A model for the uptake and release of Ca^{2+} by sarcoplasmic reticulum

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On addition of ATP to vesicles derived from the sarcoplasmic reticulum (SR) of skeletal muscle, Ca^{2+} is accumulated from the external medium. Following uptake, spontaneous release of Ca^{2+} occurs in the presence or in the absence of ATP. These processes of Ca^{2+} uptake and release were simulated by using the models derived for ATPase activity [Gould, East, Froud, McWhirter, Stefanova & Lee (1986) *Biochem. J.* **237**, 217–227; Stefanova, Napier, East & Lee (1987) *Biochem. J.* **245**, 723–730] and for Ca^{2+} release from passively loaded vesicles [McWhirter, Gould, East & Lee (1987) *Biochem. J.* **245**, 713–722]. The simulations are consistent with measurements of the effects of pH, K^+ , Ca^{2+} and Mg^{2+} on uptake and release of Ca^{2+} . The increase in maximal Ca^{2+} accumulation observed in the presence of maleate is explained in terms of complexing of Ca^{2+} and maleate within the SR. The calculated concentration of ADP generated by hydrolysis of ATP has a large effect on the simulations. The effects of an ATP-regenerating system on the measured Ca^{2+} uptake is explained in terms of both removal of ADP and precipitation of $\text{Ca}_3(\text{PO}_4)_2$ within the vesicles. It is concluded that both the process of Ca^{2+} uptake and the process of Ca^{2+} release seen with SR vesicles can be interpreted quantitatively in terms solely of the properties of the Ca^{2+} + Mg^{2+} -activated ATPase.

INTRODUCTION

It is known that vesicles prepared from the sarcoplasmic reticulum (SR) of skeletal muscle will take up Ca^{2+} from the external medium on addition of ATP. Following uptake, the SR vesicles then spontaneously release some of this accumulated Ca^{2+} in two phases, an initial slow phase and a second faster phase (Ohnishi & Ebashi, 1963; Nakamaru & Schwartz, 1970; Van Winkle, 1976; Sorenson & de Meis, 1977; Entman *et al.*, 1979; Gerdes & Møller, 1983; McWhirter *et al.*, 1987b). In the preceding paper (McWhirter *et al.*, 1987b) it was shown that the initial slow phase of Ca^{2+} release occurs in the presence of ATP but that the second phase of release occurs when the ATP concentration has been lowered to values comparable with the K_d for ATP (McWhirter *et al.*, 1987b). It is known that uptake of Ca^{2+} is driven by the Ca^{2+} + Mg^{2+} -activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) (de Meis, 1981), but there is considerably less certainty as to the protein(s) responsible for Ca^{2+} efflux. Although it has often been suggested that Ca^{2+} efflux is mediated by Ca^{2+} channels within the SR membrane, we have suggested that, in fact, the Ca^{2+} + Mg^{2+} -activated ATPase could also be responsible for efflux (McWhirter *et al.*, 1987a,b).

In the normal reaction cycle of the ATPase, on phosphorylation of the ATPase by ATP the two Ca^{2+} -binding sites per ATPase monomer transform from being of high affinity and facing the cytoplasm to being of low affinity and facing inward. We have suggested that Ca^{2+} efflux is mediated by a conformational state of the ATPase in which the Ca^{2+} -binding sites are of low affinity but are outward-facing (McWhirter *et al.*, 1987a). We have shown that Ca^{2+} efflux from SR vesicles

passively loaded with Ca^{2+} can be simulated in terms of such a model (McWhirter *et al.*, 1987a). In the preceding paper we suggested that Ca^{2+} efflux from SR vesicles actively loaded with Ca^{2+} in the presence of ATP could also be mediated by the ATPase (McWhirter *et al.*, 1987b). In the present paper we combine our previous kinetic model for ATPase activity (Gould *et al.*, 1986; Stefanova *et al.*, 1987) with that for Ca^{2+} efflux (McWhirter *et al.*, 1987a) to produce a model for Ca^{2+} uptake and release in the presence of ATP. We show that such a model is adequate to account for all the main features of the experimental data.

MATERIALS AND METHODS

AnalR reagents and murexide were obtained from BDH Chemical Co., and Hepes (Ultrol) was from Calbiochem. SR vesicles were prepared as described in McWhirter *et al.* (1987b). Ca^{2+} fluxes were monitored photometrically by using the dye murexide as described in McWhirter *et al.* (1987b). To calibrate the optical signal, portions of a concentrated stock solution of CaCl_2 were added to give Ca^{2+} concentrations of 50 μM and 100 μM : over the concentration range used here the murexide response was linear with Ca^{2+} concentration. ATPase activities were determined by using the coupled enzyme assay method described in Stefanova *et al.* (1987). When used, the Ca^{2+} ionophore A23187 was added as a stock solution in methanol (1.6 mg/ml) to a final concentration of 5 $\mu\text{g/ml}$. All experiments were carried out at 25 °C.

