Effects of inorganic phosphate and ADP on calcium handling by the sarcoplasmic reticulum in rat skinned cardiac muscles

Jian-Zhong Xiang and Jonathan C Kentish

Objective: The aim was to investigate whether, and how, increases in inorganic phosphate (P_i) and ADP, similar to those occurring intracellularly during early myocardial ischaemia, affect the calcium handling of the sarcoplasmic reticulum. **Methods:** Rat ventricular trabeculae were permeabilised with saponin. The physiological process of calcium induced calcium release (CICR) from the muscle sarcoplasmic reticulum was triggered via flash photolysis of the "caged Ca^{2+}", nitr-5. Alternatively, calcium release was induced by rapid application of caffeine to give an estimate of sarcoplasmic reticular calcium loading. The initial rate of sarcoplasmic reticular calcium pumping was also assessed by photolysis of caged ATP at saturating [Ca^{2+}]. Myoplasmic [Ca^{2+}] (using fluo-3) and isometric force were measured. **Results:** P_i (2-20 mM) significantly depressed the magnitude of CICR and the associated force transient. Sarcoplasmic reticular calcium loading was inhibited even more than CICR by P_i, suggesting that reduced calcium loading could account for all of the inhibitory effect of P_i on CICR and that P_i may slightly activate the calcium release mechanism. The reduced sarcoplasmic reticular calcium loading seemed to be due to a fall in the free energy of ATP hydrolysis (ΔG_{ATP}) available for the calcium pump, since equal decreases in ΔG_{ATP} produced by adding both P_i and ADP in various ratios caused similar falls in the calcium loading of the sarcoplasmic reticulum. The caged ATP experiments indicated that P_i (20 mM) did not affect the rate constant of sarcoplasmic reticular calcium uptake. ADP (10 mM) alone, or with 1 mM P_i, inhibited calcium loading. In spite of this, ADP (10 mM) did not alter CICR and, when 1 mM P_i was added, ADP increased CICR above control. **Conclusions:** An increase in intracellular P_i reduces sarcoplasmic reticular calcium loading and thus depresses the CICR. This could be an important contributing factor in the hypoxic or ischaemic contractile failure of the myocardium. However the detrimental effect of P_i may be offset to some extent by a stimulatory action of ADP on the calcium release mechanism of CICR.
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During the first few minutes of ischaemia in the mammalian myocardium there is a rapid fall of contractile force. Associated with this are alterations in intracellular pH and in the concentrations of intracellular metabolites (reviewed[1]). It is now clear that part of the contractile failure is due to a rise in the intracellular concentration of inorganic phosphate (P_i), from its normal value of about 1 mM to about 20 mM. P_i directly inhibits the calcium activated force production of cardiac myofilaments.[2 3] However, P_i (or indeed other metabolites) could also potentially suppress earlier steps in excitation-contraction coupling, such as calcium induced calcium release (CICR[4]) from the sarcoplasmic reticulum. Any reduction in CICR would decrease the calcium activation of the myofilaments and so contribute to the ischaemic contractile failure. At present, it is not clear whether the sarcoplasmic reticular calcium handling is compromised during ischaemia, since the inaccessibility of the sarcoplasmic reticulum in intact cardiac myocytes makes it difficult to examine the properties of the organelle under controlled changes in intracellular metabolic conditions. There have been many studies of the calcium transient in intact cells during hypoxia or ischaemia, but these have given only limited information on the handling of calcium by the sarcoplasmic reticulum because the calcium transient is also dependent upon cellular calcium binding and transsarcolemmal calcium extrusion processes, which may also be altered during hypoxia or ischaemia. An alternative approach is to study sarcoplasmic reticular calcium release in skinned cardiac cells, in which the sarcolemma has either been removed mechanically[4] or chemically (for example, by using saponin, which leaves the sarcoplasmic reticulum intact[5]), so that the intracellular milieu is under precise control. This permits the effects of metabolic changes (for example, pH, P_i) on calcium release from the organelle to be studied individually. Using saponin skinned cardiac muscle, Zhu and Nosek[6] reported that P_i decreased the caffeine induced calcium release from the sarcoplasmic reticulum (an estimate of its calcium loading) with short loading times (<1 min), but potentiated the release at longer times. On the other hand, Smith and Steele[7] found a decrease in caffeine stimulated calcium release after a two minute loading. Thus the effects of P_i on sarcoplasmic reticular calcium loading remain uncertain. Furthermore, changes in calcium loading suggest only indirectly how CICR will be affected by P_i, since the calcium release channel could also be influenced directly by P_i,[8] as it is by adenine nucleotides.[9]

In the present study, we examined directly the effects of P_i on CICR and on the resulting force transients in saponin skinned trabeculae from rat ventricle, in order to explore the possible involvement of CICR dysfunction in the contractile failure of the ischaemic myocardium. To produce the rapid rise in trigger [Ca^{2+}] that is necessary for CICR,[4] we employed flash photolysis of the "caged Ca^{2+}" nitr-5, which

Department of Pharmacology, UMDS, St Thomas' Hospital, London SE1 7EH, United Kingdom: J-Z Xiang, J C Kentish. Correspondence to Dr Kentish.

Compositions of the different types of solutions used in the experiments.

Solution	*EGTA (mmol·litre^{-1})*	*$CaCl_2$ (mmol·litre^{-1})*	*Nitr-5 (mmol·litre^{-1})*	*Fluo-3 (mmol·litre^{-1})*	*Caffeine (mmol·litre^{-1})*	*[Ca^{2+}] (μmol·litre^{-1})*
Relaxing (R)	1.0	–	–	–	–	0.003
Preactivating (P)	0.05	–	–	–	–	0.03
Activating (A)	1.0	1.1	–	0.01	3	28
Depleting (D)	1.0	–	–	–	10	0.003
Loading (L)	–	0.04–0.06[a]	0.1	0.01	–	~0.20
Caffeine (C)	0.1	0.05–0.07[b]	–	0.01	3	~0.25[b]

All solutions had the same basic composition (see text), plus the components shown. Up to 20 mM P_i and 10 mM ADP were also added. Corrections for ionic strength, allowance for EGTA purity (97%) and the principles of the calculations were as previously described.[3 39]
[a]Due to variable amounts of contaminant Ca^{2+}, the total added Ca^{2+} was varied as shown to produce a measured [Ca^{2+}] of ~0.2 μM.
[b]Varied to produce the same fluorescence as loading solution, in spite of a 16% quenching of fluo-3 fluorescence by the caffeine.

produces a rapid (<1 ms) rise in [Ca^{2+}] throughout the muscle.[10] In addition, the sarcoplasmic reticular calcium loading was estimated by rapid injection of caffeine. Both the resting [Ca^{2+}] and the calcium transients were measured using the fluorescent calcium indicator, fluo-3.[11] While trying to elucidate the mechanisms of action of P_i, we also studied the effects of ADP on calcium loading and calcium release. We found that both P_i and ADP are modulators of sarcoplasmic reticular calcium release.

Preliminary accounts of some of these results have been presented in abstract form.[12 13]

Methods

Muscle skinning and fluorescence measurement
The investigation was performed in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986*, published by HMSO, London. Male Wistar rats (200-250 g) were killed by cervical dislocation. Their hearts were removed and bathed at room temperature in oxygenated Tyrode solution as described previously.[3] Trabeculae of 100-200 μm diameter were dissected out from the right ventricle and were mounted in an apparatus for measuring force and fluorescence.[14] One end of the muscle was clamped in the tweezers of a force transducer and the other end was similarly attached to a micromanipulator. The muscle was slid into a cuvette of 1 × 1 mm quartz tubing (20 μl volume), and was skinned by a 30 min superfusion with relaxing solution (see below) containing 50 μg·ml^{-1} saponin. Saponin was then washed out with relaxing solution. Solutions could either be pumped into the cuvette via electrical valves or, when a fast perfusion was desired, could be injected from a syringe driven by a stepper motor. Perfusion solutions were maintained at 22°C using water circulating in the cuvette holder.

