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Effect of Orthophosphate on the Rate of Calcium Uptake by Red and White Muscle Sarcoplasmic Reticulum

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Summary. In the presence of added orthophosphate (P_i) there is a period during which sarcoplasmic reticulum vesicles (SR) accumulate calcium at a constant rate. This constant rate is increased and is reached sooner when the added P_i concentration is increased. A double reciprocal plot of rate and P_i concentration gives a straight line.

The P_i concentration required for half-maximum rate of calcium accumulation is the same for SR preparations from red and white rabbit muscles, although the maximum rates are widely different. During the ageing of SR preparations the P_i concentration required for half-maximum rate increases, but the maximum rate remains the same.

Inorganic orthophosphate (P_i), like oxalate, greatly increases the calcium-accumulating ability of the sarcoplasmic reticulum (SR) (Lorand & Molnar, 1962; Ebashi & Endo, 1964; Hasselbach, 1964; Martonosi & Feretos, 1964; Weber, Herz & Reiss, 1964*a*). This is apparently because of the precipitation of $CaHPO_4$ within the SR vesicles when the solubility product of Ca^{2+} and HPO_4^{2-} is reached, and the subsequent maintenance within the vesicles of a free calcium concentration below that reached in the absence of a calcium-precipitating agent. A study of the effect of varying the P_i concentration on the rate of calcium uptake is reported here.

Materials and Methods

White muscle SR preparations (WSR) were made from psoas and red muscle preparations (RSR) from the combined soleus and semimembranosus proprius muscles of New Zealand White rabbits. These preparations were made essentially as described by Martonosi, Donley and Halpin (1968) except that 1 mM dithiothreitol was present at all stages of the preparation and in the final suspending buffer. In addition, ethylene glycol bis (β-aminoethyl ether) N, N′-tetraacetic acid to a final concentration of 2 mM was added to the supernatant after the first $8{,}000 \times g$ centrifugation but not to the buffer used to wash the $8{,}000$–$28{,}000 \times g$ pellet or the final suspending medium. The WSR preparations contained

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8–12 and the RSR preparations 3–6 mg membrane protein/ml. Most preparations were used within 2 hr of completion of the preparation. Some, however, were "aged" by storing them at 0–1 °C for several days.

Calcium uptake was measured at 23 °C by the Millipore filtration method of Martonosi and Feretos (1964) using filters with an average pore diameter of 0.22 μm. The medium, pH 6.4, contained 100 mM KCl, 20 mM histidine, 5 mM sodium azide, 5 mM ATP, 5 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, pyruvate kinase (8 units/ml), 0–100 mM potassium phosphate, and 25 μM and 5 μM $^{45}CaCl_2$ for WSR and RSR, respectively. Calcium uptake was initiated by the addition of SR. In each experiment all reaction mixtures contained the same amount of SR, an amount sufficient for WSR to take up about 15% and for RSR to take up about 30% of the added calcium in 2 min in the absence of added P_i (13–22 μg SR protein/ml reaction mixture for WSR and 35–90 μg/ml for RSR). The reaction mixture was sampled for calcium uptake measurement at intervals during the first 2 min for WSR and 5 min for RSR.

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). The slopes of the linear parts of the rate curves and the slopes and intercepts of the double reciprocal plots were computed by the method of least squares.

Results

The effect of P_i concentration on the rate of calcium uptake by WSR is shown in Fig. 1 *A*, where each point is the mean value obtained for 5 different WSR preparations. When no P_i was added, the rate of calcium uptake decreased rapidly and calcium uptake soon ceased. In the presence of added P_i the rate decreased at first and then became constant.

As the P_i concentration was increased the constant rate became greater and was reached sooner. At all P_i concentrations, except perhaps 5 mM (the lowest added P_i concentration used), the constant rate was reached within 15 sec of the start of the reaction and continued until 2 min after the start or until about 70% of the calcium had been taken up. At greater than 70% uptake the rate fell off again.

