence and tension records obtained from a saponin-treated trabecula. The muscle was perfused constantly with a weakly Ca^{2+}-buffered solution (solution A) containing 4 μM Indo-1 and illuminated with light of wavelength 360 nm. The free $[Ca^{2+}]$ of the perfusing solution was 0·08 μM, below the threshold for Ca^{2+}-activated tension. Each 50 ms injection of 20 mM caffeine caused a transient decrease in the intensity of light emitted at wavelength 450 nm but appeared to have little effect on the 495 nm signal (top panel). Since both wavelengths are quenched to a similar extent by caffeine (O'Neill, Donoso & Eisner, 1990), the small changes in fluorescence at 405 nm are a result of an increase in fluorescence due to increased $[Ca^{2+}]$ cancelled out by the decrease due to the caffeine quench. The Ca^{2+}-sensitive component may be revealed by dividing the signal at 405 nm by that at 495 nm. The transient increase in the fluorescence ratio on injection of caffeine is due to Ca^{2+} release from the SR (middle panel). Reproducible Ca^{2+} transients were obtained by regular application of caffeine. The rapid increase in the $[Ca^{2+}]$ within the preparation is due to the caffeine-induced release of Ca^{2+} from the SR. However, the rate of decline in the $[Ca^{2+}]$ is a result of several processes: (i) reuptake of Ca^{2+} into the SR, (ii) the diffusion of Ca^{2+} and (iii) diffusion of caffeine out of the preparation. Therefore the time course of the decline in $[Ca^{2+}]$ after caffeine injection cannot be easily interpreted. At this bathing $[Ca^{2+}]$, the amount of Ca^{2+} released by caffeine was insufficient to activate the myofilaments, and the small tension transients (bottom panel) are artifacts of caffeine injection (see below).

After the third injection of caffeine, the $[Ca^{2+}]$ of the perfusing solution was increased to 0·6 μM, as indicated by the sustained rise in the fluorescence ratio. Subsequent applications of caffeine initiated larger Ca^{2+} transients and the increase in $[Ca^{2+}]$ within the muscle was sufficient to generate large tension responses. This is consistent with an increase in the amount of Ca^{2+} accumulated by the SR prior to release by caffeine. Ryanodine (10 μM), a drug known to inhibit SR Ca^{2+} release, abolished Ca^{2+} and tension transients leaving an artifact associated with injection. After ryanodine treatment, the caffeine-induced quench of Indo-1 fluorescence at 450 and 495 nm can be seen in the absence of a superimposed Ca^{2+} signal.

One shortcoming of this technique is that it is not possible to calibrate the transient changes in $[Ca^{2+}]$ that occur within saponin-permeabilized preparations. The fluorescence ratio is an approximately linear function of $[Ca^{2+}]$ over the range of $[Ca^{2+}]$ encountered in these experiments. Thus, it represents the mean $[Ca^{2+}]$ in a volume approximately $300 \times 100 \times 200$ μm containing the preparation and a volume of the perfusing solution. The $[Ca^{2+}]$ in the perfusing solution is known and constant, thus any changes in the fluorescence signal are due to net release or uptake of Ca^{2+} by the preparation. Without an accurate measure of the volume of preparation relative

to the total volume from which the signal is collected, $[Ca^{2+}]$ within the preparation cannot be calculated. Preliminary experiments with this technique have established that Indo-1 binds to structures within the preparation and yet remains able to signal $[Ca^{2+}]$ with a Ca^{2+} sensitivity similar to that free in solution. Thus more of the signal

