

## Interdependent effects of inorganic phosphate and creatine phosphate on sarcoplasmic reticulum $\text{Ca}^{2+}$ regulation in mechanically skinned rat skeletal muscle

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1. The effects of creatine phosphate (CP) and inorganic phosphate ( $\text{P}_i$ ) on sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  regulation were investigated in mechanically skinned muscle fibres from rat extensor digitorum longus (EDL) muscles. Changes in  $[\text{Ca}^{2+}]$  were detected using fura-2 fluorescence, during continuous perfusion or when the solution surrounding the preparation was restricted to approximately  $6 \mu\text{l}$  by stopping perfusion.
2. In solutions with 5 mM ATP and 10 mM CP, stopping the flow for 2–3 min had no effect on  $[\text{Ca}^{2+}]$  within the bath. This suggests that SR  $\text{Ca}^{2+}$  uptake is balanced by an efflux under these conditions.
3. In solutions with CP, the introduction of  $\text{P}_i$  induced a small transient rise in  $[\text{Ca}^{2+}]$ , due to  $\text{Ca}^{2+}$  loss from the SR. Following equilibration with solutions containing  $\text{P}_i$  ( $\geq 5$  mM), a maintained decrease in  $[\text{Ca}^{2+}]$  occurred when the flow was stopped. This is consistent with calcium phosphate (Ca- $\text{P}_i$ ) precipitation within the SR, resulting in maintained  $\text{Ca}^{2+}$  uptake.
4. In the absence of CP, the  $[\text{Ca}^{2+}]$  within the bath increased progressively when the flow was stopped. This rise in  $[\text{Ca}^{2+}]$  was inhibited by an alternative ATP regenerating system comprising phosphoenolpyruvate (PEP) and pyruvate kinase (PK). Therefore, the loss of  $\text{Ca}^{2+}$  from the SR may result from local ADP accumulation and the consequent reversal of the SR  $\text{Ca}^{2+}$  pump.
5. In the absence of CP, the initial  $\text{Ca}^{2+}$  release associated with the introduction of  $\text{P}_i$  increased markedly. Following prolonged equilibration with solutions containing  $\text{P}_i$ , a rise in  $[\text{Ca}^{2+}]$  occurred within the bath when the flow was stopped. Maintained  $\text{Ca}^{2+}$  uptake associated with Ca- $\text{P}_i$  precipitation was not apparent at any level of  $\text{P}_i$  tested (1–60 mM), when CP was absent.
6. These results suggest that withdrawal of CP is associated with activation of a SR  $\text{Ca}^{2+}$  efflux pathway. This may involve reversal of the SR  $\text{Ca}^{2+}$  pump, due to local ADP accumulation. In the absence of CP, the dominant influence of  $\text{P}_i$  appears to involve further  $\text{Ca}^{2+}$  efflux via the SR  $\text{Ca}^{2+}$  pump. The possible relevance of these effects to skeletal muscle fatigue is considered.

In skeletal muscle, intermittent fatiguing stimulation results in a progressive depletion of [CP], and an increase in  $[\text{P}_i]$  to 30 mM or more (Dawson *et al.* 1980; Nagesser *et al.* 1993). Experiments on isolated membrane vesicles have provided evidence of a functional link between the SR  $\text{Ca}^{2+}$  pump and creatine kinase (CK; Korge *et al.* 1993). Local rephosphorylation of ADP by bound CK serves to maintain a low ADP/ATP ratio in the vicinity of the SR  $\text{Ca}^{2+}$  pump. Withdrawal of CP or pharmacological inhibition of CK activates a  $\text{Ca}^{2+}$  efflux pathway involving reversal of the  $\text{Ca}^{2+}$  pump and impairs net SR  $\text{Ca}^{2+}$  accumulation (Duke & Steele, 1999). This suggests that CP depletion may contribute to impaired SR  $\text{Ca}^{2+}$  reuptake which has been shown to occur during fatigue (e.g. Westerblad & Allen, 1993).

Experiments on skinned fibres have shown that  $\text{P}_i$  entry into the SR lumen and subsequent precipitation of Ca- $\text{P}_i$  can influence the amount of  $\text{Ca}^{2+}$  released from the SR (Fryer *et al.* 1995). Indeed, it has been suggested that Ca- $\text{P}_i$  precipitation may underlie the decrease in SR  $\text{Ca}^{2+}$  release that occurs in the final stages of fatigue (e.g. Posterino & Fryer, 1998). However, the reported effects of  $\text{P}_i$  in skinned fibres are inconsistent; depending on the experimental conditions, exposure to  $\text{P}_i$  may increase, decrease or have little influence on releasable  $\text{Ca}^{2+}$  (Stienen *et al.* 1993; Fryer *et al.* 1995, 1997; Posterino & Fryer, 1998). Some of the variability in these results may reflect the fact that  $\text{P}_i$  has other effects on SR  $\text{Ca}^{2+}$  regulation; in isolated SR  $\text{Ca}^{2+}$  channels,  $\text{P}_i$  has been reported to facilitate activation by  $\text{Ca}^{2+}$  (Fruen *et al.*

1994). In skinned fibres,  $P_i$  produced an apparent inhibition of the SR  $Ca^{2+}$ -ATPase, particularly at low pH (Stienen *et al.* 1999). Experiments on isolated SR vesicles and skinned muscle fibres have shown that  $P_i$  can induce  $Ca^{2+}$  efflux from the SR by reversal of the  $Ca^{2+}$  pump (Hasselbach, 1978; Duke & Steele, 2000).

Work on skinned cardiac muscle suggests that the actions of  $P_i$  may also depend on the cytosolic [CP] (Steele *et al.* 1995; Smith *et al.* 2000). In the absence of CP,  $P_i$  induced an efflux of  $Ca^{2+}$  and a marked decrease in the SR  $Ca^{2+}$  content, without evidence of Ca- $P_i$  precipitation. However, in the presence of CP, the  $P_i$ -induced  $Ca^{2+}$  efflux was less pronounced and precipitation of Ca- $P_i$  was apparent. This influence of CP on the response to  $P_i$  may reflect the fact that ADP is required for reversal of the SR  $Ca^{2+}$  pump (Hasselbach, 1978). In the presence of CP, the local [ADP] is low due to rephosphorylation via the CK reaction. This should reduce  $P_i$ -induced efflux via the SR  $Ca^{2+}$  pump, and the higher luminal [ $Ca^{2+}$ ] may then favour precipitation. The possible influence of CP on Ca- $P_i$  precipitation has not yet been investigated in skeletal muscle.

The aim of the present study was to investigate the effect of  $P_i$  on  $Ca^{2+}$  fluxes across the SR membrane and the possible influence of CP on Ca- $P_i$  precipitation. Experiments were carried out on mechanically skinned skeletal muscle fibres and SR  $Ca^{2+}$  uptake and release were detected using fura-2 fluorescence. The results suggest that Ca- $P_i$  precipitation occurs within the SR when the bathing [ $P_i$ ] is  $\geq 5$  mM, and when CP is present in the cytosol. Withdrawal of CP resulted in the loss of  $Ca^{2+}$  from the SR and subsequent introduction of  $P_i$  induced a further, more pronounced  $Ca^{2+}$  efflux. Maintained stimulation of SR  $Ca^{2+}$  uptake, characteristic of Ca- $P_i$  precipitation, was not apparent in the absence of CP. The underlying mechanisms and the relevance of these results to skeletal muscle fatigue are discussed.

## METHODS

Male Wistar rats (250–300 g) were killed by a blow to the head and cervical dislocation according to standard UK Schedule 1 procedures. The extensor digitorum longus (EDL) muscle was removed rapidly and placed in a solution mimicking the intracellular milieu. Single muscle fibres were mechanically skinned with fine forceps and then attached between an isometric tension transducer (SensoNor, Norway) and a fixed support using monofilament snares (diameter 30  $\mu$ m) within stainless steel tubes (Goodfellow Metals, UK). Two muscle fibres were attached in parallel to increase the amount of light collected by the objective, thereby improving the fura-2 fluorescence ratio signal.

### Apparatus

The apparatus for simultaneous measurement of isometric force and SR  $Ca^{2+}$  release is detailed elsewhere (Duke & Steele, 1998a). Briefly, the mounted preparation was lowered close to the bottom of a shallow bath with a glass coverslip base. A Perspex column (5 mm diameter) was lowered to within a few micrometres of the muscle to

minimise the volume of the solution above the preparation. Throughout the experiments, preparations were perfused by pumping solution at 0.8 ml min<sup>-1</sup> via a narrow duct (100  $\mu$ m diameter) passing through the centre of the column. Waste solution was collected continuously at the column edge. This created a film of solution between the coverslip and the base of the column of  $\sim 6$   $\mu$ m. The perfusing solution was changed using a series of valves positioned above the column. In experiments involving detection of  $Ca^{2+}$  uptake by the SR, a solution containing ATP was rapidly applied via a narrow plastic tube, which joined the main perfusion duct close to the base of the column. The higher flow rate and the smaller dead space allowed a more rapid exchange of solutions within the bath. The volume of solution and the frequency of application were regulated using a syringe pump under computer control.

Light emitted from areas of the field not occupied by the muscle image was reduced using a variable rectangular diaphragm on the side port of the microscope. The bath was placed on the stage of a S200 Nikon Diaphot inverted microscope. The muscle was viewed via a  $\times 40$  Fluor objective (oil immersion, Nikon) and muscle length was increased to approximately 20% above slack length. In control experiments, it was found that length did not have a direct effect on SR  $Ca^{2+}$  regulation (not shown). The preparation was alternately illuminated with light of wavelengths 340 nm and 380 nm at 50 Hz frequency, using a spinning wheel spectrophotometer (Cairn Research, Faversham, Kent, UK). The average [ $Ca^{2+}$ ] within the visual field containing the preparation, was indicated by the ratio of light intensities emitted at  $> 500$  nm.