Kinetic simulations were carried out by using the FACSIMILE program (Chance *et al.*, 1977) running on an ICL 2976 computer.

Abbreviations used: ATPase, Ca^{2+} + Mg^{2+} -activated ATPase; SR, sarcoplasmic reticulum.

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Fig. 1. Effect of pH on uptake and release of Ca^{2+} by SR vesicles

Shown are the changes in external Ca^{2+} concentration, monitored by using the dye murexide, following addition of ATP ($500 \mu\text{M}$) to SR vesicles (0.3 mg of protein/ml) in buffer (40 mM -Hepes/KOH/ 100 mM -KCl/ 5 mM - MgSO_4) at an initial external Ca^{2+} concentration of $100 \mu\text{M}$, 25°C and the given pH value. Downward deflections correspond to accumulation of Ca^{2+} by the vesicles, and upward deflections correspond to release of Ca^{2+} . At each pH value the scale denotes a $10 \mu\text{M}$ change in external Ca^{2+} concentration.

RESULTS

In the preceding paper (McWhirter *et al.*, 1987b) we showed that the dye murexide could be used to monitor the concentration of Ca^{2+} in the external medium of a suspension of SR vesicles. At the Mg^{2+} concentration of 4 mM usually used here, the change in free Ca^{2+} concentration due to binding of Ca^{2+} to ATP was negligible, so that decreases in external Ca^{2+} that result from addition of ATP to the SR vesicles can be attributed to accumulation of Ca^{2+} within the SR. As shown in Fig. 1, addition of ATP to SR vesicles results in accumulation of Ca^{2+} by the vesicles followed by

Fig. 2. Simulation of the effect of pH on uptake and release of Ca^{2+} by SR vesicles

Shown are simulations of the changes in external Ca^{2+} concentration following addition of ATP ($500 \mu\text{M}$) to SR vesicles (0.3 mg of protein/ml) under the conditions of Fig. 1 at the given pH values, calculated as described in the text.

Fig. 3. Effect of maleate on uptake and release of Ca^{2+} by SR vesicles

Shown are the changes in external Ca^{2+} concentration following addition of ATP ($500 \mu\text{M}$) to SR vesicles (0.3 mg of protein/ml) in buffer (40 mM -Hepes/KOH/ 100 mM -KCl/ 5 mM - MgSO_4) containing 40 mM -maleate, at an initial external Ca^{2+} concentration of $100 \mu\text{M}$, 25°C and the given pH value. At each pH value the scale denotes a $10 \mu\text{M}$ change in external Ca^{2+} concentration. The preparation of SR vesicles was that used in the experiments illustrated in Fig. 1.

Fig. 4. Simulation of the effect of maleate on uptake and release of Ca^{2+} by SR vesicles

Shown are simulations of the changes in external Ca^{2+} concentration following addition of ATP ($500\ \mu\text{M}$) to SR vesicles ($0.3\ \text{mg}$ of protein/ml) under the conditions of Fig. 3 in the presence of $40\ \text{mM}$ -maleate at the given pH values, calculated as described in the text.

Fig. 5. Effect of external Ca^{2+} concentration on uptake and release of Ca^{2+} by SR vesicles

Shown are the changes in external Ca^{2+} concentration following addition of ATP ($500\ \mu\text{M}$) to SR vesicles ($0.15\ \text{mg}$ of protein/ml) in buffer ($40\ \text{mM}$ -Hepes/KOH/ $100\ \text{mM}$ -KCl/ $5\ \text{mM}$ - MgSO_4 , pH 6.0) at initial Ca^{2+} concentrations of $50\ \mu\text{M}$ (trace A), $150\ \mu\text{M}$ (trace B), $200\ \mu\text{M}$ (trace C) and $33\ \mu\text{M}$ (trace D). The scale denotes a $10\ \mu\text{M}$ change in external Ca^{2+} concentration. The maximal extents of Ca^{2+} accumulated are identical for traces A and D ($196\ \text{nmol/mg}$ of protein).

spontaneous release of some of the accumulated Ca^{2+} . At higher temperatures, this release is seen to occur in two phases, an initial slow phase, which occurs when ATP is still present in the system, and a faster second phase, which occurs when the ATP has been depleted (McWhirter *et al.*, 1987b). At 25°C the two-phase nature of release is less obvious, both because the rates of release decrease with decreasing temperature and because the time for depletion of ATP becomes longer.

As shown in Fig. 1 and Table 3, the maximal amount of Ca^{2+} accumulated and the apparent rate of release of Ca^{2+} increase with decreasing pH. Fig. 3 and Table 1

Fig. 6. Simulation of the effect of external Ca^{2+} concentration on uptake and release of Ca^{2+} by SR vesicles

Shown are simulations of the changes in external Ca^{2+} concentration following addition of ATP ($500\ \mu\text{M}$) to SR vesicles ($0.15\ \text{mg}$ of protein/ml) at the given initial Ca^{2+} concentrations, under the conditions of Fig. 5.