We measured [Ca^{2+}] in the skinned muscles using fluo-3, rather than fura-2 or indo-1, since fluo-3 uses longer excitation wavelengths (485-505 nm) which do not overlap the near-UV wavelengths needed for nitr-5 photolysis.[11] Excitation light from a 100 watt mercury arc lamp was directed through a 490 nm bandpass filter and an adjustable slit onto the skinned muscle in the cuvette and its intensity was monitored with a photodiode in the light path. Emitted light (fluo-3 fluorescence) was collected via an objective (20×, NA 0.3) above the cuvette, was passed through a 510 nm dichroic mirror and a bandpass filter (535 DF45, Omega Optical), and was recorded by a photomultiplier. The dichroic mirror directed the reflected excitation light to an eyepiece, through which the muscle could be observed. The slits in the excitation and emission light pathways were adjusted to ensure fluorescence was collected from all the muscle (to avoid motion artefacts) but with a minimum contribution (<15%) from the fluorescence of solution outside the muscle. The fluo-3 fluorescence signal was divided by the light signal from the photodiode to correct for changes in excitation light intensity. For flash photolysis, a flash was generated by a xenon flashlamp, filtered by a broad band filter (UG11, 310-370 nm), and focused onto the muscle. The flash energy was 100 mJ, which photolysed approximately 30% of the nitr-5, judged from the resulting rise in [Ca^{2+}]. Force and fluorescence signals were filtered at 50 Hz, displayed on a four channel chart recorder and recorded on an IBM compatible computer fitted with a 12 bit A/D board.

Solutions
All solutions (except where otherwise stated) contained (in mmol·litre^{-1}): $MgATP^{2-}$ 5, Mg^{2+} 1, Na_2 phosphocreatine 10, dithiothreitol 1, potassium propionate 83, and N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES) 100; total ionic strength 0.20 M; pH 7.10 at 22°C. Sodium azide (5 mM) and carbonylcyanide m-chlorophenylhydrazone (CCCP, 10 μM) were included to prevent mitochondrial calcium accumulation. The table shows the different types of experimental solutions. For solutions containing ADP, phosphocreatine was omitted and the myokinase inhibitor p^1,p^2-di(adenosine-5')pentaphosphate (AP_5A) was included to prevent ATP production from ADP. The concentrations of $MgCl_2$ and potassium propionate were adjusted to keep [Mg^{2+}] at 1 mM and ionic strength at 0.20 M. Free ion concentrations were calculated as previously[3] but with the binding constants[15] corrected for temperature and ionic strength effects.[16]

Nitr-5 (free acid) was from Calbiochem and fluo-3 pentapotassium salt was from Molecular Probes. Caged ATP (2-nitrophenylethyl ester) was a generous gift from Dr D Trentham (National Institute for Medical Research, Mill Hill). All other chemicals were from Sigma.

Experimental protocol
The protocols for the calcium release experiments are illustrated in fig 1. To obtain the maximum force and fluorescence responses (fig 1A), the muscle was bathed sequentially with: caffeine-containing depleting solution (D; table) for 1 min to deplete the sarcoplasmic reticulum of calcium; relaxing solution (R) for 1 min to wash out caffeine; preactivating solution (P) for 1 min to wash out EGTA and so minimise the calcium buffering capacity of the solution, thereby accelerating the subsequent calcium activation of the muscle; a maximal activating solution (A; containing 3 mM caffeine to block net calcium uptake during force development) until steady force and fluo-3 fluorescence were attained (usually within 1 min); and finally, depleting solution. After the first maximum contracture, the resting force was set at ~10% of the maximum force.

To study CICR (fig 1B), the muscle was perfused with D, R, and P solutions as above and then loading solution (L; [Ca^{2+}] ~0.21 μM buffered with 0.1 mM nitr-5) for 3 min. The perfusion was then stopped and a 100 mJ flash was directed onto the muscle (bathed throughout in solution inside the cuvette). Photolysis of nitr-5 caused transient increases in both fluorescence and force. The change in fluorescence (for example, fig 2) consisted of a fast initial increase as calcium was liberated from photolysed nitr-5 ("Ca^{2+} jump"), followed by a transient increase due to CICR from the sarcoplasmic reticulum ("Ca^{2+} transient"). Perfusion was resumed after 20 s, and the muscle was returned to depleting solution.

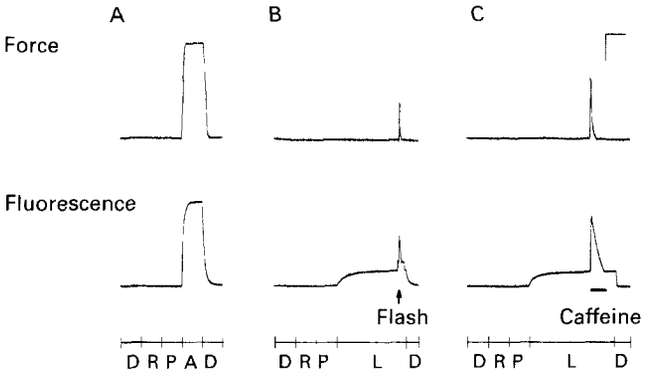

Figure 1 *Chart records of force and fluo-3 fluorescence in a saponin skinned trabecula to illustrate the major experimental protocols. The solutions changes are shown at the bottom (see the table for key). (A) The responses of fluorescence and force to maximum activating solution (solution A, 28 μM Ca^{2+}). (B) Calcium induced calcium release initiated by flash photolysis (100 mJ) of nitr-5 (100 μM) after the muscle had been perfused with loading solution (L, 0.21 μM Ca^{2+}) for 3 min. (C) Sarcoplasmic reticular calcium loading assessed by rapid injection of caffeine (3 mM) after the 3 min loading period. Horizontal bar: 1 min; vertical bar: 0.2 of maximum values.*

To examine sarcoplasmic reticular calcium loading (fig 1C), muscles were subjected to the same loading protocol as above except that after the 3 min loading of the sarcoplasmic reticulum, calcium was mobilised from the organelle by rapid injection (1 ml·s^{-1} for 80 ms) of caffeine solution (C; table), which gave more than 90% of the maximum calcium release. After force and fluorescence transients had declined to the precaffeine levels, perfusion was restarted and the muscle was returned to depleting solution.

After subtraction of the backgrounds, force and fluorescence data were expressed relative to the values in activating solution. Because 3 mM caffeine, as used in the activating solution, was found to quench fluo-3 fluorescence by 16(SEM 1)% (n = 9), a correction for this was made to the fluorescence data from CICR experiments. The relative fluorescence was then converted to [Ca^{2+}] using the parameters n and K_d derived from the fluo-3 calibration curve (see below). The magnitude of the calcium transient due to sarcoplasmic reticular calcium release was calculated from the peak [Ca^{2+}] minus the [Ca^{2+}] jump in CICR or minus the loading calcium in caffeine induced calcium release. The size of the calcium jump was checked at the end of each experiment after the muscle had been treated with 1 μM ryanodine to disable sarcoplasmic reticular function (fig 2).

To determine the effects of P_i or ADP on CICR or on calcium loading of the sarcoplasmic reticulum, P_i (1-20 mM) and/or ADP (1-10 mM) were added to the loading solution. With no P_i added, solution P_i was ~0.4 mM due to contamination from phosphocreatine.[3] Calcium releases were elicited a few times at each [P_i] or [ADP] and the mean values for calcium and force transients were then used as individual data points for each muscle. Control releases were always performed before and after the "test" releases in order to check for any decline of the responses.