Figure 1 *B* shows that a straight line is obtained when the reciprocal of the constant rate of calcium uptake obtained from the data in Fig. 1 *A* is plotted against the reciprocal of the P_i concentration. Values for the maximum velocity and the amount of P_i necessary to produce half-

Fig. 1. (*A*): Effect of P_i concentration on the rate of calcium uptake by WSR. Each point represents the mean of the values obtained in 5 experiments. The reaction mixture was as described under *Methods* and contained no added P_i, ○; 5 mM P_i, ●; 10 mM P_i, △; 15 mM P_i, ▲; 25 mM P_i, □; 35 mM P_i, ■; 50 mM P_i, ▽; or 100 mM P_i, ▼. (*B*): Double reciprocal plot of v, the rate during the linear phase of calcium uptake in *A*, and P_i concentration

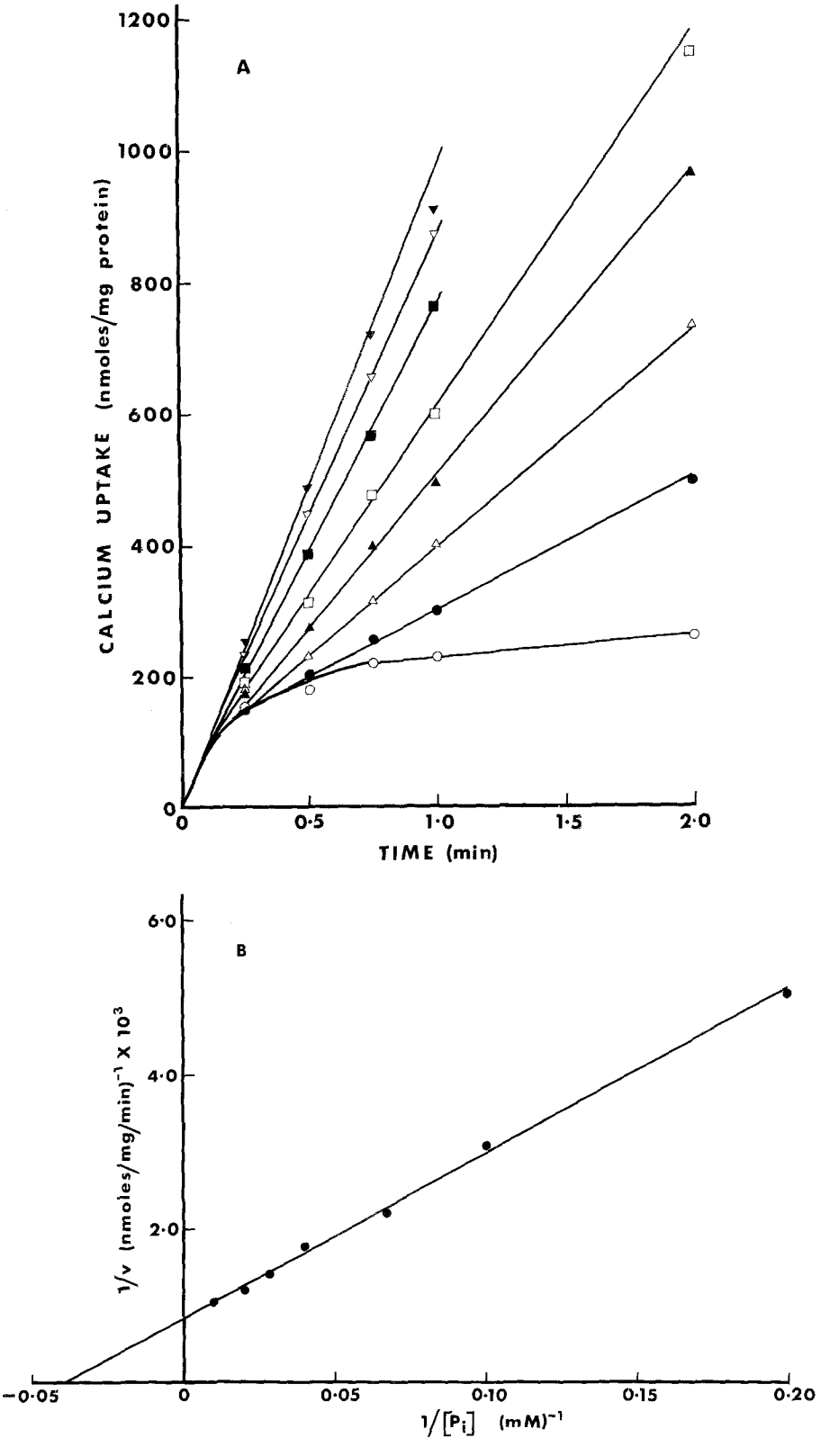

A
CALCIUM UPTAKE (nmoles/mg protein)
1200
1000
800
600
400
200
0
0
0·5
1·0
1·5
2·0
TIME (min)
B
1/v (nmoles/mg/min)$^{-1}$ X 10^3
6·0
4·0
2·0
−0·05
0
0·05
0·10
0·15
0·20
1/[P_i] (mM)$^{-1}$

Table 1. Calcium uptake characteristics of WSR and RSR

Preparation	No. of expts.	Total uptake in the absence of added P_i (nmol/mg SR protein)	Maximum velocity (nmol/mg SR protein/min)	P_i concentration for half maximum velocity (mM)
WSR	7	240±37	1008±120	25.3±3.0
RSR	9	43± 8	39± 5	18.2±2.8

Calcium uptake was measured at pH 6.4 as described in *Materials and Methods*. The total uptake in the absence of added P_i is the amount taken up in 2 min for WSR and 5 min for RSR. Maximum velocities and P_i concentrations at which the velocity was half-maximal were obtained from double reciprocal plots as described in the text. Values are means ±SEM.