Fig. 7. Effect of Mg^{2+} concentration on uptake and release of Ca^{2+} by SR vesicles

Shown are the changes in external Ca^{2+} concentration following addition of $500\ \mu\text{M}$ -ATP (traces A and B) or $2000\ \mu\text{M}$ -ATP (traces C) to SR vesicles ($0.3\ \text{mg}$ of protein/ml) in buffer ($40\ \text{mM}$ -Hepes/KOH/ $100\ \text{mM}$ -KCl, pH 7.2) at an initial Ca^{2+} concentration of $100\ \mu\text{M}$ and an Mg^{2+} concentration of $0.5\ \text{mM}$ (trace a), $0.75\ \text{mM}$ (trace b), $1.0\ \text{mM}$ (trace c), $1.0\ \text{mM}$ (trace d), $2.0\ \text{mM}$ (trace e), $3.0\ \text{mM}$ (trace f), $1.0\ \text{mM}$ (trace g), $2.0\ \text{mM}$ (trace h) and $3.0\ \text{mM}$ (trace i). The scales denote a $10\ \mu\text{M}$ change in external Ca^{2+} concentration. The artifact due to binding of Ca^{2+} to ATP at low Mg^{2+} concentrations has been subtracted from all traces (see Fig. 11): this effect is likely to make the traces at short times following addition of ATP an unreliable indicator of Ca^{2+} uptake.

show that addition of maleate both increases the extent of maximal accumulation of Ca^{2+} and increases the apparent rate of release of Ca^{2+} at any pH value.

As described in McWhirter *et al.* (1987a), the rate of efflux of Ca^{2+} from SR vesicles passively loaded with Ca^{2+} is dependent on the concentrations of Ca^{2+} and Mg^{2+} in the external medium. As shown in Fig. 5, the rate

Fig. 8. Simulation of the effect of Mg^{2+} concentration on uptake and release of Ca^{2+} by SR vesicles

Shown are simulations of the changes in external Ca^{2+} concentration following the addition of $500 \mu M$ -ATP (traces A) or $2000 \mu M$ -ATP (traces B) to SR vesicles (0.3 mg of protein/ ml) at the given Mg^{2+} concentrations, under the conditions of Fig. 7.

Fig. 9. Effect of K^+ concentration on uptake and release of Ca^{2+} by SR vesicles

Shown are the changes in external Ca^{2+} concentration following addition of ATP ($500 \mu M$) to SR vesicles (0.3 mg of protein/ ml) in buffer (40 mM -Hepes/ KOH / 4 mM - $MgSO_4$, pH 6.0) at an initial Ca^{2+} concentration of $100 \mu M$ and K^+ concentrations of 16 mM (trace A), 116 mM (trace B) and 216 mM (trace C). The scales denote a $10 \mu M$ change in external Ca^{2+} concentration.

of Ca^{2+} release from actively loaded SR vesicles increases with decreasing external Ca^{2+} concentration, although the external Ca^{2+} concentration has relatively little effect on the maximal extent of Ca^{2+} accumulation, as long as it is not so low that Ca^{2+} accumulation leads to complete

Fig. 10. Simulation of the effect of K^+ concentration on uptake and release of Ca^{2+} by SR vesicles

Shown are simulations of the changes in external Ca^{2+} concentration following addition of ATP ($500 \mu M$) to SR vesicles (0.3 mg of protein/ ml) under the conditions of Fig. 9 at the given K^+ concentrations.

Fig. 11. Effect of an ATP-regenerating system on uptake and release of Ca^{2+} by SR vesicles

Shown are the changes in external Ca^{2+} concentration following addition of ATP ($2000 \mu M$) to SR vesicles (0.3 mg of protein/ ml) in buffer (40 mM -Hepes/ KOH / 100 mM -KCl, pH 7.2) containing 2 mM -phosphoenolpyruvate and pyruvate kinase (22 units) at an initial Ca^{2+} concentration of $100 \mu M$ and an Mg^{2+} concentration of 1 mM (trace A), 2 mM (trace B) and 5 mM (trace C). Under these conditions at Mg^{2+} concentrations of 1 mM and 2 mM there is a large change in free Ca^{2+} concentration on addition of ATP in the absence of SR, attributable to binding of Ca^{2+} to ATP. The sizes of these artifacts are shown by the vertical bars. These effects are likely to make the traces at short times following addition of ATP in traces A and B unreliable indicators of Ca^{2+} uptake. The scale denotes a $50 \mu M$ change in external Ca^{2+} concentration.

loss of external Ca^{2+} . Fig. 7 shows that the rate of Ca^{2+} release increases with decreasing concentration of Mg^{2+} , particularly when the Mg^{2+} concentration falls below that of ATP, so that both ATP and $MgATP$ will be present in the system. ATPase activity is markedly

