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# Effect of Orthophosphate and Oxalate on the Cold-induced Release of Calcium from Sarcoplasmic Reticulum Preparations from Rabbit Skeletal Muscle

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*Abstract*

The cold-induced release of calcium from sarcoplasmic reticulum preparations from both white and red muscles of the rabbit was studied. Part of the release was due to the increase in pH of the reaction mixture with cooling. Calcium release was greatly reduced or completely prevented by the inclusion of oxalate or inorganic orthophosphate in the medium. No release occurred in 5 mM oxalate. With phosphate, the proportion of the calcium previously taken up at 23°C that was released at 0°C became progressively smaller as the phosphate concentration was increased. When the pH was adjusted to be the same at 0°C as at 23°C there was little release from white muscle preparations in 10 mM phosphate and no release when the phosphate concentration was 20 mM or more. With red muscle preparations calcium was released at higher phosphate concentrations, 8% of the amount previously taken up still being released at 50 mM phosphate and a smaller amount at 100 mM phosphate.

The effects of oxalate and phosphate can be explained in terms of the reduction in free calcium concentration inside the vesicles by calcium precipitants, and a difference in the temperature coefficients of calcium inflow and outflow.

## Introduction

Inorganic orthophosphate ($P_i$) and oxalate are known to greatly increase the calcium-accumulating ability of sarcoplasmic reticulum vesicles (SR) (Hasselbach and Makinose 1961; Lorand and Molner 1962) and to reduce the rate at which the accumulated calcium exchanges with calcium in the external medium (Martonosi and Feretos 1964; Weber *et al.* 1964). These effects are attributable to a decrease in the free calcium concentration inside the vesicles by the precipitation of calcium phosphate or calcium oxalate.

When SR preparations from skeletal muscle are loaded with calcium at 20–25°C and then rapidly cooled to 0°C they release some of their accumulated calcium (Taniguchi and Nagai 1970; Horgan *et al.* 1973), an effect which was observed to be smaller when the medium contained added $P_i$ than when it did not (Horgan *et al.* 1973). The present paper reports on the effects of $P_i$ concentration and also of oxalate on the cold-induced release of calcium.

In the earlier studies (Taniguchi and Nagai 1970; Horgan *et al.* 1973) buffers whose pH values were highly dependent on temperature were used and no attempt was made to keep the pH constant when the temperature was changed. Since the calcium-accumulating ability of SR in the absence of calcium-precipitating anions decreases as the pH increases above 6·4–6·5 (Carvalho and Leo 1967; Sreter 1969; Nakamaru and Schwartz 1970; Huxtable and Bressler 1974) and since calcium has

been shown to be released when the pH of vesicles loaded with calcium at pH 6·5–6·7 is abruptly increased (Nakamaru and Schwartz 1970, 1972), at least part of the calcium release observed in the earlier studies would have been due to the increase in the pH of the medium brought about by lowering the temperature. This aspect also is examined in the present paper.

## Materials and Methods

Preparations of SR were made from the psoas major (white) and combined soleus and semimembranosus proprius (red) muscles of rabbits as described by Martonosi *et al.* (1968) except that the supernatant was made 2 mM with respect to ethylene glycol bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid after the first 8000 *g* centrifugation and 1 mM dithiothreitol was present at all stages of preparation and in the final suspending medium. These SR preparations, referred to as WSR and RSR respectively, were used within 2 h after completion of the preparation unless stated otherwise. The 'aged' WSR preparations used in some experiments had been stored at 0–1°C for up to 8 days.

Calcium uptake and release were measured using $^{45}Ca$ and the Millipore filtration technique (Martonosi and Feretos 1964). The filters had an average pore diameter of 0·22 μm.

The reaction mixture contained 100 mM KCl, 20 mM histidine, 5 mM ATP, 5 mM $MgCl_2$, 5 mM sodium azide, 2·5 mM phosphoenolpyruvate, 8 units of pyruvate kinase/ml, 0–100 mM potassium orthophosphate or 5 mM potassium oxalate, and SR and $^{45}CaCl_2$ in concentrations indicated in the results section.