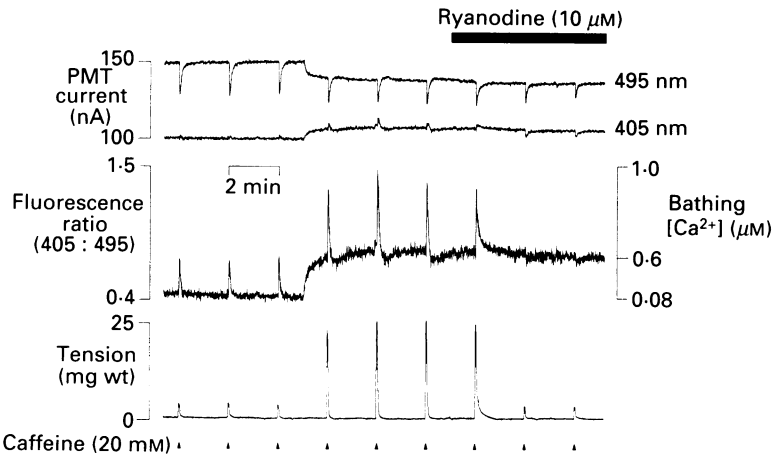

Fig. 2. Typical fluorescence and tension records from a saponin-treated trabecula. The preparation was constantly perfused with solution A (Table 1) containing 4 μM Indo-1 and illuminated with light of wavelength 360 nm. The figure shows simultaneous records of light intensity emitted at 405 and 495 nm in terms of output current from the photomultiplier tubes (upper panel), the ratio of light intensities emitted at 405 and 495 nm (middle panel) and isometric tension (lower panel). The steady-state $[Ca^{2+}]$ corresponding to the fluorescence ratio is shown on the right-hand side of the record. Caffeine (20 mM) was injected for 50 ms duration as indicated (arrowheads). Ryanodine (10 μM) was added for the period indicated by the bar.

comes from the volume contained by the preparation than from the surrounding solution. Estimates based on total fluorescence measurements suggest that the lower limit to the signal due to the preparation is 66% of the total signal. The fluorescence ratio is shown to the left of the trace and the corresponding $[Ca^{2+}]$ on the right-hand side. These values accurately reflect the steady-state $[Ca^{2+}]$ within the volume containing the preparation, dictated by the bathing $[Ca^{2+}]$. This scale does not indicate the $[Ca^{2+}]$ within the preparation during the transient release of Ca^{2+} from the SR. However, it does indicate the lower limit of the average $[Ca^{2+}]$ within the preparation and accurately reflects relative changes in Ca^{2+} release from the SR.

The effect of P_i on caffeine-induced Ca^{2+} release

Figure 3 shows the effect of P_i on caffeine-induced Ca^{2+} release in a saponin-treated trabecula. The protocol was similar to that described above, except the $[Ca^{2+}]$ was maintained at 0·2 μM throughout the experiment. Introduction of 10 mM P_i between applications of caffeine was associated with a transient increase in the fluorescence ratio (Fig. 3*A*). This was not observed in the presence of ryanodine or after removal of the muscle from the visual field (not shown). This suggests that P_i induces net Ca^{2+}

release from the SR. Caffeine-induced Ca^{2+} transients evoked in the presence of P_i were of smaller amplitude, consistent with a reduction in the Ca^{2+} content of the SR. P_i (10 mM) reduced the size of the caffeine-induced Ca^{2+} transients to $65 \pm 7{\cdot}5\%$ (mean $\pm$ S.E.M., $n = 13$) of the control value. The amplitude of the tension transients

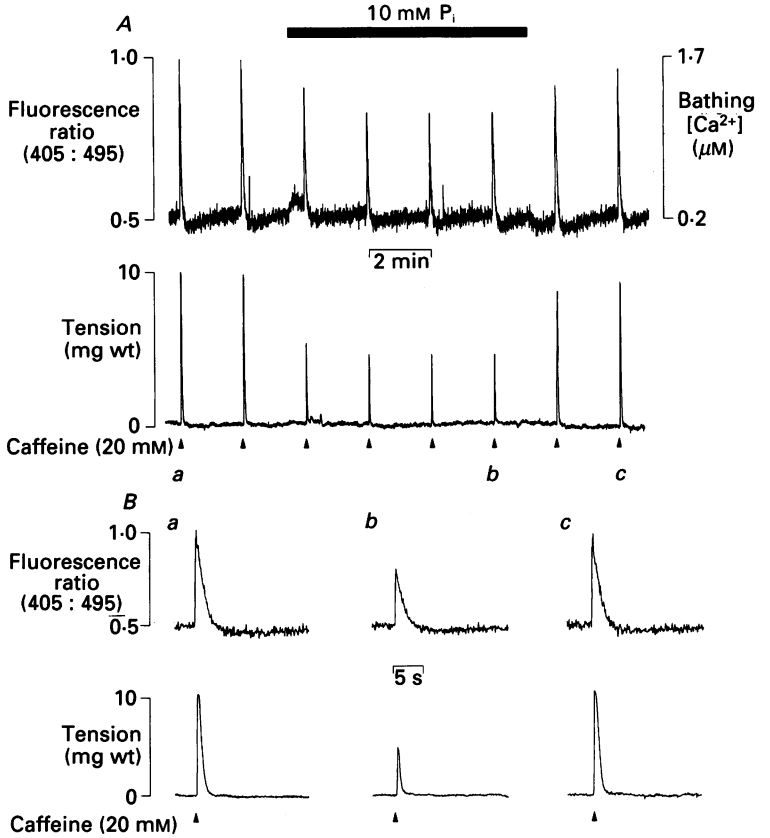

Fig. 3. Effect of P_i on caffeine-induced Ca^{2+} and tension transients. Basic solution composition and protocol as Fig. 2. Panel *A* shows simultaneous records of fluorescence ratio (upper trace) and tension (lower trace). P_i (10 mM) was added for the period indicated above the records. The steady-state $[Ca^{2+}]$ corresponding to the fluorescence ratio is shown on the right-hand side of the ratio. Panel *B* shows Ca^{2+} and tension transients selected from the period before introduction of P_i (*a*) in the presence of P_i (*b*) and after removal of P_i (*c*) on an expanded time scale.