Fig. 12. Simulations of the effects of an ATP-regenerating system on uptake and release of Ca^{2+} by SR vesicles

The continuous lines show simulations of the changes in external Ca^{2+} concentration following addition of ATP (2000 μM) to SR vesicles (0.3 mg of protein/ml) under the conditions of Fig. 11 with a constant concentration of ATP and a zero concentration of ADP, as a function of Mg^{2+} concentration and rate constant for P_i diffusion across the membrane, k_{diff} : curve a, $[\text{Mg}^{2+}] = 1 \text{ mM}$, $k_{\text{diff}} = 1.0 \times 10^{-5}$; curve b, $[\text{Mg}^{2+}] = 2 \text{ mM}$, $k_{\text{diff}} = 1 \times 10^{-4}$; curve c, $[\text{Mg}^{2+}] = 5 \text{ mM}$, $k_{\text{diff}} = 1 \times 10^{-2}$. The broken line shows a simulation for $[\text{Mg}^{2+}] = 2 \text{ mM}$ but with no diffusion of P_i across the membrane.

affected by anion and cation concentrations (Stefanova *et al.*, 1987). Fig. 9 shows that with increasing concentration of KCl there is an increase in the maximal extent of Ca^{2+} accumulation.

In McWhirter *et al.* (1987b), it was shown that ADP had important effects on the pattern of Ca^{2+} uptake and release. Fig. 11 illustrates the effect on Ca^{2+} uptake of an ATP-regenerating system, consisting of phosphoenolpyruvate and pyruvate kinase. In the presence of the regenerating system, Ca^{2+} uptake occurs in two phases at Mg^{2+} concentrations of 2 or 5 mM, the second phase leading to complete removal of the external Ca^{2+} ; at 1 mM- Mg^{2+} the effects of the regenerating system are much less marked. At the lower Mg^{2+} concentrations the measurements are complicated by an artifact attributable to the binding of Ca^{2+} to free ATP: the size of this artifact was determined in parallel experiments in the absence of SR.

We have found that the ATPase activities of SR vesicles are highly variable between preparations, and increase significantly with time of storage at 0 °C (Fig. 13). ATPase activities of SR vesicles also generally increase after freezing to -20 °C for storage (results not shown). The pH-dependence of the ATPase activity of SR vesicles (Fig. 13) is comparable with that of purified ATPase (see Gould *et al.*, 1986). The pH-dependence of the ATPase activity of SR vesicles in the presence of A23187 is similar to that of purified ATPase in that activity decreases with increasing or decreasing pH from pH 7, but is not identical because we found that at acid pH values the ionophore had a significant inhibitory effect. We have also found that A23187 causes a significant increase in ATPase activity for the purified ATPase reconstituted into bilayers of dimyristoleoyl phosphatidylcholine (C. Gutierrez-Merino, J. M. East, G. W. Gould & A. G. Lee, unpublished work). The effects of external Ca^{2+} (Fig. 14) concentrations on the

Fig. 13. Effect of pH on ATPase activity of SR vesicles

Shown are ATPase activities measured for the preparation of SR vesicles used in Figs. 1 and 3 (O) or for a preparation stored at 0 °C for 2 days (●), as a function of pH, in 40 mM-Hepes/KOH/100 mM-KCl/5 mM- MgSO_4 buffer containing 2017 μM -ATP and a maximally stimulating concentration of Ca^{2+} . The lines are simulations with the parameters in Stefanova *et al.* (1987) assuming ATPase activities for the SR preparations either 10% (—) or 16% (---) of those of the pure uncoupled ATPase.

activities of SR vesicles are also comparable in the presence and in the absence of A23187.

DISCUSSION

In previous papers we have presented a model for the ATPase activity of the Ca^{2+} + Mg^{2+} -activated ATPase at 25 °C that can account for active accumulation of Ca^{2+} in the presence of ATP (Gould *et al.*, 1986; Stefanova *et al.*, 1987). We argued that Ca^{2+} efflux from passively loaded vesicles was also mediated by the Ca^{2+} + Mg^{2+} -activated ATPase because of the many similarities between effects on ATPase activity and on Ca^{2+} efflux, and presented a model for efflux consistent with the experimentally available data (McWhirter *et al.*, 1987a).