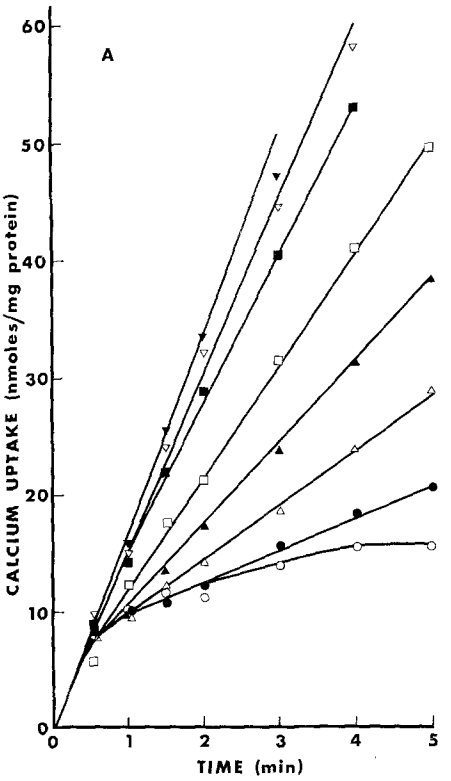
maximum velocity were obtained in 7 experiments from such plots. These values are summarized in Table 1.

Results obtained with RSR are shown in Fig. 2 and Table 1. With these preparations the rate of calcium uptake was virtually constant from about 30 sec after the start for the duration of the experiment or until 60–70% of the calcium had been taken up.

Mean values for total calcium uptake by WSR and RSR in the absence of added P_i (Table 1) were the same as those obtained by Sreter (1969). In addition, the maximum rates of calcium uptake by WSR obtained here from double reciprocal plots of rate during the linear phase and P_i concentration (Table 1) were in the same range as those obtained by other workers (Martonosi & Feretos, 1964; Weber, Herz & Reiss, 1964*b*, 1966; Sreter, 1969) by direct measurement of the rate of calcium uptake by rabbit SR preparations during the first few seconds. The maximum rate obtained in the present work for RSR, however, was only about a quarter that obtained by Sreter (1969).

Although the maximum velocity for calcium uptake by WSR was about twenty-five times that for RSR, the P_i concentrations needed by WSR and RSR to give half-maximum velocity were not significantly different.