In another set of experiments we attempted to study the effect of P_i on the rate of sarcoplasmic reticular calcium pumping. The sarcoplasmic reticulum was first depleted of calcium using solution D for 1 min. Then ATP and phosphocreatine were removed using a rigor solution containing (in mmol·litre^{-1}): BES 100, potassium propionate 95, Mg^{2+} 1.0, EGTA 1.0, and caffeine 10, with 0.1 U·ml^{-1} apyrase to break down contaminant ATP and ADP. After rigor had developed (3-5 min), a calcium rigor solution containing 1-2 μM Ca^{2+} was perfused for 3 min. Then the perfusion was stopped and calcium rigor solution containing 0.5 mM caged ATP was injected into the cuvette. At least 15 s after the injection, a 100 mJ flash was given. From the calculated photolysis of nitr-5 (30%) and the higher quantum efficiency of caged ATP, we estimate that this flash photolysed 50% or more of the caged ATP. The ATP released activated the sarcoplasmic reticular calcium pump and the rapid fall of [Ca^{2+}] as calcium was pumped into the organelle gave us a measure of the rate of calcium uptake. To check that this fall of [Ca^{2+}] was indeed due to the operation of the sarcoplasmic reticular calcium pump, we routinely repeated the protocol after treating the muscle for 60 min with the specific calcium pump inhibitor, cyclopiazonic acid (CPA, 60 μM).

Fluorescence calibration

Fluo-3 fluorescence was calibrated in saponin skinned muscles using solutions like relaxing solution but containing 10 mM EGTA, 0-10.5 mM calcium (free [Ca^{2+}]: 3 nM-125 μM), and 10 μM fluo-3. Fluorescence levels above baseline (~2% of maximum fluorescence) were fitted by non-linear least squares to a Hill equation: fluorescence = maximum fluorescence × $[Ca^{2+}]^n/(K_d + [Ca^{2+}]^n)$, where n and K_d are the Hill coefficient and the dissociation constant of fluo-3 for calcium, respectively. Fluo-3 fluorescence from the muscles was sigmoidally related to log [Ca^{2+}], with $n = 1.03$ and $K_d = 0.55$(SEM 0.02) μM (n = 14). P_i (20 mM) did not alter the K_d [0.54(0.02) μM, n = 8], nor did ADP [10 mM, 0.58(0.04) μM, n = 6]. The control K_d was also the same as that in solution without a muscle [0.54(0.02) μM, n = 4]. Force, recorded simultaneously with fluorescence, was less sensitive to [Ca^{2+}] but had a steeper relationship. The [Ca^{2+}] for 50% activation of force was 2.63(0.05) μM in control, 8.41(0.08) μM in 20 mM P_i, and 2.23(0.05) μM in 10 mM ADP.

Data analysis

Data were normally analysed using Student's unpaired or paired t test and are given as mean (SEM). $P < 0.05$ was considered statistically significant.

Results

Effects of P_i *on calcium induced calcium release*

After the 3 min loading of the sarcoplasmic reticulum in 0.2 μM free calcium, photolysis of nitr-5 induced increases in muscle force and fluo-3 fluorescence (figs 1B and 2). Following a flash, there was a rapid jump of [Ca^{2+}] from 0.18(0.04) μM to 0.37(0.03) μM (10 muscles), followed by a transient rise of [Ca^{2+}], which reached 0.78(0.03) μM. The Ca^{2+} transient was fast to rise [half time to peak, $t_{1/2\ peak}$, was 0.39(0.02) s] and slower to fall [half time to recovery, $t_{1/2\ recovery}$, was 0.61(0.06) s]. The accompanying force transient amounted to 20(5)% of maximum force and had a $t_{1/2\ peak}$ of 0.31(0.02) s and a $t_{1/2\ recovery}$ of 0.54(0.03) s. After the muscles had been treated with 1 μM ryanodine, which specifically opens the sarcoplasmic reticular calcium release channels and depletes the organelle of calcium,[17] the transients of force and fluorescence disappeared, although the jump of fluorescence remained (fig 2). This indicates that the transients induced by a flash were due to CICR from the

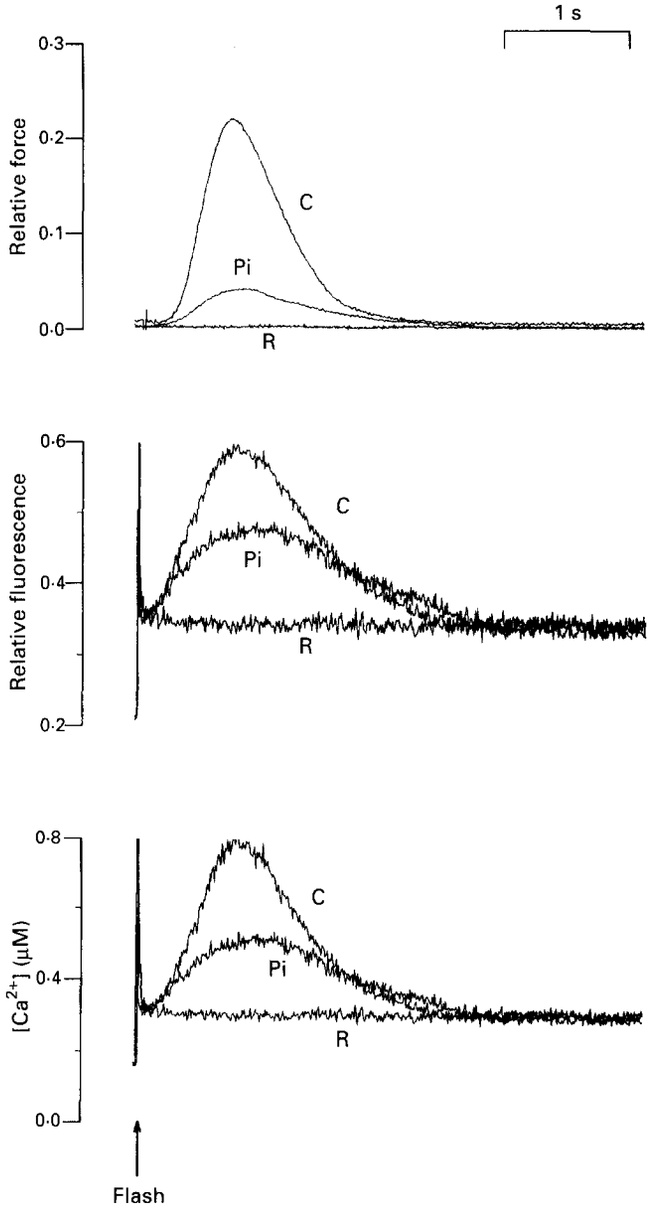

Figure 2 *Chart records illustrating the inhibitory effects of P_i on calcium induced calcium release induced by flash photolysis of nitr-5 in a skinned trabecula. The sarcoplasmic reticulum was calcium loaded in the absence (C) or presence of 20 mM P_i (P_i) before a flash was given. Force and fluorescence are given relative to the maximum values (at >28 μM Ca^{2+}) in zero added P_i. [Ca^{2+}] was converted from fluorescence according to the fluorescence-[Ca^{2+}] calibration. The loading/flash protocol was carried out six times (two control, two with P_i, then two control) and the appropriate force, fluorescence, and [Ca^{2+}] data were averaged and superimposed. Traces designated R are control traces obtained after exposing the muscle to 1 μM ryanodine for 20 min.*

sarcoplasmic reticulum and that the calcium release from nitr-5 did not itself produce any direct calcium activation of the myofibrils.