Studies of ATPase activity suggest that the ATPase can exist in one of two conformations, E1 or E2. In the E1 conformation, the two Ca^{2+} -binding sites per ATPase molecule are of high affinity and are exposed on the outer (cytoplasmic) side of the SR membrane. After phosphorylation the ATPase undergoes a conformation change in which the Ca^{2+} -binding sites become of low affinity and exposed to the inside of the SR. To explain Ca^{2+} efflux from SR vesicles mediated by the ATPase, we suggested that, after binding of Ca^{2+} to E1, the ATPase can undergo a slow conformation change in which the Ca^{2+} -binding sites transform into a low-affinity state characteristic of E2 but remain exposed to the outside of the SR. Cycling of the Ca^{2+} -binding sites in this low-affinity state between being inward-facing and outward-facing then provides a pathway for Ca^{2+} efflux. Importantly, we showed that the experimental data on

Fig. 14. Effect of external Ca^{2+} concentration on ATPase activity of SR vesicles

Shown are the fractions of the maximum ATPase activity measured in 40 mM-Hepes/KOH/100 mM-KCl/5 mM-MgSO₄ buffer containing 2000 μM -ATP as a function of $-\log[\text{free Ca}^{2+}]$ (curve A) at pH 7.2 for SR vesicles in the absence ($\circ$) and in the presence (Δ) of the Ca^{2+} ionophore A23187 and for the purified ATPase ($\square$), and (curve B) at pH 6.0 for SR vesicles ($\circ$) and for the purified ATPase (Δ). The lines are simulations with the parameters given in Stefanova *et al.* (1987).

Ca^{2+} efflux could be simulated in terms of such a model, with Ca^{2+} affinities for the ATPase as defined from studies of ATPase activity (McWhirter *et al.*, 1987a).

In McWhirter *et al.* (1987b) we studied release of actively accumulated Ca^{2+} under a variety of conditions and argued that again the data were consistent with Ca^{2+} release being mediated by the ATPase. It should then be possible to model Ca^{2+} uptake and release by SR vesicles in the presence of ATP in terms of our previously developed models.

As described below, an important parameter in modelling Ca^{2+} fluxes in these systems is the ATPase activity of sealed SR vesicles. Unfortunately, this cannot be determined directly by experiment. Steady-state ATPase activities of preparations of SR vesicles are generally about 10% of those measured in the presence of the Ca^{2+} ionophore A23187 or for purified ATPase (Fig. 13; Stefanova *et al.*, 1987; Gould *et al.*, 1987). However, it cannot be assumed that this represents the steady-state ATPase activity of sealed SR vesicles, since the same net activity would be observed if the system contained approx. 10% of unsealed leaky SR vesicles with the sealed vesicles showing essentially zero activity. Estimates of the proportion of unsealed SR vesicles in preparations from heart and skeletal muscle range from 14 to 25% (Chevallier *et al.*, 1977; Feher & Briggs, 1984). If the measured ATPase activity did, in fact, correspond to that of sealed vesicles, then ATPase activity should be comparable with the rate of passive Ca^{2+} efflux, and should vary with the concentrations of external Ca^{2+} and Mg^{2+} , as does Ca^{2+} efflux. However, as shown in Figs. 13 and 14, effects of pH and Ca^{2+} on the ATPase activity of SR vesicles are, within experimental error, identical with those measured in the presence of ionophore or measured for purified ATPase, despite the very different absolute values of activities in these systems. Effects of Mg^{2+} on the ATPase activities of SR vesicles in the presence and in the absence of ionophore are also

Table 1. Parameters used in simulations of Ca^{2+} uptake and release by SR vesicles at 25 °C

Internal volume	5 $\mu\text{l}/\text{mg}$ of protein
ATPase as % of total protein	75%
Internal Ca^{2+} -binding capacity	65 nmol/mg of protein
Percentage uncoupled SR vesicles	10%
Association constant for uncoupled vesicles:	
MgATP	1×10^5
MgADP	1×10^6
Rate constant for hydrolysis of bound MgATP by uncoupled vesicles:	
pH 5.5	1.2
pH 6.0	3.6
pH 6.5	4.8
pH 7.0–8.0	6.0

Table 2. ATPase activities of SR vesicles

ATPase activities were measured for the preparation of SR vesicles used in the experiments shown in Fig. 1 in 40 mM-Hepes/KOH/100 mM-KCl/5 mM-MgSO₄ buffer containing 2107 μM -ATP and a maximally stimulating concentration of Ca^{2+} . In the absence of Ca^{2+} a basal ATPase activity of 0.15 unit/mg of protein was measured. The measured total ATPase activity is given by the sum of the Ca^{2+} + Mg^{2+} -activated ATPase activity and the basal activity. The calculated total ATPase activity was calculated as described in the text, by using the parameters in Table 1. The calculated total ATPase activity should be compared with the measured total ATPase activity.

pH	ATPase activity (unit/mg)	
	Exptl. Ca^{2+} + Mg^{2+} -activated ATPase activity	Calc. total ATPase activity
8	0.21	0.42
7.5	0.32	0.46
7	0.44	0.48
6.5	0.26	0.44
6	0.16	0.31
5.5	0.08	0.23

comparable (results not shown). The data are therefore consistent with the ATPase activity of the SR vesicle preparation being dominated by a small fraction of unsealed leaky vesicles. Also consistent with this suggestion is the observation that the ATPase activity of a preparation of SR vesicles increases with time of storage (Fig. 13). In fact, the analysis of Ca^{2+} uptake and release by SR vesicles presented below would predict ATPase activities for sealed SR vesicles at 2107 μM -ATP of 0.03 and 0.01 unit/mg at pH 6 and 7 respectively.