also decreased in the presence of P_i due to both a decreased SR Ca^{2+} release and a depression in myofilament Ca^{2+} sensitivity. On removal of P_i the fluorescence ratio transiently decreased, consistent with net Ca^{2+} uptake by SR. This interpretation is supported by the larger Ca^{2+} release on the next injection of the caffeine and the gradual recovery of Ca^{2+} and tension transients to near control levels. Figure 3*B* shows selected Ca^{2+} and tension transients (*a*) before, (*b*) during and (*c*) after exposure to P_i on an expanded time scale. This indicates that 10 mM P_i does not significantly alter the time course of the caffeine-induced Ca^{2+} transients.

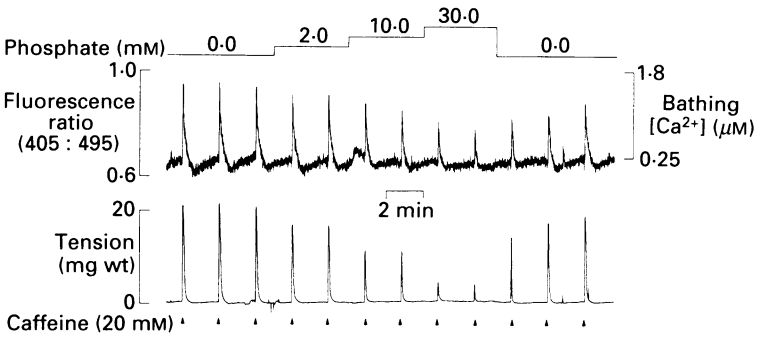

Fig. 4. Effect of progressively increasing $[P_i]$ on caffeine-induced Ca^{2+} and tension transients. Basic solution composition and protocol as Fig. 2. Simultaneous records of fluorescence ratio (upper trace) and tension (lower trace) are shown. The steady-state $[Ca^{2+}]$ corresponding to the fluorescence ratio is shown on the right of the ratio signal.

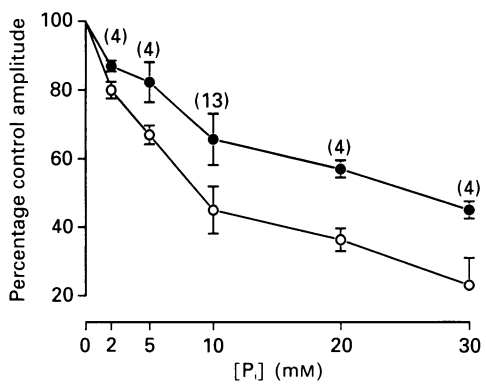

Fig. 5. Cumulated data showing the effect of P_i on the amplitude of the caffeine-induced Ca^{2+} release and tension transients. Points represent steady-state response at different P_i concentrations as a percentage of mean control response (error bars represent S.D.). Numbers in parentheses are the number of preparations used. ●, amplitude of the caffeine-induced Ca^{2+} transient relative to control. ○, accompanying tension transients relative to control.

Figure 4 shows the effects of P_i at a range of concentrations on caffeine-induced Ca^{2+} and tension transients. At 2 mM P_i caused only a small reduction in the amplitude of the Ca^{2+} transient, but a proportionally larger decrease in the amplitude of the tension transient. Increasing the $[P_i]$ to 10 mM caused a transient release of Ca^{2+} from the SR. Both the amplitude of the following caffeine-induced Ca^{2+} transient and the associated tension transient were reduced. Increasing the P_i concentration to 30 mM further reduced caffeine-induced Ca^{2+} release and the tension transients were almost abolished. After removal of P_i the amplitude of caffeine-induced Ca^{2+} and tension transients returned towards control levels.

Figure 5 summarizes data from several experiments. The amplitudes of caffeine-induced Ca^{2+} and tension transients are expressed as a percentage of control values. Only a small depression of caffeine-induced Ca^{2+} transients was seen at 2–5 mM P_i,

but the accompanying tension transients were markedly inhibited. Caffeine-induced Ca^{2+} release was markedly depressed at 10 mM P_i, and was further inhibited at 20 and 30 mM P_i.