### Solution composition and data recording

All chemicals were obtained from Sigma unless otherwise stated. The basic perfusing solution contained (mM):  $K^+$ , 130;  $Na^+$ , 32; Hepes, 25; BAPTA, 0.1; ATP, 5; creatine phosphate (CP), 10; free  $Mg^{2+}$ , 1; fura-2, 2  $\mu$ M. Two millimolar  $NaN_3$  was added routinely to inhibit mitochondrial activity. Where necessary, the equilibrium concentrations of metal ions in the calibration solutions were calculated using the affinity constants for  $H^+$  and  $Ca^{2+}$  for EGTA as previously reported (Fabiato & Fabiato, 1979; Smith & Miller, 1985) using the Windows-based REACT program (Duncan *et al.* 1999). Correction for ionic strength, details of pH measurement, allowance for EGTA purity and the principles of the calculations are as described in Miller & Smith (1984).

Measurement of [ $Mg^{2+}$ ] with fura-2 showed that an extra 0.2 mM  $Mg^{2+}$  was needed to compensate for binding to 10 mM CP, an extra 1 mM  $Mg^{2+}$  to compensate for binding to 40 mM  $P_i$ , an extra 0.5 mM  $Mg^{2+}$  for binding to 2 mM oxalate and 0.75 mM  $Mg^{2+}$  for binding to 5 mM ADP.  $Mg^{2+}$  was added as  $MgCl_2$ . The free [ $Ca^{2+}$ ] of each solution was adjusted (to 100 nM unless stated otherwise) by addition of  $CaCl_2$  (BDH). CP and ATP were added as disodium salts,  $P_i$  was added as potassium dihydrogen orthophosphate and oxalate as a dipotassium salt. In some experiments PEP (5 mM, monosodium salt) and PK (50 U ml<sup>-1</sup>) were added as an alternative regenerating system. pH was adjusted in all solutions to 7.0 by addition of KOH. In solutions lacking CP or ATP, [ $Na^+$ ] was maintained at a constant level by addition of NaCl. In all solutions, [ $K^+$ ] was adjusted to 130 mM by addition of KCl. The [ $Cl^-$ ] ranged from  $\sim 60$  to  $\sim 130$  mM. However, this difference in [ $Cl^-$ ] had no influence on the results. In further control experiments, the effects of  $P_i$  were studied when potassium propionate or potassium 1,6-diaminohexane-*N,N,N',N'*-tetraacetic acid (K-HDTA) was used in place of KCl. However, the effects of  $P_i$  were the same when propionate or K-HDTA replaced  $Cl^-$  as the principal anion. The CK inhibitor 2,4-dinitro-fluorobenzene (DNFB) was added from a 50 mM stock solution. Cyclopiazonic acid (CPA) was added from a 20 mM stock made up in DMSO. The final [DMSO] never exceeded 0.1%, and this concentration had no apparent influence on

the results. All experiments were performed at room temperature (22–24 °C).

In all experiments, the ratio and individual wavelength intensities were low-pass filtered (–3 dB at 30 Hz) and digitised for later analysis using an IBM-compatible 80486 computer with a Data Translation 2801A card.

## RESULTS

### Effects of $P_i$ on net SR $Ca^{2+}$ uptake in the presence of 10 mM CP

Figure 1A shows a continuous record of the fura-2 fluorescence ratio from two mechanically skinned EDL muscle fibres attached in parallel. The resting fluorescence ratio corresponds to a free  $[Ca^{2+}]$  of 100 nM. The preparation was perfused for 10 min with a solution containing 10 mM CP. When the volume of solution surrounding the preparation was restricted (to  $\sim 6 \mu\text{l}$ ) by stopping perfusion,  $[Ca^{2+}]$  remained constant (left), suggesting that  $Ca^{2+}$  uptake is balanced by efflux. This is consistent with work showing that the SR  $Ca^{2+}$  content reaches a steady state within 2 min under these conditions (Duke & Steele, 1998b).

As shown previously by Duke & Steele (2000), the introduction of 20 mM  $P_i$  resulted in a small transient increase in the fluorescence ratio, due to  $Ca^{2+}$  release from the SR (Fig. 1A, middle). After the  $P_i$ -induced  $Ca^{2+}$  transient decayed, the baseline  $[Ca^{2+}]$  fell below that recorded in the absence of  $P_i$ . After 3 min of exposure to 20 mM  $P_i$ , perfusion was stopped. This was followed by a further, maintained decrease in bathing  $[Ca^{2+}]$ , which approached a new steady-state level within 2–3 min. When perfusion was resumed, the bathing  $[Ca^{2+}]$  returned to the lower baseline level. The same preparation was then perfused with a  $P_i$ -free solution for 10 min. Following removal of  $P_i$ , the baseline  $[Ca^{2+}]$  returned to the control level.

As considered previously (Steele *et al.* 1996), the decrease in  $[Ca^{2+}]$  shown in Fig. 1A is consistent with  $Ca$ - $P_i$  precipitation within the SR. Precipitation occurs when the product of the luminal  $[P_i]$  and  $[Ca^{2+}]$  exceeds the  $Ca$ - $P_i$  solubility product. The continual entry of  $P_i$  followed by precipitation will reduce the free luminal  $[Ca^{2+}]$ , thereby maintaining SR  $Ca^{2+}$  uptake. During perfusion,  $P_i$ -induced stimulation of  $Ca^{2+}$  uptake produced only a small, steady-state decrease in  $[Ca^{2+}]$  within the preparation. This may be explained if the constant inward diffusion of  $Ca^{2+}$  maintains the free  $[Ca^{2+}]$ , within the preparation, close to that of the perfusate. However, when flow was stopped and the bath volume restricted,  $P_i$ -stimulated SR  $Ca^{2+}$  uptake resulted in a pronounced and maintained decrease in  $[Ca^{2+}]$ .

Similar changes in  $[Ca^{2+}]$  occurred in the presence of oxalate, which also enters the SR precipitates with  $Ca^{2+}$

(Fig. 1A, right). A lower [oxalate] (e.g. 2 mM) produced an effect comparable with that of 20 mM  $P_i$ . This may reflect the lower solubility product for calcium oxalate (Lide, 1995), although the threshold for calcium oxalate precipitation was not defined in this study. Unlike  $P_i$ , the introduction of oxalate was not associated with a transient release of  $Ca^{2+}$  from the SR.

Figure 1B shows the reproducibility and reversibility of responses obtained following the addition of  $P_i$ . The preparation was initially perfused with a solution containing 10 mM CP and again  $[Ca^{2+}]$  remained constant, when the flow was stopped (left). Introduction of  $P_i$  produced an efflux of  $Ca^{2+}$  from the SR and  $[Ca^{2+}]$  then decreased to a new lower level (middle). A characteristic decrease in  $[Ca^{2+}]$  occurred within the preparation when the flow was stopped.  $[Ca^{2+}]$  returned to the new steady-state level when perfusion was resumed. After approximately 10 min, the flow was again stopped and  $[Ca^{2+}]$  decreased towards the same steady-state level, over a similar time course. The preparation was then exposed to a  $P_i$ -free solution for 10 min (not shown). Following removal of  $P_i$ , there was no apparent change in  $[Ca^{2+}]$  within the bath, when the flow was stopped (right).

If the  $P_i$ -induced fall in resting  $[Ca^{2+}]$  reflects maintained SR  $Ca^{2+}$  uptake, then inhibition of the SR  $Ca^{2+}$  pump should reduce or abolish this effect. In Fig. 1C, the introduction of 20 mM  $P_i$  again caused a  $Ca^{2+}$  efflux and a decrease in bathing  $[Ca^{2+}]$ , which was more pronounced when the flow was stopped. On restarting the flow,  $[Ca^{2+}]$  within the bath rapidly returned to a lower baseline level. Subsequent introduction of the SR  $Ca^{2+}$  pump inhibitor cyclopiazonic acid (CPA) was followed by a slow transient release of  $Ca^{2+}$  from the SR and the  $[Ca^{2+}]$  returned to the original control level. As previously suggested, this probably reflects the loss of  $Ca^{2+}$  via a leak pathway, which is revealed by inhibition of the  $Ca^{2+}$  pump (Duke & Steele, 1998a). Following exposure to CPA,  $[Ca^{2+}]$  increased slightly when the flow was stopped. This shows that the reduction in  $[Ca^{2+}]$  in the presence of 20 mM  $P_i$  requires active  $Ca^{2+}$  uptake via the SR  $Ca^{2+}$  pump. The rise in  $[Ca^{2+}]$ , which occurred when the flow was stopped, may reflect the continued loss of  $Ca^{2+}$  from the SR via  $Ca^{2+}$  leak pathways, and its accumulation in the restricted bath volume. In Fig. 1D, the preparation was exposed to 2% Triton X-100, which disrupts all membranes, including the SR. This resulted in a large  $Ca^{2+}$  release, confirming that the SR was not fully  $Ca^{2+}$  depleted by exposure to CPA (not shown). Following treatment with Triton X-100, stopping the flow had no influence on  $[Ca^{2+}]$  in the presence or absence of  $P_i$ . Qualitatively similar results to those shown in Fig. 1 were obtained in six other preparations.