It has been shown that ADP has important effects on the pattern of uptake and release of Ca^{2+} by SR vesicles (McWhirter *et al.*, 1987b). In the simulations it is therefore necessary to take into account the ADP generated by hydrolysis of ATP, both by the coupled and uncoupled SR vesicles present in the system and by the small basal ATPase activity observed in the absence of Ca^{2+} . It is not possible to describe the ATPase activity

Table 3. Ca^{2+} accumulation by SR vesicles

Extents of maximal Ca^{2+} accumulation were determined from the traces in Figs. 1 and 3 in the absence and in the presence of maleate respectively. Calculated extents of maximal Ca^{2+} accumulation and maximal internal Ca^{2+} concentrations were obtained from the simulations shown in Figs. 2 and 4 in the absence and in the presence of maleate respectively.

pH		Maximal Ca^{2+} accumulation (nmol/mg of protein)		Calc. maximal internal free Ca^{2+} concentrations (mM)	
		– Maleate	+ Maleate	– Maleate	– Maleate
5.5	Exptl.	180	249	–	–
	Calc.	197	230	25.7	19.3
6.0	Exptl.	147	244	–	–
	Calc.	146	189	15.4	11.4
6.5	Exptl.	132	191	–	–
	Calc.	116	163	9.6	7.3
7.0	Exptl.	103	135	–	–
	Calc.	94	131	5.7	4.4
7.5	Exptl.	63	66	–	–
	Calc.	76	100	3.3	2.7
8.0	Exptl.	38	88	–	–
	Calc.	61	75	1.9	1.6

of the uncoupled SR vesicles present in the preparation of SR vesicles by the full set of equations derived elsewhere because of limitations of computational time (Gould *et al.*, 1986; Stefanova *et al.*, 1987). A simplified approach was therefore adopted, with the use of the parameters listed in Table 1. It was assumed that 10% of the SR vesicles were uncoupled and that MgATP bound to the ATPase in these uncoupled vesicles with an association constant of 1×10^5 . It was also assumed that binding of MgADP with an association constant of 1×10^5 was competitive with the binding of MgATP (see Stefanova *et al.*, 1987). Hydrolysis of bound MgATP by the ATPase in the unsealed vesicles was assumed to occur in a single irreversible step, with a rate constant chosen to match typical ATPase activities measured at each pH. The small basal ATPase activities were included in the calculation of uncoupled ATPase activities. A comparison of the ATPase activities measured for the SR preparation used in the experiments shown in Figs. 1 and 3 and the ATPase activities calculated by using the parameters listed in Table 1 is given in Table 2.

Ca^{2+} uptake and release and ATP hydrolysis by the preparation of SR vesicles were then simulated by combining the full set of equations for the ATPase activity of sealed vesicles derived in Gould *et al.* (1986) and Stefanova *et al.* (1987), the simplified equations for ATP hydrolysis by uncoupled vesicles described above and the equations for Ca^{2+} efflux derived in McWhirter *et al.* (1987a). In the simulations, all parameters were fixed at their previously determined values except for the maximal extent of Ca^{2+} binding within the SR vesicles. As described in McWhirter *et al.* (1987a), there is considerable uncertainty as to the correct value for this parameter. For our preparations of SR vesicles we found the best agreement with the experimentally determined maximal extents of Ca^{2+} accumulation by assuming a maximal internal Ca^{2+} -binding capacity of 65 nmol of Ca^{2+} /mg of protein (Figs. 1 and 3 and Table 3). This is well within the range of values determined from

Fig. 15. Variability between preparations of SR vesicles

Shown are the changes in external Ca^{2+} concentration following addition of ATP (2.0 mM) to SR vesicles (0.5 mg of protein/ml) in buffer (40 mM-Hepes/KOH/100 mM-KCl/4 mM-MgSO₄, pH 5.8) at an initial external Ca^{2+} concentration of 200 μM . Trace A is representative of a preparation showing high maximal accumulation of Ca^{2+} (220 nmol of Ca^{2+} /mg of protein), trace C is from an intermediate preparation (160 nmol of Ca^{2+} /mg of protein) and trace D is from a low-uptake preparation (80 nmol of Ca^{2+} /mg of protein). Trace B is from the preparation used to prepare the SR vesicles used for trace A, but with dithiothreitol excluded from the final wash and storage buffers. The scale denotes a 50 μM change in external Ca^{2+} concentration.

passive- Ca^{2+} -binding experiments (see McWhirter *et al.*, 1987a) and was the value used in all the simulations shown in the present paper.