Effect of 10 mM ADP on caffeine-induced Ca^{2+} release

As one of the products of ATP hydrolysis, P_i may affect SR functioning by interfering with the active transport of Ca^{2+} across the SR membrane by the Ca^{2+}

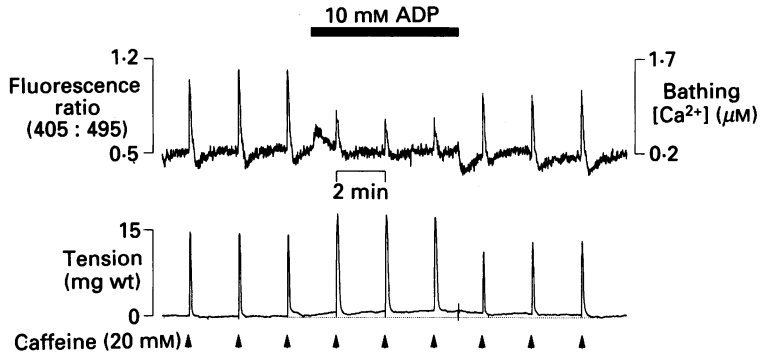

Fig. 6. Effects of ADP on caffeine-induced Ca^{2+} and tension transients. Basic solution composition and protocol as Fig. 2. Simultaneous records of fluorescence ratio (upper trace) and tension are shown. The steady-state $[Ca^{2+}]$ corresponding to the fluorescence ratio is shown on the right of the ratio.

pump. If P_i has its effect through a reduction of the free energy available from ATP hydrolysis (ΔG_{ATP}, see Discussion) then ADP should have equivalent effects to P_i on the Ca^{2+} content of the SR. Figure 6 shows that introduction of 10 mM ADP (like 10 mM P_i) caused a net release of Ca^{2+} from the SR and subsequent caffeine-induced Ca^{2+} transients also decreased in size. However, despite the decrease in Ca^{2+} release, the tension transients increased in amplitude and were of longer duration in the presence of ADP. Furthermore, introduction of ADP was generally associated with a slight increase in baseline tension which was not always fully reversible. These effects of ADP are consistent with an increase in Ca^{2+} sensitivity of myofilament tension production. On removal of ADP, caffeine-induced Ca^{2+} transients returned towards control levels.

Effects of simultaneously changing $[P_i]$ and $[ADP]$ on caffeine-induced Ca^{2+} release

The equivalence of the action of P_i and ADP was further tested by simultaneously lowering [ADP] and raising $[P_i]$ while maintaining the product of their concentrations constant. Figure 7 shows caffeine-induced Ca^{2+} and tension transients in a perfusing solution containing 1 mM ADP and 10 mM P_i. At the point indicated, the solution was changed to one containing 10 mM ADP and 1 mM P_i. This caused a small transient increase in the fluorescence ratio indicating a net release of Ca^{2+} from the SR. As in Fig. 6, although subsequent Ca^{2+} transients were smaller, the accompanying tension transients were much increased. Presumably this was due to the reduction of the inhibitory effects of P_i and the additional Ca^{2+}-sensitizing effects

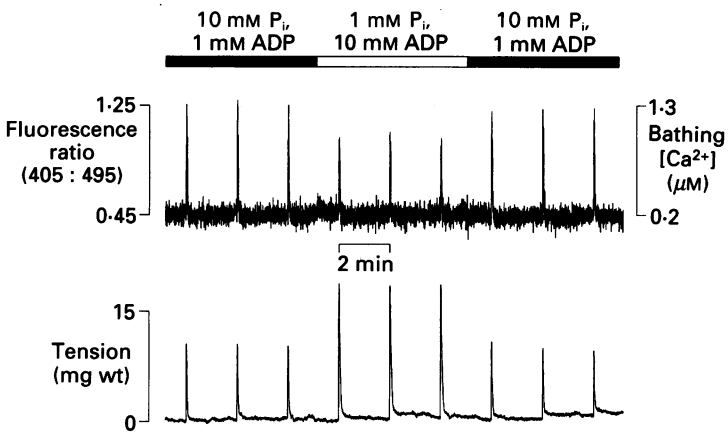

Fig. 7. Comparative effects of solutions containing 10 mM ADP and 1 mM P_i and solutions containing 10 mM P_i and 1 mM ADP on caffeine-induced Ca^{2+} release. Basic solution composition and protocol as Fig. 2. Simultaneous records of fluorescence ratio (upper trace) and tension are shown. The steady-state [Ca^{2+}] corresponding to the fluorescence ratio is shown on the right of the ratio.