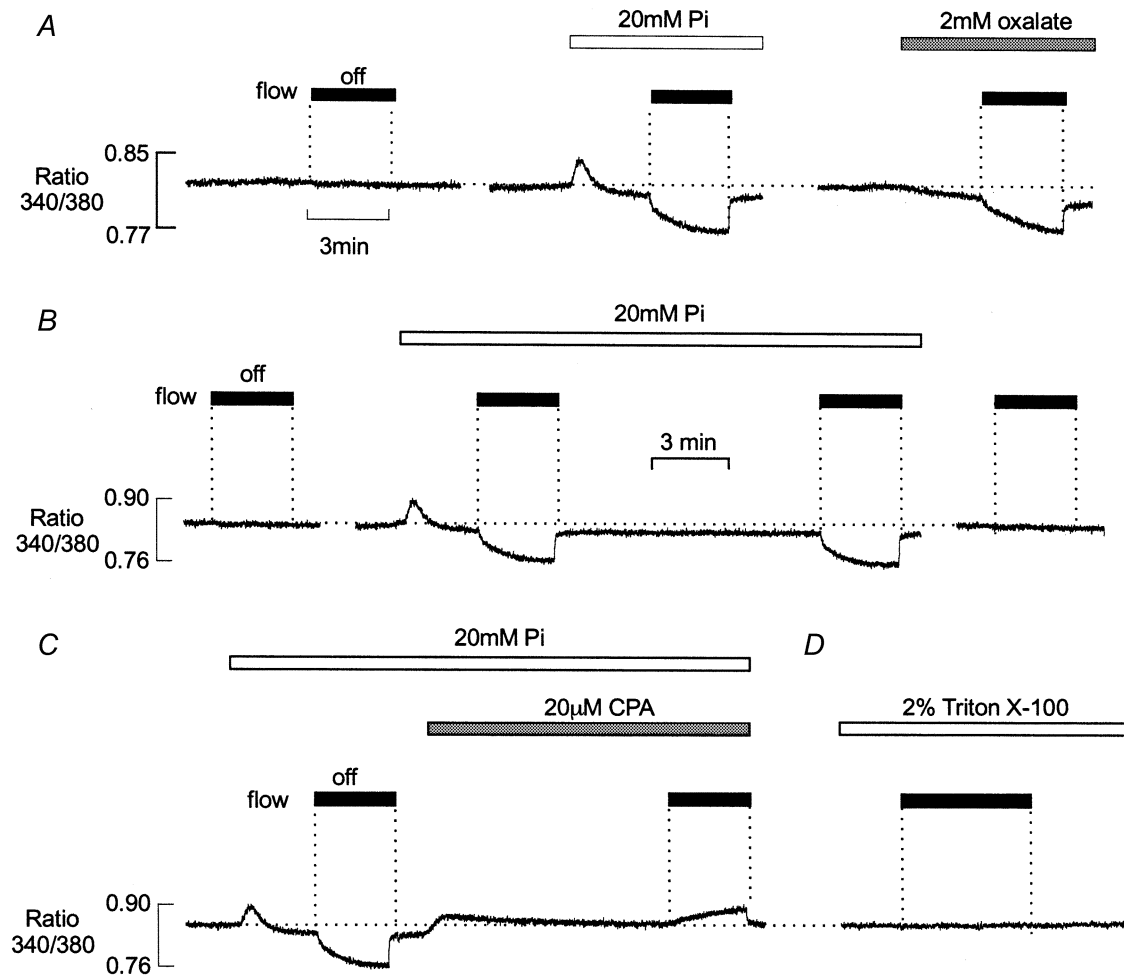
In further experiments, it was found that the decrease in  $[Ca^{2+}]$  was not influenced by azide, confirming that mitochondrial  $Ca^{2+}$  accumulation does not contribute significantly to the 'stop flow'

response. The decrease in  $[Ca^{2+}]$  was not influenced by ruthenium red, suggesting that SR  $Ca^{2+}$  channel activation is not significant over the range of  $[Ca^{2+}]$  used in this study. Similar results were obtained when potassium propionate or K-HDTA was used in place of KCl. The rate of decline of  $[Ca^{2+}]$  slowed progressively and approached a new steady state after 1–2 min and several factors may influence the magnitude and time course of this response. First, net  $Ca^{2+}$  uptake would be expected to reduce progressively as  $[Ca^{2+}]$  falls further below the  $K_m$  of the  $Ca^{2+}$  pump. Second, the initial rate

of  $Ca^{2+}$  uptake may be influenced by the rate of  $P_i$  (or oxalate) entry into the SR, which appears to occur relatively slowly over minutes (Fryer *et al.* 1997). Finally, as in intact cells during fatigue, accumulation of metabolites may also influence net  $Ca^{2+}$  uptake, particularly in the absence of CP (see below).

#### Ca- $P_i$ precipitation threshold in the presence of CP

As considered above, several factors may influence the magnitude and time course of the fall in  $[Ca^{2+}]$ , which



**Figure 1.** Effects of  $P_i$  on net SR  $Ca^{2+}$  uptake in the presence of 10 mM CP

Typical records of fura-2 fluorescence ratio from a preparation comprising two mechanically skinned EDL muscle fibres. *A*, after 10 min equilibration in the presence of 10 mM CP, stopping perfusion had no effect on fluorescence ratio (left). During continuous perfusion, introduction of 20 mM  $P_i$  caused a transient SR  $Ca^{2+}$  efflux, followed by a sustained reduction in bathing  $[Ca^{2+}]$  below control levels. After 3 min exposure to 20 mM  $P_i$ , the flow was stopped. This resulted in a maintained decrease in bathing  $[Ca^{2+}]$  due to net SR  $Ca^{2+}$  uptake (middle). The preparation was then exposed to a  $P_i$ -free solution for over 10 min. Subsequent addition of 2 mM oxalate caused a sustained reduction in bathing  $[Ca^{2+}]$  below control levels, which was more pronounced when the flow was stopped (right). *B*, after a control 'stop flow' response (left) introduction of 20 mM  $P_i$  induced a transient release of  $Ca^{2+}$ , followed by a small maintained decrease in the resting  $[Ca^{2+}]$ , which became more pronounced when the flow was stopped. When the flow was resumed,  $[Ca^{2+}]$  returned rapidly to the lower steady-state level. After a further 10 min perfusion, a similar decrease in  $[Ca^{2+}]$  occurred when the flow was stopped (middle). Following perfusion with a  $P_i$ -free solution for 10 min,  $[Ca^{2+}]$  remained constant when the flow was stopped (right). *C*, a control response to introduction of  $P_i$  and stopping the flow was followed by addition of 20  $\mu$ M CPA (left). This resulted in a slow transient release of  $Ca^{2+}$  from the SR and the  $[Ca^{2+}]$  returned to the original control level. When the flow was then stopped, a further slow increase in  $[Ca^{2+}]$  occurred. *D*, after exposure to Triton X-100 to disrupt the SR membrane, the  $[Ca^{2+}]$  remained constant when the flow was stopped.

occurs when the flow is stopped in the presence of  $P_i$ . However, the absence or presence of this response can be used to assess whether or not  $Ca-P_i$  precipitation occurs under any given condition. Precipitation of  $Ca-P_i$  should occur with a distinct threshold when the solubility product is exceeded within the SR. Figure 2 shows the protocol used to determine the apparent threshold for  $Ca-P_i$  precipitation in the presence of CP and 100 nM bathing  $Ca^{2+}$ . The basic protocol was similar to that shown in Fig. 1. When the flow was stopped in the absence of  $P_i$ ,  $[Ca^{2+}]$  within the bath remained constant. A small transient  $Ca^{2+}$  release was apparent on the introduction of 1 or 2 mM  $P_i$ . However, when the flow was stopped,  $[Ca^{2+}]$  remained constant, suggesting that 1–2 mM  $P_i$  was insufficient to produce  $Ca-P_i$  precipitation under these circumstances. In the presence of 5 mM  $P_i$ , a small maintained decrease in  $[Ca^{2+}]$  was apparent when the flow was stopped. This suggests that the solubility product was exceeded at 5 mM  $P_i$ , resulting in maintained SR  $Ca^{2+}$  uptake. At 10, 20 and 40 mM  $P_i$ , the initial  $Ca^{2+}$  release, on addition of  $P_i$ , was also followed by a maintained decrease in the steady-state  $[Ca^{2+}]$  during perfusion. Hence, at 10–40 mM  $P_i$ , steady-state SR  $Ca^{2+}$  uptake was sufficiently rapid to decrease  $[Ca^{2+}]$  within the muscle, despite the constant inward diffusion of  $Ca^{2+}$  from the perfusate. As previously reported, the amplitude and time course of the  $P_i$ -induced  $Ca^{2+}$  efflux was concentration dependent over the range 1–40 mM (Duke & Steele, 2000). Similar results were obtained in eight other preparations.

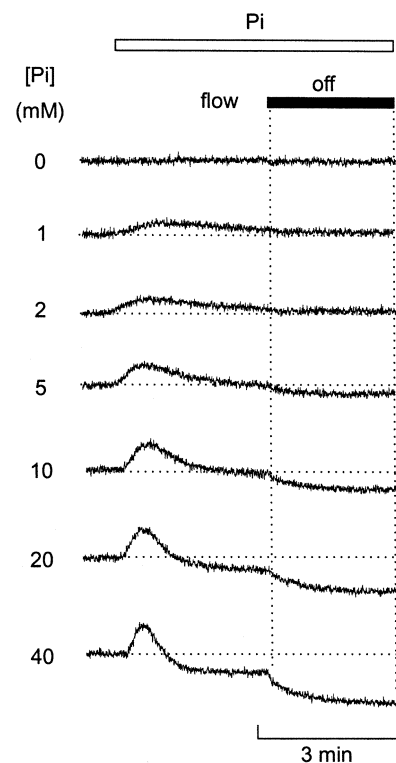
#### Effects of CP withdrawal on SR $Ca^{2+}$ regulation

Figure 3A shows the effects of CP withdrawal on SR  $Ca^{2+}$  regulation. The preparation was initially perfused with a solution containing 10 mM CP and the  $[Ca^{2+}]$  remained constant when the flow was stopped (left). Subsequent withdrawal of CP resulted in a slow, transient  $Ca^{2+}$  efflux from the SR, as reported previously (Duke & Steele, 1999). After  $[Ca^{2+}]$  returned to baseline levels, a marked increase in bathing  $[Ca^{2+}]$  occurred, when perfusion was stopped (middle). Following the re-introduction of CP,  $[Ca^{2+}]$  again remained constant when the flow was stopped (right).