As shown in Fig. 2 and Table 3, the model presented here can successfully simulate the pH-dependence of the extent of Ca^{2+} uptake and the pH-dependence of the rate

Fig. 16. Effects of transition rates on the simulations of Ca^{2+} uptake and release by SR vesicles

Shown are simulations of the change in external Ca^{2+} concentration under the conditions of Fig. 1 with the rates of the transitions $\text{E1'Ca}_2\text{-E2'Ca}_2$ and $\text{E3'Ca}_2\text{-E4'Ca}_2$ given in McWhirter *et al.* (1987a) having been multiplied by the factors 0.1, 0.5, 2, 5 and 10. Traces A, pH 6.0; traces B, pH 7.0.

of Ca^{2+} release, and, in particular, simulations demonstrate the overshoot in Ca^{2+} uptake observed experimentally at acid pH values (Fig. 1). The rate of Ca^{2+} release shown in the simulations is somewhat higher than that observed experimentally, but, as described in McWhirter *et al.* (1987b), we have found that the rate of Ca^{2+} release is highly variable. Fig. 15 illustrates the range of Ca^{2+} uptake and release profiles that are typically observed. Trace A in Fig. 15 is typical of preparations showing the highest extents of uptake (220 nmol of Ca^{2+} /mg of protein) that we observed, trace C in Fig. 15 represents a preparation of intermediate uptake (150 nmol of Ca^{2+} /mg of protein) and trace D in Fig. 15 shows a preparation with low uptake (80 nmol of Ca^{2+} /mg of protein). In Gould *et al.* (1986) we described the variable ATPase activities found for the Ca^{2+} + Mg^{2+} -activated ATPase purified from SR, and suggested that this variability could arise from oxidation of protein thiol groups. The possibility that this could, at least in part, explain the variability in Ca^{2+} uptake by SR vesicles was explored by preparing SR vesicles either in the presence of dithiothreitol throughout the preparation, as described in McWhirter *et al.* (1987b), or excluding dithiothreitol from the final wash and storage buffers. As illustrated in Fig. 15 (traces A and B), exclusion of dithiothreitol converts an SR preparation from one showing high uptake into one showing intermediate extents of uptake. This observation is also consistent with the experiments of Bindoli & Fleischer (1983), who showed that water-soluble thiol-blocking reagents caused rapid release of Ca^{2+} from loaded SR vesicles.

In Gould *et al.* (1986) and Stefanova *et al.* (1987) it was shown that differences in ATPase activities between different preparations of the ATPase could be simulated in terms of differences in the rates of the $\text{E1'PCa}_2\text{-E2'PCa}_2$ transitions. By analogy, it would then seem possible that the differences in Ca^{2+} release rates seen

between different preparations of SR could be due to differences in the rates of the conformational changes $\text{E1'Ca}_2\text{-E2'Ca}_2$ and $\text{E3'Ca}_2\text{-E4'Ca}_2$ (see Scheme 1 in McWhirter *et al.*, 1987a). Fig. 16 illustrates the effects of varying these parameters on the Ca^{2+} uptake and release profiles at pH 6 and 7. As expected, decreasing the rates of these parameters slightly increases the extent of maximal accumulation of Ca^{2+} and decreases the rate of Ca^{2+} release, whereas increasing the transition rates decreases the extent of maximal Ca^{2+} accumulation and increases the rates of Ca^{2+} release.

It is known that the concentrations of Ca^{2+} and Mg^{2+} in the external medium have significant effects on the rates of Ca^{2+} efflux from passively loaded vesicles (see McWhirter *et al.*, 1987a). For SR vesicles actively accumulating Ca^{2+} in the presence of ATP, increasing the external Ca^{2+} concentration leads to a decreased rate of Ca^{2+} release with relatively little effect on maximal Ca^{2+} accumulation (Fig. 5). Simulations show a very similar effect (Fig. 6). For passively loaded SR vesicles in the presence of ATP, increasing the concentration of Mg^{2+} leads to a decrease in the rate of Ca^{2+} efflux, both because of inhibition by Mg^{2+} itself and because, though free ATP stimulates efflux, MgATP does not (McWhirter *et al.*, 1987a). As shown in Fig. 7, decreasing the total Mg^{2+} concentration also leads to an increase in the rate of Ca^{2+} release from actively loaded vesicles, particularly when the concentration of Mg^{2+} falls below that of ATP, so that significant amounts of free ATP will be present. Addition of ATP to solutions containing Ca^{2+} at low concentrations of Mg^{2+} leads to a decrease in free Ca^{2+} concentration due to the formation of CaATP (see Fig. 11). In Fig. 7, the change in murexide absorption attributable to this effect has been subtracted out, but the result is that traces at short times following addition of ATP will be unreliable indicators of Ca^{2+} uptake by the SR vesicles. However, the slopes recorded at longer times should be reliable indicators of the effects of Mg^{2+} on spontaneous Ca^{2+} release, and these effects are reproduced well in the simulations (Fig. 8). As shown in Fig. 9 and Table 4, increasing the concentration of KCl leads to an increase in the extent of maximal Ca^{2+} accumulation at pH 6.0, and a more distinct overshoot in Ca^{2+} uptake. The effects on the extent of maximal accumulation (Table 4) are also reproduced well in the simulations. Exact comparison between the observed and simulated rates of Ca^{2+} release were not attempted because, as described above, rates of Ca^{2+} release were highly variable between preparations. However, the increase in the rate of Ca^{2+} release observed experimentally with increasing K^+ concentrations (Fig. 9) is matched by the simulations (Fig. 10). In trace C in Fig. 9 a second phase of rapid Ca^{2+} release is apparent, starting approx. 7 min after the addition of ATP. In McWhirter *et al.* (1987b) it was shown that the start of the second phase of release corresponds to the time when the ATP concentration falls below the K_d of the ATPase for ATP. As described above, this time is highly variable between preparations owing to variations in basal ATPase activity and in ATPase activity attributable to leaky vesicles. These effects of KCl follow from the effects of K^+ on the rate of dephosphorylation of the phosphorylated ATPase and on the rates of the transitions between E1'PCa_2 and E2'PCa_2 , as described in Stefanova *et al.* (1987).