Fig. 2. (*A*): Effect of P_i concentration on the rate of calcium uptake by RSR. The medium was as described under *Methods* and contained 5 μM added $^{45}CaCl_2$, 77 μg SR protein/ml and no added P_i, ○; 5 mM P_i, ●; 10 mM P_i, △; 15 mM P_i, ▲; 25 mM P_i, □; 35 mM P_i, ■; 50 mM P_i, ▽; or 100 mM P_i, ▼. (*B*) Double reciprocal plot of v, the rate during the linear phase of calcium uptake in *A*, and P_i concentration


A
CALCIUM UPTAKE (nmoles/mg protein)
TIME (min)
0
10
20
30
40
50
60
0
1
2
3
4
5

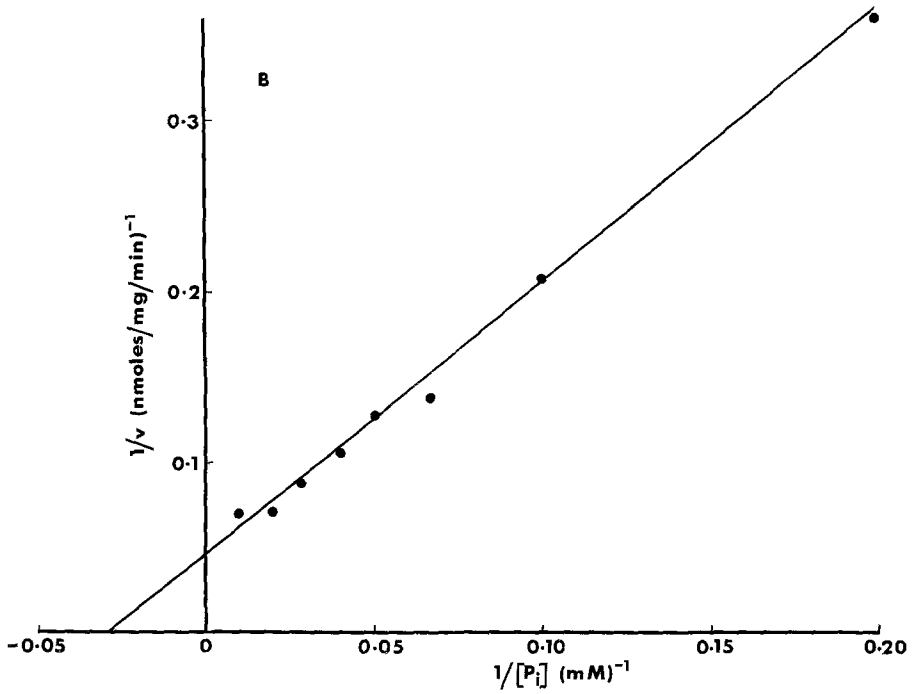

B
1/v (nmoles/mg/min)$^{-1}$
1/[Pi] (mM)$^{-1}$
0·3
0·2
0·1
-0·05
0
0·05
0·10
0·15
0·20

Table 2. Effect of ageing on the calcium uptake characteristics of WSR

Expt. No.	Days aged	Maximum velocity (nmol/mg SR protein/min)	P_i concentrations for half maximum velocity (mM)
1	0	630	25
	4	645	64
2	0	1100	42
	2	1190	74

Calcium uptake was measured at pH 6.4 as described in *Materials and Methods*. All conditions, including SR concentration, were the same for fresh and aged samples. Aged samples had been stored at 0–1 °C for the number of days indicated. Maximum velocities and P_i concentrations at which the velocity was half maximal were obtained from double reciprocal plots as described in the text. Measurements of calcium uptake in the absence of added P_i, made only in the first of these experiments, showed that the aged preparation had only about 40% of the calcium uptake acitivity of the fresh preparation.

Table 2 gives the results of two experiments in which measurements of the effect of added P_i on calcium uptake were made on the same SR preparations before and after ageing. These results show that ageing did not affect the maximum velocity but did lead to an increase in the amount of P_i needed for half-maximum velocity.