The concentration dependence of this effect of CP is shown in Fig. 3B. The preparation was initially perfused with a solution containing 10 mM CP and  $[Ca^{2+}]$  remained constant when the flow was stopped. The [CP] was then decreased in a stepwise manner and the preparation exposed to each [CP] for 15 min before the flow was stopped. In the presence of 5 mM CP,  $[Ca^{2+}]$  remained constant when the flow was stopped. However, when the flow was stopped in the presence of 2 mM CP, a small rise in resting  $[Ca^{2+}]$  was apparent. This rise in  $Ca^{2+}$  became progressively more pronounced when [CP] was further reduced to 1, 0.5 and 0 mM. Similar results were obtained in six other preparations.

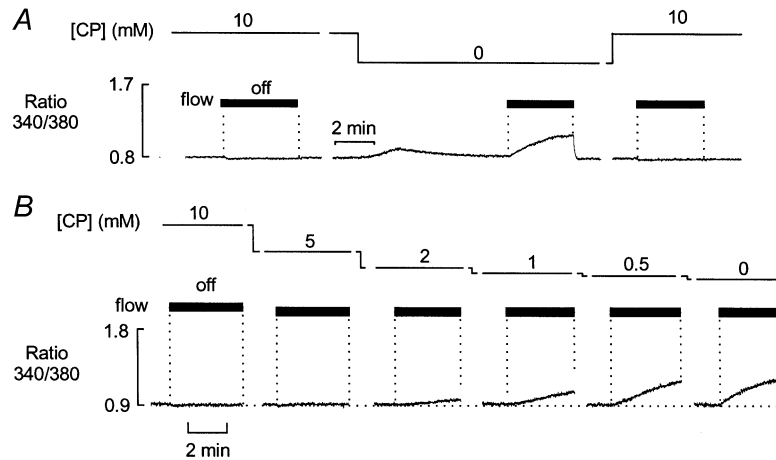
#### Responses obtained in the presence of an alternative ATP regenerating system

On withdrawal of CP, rephosphorylation of ADP via the creatine kinase reaction will cease and the local [ADP] will rise. Previous studies on skinned fibres suggest that the  $Ca^{2+}$  efflux on removal of CP, results from a local rise in [ADP], which induces reversal of the SR  $Ca^{2+}$  pump (Duke & Steele, 1999). Therefore, the increase in  $[Ca^{2+}]$  that occurs when the flow is stopped in the absence of CP, might reflect further accumulation of ADP in the restricted bath volume. This would be expected to alter the balance between  $Ca^{2+}$  uptake and efflux, resulting in



**Figure 2. Apparent threshold for  $Ca-P_i$  precipitation**

Effect of stopping bath perfusion following equilibration with solutions containing various  $[P_i]$ . At each  $[P_i]$ , introduction of  $P_i$  resulted in a transient increase in  $Ca^{2+}$  due to SR  $Ca^{2+}$  release. At 1 and 2 mM  $P_i$ , the  $[Ca^{2+}]$  remained constant when the flow was subsequently stopped. At 5 mM  $P_i$ , a small maintained decrease in  $[Ca^{2+}]$  occurred when the flow was stopped. At higher  $[P_i]$ , the initial  $[Ca^{2+}]$  release was followed by a maintained decrease in the steady state  $[Ca^{2+}]$ . The fall in  $[Ca^{2+}]$  that occurred when the flow was stopped became progressively greater as the  $[P_i]$  was increased above 5 mM. All responses were obtained in the same mechanically skinned preparation. The  $[Ca^{2+}]$  of the bathing solution was 100 nM and the free  $[Mg^{2+}]$ , 1 mM. The vertical bar indicates a change in ratio of 0.1 units. All responses were obtained in the same preparation.



**Figure 3.** Effects of 20 mM  $P_i$  on net SR  $Ca^{2+}$  uptake in the presence and absence of CP

*A*, after 10 min equilibration with solution containing 10 mM CP,  $[Ca^{2+}]$  remained constant when the flow was stopped (left). During constant perfusion, withdrawal of CP resulted in a transient increase in  $[Ca^{2+}]$ . Once the  $[Ca^{2+}]$  had returned to the control level, stopping the flow resulted in a maintained increase in  $[Ca^{2+}]$  (middle). *B*, the preparation was exposed to solutions containing a range of [CP]. In each case, the flow was stopped after 15 min exposure to each [CP]. The increase in  $[Ca^{2+}]$  associated with stopping the flow became apparent at 2 mM CP and increased progressively as the [CP] was reduced further.

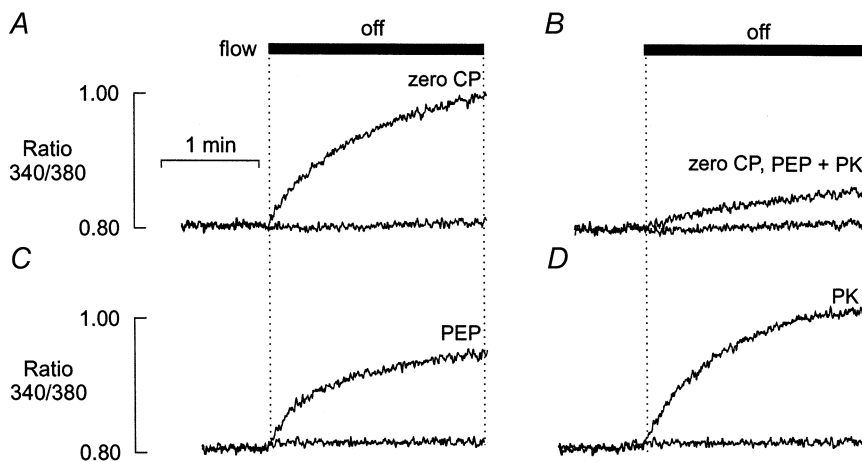
a progressive loss of  $Ca^{2+}$  from the SR. If this interpretation is correct, then an alternative ATP regenerating system should inhibit the rise in  $[Ca^{2+}]$ .

In each panel of Fig. 4, the baseline record is the control response obtained when the flow was stopped in the presence of 10 mM CP. In Fig. 4*A*, the characteristic rise in  $[Ca^{2+}]$  obtained when the flow was stopped in complete absence of CP is shown superimposed. As shown in Fig. 4*B*, this rise in  $Ca^{2+}$  was markedly reduced when an alternative regenerating system comprising PEP and PK was included in the zero CP solution. Inclusion of PEP

without exogenous PK also partially inhibited the rise in  $[Ca^{2+}]$  (Fig. 4*C*). This may reflect the presence of endogenous bound PK within the preparation. However, as expected, addition of PK in the absence of PEP was without effect (Fig. 4*D*). Similar results were obtained in four other preparations.

#### Effects of $P_i$ in the absence of CP

Figure 5*A* (upper left) shows a typical response to the introduction of 20 mM  $P_i$  in the presence of 10 mM CP. In this experiment, the free  $[Ca^{2+}]$  was increased to 250 nM to

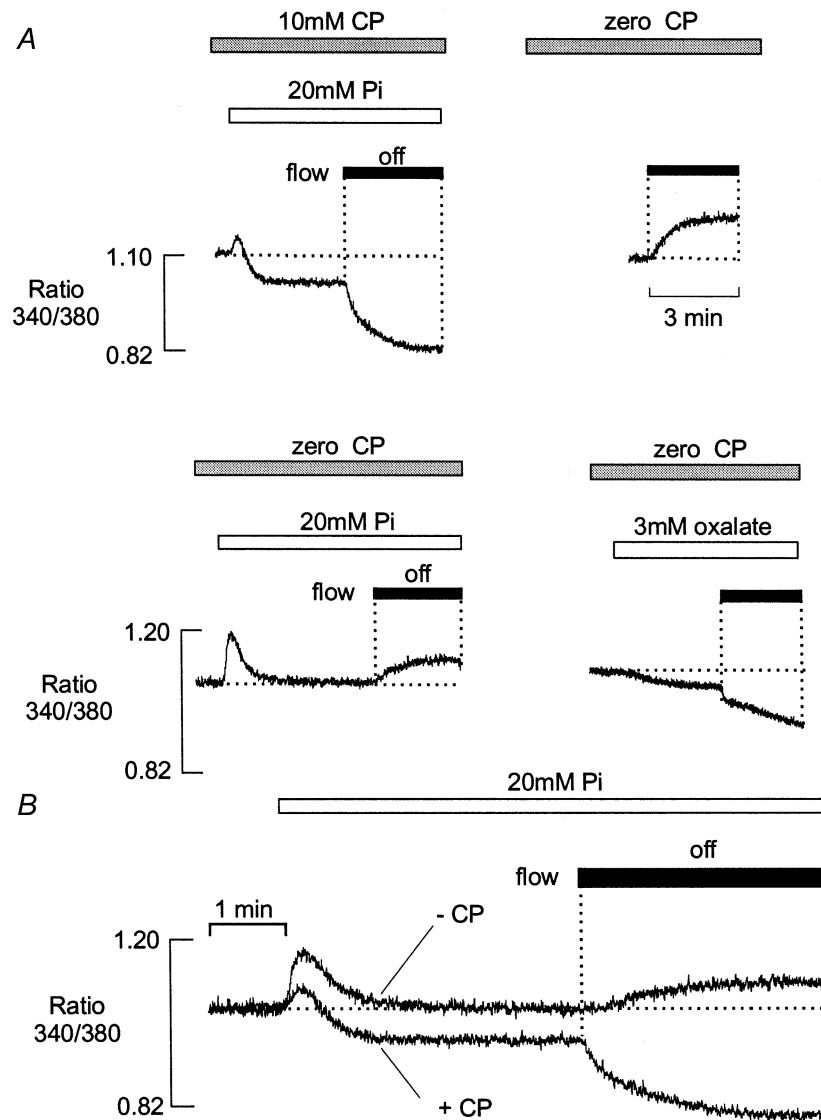


**Figure 4.** Effects of an alternative regenerating system on stop flow response

*A*, effect of stopping flow on  $[Ca^{2+}]$  in the absence of CP. *B*, stopping flow in the presence of an alternative regenerating system comprising PEP (5 mM) and PK (50 units  $ml^{-1}$ ). *C*, stopping flow in the presence of PEP, but in the absence of PK. *D*, stopping flow in the presence of PK, but in the absence of PEP. In each example, the control response to stopping the flow in the presence of 10 mM CP is superimposed (lower trace). All responses were obtained from the same preparation.