Measurements of pH were made at room temperature (23°C) or 0°C using a Radiometer (Copenhagen, Denmark) model 26 pH-meter standardized against a phosphate buffer at the appropriate temperature. All reaction mixtures had a pH of 6·4 at 23°C. The unadjusted pH of the reaction mixtures that contained no oxalate or added $P_i$ was about 6·8 at 0°C. When the pH was adjusted at the time of cooling to be the same at 0°C as at 23°C, known volumes (2–5 ml) of the reaction mixtures were dispensed into cooled tubes containing predetermined amounts of 1 M HCl. When the pH was raised or lowered at either 23°C or 0°C, known volumes of the reaction mixture were dispensed into tubes containing predetermined amounts of 1 M KOH or 1 M HCl respectively.

The reaction was started by the addition of SR and unless noted otherwise was allowed to proceed at 23°C and pH 6·4 for 2 min before alkali was added to raise the pH, or the reaction mixture was cooled rapidly in melting ice with or without addition of acid.

Protein concentration was measured by the method of Lowry *et al.* (1951).

## Results

### *Calcium Release in the Absence of Added $P_i$ or Oxalate*

Table 1 shows the effects of changes in temperature, pH, or both on the release of calcium from calcium-loaded SR preparations. When the temperature was lowered and the pH raised to 6·8, 43–45% of the calcium present in the SR was released. The total amount released was independent of whether the increase in pH was a consequence of lowering the temperature (incubation 2*a*), whether the temperature was first decreased at constant pH and the pH was then increased (incubation 3*b*), or whether the pH was first increased at 23°C and the temperature was then reduced while the pH was kept constant (incubation 1*b*). About 15% of the total calcium release was due to the increase in pH (cf. incubations 2*b* and 3*a* with incubations 2*a* and 3*b*).

Increasing the pH from 6·4 to 6·8 at constant temperature resulted in the release of nearly twice as much calcium at 23°C (cf. control and incubation 1*a*) as at 0°C (cf. incubations 2*b* and 3*a* with incubation 2*a* and 3*b*). The reversibility of the pH effect at 0°C is apparent from comparisons of incubations 2*a* and 2*b* and of incubations 3*a* and 3*b*. These show that when the pH is lowered from 6·8 to 6·4 the same amount of calcium is taken up as is released when the pH is raised from 6·4 to 6·8.

In other experiments the overall effect of lowering the temperature without adjusting the pH was shown to be reversible, i.e. after cooling, calcium was taken up again when the temperature was raised.

**Table 1. Effects of changes in temperature, pH or both on the release of calcium from SR preparations**
A WSR preparation was loaded with calcium at 23°C and pH 6·4 as described under Methods. The reaction mixture contained 25 $\mu$M $^{45}CaCl_2$ and 78 $\mu$g SR protein/ml. In the first 2 min 54% of the added calcium was taken up. At the end of 2 min aliquots of this control incubation were (1) added to and rapidly mixed with enough 1 M KOH to raise the pH to 6·8 (incubation 1*a*), (2) rapidly cooled to 0°C without adjusting the pH (incubation 2*a*), or (3) rapidly cooled to 0°C with enough 1 M HCl to maintain the pH at 6·4 (incubation 3*a*). The calcium content of the SR was determined (as described under Methods) in the control incubation 2, 7, and 10 min after the start of the reaction and in incubations 1*a*, 2*a*, and 3*a* when they were 5 and 8 min old. Seven minutes after the start of the reaction an aliquot of incubation 1*a* was rapidly cooled to 0°C in the presence of sufficient 1 M HCl to maintain the pH at 6·8 (incubation 1*b*), an aliquot of incubation 2*a* was mixed with sufficient 1 M HCl to lower the pH to 6·4 (incubation 2*b*), and an aliquot of incubation 3*a* was mixed with sufficient 1 M KOH to raise the pH to 6·8 (incubation 3*b*). The calcium contents of the SR in incubations 1*b*, 2*b*, and 3*b* were measured at the end of 3 min (i.e. 10 min after the start of the experiment)