Inclusion of P_i (20 mM) in loading solution substantially decreased the calcium and force transients elicited by photolysis of nitr-5, although the initial $[Ca^{2+}]$ jump was unaffected (fig 2). These effects of P_i were reversed after returning to control solution. In eight muscles (fig 4), the inhibition by P_i of the calcium transient (filled triangles) was statistically significant with as little as 2 mM added P_i. The inhibition increased non-linearly as $[P_i]$ rose to 20 mM, at which the calcium transient was reduced to 55(4)% of control. The force transients (filled circles) were inhibited even more strongly; for example, force in 20 mM P_i was 7(3)% of control. Some of the fall of force was certainly due to the direct depression by P_i of myofibrillar force production, but some was also due to the diminution of the calcium transient, resulting in less calcium activation of the myofibrils. The timecourses of the calcium and force transients were not significantly altered in the presence of P_i; for example, $t_{1/2\ recovery}$ for the calcium transient was 0.58(0.07) s in 20 mM P_i, compared with 0.61(0.06) s for control.

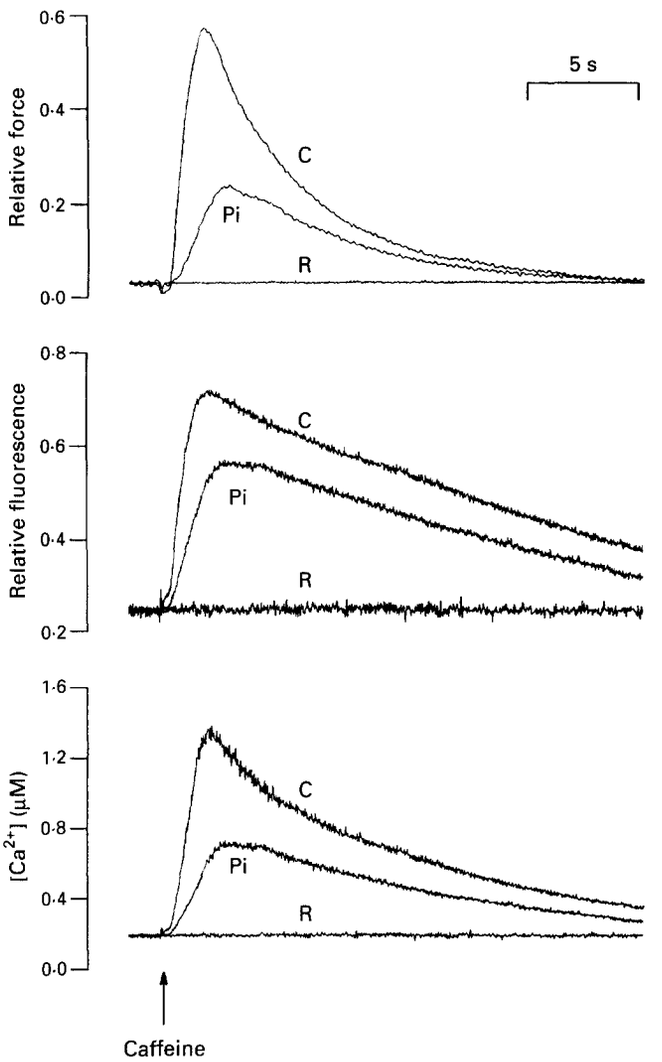

Figure 3 *Chart records showing the inhibitory effects of 20 mM P_i on sarcoplasmic reticular calcium loading, assessed by depletion of the sarcoplasmic reticulum using caffeine (3 mM). The superimposed traces were obtained using a bracketing procedure as in fig 2 and represent the average of four traces for control (C) and two traces for 20 mM P_i (Pi). Traces designated R are control traces obtained after exposing the muscle to 1 μM ryanodine for 20 min.*

Effects of P_i on the releasable calcium in the sarcoplasmic reticulum

The inhibition of CICR by P_i could have been due to (1) a decrease in the amount of releasable calcium inside the sarcoplasmic reticulum, or (2) a decrease in the calcium activation of the sarcoplasmic reticular calcium release channels. To distinguish between these mechanisms, we used caffeine to measure the releasable calcium in the absence and presence of P_i. Previous work had shown that after a 3 min loading period, as we used, caffeine-induced calcium release was slightly potentiated[6] or markedly inhibited[7] by P_i, so it was important to measure caffeine induced release under our conditions. After the loading period, the rapid injection of caffeine caused substantial calcium and force transients (figs 1C and 3), reaching 1.40(0.09) μM (n = 10) and 63(4)% of maximum force, respectively. The timecourses of the caffeine induced transients were slower than those induced by flash photolysis; for example, $t_{1/2\ recovery}$ for $[Ca^{2+}]$ was 1.98(0.31) s. This probably reflects the fact that, in the continued presence of caffeine, net reuptake of calcium into the sarcoplasmic reticulum is largely prevented, so $[Ca^{2+}]$ declines only by the slow diffusion of calcium out of the muscle. The faster decline after CICR (fig 2) shows that most of the fall of $[Ca^{2+}]$ after CICR was due to calcium reuptake into the sarcoplasmic reticulum.

As fig 3 shows, the presence of P_i (20 mM) in loading solution reduced the caffeine induced Ca^{2+} and force transients. In six muscles (fig 4), the caffeine induced Ca^{2+} transient (empty triangles) was reduced significantly at all P_i concentrations studied. The inhibition tailed off at higher $[P_i]$ and at 20 mM P_i the calcium transient was 45(9)% of control. The force transient (empty circles) was reduced to 18(3)% of control at 20 mM P_i. These results suggested that P_i had decreased the releasable calcium in the sarcoplasmic reticulum. To check that P_i was not merely acting by reducing the effectiveness of 3 mM caffeine, which may induce calcium release by sensitising the calcium release channel to calcium,[18] we also used 10 mM caffeine, which releases calcium by a calcium independent

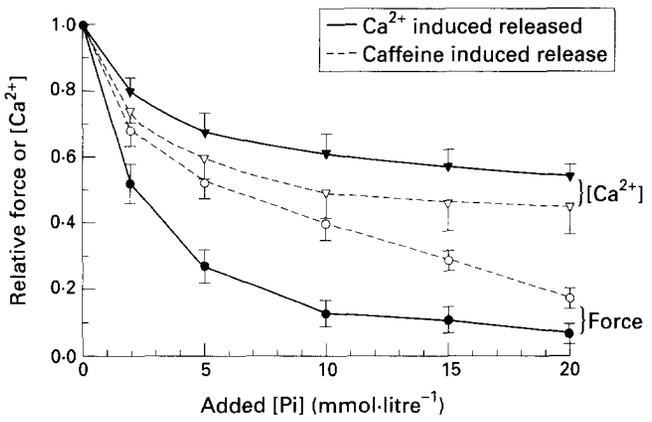

Figure 4 *Comparison of the effects of P_i on caffeine induced and calcium induced calcium release. The transients in the presence of P_i are expressed relative to those in bracketing controls, as in figs 2 and 3. ▼ = $[Ca^{2+}]$ and ● = force in calcium induced transients (n = 8). ▽ = $[Ca^{2+}]$ and ○ = force in caffeine induced transients (n = 6). The curves passing through the calcium data are double exponential fits to all the individual data points from all muscles. All points were significantly different from control ($P < 0.01$, paired t test).*

mechanism.[18] The results (not shown) were the same as with 3 mM caffeine, demonstrating that P_i had indeed reduced the amount of releasable calcium in the sarcoplasmic reticulum.