increase the probability of Ca-P<sub>i</sub> precipitation within the SR. Following the initial Ca<sup>2+</sup> efflux, [Ca<sup>2+</sup>] decreased to a lower steady-state level. When the flow was stopped, a further maintained decrease in [Ca<sup>2+</sup>] occurred, consistent with precipitation of Ca-P<sub>i</sub> within the SR. The flow was then re-started and the preparation equilibrated for 15 min with a solution lacking both P<sub>i</sub> and CP. When the flow was then stopped, [Ca<sup>2+</sup>] within the bath increased as shown previously (upper right). When 20 mM P<sub>i</sub> was

introduced in the absence of CP, the initial transient rise in [Ca<sup>2+</sup>] was markedly increased (lower left). Furthermore, after the initial Ca<sup>2+</sup> release, [Ca<sup>2+</sup>] returned to control levels; a decrease below the baseline level did not occur during constant perfusion, even when [P<sub>i</sub>] was increased to 60 mM (not shown). In contrast to the effects of P<sub>i</sub>, introduction of oxalate resulted in a maintained decrease in [Ca<sup>2+</sup>], consistent with calcium oxalate precipitation within the SR and consequent



**Figure 5.** Effects of 20 mM P<sub>i</sub> in the presence and absence of CP

*A*, after 10 min equilibration in a solution containing 10 mM CP, introduction of 20 mM P<sub>i</sub> caused a small transient rise in [Ca<sup>2+</sup>] and a maintained decrease in resting [Ca<sup>2+</sup>]. A further reduction in bathing [Ca<sup>2+</sup>] occurred when the flow was stopped (left). Following equilibration with a solution lacking CP, [Ca<sup>2+</sup>] increased when the flow was stopped (right). In the absence of CP, introduction of 20 mM P<sub>i</sub> induced a larger transient increase in [Ca<sup>2+</sup>], which then declined to the control level. A maintained increase in [Ca<sup>2+</sup>] occurred when the flow was subsequently stopped (lower left). Introduction of 3 mM oxalate resulted in a maintained decrease in the resting [Ca<sup>2+</sup>], which became more pronounced when the flow was stopped (lower right). *B*, superimposed fluorescence records showing changes in [Ca<sup>2+</sup>] on exposure to 20 mM P<sub>i</sub> in the presence and absence of 10 mM CP. All responses were obtained from the same preparation. In this example, the free [Ca<sup>2+</sup>] was increased to 250 nM to increase the possibility of Ca-P<sub>i</sub> precipitation. All results were obtained from the same preparation.

stimulation of the  $\text{Ca}^{2+}$  pump (lower right). Figure 5B shows the responses to 20 mM  $\text{P}_i$  in the presence and absence of CP, on an expanded time scale.

In the absence of CP, the increase in bathing  $[\text{Ca}^{2+}]$  was less pronounced when the flow was stopped in the presence of  $\text{P}_i$ , than in absence (compare Fig. 5A upper right and lower left). This might reflect a decrease in SR  $\text{Ca}^{2+}$  content, due to the larger  $\text{P}_i$ -induced  $\text{Ca}^{2+}$  efflux that occurs in the absence of CP. Figure 6 shows that the general characteristics of the  $\text{P}_i$  response could be mimicked by the addition of millimolar levels of ADP. A control response in the presence of 10 mM CP is shown (left). This was followed by a transient release of  $\text{Ca}^{2+}$  on withdrawal of CP and a maintained increase in  $[\text{Ca}^{2+}]$  when the flow was stopped (middle). Subsequent introduction of 2 mM ADP resulted in a further transient increase in  $[\text{Ca}^{2+}]$ , which then returned to baseline levels. However, when the flow was then stopped in the presence of 5 mM ADP, the maintained rise in  $[\text{Ca}^{2+}]$  was markedly reduced. This supports the suggestion that the release of SR  $\text{Ca}^{2+}$  by  $\text{P}_i$  may explain the smaller rise in  $[\text{Ca}^{2+}]$  that occurred when the flow was stopped. Similar results were obtained in five other preparations.

#### Effects of creatine kinase inhibition

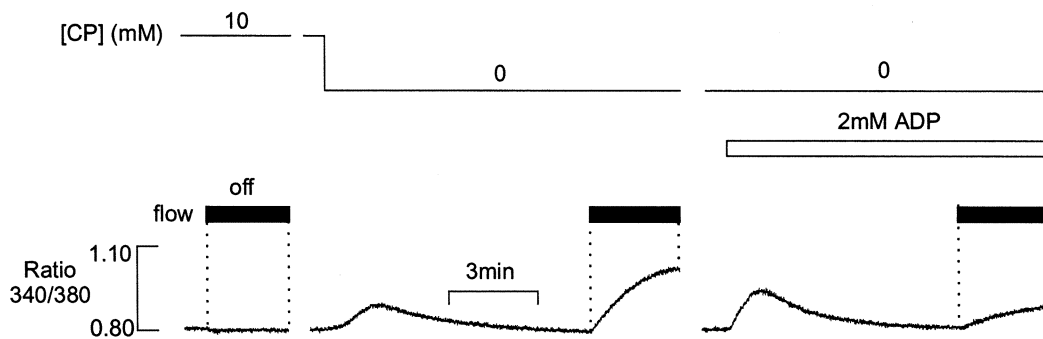
Figure 7A shows the effects of CK inhibition on SR  $\text{Ca}^{2+}$  regulation. In the presence of 10 mM CP,  $[\text{Ca}^{2+}]$  remained constant when the flow was stopped (left). As shown previously (Duke & Steele, 1999), introduction of the CK inhibitor DNFB (50  $\mu\text{M}$ ) resulted in a slow transient  $\text{Ca}^{2+}$  efflux from the SR, similar to that observed on removal of CP (middle). After exposure to DNFB, a pronounced increase in  $[\text{Ca}^{2+}]$  occurred when the flow was stopped (right).

Figure 7B shows the response to 20 mM  $\text{P}_i$  in the presence of 10 mM CP, before and after treatment with DNFB. As in Fig. 5, the  $[\text{Ca}^{2+}]$  of the perfusing solution was 250 nM

throughout. During perfusion in the presence of 10 mM CP, the introduction of 20 mM  $\text{P}_i$  induced a small transient  $\text{Ca}^{2+}$  efflux from the SR and a decrease in  $[\text{Ca}^{2+}]$  to a new lower level. A further maintained fall in  $[\text{Ca}^{2+}]$  occurred when the flow was stopped (left). However, when 20 mM  $\text{P}_i$  was introduced following inhibition of CK, the initial rise in  $[\text{Ca}^{2+}]$  increased markedly (right). As in the complete absence of CP,  $[\text{Ca}^{2+}]$  returned to baseline levels after the initial  $\text{Ca}^{2+}$  release. A similar response was obtained when DNFB was applied in the absence of CP (not shown). The superimposed responses to addition of  $\text{P}_i$ , before and after inhibition of CK with DNFB, are shown in Fig. 7C. As previously reported, the effects of DNFB were not reversible within 20 min. These results suggest that the effects of CP withdrawal can be mimicked by inhibition of CK. This also confirms that the effects of CP withdrawal reflect the abolition of ATP buffering via the CK reaction, rather than a direct effect of CP on the SR. Similar results were obtained in five other preparations.