| Incubation | Temp. (°C) and pH of reaction mixture during the period: 0–2 min | 2–7 min | 7–10 min | Calcium content of SR (nmol/mg protein) at the end of: 2 min | 7 min | 10 min |
|---|---|---|---|---|---|---|
| Control | 23, 6·4 | 23, 6·4 | 23, 6·4 | 193 | 168 | 161 |
| 1*a* | | 23, 6·8 | 23, 6·8 | | 143 | 143 |
| 1*b* | | | 0, 6·8 | | | 92 |
| 2*a* | | 0, 6·8 | 0, 6·8 | | 97 | 89 |
| 2*b* | | | 0, 6·4 | | | 100 |
| 3*a* | | 0, 6·4 | 0, 6·4 | | 107 | 101 |
| 3*b* | | | 0, 6·8 | | | 91 |

*Calcium Release in the Presence of Added $P_i$*

The effect of added $P_i$ on calcium release from WSR was studied in reaction mixtures containing 25 $\mu$M added $^{45}CaCl_2$ and an amount of SR (0·10–0·20 mg protein/ml) which, in the absence of added $P_i$, accumulated 60–80% of the calcium in the first 15 s at 23°C and over 90% in the first 2 min. Under these conditions the calcium content of the SR at the time of cooling was about the same at all $P_i$ concentrations.

With RSR the reaction mixtures contained 5 $\mu$M added calcium and an amount of SR (0·10–0·40 mg SR protein/ml) sufficient to take up about 50% of the calcium in the first 30 s at 23°C and 70–90% in the first 2 min at this temperature in the absence of added $P_i$.

In some experiments the pH (6·4 at 23°C) was not adjusted on cooling but in others it was. With both WSR and RSR preparations, as the concentration of added $P_i$ increased the amount of calcium released on cooling decreased. This is exemplified in Fig. 1.

Results obtained in three other experiments with WSR and three experiments with RSR are shown in Fig. 2. In these experiments the pH was adjusted to 6·4 on cooling. At the time of cooling, although the WSR had accumulated about 190 nmol calcium/mg SR protein and the RSR only about 30 nmol/mg, when no $P_i$ was added both types of preparation released about 40% of this calcium on cooling. The amount

of calcium released from WSR decreased rapidly as the $P_i$ concentration was increased until at $P_i$ concentrations of 20 mM and more there was little if any release. With RSR the percentage release fell off more slowly with increase in $P_i$ concentration than it did with WSR.

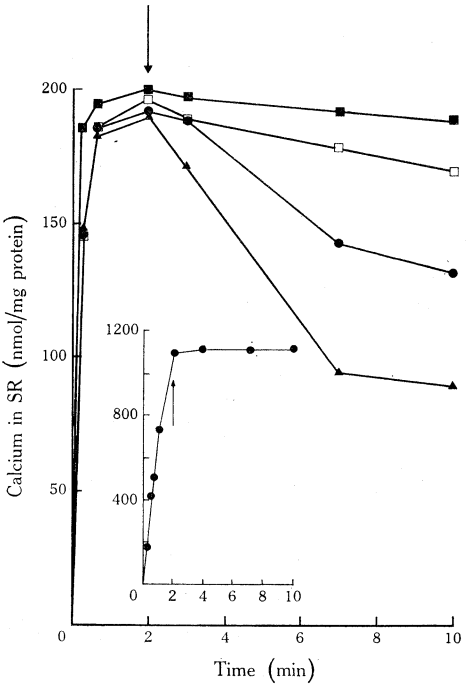


**Fig. 1.** Effect of $P_i$ concentration on the release of calcium from WSR loaded with calcium for 2 min at 23°C and then cooled to 0°C (arrow). Each value is the mean obtained from three experiments with three different WSR preparations. The reaction mixtures contained 25 μM added calcium and in two of the experiments 0·16 mg SR protein/ml. In the third experiment the SR concentration was 0·11 mg/ml. The pH of all reaction mixtures was 6·4 at 23°C and was not adjusted back to 6·4 on cooling. The mean value for maximum possible calcium uptake was 205 nmol/mg protein. ▲ No $P_i$. ● 5 mM $P_i$. □ 10 mM $P_i$. ■ 25 mM $P_i$.
*Inset.* Effect of oxalate on the release of calcium from WSR. The conditions were as described in the figure except that 5 mM oxalate was used instead of $P_i$, the calcium concentration was 100 μM, and the SR concentration was 0·09 mg SR protein/ml.