The fall in both caffeine and calcium induced calcium release in the presence of P_i were very similar over the entire $[P_i]$ range studied (fig 4), and there was no significant difference between each pair of points at a given $[P_i]$ (Student's *t* test). This suggested that the suppression of CICR by P_i could be explained entirely by a reduction in the releasable calcium in the sarcoplasmic reticulum. There was no evidence for an additional inhibition by P_i on the calcium release mechanism of CICR. Indeed, there was a tendency for P_i to have a smaller effect on CICR than would be expected from the reduced calcium loading. To analyse this further, we fitted double exponential curves to the collected, individual data points from all muscles in caffeine and CICR experiments (these curves are shown in fig 4) and calculated the variance of the points about each curve. We then compared these with the variance of points about a *single* curve fitted to both caffeine and CICR data (not shown). The variances were significantly different (F test, $P < 0.05$), indicating that there was a significantly smaller effect of P_i on calcium induced than on caffeine induced calcium transients. These results indicate that the effect of P_i on CICR is indeed somewhat less than expected from the reduction in calcium loading.

With the force transients, P_i affected the caffeine induced contractions less than the calcium induced contractions, particularly at $[P_i]$ of 5-15 mM (fig 4). This is probably related to the direct inhibitory actions of P_i on the myofibrils, which would be expected to be less with caffeine than with CICR, since with caffeine the calcium activation of the myofibrils was closer to saturation, and the direct action of P_i on myofibrillar calcium sensitivity[3] would then have a smaller overall effect.

Calcium release in the presence of ADP and P_i

We considered three possible mechanisms by which P_i could have decreased the releasable calcium in the sarcoplasmic reticulum: (1) a decrease in the free energy available from ATP hydrolysis, resulting in less uptake of calcium into the organelle[19 20]; (2) a direct inhibition of sarcoplasmic reticular calcium pump activity; and (3) the formation of Ca-P_i precipitates inside the sarcoplasmic reticular lumen.[21]

On the first mechanism, thermodynamics predict that the equilibrium concentration ratio of calcium generated across the sarcoplasmic reticular membrane will be reduced by a rise in cytosolic $[P_i]$. The free energy change available from ATP hydrolysis, ΔG_{ATP}, is given by:

$$\Delta G_{ATP} = \Delta G_o - RT \ln [ATP]/([ADP][P_i]) \quad (1)$$

where ΔG_o is the standard free energy change under our conditions (37 $kJ \cdot mol^{-1}$ [22]), R is the gas constant, and T is the temperature. Thus an elevation of $[P_i]$ will reduce ΔG_{ATP} and so make less energy available for pumping. If two calcium ions are pumped per ATP molecule hydrolysed,[20] and assuming that there is no electrical gradient across the sarcoplasmic reticular membrane, the equilibrium ratio of $[Ca^{2+}]$ that can be generated by ATP hydrolysis is given by:

$$\Delta G_{ATP} = -2RT \ln [Ca^{2+}]_{lumen}/[Ca^{2+}]_{cytosol} \quad (2)$$

Thus, if cytosolic $[Ca^{2+}]$ is constant, as in this study (loading $[Ca^{2+}]$ was ~0.2 μM throughout), the smaller ΔG_{ATP} in the presence of P_i would decrease the equilibrium value for sarcoplasmic reticular luminal $[Ca^{2+}]$. One way to test whether the inhibition by P_i is indeed due to a fall in ΔG_{ATP} is to vary $[P_i]$ and [ADP] but keep their product constant[3 7]; then if ΔG_{ATP} is the critical factor, the depression of calcium release should be constant.

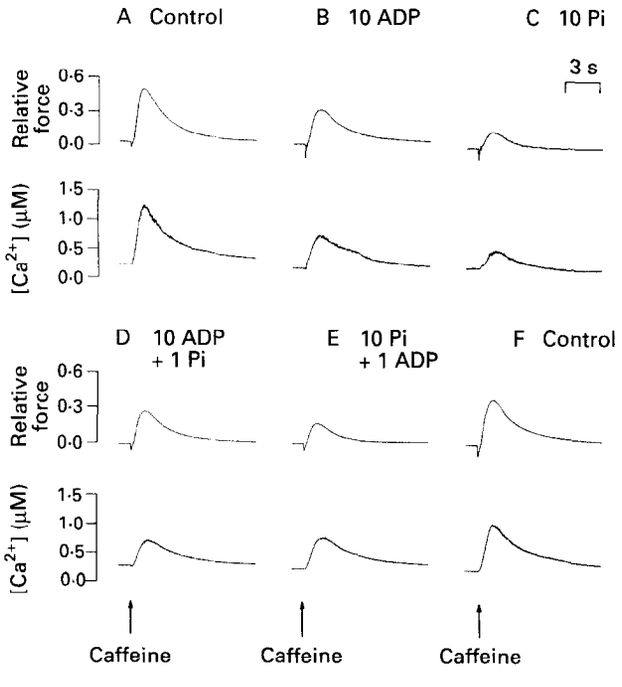

Figure 5 *The effects of ADP and P_i on the releasable sarcoplasmic reticulum calcium in a skinned trabecula. A protocol similar to that in fig 3 was used. The concentrations of P_i and ADP are in mmol·litre^{-1}.*

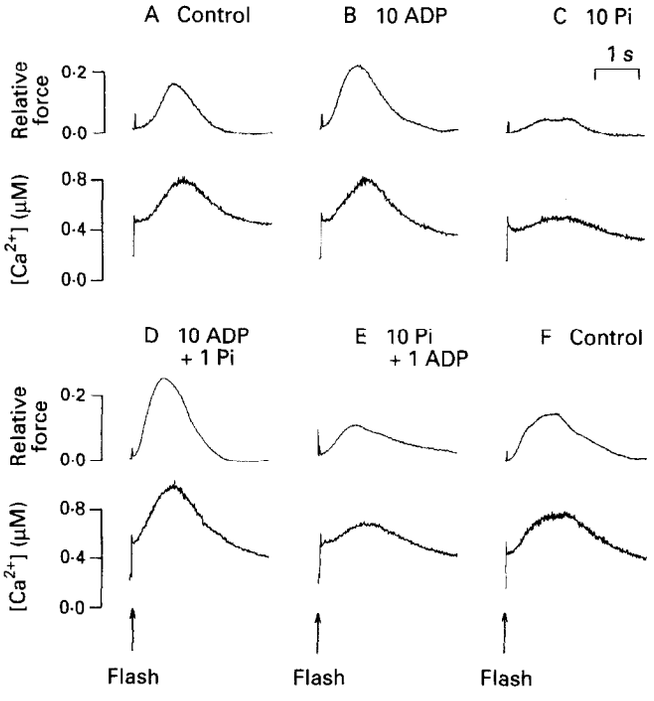

Figure 6 *The effects of ADP and P_i on calcium induced calcium release in a skinned trabecula. A protocol similar to that in fig 2 was used. The concentrations of P_i and ADP are in mmol·litre^{-1}.*

Figures 5 and 7A show how sarcoplasmic reticular calcium loading was affected by variations of [ADP] and/or [P_i] (in the absence of phosphocreatine). We found that 10 mM ADP alone reduced caffeine induced calcium release, though the fall in loading (to 68% of control) was only about one half of that produced by 10 mM P_i (to 38%). When [ADP] and [P_i] were varied in combination (of 10:1 or 1:10), the calcium transient was decreased to similar extents [to 43(3)% and 45(4)% of control, respectively; $P > 0.05$]. This similarity suggests that the action of P_i to diminish the amount of releasable calcium in the sarcoplasmic reticulum may indeed be due to a fall of ΔG_{ATP}.