#### Net SR $\text{Ca}^{2+}$ uptake following depletion of SR $\text{Ca}^{2+}$

In previous figures, CP was withdrawn or  $\text{P}_i$  introduced after the SR content had reached steady state. However, such experiments do not provide information regarding the relative decrease in SR  $\text{Ca}^{2+}$  content associated with CP withdrawal or addition of  $\text{P}_i$ . Therefore, the protocol shown in Fig. 8A was designed to investigate the effects of CP and  $\text{P}_i$  on net  $\text{Ca}^{2+}$  uptake following complete depletion of SR  $\text{Ca}^{2+}$ . Simultaneous records of the fluorescence ratio (upper panel) and force (lower panel) are shown. During continuous perfusion with a solution containing 5 mM ATP and 10 mM CP, the introduction of 20 mM caffeine caused a large increase in fluorescence and force due to SR  $\text{Ca}^{2+}$  release. After 10 min exposure to caffeine, ATP was withdrawn to prevent re-accumulation of  $\text{Ca}^{2+}$  by the SR. ATP withdrawal caused the development of rigor and a further slow, transient efflux



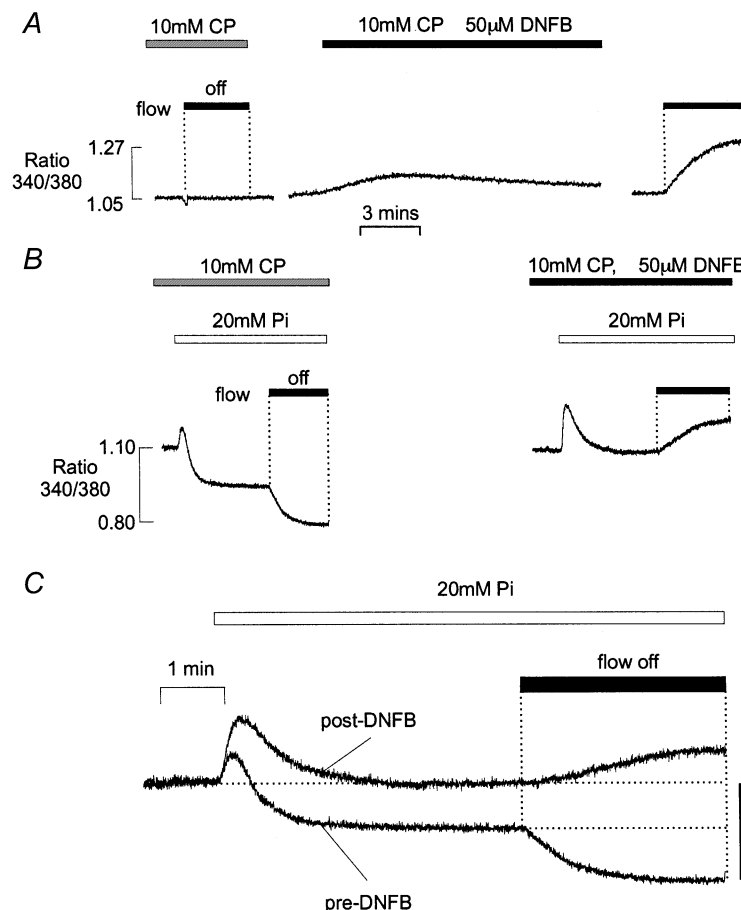
**Figure 6.** Effects of ADP in the absence of CP

After 10 min equilibration in the presence of 10 mM CP,  $[\text{Ca}^{2+}]$  remained constant when the flow was stopped (left). Withdrawal of CP resulted in a transient  $\text{Ca}^{2+}$  efflux. In the continued absence of CP, the  $[\text{Ca}^{2+}]$  increased markedly when the flow was stopped (middle). Subsequent introduction of 2 mM ADP resulted in a further transient  $\text{Ca}^{2+}$  efflux (right). However, when perfusion was then stopped, the  $\text{Ca}^{2+}$  efflux was markedly reduced relative to that obtained in the absence of CP and  $\text{P}_i$ .



of  $\text{Ca}^{2+}$  remaining in the SR (see Duke & Steele, 1998). Caffeine was then removed for 5 min and perfusion stopped. Following this, the solution within the bath was rapidly replaced (within 10 ms) with one containing 5 mM ATP and the flow stopped. This resulted in relaxation from rigor (lower panel) and a maintained fall in bathing  $[\text{Ca}^{2+}]$ . This decrease in  $[\text{Ca}^{2+}]$  did not occur following disruption of the SR membrane with Triton X-100, or after treatment of the fibre with the pump inhibitor CPA (not shown). This confirms that the maintained decrease in  $[\text{Ca}^{2+}]$  is due to ATP-activated SR  $\text{Ca}^{2+}$  uptake. The uptake of  $\text{Ca}^{2+}$  on introduction of ATP was reproducible. However, some deterioration in the myofilament response was generally apparent with repeated exposures to zero ATP solution (not shown).

The protocol shown in Fig. 8A was modified to assess the effects of 20 mM  $\text{P}_i$  on SR  $\text{Ca}^{2+}$  uptake in the presence and absence of 10 mM CP. In all cases,  $\text{P}_i$  was introduced in the absence of ATP, immediately after removal of caffeine. Figure 8B (left) shows that in the presence of 10 mM CP, net  $\text{Ca}^{2+}$  uptake was similar in the presence and absence of 20 mM  $\text{P}_i$ . However, in the absence of CP (right), net SR  $\text{Ca}^{2+}$  uptake was reduced. In the absence of CP, 20 mM  $\text{P}_i$  further reduced net  $\text{Ca}^{2+}$  uptake by the SR. Furthermore, in the presence of 20 mM  $\text{P}_i$ , the initial decrease in  $[\text{Ca}^{2+}]$  within the bath was followed by a slower rise. This may result from the loss of  $\text{Ca}^{2+}$  from the SR as ADP progressively accumulates within the bath, facilitating (with  $\text{P}_i$ ) reversal of the SR  $\text{Ca}^{2+}$  pump. The slow rise in  $[\text{Ca}^{2+}]$  is unlikely to reflect inhibition of the SR  $\text{Ca}^{2+}$  pump due to ATP depletion, because (i) the myofilaments have



**Figure 7.** Effects of 20 mM  $\text{P}_i$  following inhibition of CK

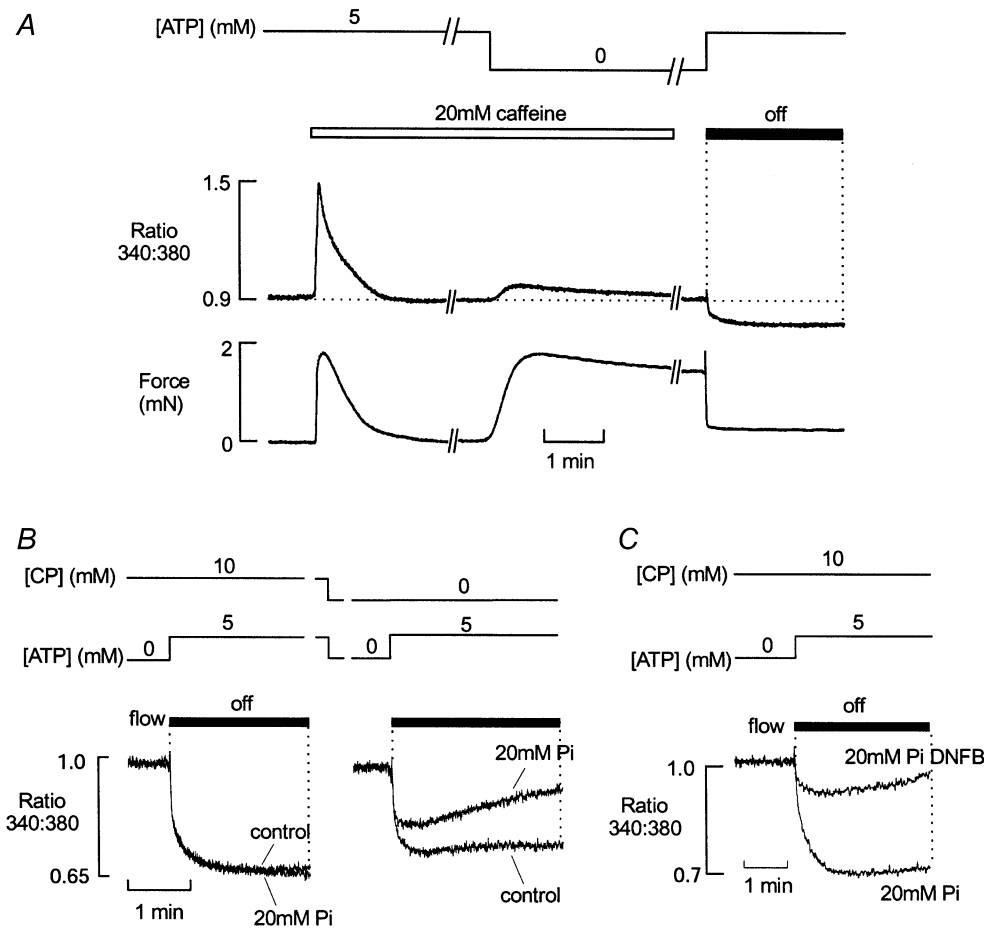
*A*, following 10 min exposure to a solution containing 10 mM CP, the flow was stopped and the  $[\text{Ca}^{2+}]$  remained constant (left). Subsequent addition of 50  $\mu\text{M}$  DNFB caused a transient efflux of  $\text{Ca}^{2+}$  from the SR (middle). After 30 min exposure to DNFB, a maintained increase in bathing  $[\text{Ca}^{2+}]$  occurred when the flow was stopped (right). *B*, a control response to the addition of  $\text{P}_i$  and stopping the flow in the presence of CP (left) was followed by a response to the introduction of  $\text{P}_i$  30 min after the introduction of DNFB (right). *C*, superimposed fluorescence records showing the effects of 20 mM  $\text{P}_i$  in the presence of 10 mM CP, before and after exposure to 50 mM DNFB. All responses were obtained from the same preparation. In this example, the free  $[\text{Ca}^{2+}]$  was increased to 250 nM to increase the possibility of Ca- $\text{P}_i$  precipitation. All results were obtained from the same preparation. In *C*, the bar represents a change in ratio of 0.23 units.

a higher sensitivity to ATP than the SR  $\text{Ca}^{2+}$  pump and re-development of rigor force did not occur during this period, and (ii) [ATP] is unlikely to decrease from 5 mM to  $<100 \mu\text{M}$  (as required to inhibit SR  $\text{Ca}^{2+}$  uptake) within 20–30 s, due to SR  $\text{Ca}^{2+}$ -ATPase activity alone. Similar results were obtained in nine other preparations.

Figure 8C shows that the effect of 20 mM  $\text{P}_i$  on net  $\text{Ca}^{2+}$  uptake in the absence of CP could be mimicked by exposure of the preparation to  $50 \mu\text{M}$  DNFB. The control response shows ATP-induced SR  $\text{Ca}^{2+}$  uptake in the presence of 20 mM  $\text{P}_i$  and then the same effect after exposure to

DNFB. Both responses were in the constant presence of 10 mM CP. Similar results were obtained in four other preparations.