TABLE 2. Calculated and experimental data concerning the effects of P_i and ADP on ΔG_{ATP}, caffeine-induced Ca^{2+} transients and developed tension

[P_i]	[ADP]	ΔG_{ATP} (kJ mol^{-1})	Ca^{2+} transient (%)	Tension transient (%)
0·02*	0·07*	−65·6	100	100
2·0	0·07*	−54·7	87±1·6	80±2·4
5·0	0·07*	−53·3	78±5·8	67±2·8
10·0	0·07*	−51·6	65±7·5	45±6·9
20·0	0·07*	−49·9	57±2·5	36±3·3
30·0	0·07*	−48·5	45±7·5	23±8·0
0·02*	10·0	−54·7	55±5·1	140±15·0
10·0	1·00	−44·8	85±2·5	56±7·0
1·0	10·0	−44·8	65±5·0	96±18·1

Bathing [ATP] was 5 mM and [Ca^{2+}] 0·2 μM in all cases. Asterisks indicate the concentrations calculated from basal ATPase rates. ΔG_{ATP} values were calculated from eqn (3). ΔG° at 20 °C, 2 mM Mg^{2+} was taken from Alberty (1972). Errors are S.D.; $n = 4$ for all entries except for 10 mM P_i alone, where $n = 13$.

of ADP on Ca^{2+}-activated force. On reperfusing with 1 mM ADP and 10 mM P_i, the caffeine-induced Ca^{2+} and tension transients returned towards the original levels. Similar results were seen in three other experiments; these and the other experimental results are summarized in Table 2.

The effect of 10 mM P_i on spontaneous Ca^{2+} release from the SR

As shown in Fig. 3, introduction and withdrawal of P_i, between applications of caffeine, was associated with transient changes in [Ca^{2+}] within the saponin-treated preparations. Figure 8 shows this effect in more detail without regular injection of caffeine. In the presence of 0·2 μM Ca^{2+}, addition of 10 mM P_i caused a transient increase and withdrawal of P_i a transient decrease in the [Ca^{2+}] (Fig. 7*A*). The tension record has been omitted because throughout this procedure, the [Ca^{2+}] within

the muscle was below the threshold for Ca^{2+}-activated force. When the $[Ca^{2+}]$ of the bathing solution was increased to about 0·5 μM, oscillations of $[Ca^{2+}]$ and tension occurred (Fig. 8*B*). Such oscillations result from the cyclic uptake and release of Ca^{2+} from the SR. Again, introduction of 10 mM P_i caused a transient increase in $[Ca^{2+}]$

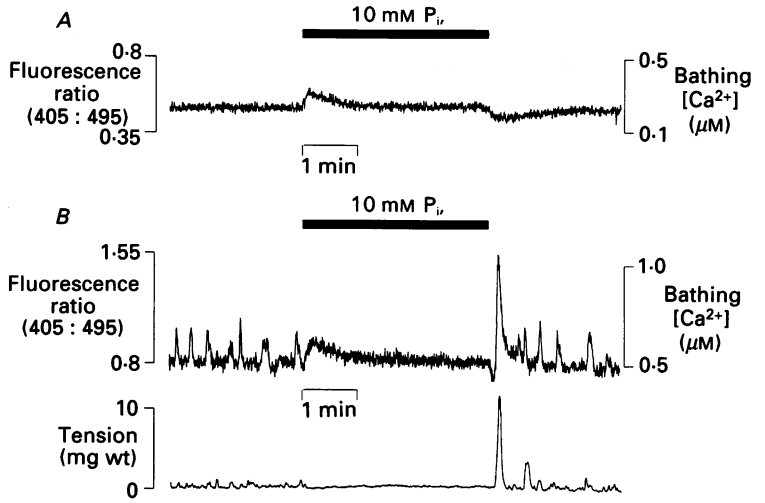

Fig. 8. Effects of 10 mM P_i on $[Ca^{2+}]$ without regular injection of caffeine. Panel *A* shows the effects of addition and removal of P_i on the fluorescence ratio (solution A, Table 1). The free $[Ca^{2+}]$ of the perfusing solution was 0·2 μM, below the threshold for tension. Panel *B* shows the effects of addition and removal of P_i on fluorescence ratio (upper trace) and tension (lower trace) at 0·5 μM. In *A* and *B*, the steady-state $[Ca^{2+}]$ corresponding to the fluorescence ratio is shown.

indicating net Ca^{2+} release from the SR. This was not accompanied by a transient increase in tension, presumably because of the depressive effect of P_i on Ca^{2+}-activated force. Oscillations of $[Ca^{2+}]$ and tension were completely abolished in the presence of 10 mM P_i. Removal of P_i caused a rapid decrease in the $[Ca^{2+}]$, consistent with net Ca^{2+} accumulation by the SR. This was followed immediately by a large transient release of Ca^{2+} and an accompanying tension transient before both records returned towards control levels. Similar results were seen in another three experiments.