When the pH was not adjusted on cooling, the percentage calcium release was higher with both WSR and RSR at all $P_i$ concentrations. In the absence of added $P_i$ about 52% of the calcium taken up was released from both WSR (8 experiments) and RSR (3 experiments), that is about 12% more of the calcium taken up was released when the pH was not adjusted than when it was adjusted. This difference became progressively smaller until at 50 mM $P_i$ the extra calcium release when the pH was not adjusted amounted to only about 3% of the 2-min uptake (data not shown).

*Calcium Release in the Presence of Oxalate*

When 5 mM oxalate was present, the reaction mixture contained 25 or 100 μM $^{45}CaCl_2$ with WSR, 5 or 20 μM $^{45}CaCl_2$ with RSR, and 0·004–0·40 mg WSR or RSR protein/ml. With SR protein concentrations of less than 0·01 mg/ml the reaction mixture was incubated at 23°C for 30 min before cooling. In these experiments the pH was not adjusted on cooling.

As a result of varying the calcium and SR concentrations and the incubation time at 23°C the calcium content at the time of cooling ranged from 0·07 to 14·5 μmol/mg protein and from 0·02 to 2·4 μmol/mg protein for WSR and RSR preparations respectively. Regardless of the calcium content, none was released on cooling. A typical result for WSR is shown in Fig. 1 (inset).

*Calcium Release from Aged WSR*

In some experiments WSR preparations were examined on the day of preparation and again after they had aged for several days. The experimental conditions used for the fresh preparations were as described earlier. The SR concentrations and all other conditions were the same for the aged preparations as for the corresponding fresh preparations. In the absence of added $P_i$ or in the presence of 5 mM $P_i$, less calcium was taken up in 2 min by aged than by fresh SR, whilst at higher $P_i$ concentrations the 2-min uptake was the same in fresh and aged preparations.

The effect of $P_i$ on calcium release from aged WSR, in addition to its effect on calcium release from fresh SR, is shown in Fig. 2. At all $P_i$ concentrations from 0 to 100 mM the amount of calcium released, as a percentage of the 2-min uptake, was greater from aged than from fresh SR.

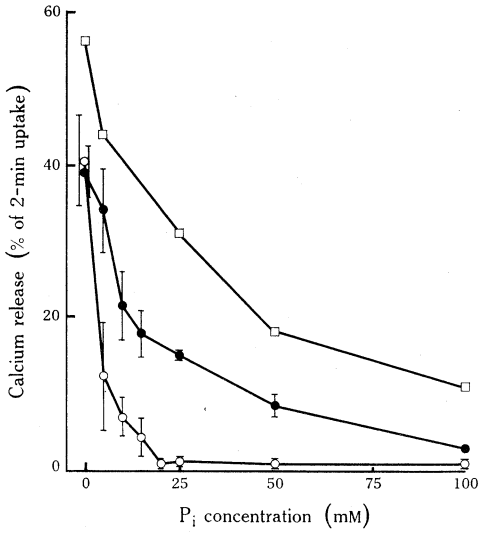


**Fig. 2.** Effect of $P_i$ on the cold-induced release of calcium from fresh and aged SR. The SR was loaded with calcium at pH 6·4 and 23°C for 2 min before being rapidly cooled to 0°C in the presence of sufficient 1 M HCl to keep the pH at 6·4. The amount of calcium released in 8 min at 0°C was determined. Each value for freshly prepared WSR and RSR represents the mean ± s.e.m. for three different preparations except the value for RSR at 100 mM $P_i$ which is for one preparation only. The values for aged WSR are those obtained in a single experiment using WSR stored at 0–1°C for 5 days. ○ Freshly prepared WSR. ● Freshly prepared RSR. □ Aged WSR.