While net  $\text{Ca}^{2+}$  uptake was reduced in the absence of CP and in the presence of  $\text{P}_i$ , there was little effect on the initial decrease in  $[\text{Ca}^{2+}]$  following the introduction of ATP. One possible explanation for this is that the initial rate of decline may be influenced by the inward diffusion of ATP. Alternatively, pump reversal may occur when the luminal  $[\text{Ca}^{2+}]$  has risen significantly.



**Figure 8.** Effects of CP and  $\text{P}_i$  on net SR  $\text{Ca}^{2+}$  uptake

A, simultaneous records of fluorescence ratio (upper panel) and force (lower panel) from a muscle preparation comprising two mechanically skinned fibres. The preparation was initially perfused with a solution containing 5 mM ATP and 10 mM CP. Introduction of 20 mM caffeine into the perfusate resulted in  $\text{Ca}^{2+}$  release from the SR and an associated force response. SR  $\text{Ca}^{2+}$  uptake was then abolished by withdrawal of ATP. This resulted in the development of a rigor tension response and a further, transient SR  $\text{Ca}^{2+}$  efflux. Perfusion was stopped and the bath solution replaced rapidly with one containing 5 mM ATP. This resulted in relaxation from rigor and a maintained reduction in  $[\text{Ca}^{2+}]$  due to net SR  $\text{Ca}^{2+}$  uptake. B, superimposed fluorescence ratio records of ATP-induced SR  $\text{Ca}^{2+}$  uptake under control conditions, or in the presence of 20 mM  $\text{P}_i$  (left). In the presence of CP, 20 mM  $\text{P}_i$  had little effect on SR  $\text{Ca}^{2+}$  uptake. However, when CP was absent from the perfusate,  $\text{Ca}^{2+}$  reuptake was reduced. When 20 mM  $\text{P}_i$  was present (but CP absent),  $\text{Ca}^{2+}$  uptake was further reduced and a slow increase in  $[\text{Ca}^{2+}]$  followed the initial rapid decline (right). C, in the presence of 10 mM CP and 20 mM  $\text{P}_i$ ,  $\text{Ca}^{2+}$  reuptake was markedly reduced following inhibition of CK with  $50 \mu\text{M}$  DNFB. Responses shown in A and B were from the same preparation.

## DISCUSSION

In most previous studies on skinned preparations, the presence and influence of Ca-P<sub>i</sub> precipitation was assessed indirectly from changes in the amount of Ca<sup>2+</sup> available for release from the SR following washout of P<sub>i</sub>. In the present study, measurement of cytosolic [Ca<sup>2+</sup>] allowed changes in SR Ca<sup>2+</sup> regulation to be studied in the continued presence of P<sub>i</sub>.

### Ca<sup>2+</sup> flux pathways across the SR membrane

Experiments on isolated SR vesicles have shown that Ca<sup>2+</sup> transport across the SR membrane is initially coupled to ATP hydrolysis with a molar ratio of 2:1. However, as the luminal [Ca<sup>2+</sup>] rises, SR ATPase activity and Ca<sup>2+</sup> uptake progressively decrease (Inesi & de Meis, 1989). Inhibition of pump activity by raised luminal [Ca<sup>2+</sup>] appears to result from the occupation of low affinity Ca<sup>2+</sup> binding sites at the inner surface of the SR membrane. In the absence of precipitating anions, the Ca<sup>2+</sup> content of the SR will reach a steady state, when uptake by the Ca<sup>2+</sup> pump is balanced by Ca<sup>2+</sup> efflux. Efflux can occur via ryanodine-sensitive Ca<sup>2+</sup> channels, a Ca<sup>2+</sup> 'leak' pathway, or in some circumstances by reversal of the SR Ca<sup>2+</sup> pump (for review see Feher & Fabiato, 1990). Previous work on skinned skeletal muscle fibres suggests that in the presence of 100–200 nM Ca<sup>2+</sup> and millimolar Mg<sup>2+</sup>, the steady-state Ca<sup>2+</sup> leak is small and may occur via the SR Ca<sup>2+</sup> channel (Kabbara & Stephenson, 1994) and via a ryanodine-insensitive Ca<sup>2+</sup> efflux pathway (Duke & Steele, 1998*a*). In the presence of CP, the local [ADP] is low and reversal of the SR Ca<sup>2+</sup> pump would not be expected to constitute a major Ca<sup>2+</sup> efflux pathway.

### Effects of P<sub>i</sub> in the presence of CP

In the protocol shown in Fig. 1, the preparation was initially perfused with a solution containing 10 mM CP. Previous studies involving caffeine application in skinned fibres have shown that the SR Ca<sup>2+</sup> content reaches a steady state within 2 min under these conditions (Duke & Steele, 1998*b*). Thereafter, Ca<sup>2+</sup> uptake must equal efflux. When the flow was stopped for 3 min in the presence of CP, [Ca<sup>2+</sup>] remained constant, suggesting that the balance of Ca<sup>2+</sup> fluxes was unaltered. However, the introduction of P<sub>i</sub> during perfusion resulted in a transient, concentration-dependent increase in [Ca<sup>2+</sup>] within the preparation (e.g. Fig. 1). We have shown previously that P<sub>i</sub>-induced Ca<sup>2+</sup> release is insensitive to ryanodine or other modulators of the SR Ca<sup>2+</sup> channel, blocked by the Ca<sup>2+</sup> pump inhibitor CPA and mimicked by addition of ADP (Duke & Steele, 2000). These characteristics are consistent with work on isolated SR vesicles showing that P<sub>i</sub> can induce Ca<sup>2+</sup> efflux by reversal of the SR Ca<sup>2+</sup> pump (e.g. Hasselbach, 1978).

At low levels of P<sub>i</sub> (< 5 mM), [Ca<sup>2+</sup>] returned to baseline levels following the initial P<sub>i</sub>-induced Ca<sup>2+</sup> release (Fig. 2). The [Ca<sup>2+</sup>] also remained constant when the flow was

stopped in the presence of low [P<sub>i</sub>]. This suggests that, following the initial P<sub>i</sub>-induced [Ca<sup>2+</sup>] release from the SR, a new equilibrium state is rapidly achieved, where Ca<sup>2+</sup> uptake again equals efflux. However, in the presence of higher levels of P<sub>i</sub> (and CP), a maintained decrease in the baseline [Ca<sup>2+</sup>] was apparent during perfusion. A further, more pronounced decrease in [Ca<sup>2+</sup>] occurred when the flow was stopped. This decrease in Ca<sup>2+</sup> was mimicked by oxalate and abolished by treatment with CPA or disruption of the SR membrane with Triton X-100, confirming maintained SR Ca<sup>2+</sup> uptake underlies the fall in [Ca<sup>2+</sup>] (Fig. 1). As previously considered, it seems likely that the maintained uptake of Ca<sup>2+</sup> results from Ca-P<sub>i</sub> precipitation within the SR lumen. When P<sub>i</sub> enters the SR, precipitation will occur when the Ca-P<sub>i</sub> solubility product is exceeded. The resulting decrease in luminal [Ca<sup>2+</sup>] disinhibits the Ca<sup>2+</sup> pump and Ca<sup>2+</sup> uptake persists due to the continual entry of P<sub>i</sub> from the surrounding medium. This phenomenon is commonly utilised in studies on isolated SR to maintain Ca<sup>2+</sup> uptake and prevent inhibition of the Ca<sup>2+</sup>-ATPase by rising luminal Ca<sup>2+</sup>. Under these conditions, the capacity of the SR may be limited only by the eventual rupture of the SR membrane by solid precipitates (Feher & Lipford, 1995).

The results shown in Fig. 2 suggest that precipitation of Ca-P<sub>i</sub> and consequent stimulation of the SR Ca<sup>2+</sup> pump occurred at a threshold of ~5 mM bathing P<sub>i</sub> when the [Ca<sup>2+</sup>] was 100 nM (Fig. 2). A similar threshold for precipitation has been reported previously in skinned EDL fibres under comparable Ca<sup>2+</sup> loading conditions (Fryer *et al.* 1995). Although the level of bathing P<sub>i</sub> required for precipitation would be expected to change depending on the Ca<sup>2+</sup> loading conditions, the apparent threshold remained consistent under the conditions of this study.

While the P<sub>i</sub> and oxalate both produced effects consistent with precipitation, the responses also differed in a number of important respects. First, the initial Ca<sup>2+</sup> release on addition of P<sub>i</sub> was not apparent on addition of oxalate (compare Fig. 1*A*, centre and right). This presumably reflects the fact that unlike P<sub>i</sub>, oxalate does not induce a reversal of the SR Ca<sup>2+</sup> pump. Second, precipitation was apparent at lower levels of oxalate, below that producing maintained Ca<sup>2+</sup> uptake in the presence of P<sub>i</sub> (Fig. 1). This is consistent with the fact that the solubility product for oxalate is lower than that of P<sub>i</sub> (Lide, 1995), and the fact that oxalate does not deplete SR Ca<sup>2+</sup> by pump reversal.

### Effects of CP withdrawal on SR Ca<sup>2+</sup> regulation

As shown in Fig. 3, withdrawal of CP during perfusion resulted in loss of Ca<sup>2+</sup> from the SR. This is consistent with previous work on isolated SR and skinned fibres suggesting that local rephosphorylation of ADP via the creatine kinase reaction is important for efficient Ca<sup>2+</sup> transport by the SR ATPase. Previously, we have shown

that the loss of  $\text{Ca}^{2+}$  from the SR on withdrawal of CP can be inhibited by CPA, which blocks the reversal of the SR  $\text{Ca}^{2+}$  pump (Duke & Steele, 1999). This suggests that the  $\text{Ca}^{2+}$  efflux associated with CP withdrawal may involve reversal of the SR  $\text{Ca}^{2+}$  pump due to local ADP accumulation.