Discussion

The shape of the calcium uptake curves in Figs. 1*A* and 2*A* can be explained on the basis of the calcium-pump protein of the SR being inhibited when a low affinity calcium-binding site located on the inner surface of the membrane is saturated with calcium (de Meis & Carvalhõ, 1974; Ikemoto, 1975; de Meis & Sorenson, 1975). Initially the free calcium concentration inside the vesicles is very low and calcium-pump activity is maximal. In the absence of calcium-precipitating ions, the internal free calcium concentration increases continuously and the calcium pump protein becomes increasingly inhibited until finally there is no further accumulation of calcium. In the presence of calcium precipitants such as oxalate or P_i, to which the membrane is apparently freely permeable, the free calcium concentration inside the vesicles increases and the calcium pump activity decreases only until the solubility product of the calcium and precipitant ions is reached. Then calcium oxalate

or calcium phosphate precipitates, the internal free calcium concentration is maintained constant at a lower level than it would otherwise have reached, and calcium uptake continues at a constant rate. The internal free calcium concentration at which precipitation of calcium oxalate or calcium phosphate starts is reached sooner and becomes lower as the concentration of oxalate or P_i is increased. Consequently, the rate of calcium accumulation becomes constant sooner and is greater the higher the oxalate or P_i concentration. When the calcium precipitant is present in high enough concentrations, the constant rate is reached so quickly and is so great that it is indistinguishable from the initial rate. In the present experiments this occured when the P_i concentration was about 50 mM, indicating that at this and higher concentrations phosphate enters the vesicles so fast that the free calcium concentration inside the vesicles does not at any time increase sufficiently to cause detectable inhibition of the calcium-pump protein.

The effect of ageing on the P_i concentration (and hence on the internal free calcium concentration) at which the rate of calcium uptake is half maximal could be a consequence of an increase in the leakiness of the membrane during ageing.

The present findings are in keeping with those of Makinose and Hasselbach (1965) and Weber *et al.* (1966), who found that the rate of calcium uptake was greater the higher the oxalate concentration of the medium and concluded that the rate of uptake is dependent on the internal free calcium concentration. The present results are also in keeping with those of Ogawa (1970) who, using the murexide method for measuring calcium uptake and oxalate or P_i as the calcium precipitant, found the time course of calcium uptake to be biphasic with the rate during the first phase falling off continuously in the same way as it did in the absence of a calcium precipitant. In the second phase the rate was constant. The duration of the initial phase and the rate during the linear phase depended on the concentration of precipitant. These effects were observed with oxalate both in the presence and absence of an ATP regenerating system but with P_i only in the presence of a regenerating system. Biphasic calcium uptake in the presence of P_i but absence of a regenerating system has recently been reported by Mermier & Hasselbach (1975).

On the basis of his results with oxalate as calcium precipitant Ogawa (1970) concluded that the rate of calcium uptake during the linear phase decreased linearly as the reciprocal of the oxalate concentration increased, that is, that the rate of uptake decreased linearly as the internal free

calcium concentration increased. On the other hand our results with P_i as calcium precipitant show a linear relationship between the reciprocal of the rate of calcium uptake during the linear phase and the reciprocal of the P_i concentration; that is, they show a linear increase in the reciprocal of the rate of uptake as the internal free calcium concentration increases. In contrast Mermier & Hasselbach (1975) have reported a linear relationship between the rate of calcium uptake and the phosphate concentration.

The reasons for these diverse results may lie, at least in part, in the different experimental conditions used. For example, Ogawa (1970) used oxalate, Mermier & Hasselbach (1975) used P_i concentrations up to 4 mM and we used P_i concentrations ranging from 5 mM to 100 mM. In calcium transport studies oxalate acts only as a calcium precipitant but P_i is one of the necessary substrates for reversal of the calcium uptake process (Barlogie, Hasselbach & Makinose, 1971; Makinose, 1971, 1972; Makinose & Hasselbach, 1971; Panet & Selinger, 1972) as well as being a calcium precipitant. Another of the necessary substrates for reversal of the calcium pump is ADP, accumulation of which was prevented in our experiments but not in those of Mermier and Hasselbach (1975) by inclusion of an ATP-regenerating system in the media.

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