DISCUSSION

Experiments using ^{31}P nuclear magnetic resonance on isolated rat and ferret heart have shown that intracellular $[P_i]$ is normally 2–3 mM and rises to 30–40 mM during ischaemia and metabolic blockade (Bailey *et al.* 1981; Allen, Morris, Orchard & Pirolo, 1985). This study shows that increasing the $[P_i]$ over this range markedly reduces caffeine-induced Ca^{2+} released from the SR within 2–4 min. Since a supramaximal concentration of caffeine was used, it is likely that the reduction reflects a decrease in the Ca^{2+} content of the SR and not an altered sensitivity of the SR to caffeine. This interpretation is supported by the transient rise in $[Ca^{2+}]$ within the saponin-permeabilized preparation on addition of P_i (10 mM or above) suggesting a net Ca^{2+} release from the SR. The rise in Ca^{2+} within the preparation is transient

for two reasons. First, if we assume Ca^{2+} efflux from the SR is a function of the $[Ca^{2+}]$ gradient across the SR membrane, then Ca^{2+} release will continue until the luminal $[Ca^{2+}]$ falls and efflux is equal to influx. Second, net release of Ca^{2+} from the SR will raise the $[Ca^{2+}]$ in the preparation. However, this effect will be only transient as Ca^{2+} is lost from the preparation by diffusion. Both of these factors will determine the amplitude and time course of P_i-induced Ca^{2+} release.

Addition of less than 10 mM P_i did not produce a detectable Ca^{2+} release, yet the subsequent caffeine-induced Ca^{2+} transients were reduced suggesting that an undetected Ca^{2+} release had occurred. Under these circumstances the Ca^{2+} release induced by P_i was probably too small or low to be measured with this technique.

As shown in Figs 4 and 5, increasing the $[P_i]$ from 2 to 30 mM caused a progressive decrease in the amplitude of the Ca^{2+} and tension transients. At $[P_i]$ values less than 10 mM, the reduction in tension transients reflects the combined depressive effect of P_i on SR Ca^{2+} content illustrated in this study and the inhibitory action of P_i on Ca^{2+}-activated tension (Kentish, 1986). However, above 10 mM P_i the additional inhibitory effects of P_i on Ca^{2+}-activated force are small (Kentish, 1986). Over this concentration range, the reduction in the size of the tension transients is mainly due to the reduced Ca^{2+} release. These results suggest that an increase in $[P_i]$ over the range observed during anoxia and ischaemia would cause a marked reduction in SR Ca^{2+} content at a given diastolic $[Ca^{2+}]$ and therefore may reduce Ca^{2+} release during systole.

Possible actions of P_i and ADP on the SR Ca^{2+} pump

P_i may inhibit Ca^{2+} uptake by the SR. A rise in $[P_i]$ could cause the observed transient rise in $[Ca^{2+}]$ within the saponin-permeabilized preparation if, in the absence of P_i, a high Ca^{2+} efflux from the SR was balanced by high Ca^{2+} uptake. Partial inhibition of Ca^{2+} uptake would cause efflux temporarily to exceed influx giving rise to a net Ca^{2+} release until the luminal $[Ca^{2+}]$ fell to a new equilibrium level. This explanation implies that at a $[Ca^{2+}]$ close to diastolic levels in cardiac cells there is a significant 'leak' of Ca^{2+} from the SR, which is balanced by Ca^{2+} uptake. Under these conditions, any change in the intracellular conditions that would reduce Ca^{2+} uptake by the SR would rapidly affect the Ca^{2+} content of the SR and hence Ca^{2+} released during systole. Ca^{2+} uptake into the SR is via the Ca^{2+} pump; eqn (1) summarizes the reaction catalysed by the SR Ca-ATPase (Knowles & Racker, 1975).

$$2[Ca^{2+}]_{o,SR} + ATP = 2[Ca^{2+}]_{i,SR} + ADP + P_i, \quad (1)$$

where $[Ca^{2+}]_{o,SR}$ and $[Ca^{2+}]_{i,SR}$ represents $[Ca^{2+}]$ outside and inside the SR respectively.

Two possible mechanisms for the inhibitory effect of P_i on the Ca-ATPase are proposed. (1) Product inhibition: products of an enzyme-catalysed reaction reduce the activity of an enzyme if present at higher concentrations. This explanation would not necessarily predict that changing [ADP] would have similar effects to altering $[P_i]$. (2) Reduced free energy for hydrolysis of ATP: in all chemical reactions, the relative concentrations of reactants and products dictate the maximum energy available from the reaction (ΔG). In the case of the Ca^{2+} pump, assuming the membrane potential across the SR is zero (Somlyo, Gonzalez-Serratos, Schuman,