Previous studies have shown that the SR  $\text{Ca}^{2+}$  content declines to a lower steady-state level within 2–3 min of CP withdrawal (Duke & Steele, 1999). At this point,  $\text{Ca}^{2+}$  uptake must again balance efflux. However, even after much more prolonged exposure to solutions lacking CP, a slow increase in  $[\text{Ca}^{2+}]$  was apparent when the flow was stopped (Fig. 3). The rise in  $[\text{Ca}^{2+}]$  was detectable when [CP] was reduced to 2 mM, and increased progressively as the concentration was further reduced to zero (Fig. 3). One possible explanation for the rise in  $[\text{Ca}^{2+}]$  is that the balance of  $\text{Ca}^{2+}$  fluxes is altered when the flow is stopped, due to further ADP accumulation in the limited bath volume. Hence, on stopping the flow,  $\text{Ca}^{2+}$  efflux via the pump may increase progressively, resulting in accumulation of  $\text{Ca}^{2+}$  within the bath. This interpretation is supported by the observation that the rise in  $\text{Ca}^{2+}$  was markedly reduced by an alternative ATP regenerating system (Fig. 4). The inability to abolish completely the rise in  $[\text{Ca}^{2+}]$  associated with stopping the flow is consistent with work on isolated SR vesicles suggesting that (i) bound CK is functionally linked to the SR  $\text{Ca}^{2+}$  ATPase and (ii) endogenous bound CK is more efficient than exogenous enzyme systems at maintaining a low ATP/ADP ratio in the vicinity of the SR ATPase (Korge *et al.* 1993).

#### Effects of $\text{P}_i$ in the absence of CP

As shown in Fig. 5, the effects of  $\text{P}_i$  were markedly altered when CP was absent from the cytosolic environment. First, as previously reported (Duke & Steele, 2000), the  $\text{P}_i$ -induced  $\text{Ca}^{2+}$  efflux was larger in the absence of CP. Second, during continuous perfusion, the bathing  $[\text{Ca}^{2+}]$  did not fall below control levels in the presence of  $\text{P}_i$ . Finally, when perfusion was stopped in the presence of  $\text{P}_i$ ,  $[\text{Ca}^{2+}]$  progressively *increased*. Even at the highest  $[\text{P}_i]$  tested (40 mM), a maintained decrease in  $[\text{Ca}^{2+}]$  consistent with precipitation did not occur when CP was absent from the solutions. This might be explained if precipitation of Ca- $\text{P}_i$  occurs, but maintained stimulation of SR  $\text{Ca}^{2+}$  uptake does not result, when CP is absent from the solutions. However, maintained stimulation of the SR  $\text{Ca}^{2+}$  pump was apparent when oxalate was introduced in the absence of CP (Fig. 5). This may reflect the fact that (i) oxalate does not deplete SR  $\text{Ca}^{2+}$  by reversal of the SR  $\text{Ca}^{2+}$  pump and (ii) calcium-oxalate has a lower solubility product. The effects of oxalate suggest that the inability of  $\text{P}_i$  to stimulate and maintain  $\text{Ca}^{2+}$  uptake in solutions lacking CP reflects the absence of Ca- $\text{P}_i$  precipitation within the SR.

Several factors may contribute to the apparent absence of Ca- $\text{P}_i$  precipitation in solutions lacking CP. First, the  $\text{Ca}^{2+}$

efflux, associated with CP withdrawal, results in a decrease in the steady-state SR  $\text{Ca}^{2+}$  content, as assessed by caffeine application (Duke & Steele, 2000). This efflux also decreases the capacity of the SR to re-accumulate  $\text{Ca}^{2+}$ , when ATP is re-introduced following  $\text{Ca}^{2+}$  depletion (compare Fig. 8B, left and right). It has also been shown that ADP is required for pump reversal by  $\text{P}_i$  (Hasselbach, 1978). Hence, in the presence of CP, the low  $[\text{ADP}]$  may limit pump reversal, thereby reducing SR  $\text{Ca}^{2+}$  depletion by  $\text{P}_i$ . This suggestion is supported by the observation that  $\text{P}_i$  had little effect on net  $\text{Ca}^{2+}$  uptake on re-introduction of ATP, following depletion of SR  $\text{Ca}^{2+}$  (Fig. 8B).

In the absence of CP, the higher local  $[\text{ADP}]$  may facilitate a larger and maintained  $\text{P}_i$ -induced  $\text{Ca}^{2+}$  efflux from the SR. Consistent with this, the initial  $\text{P}_i$ -induced  $\text{Ca}^{2+}$  efflux was more pronounced in the absence of CP (Fig. 5). ATP-activated  $\text{Ca}^{2+}$  uptake by the SR was also markedly reduced by  $\text{P}_i$ , in solutions lacking CP (Fig. 8B). Furthermore, in the presence of  $\text{P}_i$ , the initial rapid decrease in  $[\text{Ca}^{2+}]$  was followed by a slow increase over 2–3 min. This may reflect a progressive increase in  $\text{Ca}^{2+}$  efflux via the SR  $\text{Ca}^{2+}$  pump, as ADP accumulates within the bath. Together, these results suggest that when CP is depleted and  $[\text{P}_i]$  rises, efflux via the SR  $\text{Ca}^{2+}$  pump will increase progressively and the free luminal  $[\text{Ca}^{2+}]$  will decline. If the free SR  $[\text{Ca}^{2+}]$  decreases sufficiently, the Ca- $\text{P}_i$  solubility product may not be exceeded in the absence of CP.

#### Physiological significance and limitations of the present study

Under physiological conditions, it has been shown that cytosolic CP can be depleted rapidly during fatigue induced by repeated tetanic stimulation (e.g. Nagesser *et al.* 1993). The present study has shown that the effects of  $\text{P}_i$  are markedly influenced by CP. This apparent interdependence of CP and  $\text{P}_i$  may have a bearing on previous studies addressing the effects of  $\text{P}_i$  in intact and skinned preparations. For example, in most previous studies on skinned fibres,  $\text{P}_i$  was introduced in the presence of millimolar levels of CP (e.g. Fryer *et al.* 1995). This would tend to minimise efflux via the SR  $\text{Ca}^{2+}$  pump and facilitate Ca- $\text{P}_i$  precipitation. Similarly, in intact skeletal muscle, injection of  $\text{P}_i$  into the cytosol of unfatigued fibres produced a maintained decrease in resting  $\text{Ca}^{2+}$  and acceleration of  $\text{Ca}^{2+}$  uptake, consistent with Ca- $\text{P}_i$  precipitation (Westerblad & Allen, 1996). This result appears consistent with the present study, where  $\text{P}_i$  produced a maintained decrease in resting  $\text{Ca}^{2+}$  in the presence of CP. However, this contrasts with the observation that resting  $[\text{Ca}^{2+}]$  *increases* progressively throughout fatiguing stimulation (e.g. Allen & Westerblad, 1991). This rise in  $[\text{Ca}^{2+}]$  might be explained if  $\text{Ca}^{2+}$  is progressively redistributed from the SR to the cytosol due to pump reversal associated with CP depletion and rising levels of  $\text{P}_i$ .

One limitation of the present study is that two extreme conditions were examined;  $P_i$  was introduced in the complete absence or presence of CP. However, progression to a state of fatigue involves reciprocal changes in  $[P_i]$  and  $[CP]$  (Dawson *et al.* 1980). Therefore, as fatigue develops, the levels of  $P_i$  within the SR might be high enough to cause precipitation, before complete CP depletion occurs. This may depend upon a number of factors such as the SR  $Ca^{2+}$  load and the rate of  $P_i$  entry (Fryer *et al.* 1997). In support of the Ca- $P_i$  precipitation hypothesis, recent experiments in intact fibres showed that the amount of  $Ca^{2+}$  released from the SR in response to caffeine or 4-chloro-*m*-cresol (4-CmC) is reduced in fatigue. This was interpreted as evidence that the precipitation of  $Ca^{2+}$  within the SR decreases the amount of  $Ca^{2+}$  available for release. However, the sensitivity of the SR  $Ca^{2+}$  channel to caffeine (Duke & Steele, 1989*b*) and 4-CmC (A. M. Duke & D. S. Steeles, unpublished observations) is markedly affected by cytosolic levels of ATP. The local  $[ATP]$  may fall substantially in the final stages of fatigue, which could contribute to the apparent reduction in releasable  $Ca^{2+}$ . Furthermore, based on the present data, precipitation of Ca- $P_i$  should result in a sudden decrease in resting  $Ca^{2+}$ , as the solubility product is exceeded and the SR  $Ca^{2+}$  pump is stimulated. This does not appear to occur in intact cells during any stage of the fatigue process. The results of the present study suggest that the dominant influence of  $P_i$ , when CP is depleted, is the activation of  $Ca^{2+}$  efflux via pump reversal and that precipitation of Ca- $P_i$  does not occur under these conditions. This does not support the suggestion that precipitation of Ca- $P_i$  underlies the failure of the SR  $Ca^{2+}$  release mechanism in end-stage fatigue (e.g. Posterino & Fryer, 1998).

### Conclusions

These experiments suggest that under the conditions of this study, precipitation of Ca- $P_i$  occurs within the SR when the bathing  $[P_i]$  is  $\geq 5$  mM and when CP is present in the solutions. Both withdrawal of CP and subsequent addition of  $P_i$  resulted in the activation of a  $Ca^{2+}$  efflux pathway, which may involve the reversal of the SR  $Ca^{2+}$  pump. In the absence of CP, maintained SR  $Ca^{2+}$  uptake characteristic of Ca- $P_i$  precipitation did not occur.

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