We also studied the effects of the ADP/P_i combinations on CICR. As figs 6 and 7B show, their effects on CICR were different from those on calcium loading. Although ADP (10 mM) depressed loading (fig 7A), it did not alter CICR significantly from control (figs 6 and 7B). This suggested that ADP could increase the proportion of sequestered calcium that was released by CICR. Even more striking, the addition of 1 mM P_i to 10 mM ADP significantly *increased* the calcium transient (to 131% of control; fig 7B), even though 1 mM P_i enhanced the inhibitory action of 10 mM ADP on sarcoplasmic reticular calcium loading (fig 7A). Thus 10 mM ADP appeared to inhibit the sarcoplasmic reticular calcium pump but stimulate CICR, particularly if 1 mM P_i was present. However, when P_i was present at 10 mM, CICR was significantly reduced (as in fig 4), with or without 1 mM ADP (fig 7B).

The effects on the force transients were generally similar to, and could be explained by, the effects on calcium release, except that P_i (10 mM) had greater effects on the force than on the calcium transients, presumably because it directly inhibited myofibrillar force production. None of the solutions affected the timecourses of the caffeine induced or calcium induced calcium transients (data not shown).

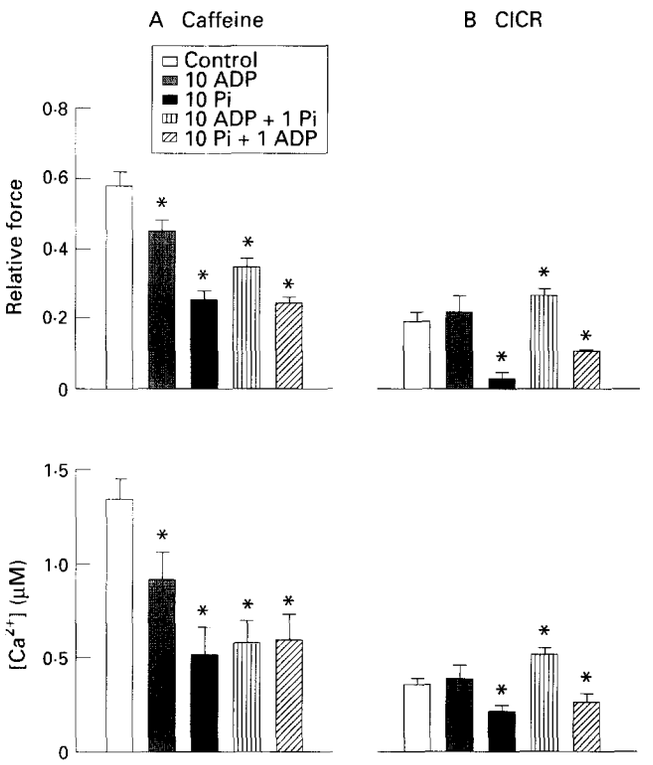

Figure 7 *Effects of ADP and P_i on (A) caffeine induced and (B) calcium induced calcium release in skinned trabeculae. Top: relative force; bottom: [Ca^{2+}]. The [Ca^{2+}] data represent the net transients, that is, peak [Ca^{2+}] minus loading [Ca^{2+}] (for caffeine) or minus the jump of [Ca^{2+}] due to nitr-5 photolysis itself (for CICR). The concentrations of P_i and ADP are in mmol·litre^{-1}; n = 9 in A and n = 6 in B.*
**$P < 0.01$ v control (paired t test).*

Effects of P_i on the rate of calcium uptake by the sarcoplasmic reticulum

Indirect evidence that P_i did not affect the rate of sarcoplasmic reticular calcium uptake was that the decay of the calcium transient after CICR was not slowed down significantly in the presence of P_i (above). To examine more directly whether P_i altered the rate of calcium pumping, we depleted the sarcoplasmic reticulum of calcium, removed the ATP using a rigor solution, and then activated the pump by the photolytic production of ATP from caged ATP in the presence of near-saturating [Ca^{2+}] (>1 μM). The photorelease of ATP (fig 8) caused a small immediate increase in force, which was probably due to the activation of the

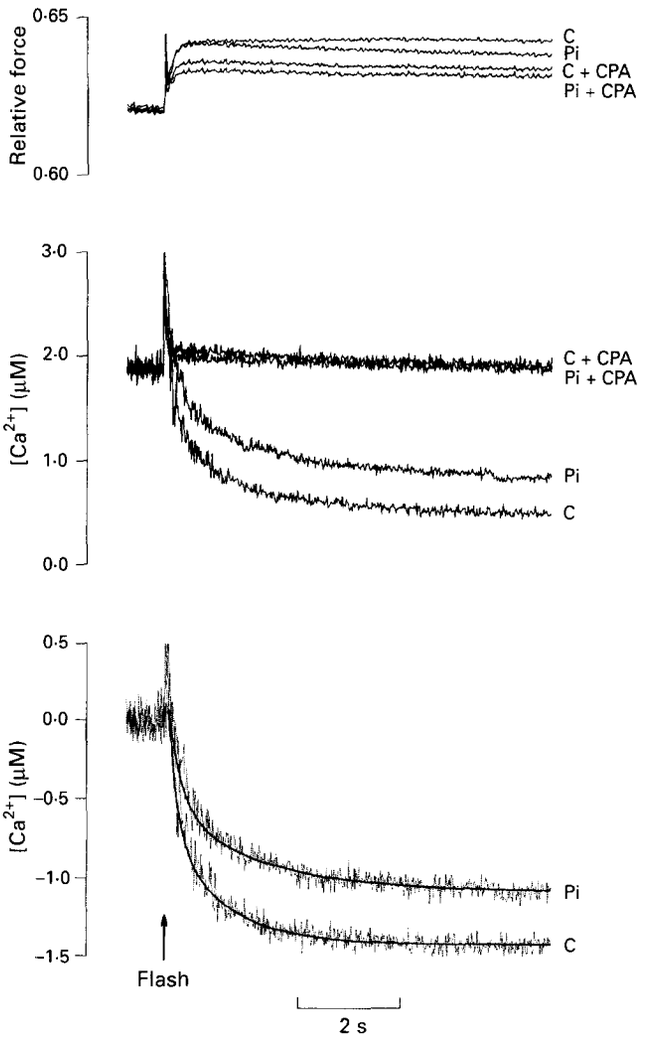

Figure 8 *Effects of 20 mM P_i on the rate of sarcoplasmic reticular calcium uptake in a skinned trabecula. Responses in force (top panel) and [Ca^{2+}] (middle panel) induced by flash photolysis of caged ATP (500 μM) in the absence (C) and presence of 20 mM P_i (Pi), before and after muscle treatment with cyclopiazonic acid (CPA; 60 μM) for 60 min. Traces were obtained using a bracketing procedure (2 C-2 Pi-2 C), were averaged and superimposed. The responses after CPA were subtracted from those before CPA; the resulting difference traces (bottom panel) represent sarcoplasmic reticular calcium uptake. The initial fall of [Ca^{2+}] (dotted traces) and the later slow recovery (not shown) were fitted by a double exponential equation (solid lines).*

myofibrils by ATP in the presence of calcium. In response to the flash, fluorescence rose transiently (probably a flash artefact, as it was also produced by photolysis of caged acetate; not shown), then declined to well below the preflash level. This fall in $[Ca^{2+}]$ was due entirely to activation of the sarcoplasmic reticular calcium pump, because it disappeared completely after the muscle was incubated with 60 μM cyclopiazonic acid for 60 min (fig 8). The fall of $[Ca^{2+}]$ was then followed by a gradual recovery towards the preflash level (not shown), possibly because the depletion of [ATP] and the corresponding rises in [ADP] and $[P_i]$ allowed reversal of the sarcoplasmic reticular calcium pump. The time course of the CPA sensitive change of $[Ca^{2+}]$ after the flash was fitted to a double exponential equation by non-linear least squares. As fig 8 (bottom panel) illustrates, 20 mM P_i reduced the extent of the fall in $[Ca^{2+}]$ from 1.01(0.11) μM to 0.52(0.09) μM ($P < 0.05$, $n = 5$) but did not alter the rate constant of the fall of $[Ca^{2+}]$ resulting from sarcoplasmic reticular calcium uptake [2.92(0.94) s^{-1} *v* 2.83(0.60) s^{-1} for control, $P > 0.05$]. The rate constant of the subsequent, slower rise of $[Ca^{2+}]$ was also unaffected by P_i [0.24(0.12) s^{-1} *v* 0.19(0.11) s^{-1} for control, $P > 0.05$]. Thus, once again the main effect of P_i was on the amplitude rather than on the rate of calcium uptake by the sarcoplasmic reticulum.