McCellan & Somlyo, 1981), the expression describing free energy available for the overall reaction is given by

$$\Delta G = 2RT\ln([Ca^{2+}]_{i,SR}/[Ca^{2+}]_{o,SR}) + \Delta G^{o} + RT\ln([ADP][P_i]/[ATP]), \quad (2)$$

where ΔG^{o} is the standard free energy of ATP hydrolysis under the appropriate conditions of pH and $[Mg^{2+}]$, R is the gas constant and T is the temperature (°K). Ca^{2+} uptake and associated ATP hydrolysis will proceed if ΔG is < 0. Equation (2) predicts a fall in luminal $[Ca^{2+}]$ ($[Ca^{2+}]_{i,SR}$) as the $[P_i]$ is increased given a constant $[Ca^{2+}]$ outside the SR ($[Ca^{2+}]_{o,SR}$). In addition, increases in $[P_i]$ or [ADP] have equivalent effects, assuming the $[P_i]$ and [ADP] initially have the same basal concentrations. In the absence of added P_i, the average $[P_i]$ within the preparation depends upon the ATPase activity of the preparation, the diffusion coefficient of P_i and the thickness of the preparation. If the preparation is assumed to be cylindrical, by using myofibrillar ATPase rates of 30 μmol cm^{-3} s^{-1} (J. C. Kentish & G. J. M. Stienen, personal communication) and the diffusion coefficients for the metabolites from Kushmerick & Podolsky (1969), a mean $[P_i]$ of 0·02 mM and [ADP] of 0·07 mM are calculated for a preparation of 100 μm diameter. From these values and the concentrations of [ATP], [ADP] and $[P_i]$ used in this study it is possible to calculate the free energy for hydrolysis of ATP (ΔG_{ATP}), where

$$\Delta G_{ATP} = \Delta G_{o} + RT\ln([ADP][P_i]/[ATP]). \quad (3)$$

Table 2 shows the calculated ΔG_{ATP} values for the combinations of $[P_i]$ and [ADP] present within the saponin-permeabilized preparations. The relative amplitudes of the caffeine-induced Ca^{2+} and tension transients are also shown.

Table 2 shows that raising $[P_i]$ causes a fall in amplitude of the caffeine-induced Ca^{2+} transient from the SR in parallel with a reduction in ΔG_{ATP}. However, the addition of 10 mM ADP produced a more profound reduction of the caffeine-induced Ca^{2+} released despite a calculated ΔG value that was slightly higher than that predicted for 10 mM P_i. This discrepancy between the effect on the SR and the ΔG values was confirmed when the responses in solutions containing 1 mM ADP and 10 mM P_i were compared with those in 10 mM ADP and 1 mM P_i. Both solutions would generate the lowest ΔG values of all solutions used in this study, yet the depression of the caffeine-induced Ca^{2+} release was no larger than that seen after 30 mM P_i alone. Furthermore, 10 mM ADP and 1 mM P_i was more effective than 1 mM ADP and 10 mM P_i in reducing the amplitude of the caffeine-induced Ca^{2+} release from the SR. These results suggest that the effects of P_i and ADP on the Ca^{2+} content of the SR cannot be explained simply in terms of an altered free energy for the hydrolysis of ATP. As described above, product inhibition by both P_i and ADP on the activity of the Ca^{2+} pump may explain this behaviour. Inhibitory constants were 4–6 mM for P_i and 1·6–2·4 mM for ADP measured in skeletal muscle SR Ca-ATPase (reviewed by Haynes, 1983). These values would predict that 10 mM ADP would be more effective than P_i at reducing the activity of the enzyme, but because of the complexity of the Ca-ATPase reaction, it is difficult to predict the actions of P_i and ADP simply on the basis of the inhibitory constants.

Work on isolated SR vesicles has shown that reversal of the Ca^{2+} pump can act as a significant efflux pathway. This process is evident when [ADP] in the solution

bathing the vesicle is raised (Suko, Hellman & Winkler, 1977). Such a mechanism may be responsible for the effect of ADP seen in this study. Raising the [P_i] may also transiently reverse the SR Ca^{2+} pump, although this effect has yet to be observed in isolated SR vesicles.

Effects of ADP and P_i on SR Ca^{2+} efflux pathway

Shoshan-Barmatz (1987) showed that 100 mM P_i stimulated Ca^{2+} loss from isolated SR vesicles from skeletal muscle by increasing the Ca^{2+} efflux through ATP-sensitive Ca^{2+} channels. Exposure to similar concentrations of ADP did not produce an increase in efflux. The present set of experiments could not distinguish between a P_i-stimulated Ca^{2+} efflux and effects on the Ca^{2+} pump, and both mechanisms may be involved in the response. Meissner (1984) has shown that ADP stimulates ATP-dependent Ca^{2+} channel activity of skeletal muscle SR. If ADP was more effective than P_i at stimulating Ca^{2+} efflux from the SR (contrary to the results of Shoshan-Barmatz, 1987) then this may explain the more profound effect of similar concentrations of ADP on cardiac SR observed in this study.