## Discussion

The present results show that in the absence of oxalate or added $P_i$ and under the experimental conditions used, about 85% of the calcium release from WSR vesicles cooled to 0°C is due to the change in temperature and the remainder to the change in pH brought about by lowering the temperature. The effect of temperature on pH becomes smaller as the $P_i$ concentration increases, for $P_i$ is less sensitive to temperature than is histidine. This explains the observation that the difference between the amount of calcium released when the pH is adjusted to be the same at 23 and 0°C and the amount released when the pH is not adjusted in this way becomes smaller as the $P_i$ concentration is increased. In the following discussion, cold-induced release of calcium refers to the calcium released on cooling when the pH is kept constant.

The observed effects of oxalate and $P_i$ on the cold-induced release of calcium can be explained in terms of their reduction of the free calcium concentration inside the

vesicles and a lower temperature coefficient for the outward than for the inward movement of calcium.

The transport of calcium into sarcoplasmic reticulum vesicles against an activity gradient depends on the activity of the calcium pump ATPase. On the other hand calcium may move out of the vesicles by passive diffusion along the activity gradient or by a carrier-mediated mechanism which is probably the reverse of the calcium pump (Barlogie *et al.* 1971; Makinose 1971; Makinose and Hasselbach 1971; Panet and Selinger 1972; Hasselbach *et al.* 1974; Masuda and de Meis 1974). A steady state is reached when outward and inward rates are the same. Carrier-mediated calcium efflux depends on the presence of ADP and is arrested when the ADP concentration is kept very low by including an ATP regenerating system in the medium (de Meis and Carvalho 1974). Thus in our experiments, which were carried out in the presence of a regenerating system, the movement of calcium outwards was most likely by passive diffusion only.

Using the apparent energies of activation reported by Sreter (1969) it can be calculated that, between 0 and 23°C, the $Q_{10}$ of the calcium pump ATPase (extra ATPase) is close to 2 for RSR and greater than 3 for WSR. On the other hand the $Q_{10}$ of diffusion for biological membranes is about 1·4 (Lehninger 1970). Consequently, when the temperature of loaded vesicles is lowered from 23 to 0°C the rate of the enzyme-mediated inwards transport is reduced more than the rate of passive movement outwards with the result that there is a net outward movement of calcium. This leads to a reduction in the rate of outward movement and to stimulation of calcium pump activity and stops when a new steady state is reached.

When vesicles are loaded with calcium at room temperature in the absence of added $P_i$ or oxalate the free calcium concentration inside the vesicles is relatively high. Under these conditions, the differential effect of lowering the temperature on outward movement and inward pumping is easily measurable, a new steady state being reached at 0°C when about 40% of the total amount of calcium taken up has been released.

As progressively higher concentrations of $P_i$ are used, the concentration of free calcium inside the vesicles at room temperature is reduced and the difference between the influx and efflux rates brought about by cooling diminishes until it is no longer detectable. The solubility product of calcium oxalate is considerably lower than that of calcium monohydrogen phosphate, and 5 mM oxalate reduces the free calcium concentration inside the vesicles at room temperature to very low levels. On cooling, the rate of diffusion is decreased not only because of the effect of temperature on the rate of the diffusion process itself but also because the solubility product of calcium oxalate and hence the free calcium concentration inside the vesicles decreases with fall in temperature.

Like treatment with proteolytic enzymes, phospholipase A, detergents, organic solvents or a number of other reagents (for review, see Martonosi 1971), aging results in the loss of calcium-uptake activity but not of ATPase activity (Ebashi and Lipmann 1962). The effects of aging on the cold-induced release of calcium in the presence of added $P_i$ are in keeping with the view that, like the other treatments, aging results in an increase in the permeability or 'leakiness' of the membrane. With freshly prepared SR, 20 mM $P_i$ is sufficient to prevent the cold-induced release of calcium. With aged SR, however, substantial amounts of calcium are still released in the presence of 100 mM $P_i$, when the free calcium concentration inside the vesicles is only one-fifth the amount needed to reduce the cold-induced release of calcium from

fresh SR to negligible proportions. This suggests faster diffusion from the aged preparation.

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