As the calcium buffering power of the rigor solutions was very low, it was possible that additional calcium buffering provided by P_i itself could have caused the smaller change in the free $[Ca^{2+}]$ in 20 mM P_i. To check this, we calculated from the ionic equilibria computer program by how much the *total* [Ca] of the solutions must have fallen to give the observed changes in $[Ca^{2+}]$ in the absence and presence of P_i (the other calcium buffers present were taken to be 10 μM EGTA and 0.25 mM ATP). Total [Ca] was calculated to fall from 27.0 μM to 18.6 μM in zero P_i and from 28.6 μM to 23.9 μM in 20 mM P_i, respectively. Thus 20 mM P_i also reduced (by 44%) the fall in *total* $[Ca^{2+}]$ as a result of calcium uptake by the sarcoplasmic reticulum; this cannot be explained by calcium binding to P_i.

We did not use muscles to examine directly the third possibility above – that formation of Ca-P_i precipitates was responsible for the sarcoplasmic reticular dysfunction in the presence of P_i – but we did estimate the time course of formation and dissolution of these precipitates by measuring transmittance in a spectrophotometer. Precipitates were formed by titrating calcium with 20 mM P_i in a solution like relaxing solution but lacking EGTA. Precipitates formed (with a latency of <10 min) when free $[Ca^{2+}]$ exceeded ~0.7 mM, which gave a calculated solubility product ($[Ca^{2+}].[HPO_4^{2-}]$) of 6.2 mM. However, the subsequent addition of 3 mM K_2EGTA dissolved the precipitates within a few seconds. Thus any Ca-P_i precipitates formed in the muscle sarcoplasmic reticulum during the loading period would be expected to be readily reversible during calcium release; this mechanism would *increase* the net amount of releasable calcium and so cannot readily explain the observed fall of releasable calcium.

Discussion

Calcium release in the presence of P_i

In the present work we show for the first time that the combination of nitr-5 photolysis and fluo-3 fluorescence provides a convenient way to trigger CICR in skinned cardiac muscles and to quantify the changes in $[Ca^{2+}]$ due to nitr-5 photolysis itself and to the subsequent calcium release from the sarcoplasmic reticulum. It was essential to measure the calcium transients directly, rather than indirectly from the force transients, since the latter would be complicated by the direct actions of P_i (and ADP) on the myofibrils. As noted previously for the force transients,[10] the calcium transients after nitr-5 photolysis were of a similar shape to those in electrically stimulated intact cells but were about twice as slow. This may be due to calcium buffering by nitr-5 and its photoproducts, which could slow calcium release from the sarcoplasmic reticulum by reducing any positive feedback of calcium on CICR and would also slow reuptake of calcium into the sarcoplasmic reticulum.

We found that the addition of P_i (2-20 mM) to loading solution significantly decreased the calcium transients produced by CICR (figs 2 and 4). Thus a major conclusion from this study is that increases of $[P_i]$ over the range found in normal and ischaemic hearts can substantially inhibit the cardiac CICR. This is the first time that the effect of P_i on the physiological process of CICR has been directly examined, although the effect of P_i on calcium loading of the sarcoplasmic reticulum had been studied previously. Zhu and Nosek[6] reported that caffeine-induced calcium release could be decreased by P_i (17 mM) at short loading times (below 30 s), but was stimulated with three minute loading (as used by us) in saponin skinned rat cardiac muscles. In contrast, Smith and Steele[7] found that P_i (20 mM) decreased caffeine releasable calcium by 60%. Our study shows that 20 mM P_i caused a fall of sarcoplasmic reticular calcium loading by 45%, which largely confirms the results of Smith and Steele.[7] Why our results differ from those of Zhu and Nosek[6] is unclear, although it is possible that their higher loading $[Ca^{2+}]$ of 1 μM enabled the $[Ca^{2+}]$ in the sarcoplasmic reticular lumen to reach the level at which Ca-P_i precipitates form, and this could enhance release. It should be noted, however, that Smith and colleagues[23] have recently reported that the inhibitory effect of P_i on caffeine induced calcium release seen in the absence of phosphocreatine[7] was not seen when they included a physiological phosphocreatine concentration. This is clearly different from our results (fig 4). Although ours is the first direct study on CICR, Zhu and Nosek[6] did report that P_i had no effect on CICR, as judged indirectly from the sarcoplasmic reticular calcium remaining after perfusion with calcium containing solutions; however, it is not clear that they were studying the same process as reported here, as their application of calcium was unavoidably slow.

The similarity in the effects of P_i on CICR and on caffeine induced calcium release (fig 4) indicates that the decrease of CICR produced by P_i can be explained entirely by a reduced amount of releasable calcium inside the sarcoplasmic reticulum; there was no evidence from this comparison for an additional inhibition by P_i of the calcium release channel. In fact, variance analysis showed that P_i had a significantly smaller effect on CICR than on calcium loading. This suggests there may be a small, direct stimulation of the calcium release mechanism by P_i. Recently Fruen *et al*[8] reported that P_i stimulates the calcium release channel in isolated skeletal sarcoplasmic reticulum preparations, although they found no such effect with cardiac sarcoplasmic reticulum.

The mechanisms by which P_i *reduces calcium loading by the sarcoplasmic reticulum*

Of the three possible explanations that we considered for the fall in releasable sarcoplasmic reticular calcium in the presence of P_i, our evidence suggests that the major

mechanism is a fall in the free energy of ATP hydrolysis (ΔG_{ATP}), rather than a reduced rate of calcium uptake or the formation of Ca-P_i precipitates. Thermodynamics predict that, for a constant cytosolic [Ca^{2+}], as used here, the fall in ΔG_{ATP} produced by a rise in [P_i] (equation 1) would decrease luminal [Ca^{2+}] (equation 2).[19 20] To study this possibility, we varied [P_i] and [ADP] 10-fold but kept their product, and thus ΔG_{ATP}, constant.[cf 3 7] We found that 10 mM ADP + 1 mM P_i or 10 mM P_i + 1 mM ADP had similar depressive effects on sarcoplasmic reticular Ca^{2+} loading (fig 7A), even though [P_i] was changed 10-fold. This supports the idea that a reduction in ΔG_{ATP} is a major mechanism by which P_i reduces the releasable calcium in the cardiac sarcoplasmic reticulum. This contrasts with the conclusion of Smith and Steele,[7] who found that, at equal ΔG_{ATP}, ADP and P_i exerted different inhibitory effects on calcium loading of sarcoplasmic reticulum in skinned trabeculae. The reason for these divergent results is not clear, although it may be noted that in their study the utilisation of ADP by endogenous myokinase was not prevented.