However, there may be other effects of these metabolites that complicate the interpretation of the results. One possibility is that the affinity of the contractile proteins for Ca^{2+} may be altered by P_i or ADP. The lowered Ca^{2+}-activated force on the addition of P_i may be accompanied by a fall in the affinity of troponin-C for Ca^{2+}. Therefore, less of the Ca^{2+} released from the SR would bind to troponin-C in the presence of P_i allowing a higher fraction to be free [Ca^{2+}] within the preparation. There is no direct evidence, however, to suggest that P_i may reduce Ca^{2+} binding to myofilaments. On the contrary, work on isolated troponin-C by Kentish & Palmer (1989) failed to reveal an effect of P_i on Ca^{2+} binding. Similar arguments may also apply to effects of ADP. Potentiation of Ca^{2+}-activated force by ADP has been observed in skeletal and cardiac muscle (Godt & Nozek, 1989). Evidence from work by Guth & Potter (1987) suggests that this potentiation may be accompanied by an increase in the Ca^{2+} binding to troponin-C, so more of the Ca^{2+} released from the SR would bind to troponin C and thereby reduce the free [Ca^{2+}]. It is not known to what degree changes in myofilament Ca^{2+} binding alter the amplitude of the caffeine-induced Ca^{2+} release in the presence of 0·2 mM EGTA. However, compounds that are known to act specifically to enhance Ca^{2+} binding to myofilaments have little or no effect on caffeine-induced Ca^{2+} transients (D. S. Steele & G. L. Smith, unpublished observation).

Effects of P_i on spontaneous Ca^{2+} release

Figure 8*A* shows that addition of 10 mM P_i in the absence of caffeine injections caused a transient rise of [Ca^{2+}] within the preparation. Removal of P_i caused a transient fall in the [Ca^{2+}] (also shown in Fig. 3). This is consistent with P_i causing transient release of Ca^{2+} from the SR and removal of P_i causing a transient uptake of Ca^{2+}. On increasing the [Ca^{2+}] of the bathing solution to 0·5 μM, the SR spontaneously released Ca^{2+} periodically resulting in oscillations in the [Ca^{2+}] within the preparation. Similar behaviour has been reported by Fabiato & Fabiato (1975) in mechanically skinned cardiac cells. Spontaneous Ca^{2+} release is thought to occur when cardiac SR has a high Ca^{2+} content. Spontaneous release of Ca^{2+} would increase

the $[Ca^{2+}]$ next to the SR surface triggering further Ca^{2+} release (Fabiato & Fabiato, 1975). Both the transient release observed on addition of 10 mM P_i and the absence of oscillations in $[Ca^{2+}]$ in the presence of P_i are consistent with P_i decreasing the SR Ca^{2+} content. Under these conditions, any Ca^{2+} efflux will be less since the $[Ca^{2+}]$ gradient across the SR is reduced. The reduced spontaneous release may be insufficient to trigger further Ca^{2+} release from the SR. Removal of P_i caused an initial rapid fall in $[Ca^{2+}]$ within the preparation, consistent with a net Ca^{2+} uptake by the SR. However, this was followed by a transient increase in the $[Ca^{2+}]$ due to spontaneous release of Ca^{2+} from the SR. After this, the oscillations of $[Ca^{2+}]$ returned to the pattern seen prior to P_i addition. These results show that in the presence of a high $[P_i]$, spontaneous release of Ca^{2+} from the SR is reduced. This is consistent with measurements of intracellular $[Ca^{2+}]$ in intact preparations during metabolic blockade and ischaemia where, despite high intracellular $[Ca^{2+}]$, oscillations of intracellular $[Ca^{2+}]$ were absent or small in amplitude (Smith & Allen, 1987; Allen *et al.* 1989). Large oscillations of intracellular $[Ca^{2+}]$ occurred when metabolism was restarted after metabolic blockade (Smith & Allen, 1987). This was originally thought to be due to resynthesis of ATP. However, the results of this study suggest that a concomitant fall in intracellular $[P_i]$ would increase the Ca^{2+} content of the SR and give rise to spontaneous Ca^{2+} release. Oscillations of intracellular $[Ca^{2+}]$ cause arrythmic behaviour in cardiac muscle (Capogrossi, Houser, Bahinski & Lakatta, 1987; Smith & Allen, 1987). Thus spontaneous release of Ca^{2+} from the SR as a result of a fall in intracellular $[P_i]$ may trigger arrhythmias frequently seen on reperfusion of a previously ischaemic myocardium (Opie, 1989).

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