As judged from the decay of the calcium transient, P_i did not affect the rapid calcium uptake by the sarcoplasmic reticulum. This was supported by the experiments with caged ATP (fig 8), which suggested that P_i (20 mM) did not affect the rate constant of calcium uptake by the sarcoplasmic reticulum under conditions where [Ca^{2+}] and [ATP] should not be rate limiting (>1 μM and >0.1 mM, respectively), although P_i did reduce the net uptake of calcium. These observations are consistent with the ΔG_{ATP} hypothesis, since the intrinsic rate of uptake of calcium by the sarcoplasmic reticulum immediately after ATP photorelease would be governed by the kinetics of the sarcoplasmic reticular pump, rather than by equilibrium considerations (that is, ΔG_{ATP}), whereas the equilibrium ratio of [Ca^{2+}] reached across the sarcoplasmic reticular membrane, and thus the net uptake of calcium, would depend upon ΔG_{ATP}, and this would be decreased in the presence of a high [P_i]. In agreement with this, in some sarcoplasmic reticulum vesicle studies P_i does not appear to affect the *initial* rate of calcium uptake.[eg 24] The results from the caged ATP experiments are also not consistent with the hypothesis that Ca-P_i precipitation inside the sarcoplasmic reticulum caused the decrease in calcium loading, because in that case either P_i should have not affected calcium uptake (if there was no precipitate formation under the conditions of this experiment) or P_i should have enhanced the net uptake of calcium (if precipitation had occurred), that is, this hypothesis does not explain the observed reduction in sarcoplasmic reticular calcium uptake. Furthermore, we found that Ca-P_i precipitates in vitro were readily soluble when [Ca^{2+}] was lowered; this suggests that these precipitates would be expected to increase, rather than decrease, the caffeine releasable Ca^{2+} in the muscle sarcoplasmic reticulum (assuming that any precipitates formed within the sarcoplasmic reticular lumen would have similar physicochemical properties to those of precipitates formed in vitro). Thus our overall conclusion is that the decline in sarcoplasmic reticular calcium loading in the presence of P_i is most likely to be due to a decrease in ΔG_{ATP}, rather than to a decrease in pump rate or to the formation of Ca-P_i precipitates.

At first sight, our finding that P_i decreases the amount of releasable sarcoplasmic reticular calcium is not consistent with the long established use of P_i to increase net calcium uptake into sarcoplasmic reticular vesicles by forming Ca-P_i precipitates in the sarcoplasmic reticular lumen, thereby keeping luminal [Ca^{2+}] low.[20] However, the vesicle studies utilise high external [Ca^{2+}], and even then precipitation does not occur, and the rate is not affected by P_i, until 15-30 s after the start of calcium uptake.[eg 24] Our evidence (albeit indirect) is that Ca-P_i precipitates are not formed under our conditions, probably because the external [Ca^{2+}] is so low (~0.2 μM) that the luminal [Ca^{2+}] does not reach that required for precipitation. In this case, there is no a priori reason why P_i should increase calcium uptake by the sarcoplasmic reticulum.

Calcium induced calcium release in the presence of P_i and ADP

Combinations of ADP and P_i were used initially to test whether ΔG_{ATP} affected the amount of caffeine releasable calcium, but as it turned out their effects on CICR were equally interesting, and somewhat unexpected (fig 7B): whereas ADP alone depressed calcium loading, it enhanced both the force and calcium transients induced by CICR, particularly if P_i was present. Although some force potentiation would have been due to the action of ADP to increase myofibrillar calcium sensitivity (see Methods; and [25–27]), most seemed to be due to an increased release of calcium. Nucleotides such as ATP and AMP-PCP are known to activate the sarcoplasmic reticular calcium release channels in isolated cardiac and skeletal sarcoplasmic reticular vesicles or bilayers,[9] and ADP increases cytosolic [Ca^{2+}] in skeletal muscle, probably by promoting sarcoplasmic reticular calcium release.[27] To our knowledge, the effect of ADP on the cardiac calcium release channel has not been examined previously. Our results suggest that ADP can potentiate calcium activation of the cardiac sarcoplasmic reticular calcium release channels and the effect of ADP can be enhanced by low (that is, physiological) [P_i]. There was evidence that P_i alone could stimulate the calcium activation of calcium release (fig 4), and it seems that the combination of P_i and ADP produce a substantial stimulatory effect. In fact, although P_i decreased CICR if ADP was absent, it increased it if ADP (10 mM) was present. One possible explanation is that when both P_i and ADP are present they can occupy the adenine nucleotide binding site on the calcium release channel and thus act as if the [ATP] had been increased, producing an enhancement of calcium activation of the channel. A similar potentiation of calcium release may not have been apparent with caffeine induced calcium release because caffeine alone may release practically all of the releasable calcium in the sarcoplasmic reticulum.

Effects of P_i and ADP on sarcoplasmic reticular calcium release in intact cells

In well oxygenated hearts, the intracellular [P_i] is approximately 1-3 mM, but this can rise during interventions such as an increase in heart rate.[28] We find that even these low concentrations of P_i can strongly inhibit CICR (fig 4). This suggests that small increases in [P_i] resulting from inotropic interventions may tonically depress calcium release, and thereby limit the observed increase of force.

P_i rises to 10 or 20 mM in the early stages of myocardial hypoxia or ischaemia[1] and the direct effects of this rise in [P_i] on calcium release from the sarcoplasmic reticulum are poorly defined. In intact cells, the results have been contradictory: the decrease in force in the first few minutes of hypoxia or simulated ischaemia is associated with an increase,[29–31] decrease,[29 32–36] or no change[37] in the calcium transient. These discrepancies can be ascribed to the use of different preparations and conditions, and to the fact that

there would be many intracellular changes that would affect Ca^{2+} release from the sarcoplasmic reticulum and, perhaps independently, the size of the calcium transient (which is also affected by calcium binding and transsarcolemmal extrusion processes, etc.). Our use of skinned muscles provides a way to study the effects of one intervention on sarcoplasmic reticular calcium handling with all other variables under experimental control. Our main conclusion is that increases of P_i over the concentration range found in hypoxia and ischaemia (up to 20 mM) decrease CICR by up to 45%, as a result of a decrease in the amount of releasable calcium in the sarcoplasmic reticulum. If other factors are constant, we would expect this inhibitory action of P_i to contribute to ischaemic contractile failure of the myocardium (see also [7]). This effect of P_i could be partly offset by a build up of ADP, which would promote CICR (particularly in the presence of P_i) in spite of a reduced sarcoplasmic reticular calcium loading. This stimulation by ADP would probably be small during early ischaemia, due to the low [ADP], but may become more important during prolonged ischaemia, when the total [ADP] could rise into the millimolar range.[1]

Of course, in intact cells during ischaemia there are complex ionic changes. Action potential shortening causes a fall in the amplitude of I_{Ca}, which results in decreases in the calcium trigger for CICR and sarcoplasmic reticular calcium loading,[33 34] whereas a raised diastolic $[Ca^{2+}]$ [eg 29 30] promotes calcium loading of the sarcoplasmic reticulum. The effects of the accumulation of other metabolic products such as lactic acid and AMP on calcium handling by the sarcoplasmic reticulum remain to be elucidated. Nevertheless, a considerable contribution to contractile failure would be made by the effects of P_i on CICR, which would add to the direct effects of P_i on myofibrillar force production. This effect of P_i on excitation-contraction coupling is likely to be particularly significant in species such as the rat, in which the sarcoplasmic reticulum, rather than calcium influx into the cell, provides most of the calcium for activation of the myofilaments.[4] The relative role of the sarcoplasmic reticulum is less clear in man, but it may be about midway in the sequence of sarcoplasmic reticulum dependent to I_{Ca} dependent species.[38] This suggests that the action of P_i on sarcoplasmic reticular calcium handling would also be of considerable significance in the contractile effects of ischaemia in man.

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Key terms: calcium transient; cardiac muscle; phosphate; ADP; heart; sarcoplasmic reticulum; ischaemia.

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