As shown in Fig. 3, inclusion of 40 mM-maleate in the

Table 4. Effect of K^+ on Ca^{2+} accumulation by SR vesicles

Extents of maximal Ca^{2+} accumulation were determined from the traces in Fig. 9, and calculated extents of maximal Ca^{2+} accumulation were obtained from the simulations shown in Fig. 10.

Concn. of K^+ (mM)	Maximal Ca^{2+} accumulation (nmol/mg of protein)	
	Exptl.	Calc.
16	98	112
116	123	146
216	150	158

incubation medium leads to increased Ca^{2+} accumulation and a more pronounced overshoot in Ca^{2+} uptake. This effect is also observed with other dicarboxylates that can chelate Ca^{2+} , such as succinate (Chu *et al.*, 1983; McWhirter *et al.*, 1987b). Chu *et al.* (1983) have observed that, in the presence of succinate, succinate is accumulated within the SR during Ca^{2+} uptake, presumably as a result of complexing between Ca^{2+} and succinate within the SR. The effects of maleate shown in Fig. 3 could follow both from complexing with Mg^{2+} in the external medium and from complexing with Ca^{2+} within the SR; the concentration of Ca^{2+} in the external medium is too low to interact significantly with maleate. To calculate the effects of maleate on the concentrations of Ca^{2+} and Mg^{2+} , the association constants of Ca^{2+} and Mg^{2+} with maleate are required, but it is known that these are highly dependent on ionic strength. We find the best agreement with our experimental data by using the association constants reported for a medium of ionic strength 0.2: the association constants used were 63.0 and 35.3 for Ca^{2+} and Mg^{2+} respectively with the maleate dianion, and 10.0 and 5.9 for Ca^{2+} and Mg^{2+} respectively with the maleate monoanion, with a pK value of 6.22 for maleate (Sillén & Martell, 1964). Assuming in this way that maleate lowers the external free concentration of Mg^{2+} and assuming fast diffusion of maleate into the SR vesicles where it can complex the internal Ca^{2+} , it is possible to reproduce the experimental data on the effects of maleate both on the maximal extent of accumulation (Table 3) and on the rate of release (Fig. 4).

It is very difficult to determine experimentally the free Ca^{2+} concentration reached within the SR during active transport because of extensive binding of Ca^{2+} to proteins within the SR. Further, as described above and in McWhirter *et al.* (1987a), it is difficult to determine binding parameters for Ca^{2+} to the proteins within the SR vesicles with sufficient accuracy to allow calculation of free Ca^{2+} concentrations from the total measured uptake of Ca^{2+} . Since the effect of maleate on the extent of maximal Ca^{2+} accumulation follows largely from complexing Ca^{2+} within the SR vesicle, the good agreement obtained here serves as a check that the concentrations of free Ca^{2+} within the SR obtained in the simulations are reasonable. Unfortunately, the calculated concentrations of free Ca^{2+} in the presence of maleate are very sensitive to the assumed Ca^{2+} -maleate binding

Fig. 17. Simulations of the effects of added ADP on Ca^{2+} uptake and release by SR vesicles

Shown are simulations of the changes in external Ca^{2+} concentration under the conditions of Fig. 1 at pH 6, but in the presence of added ADP. Simulations are for added ADP concentrations of 0.1 mM, 0.2 mM, 0.5 mM, 0.75 mM and 1 mM.

Fig. 18. Simulations of the effects of ADP generated by hydrolysis of ATP

The simulations compare the changes in external Ca^{2+} concentrations calculated for a fixed ATP concentration of 500 μM with the ADP concentration either allowed to vary (curves a and b) or fixed at zero (curves c and d) at pH 6 (curves a and d, continuous lines) and at pH 7 (curves b and c, broken lines), under the conditions of Fig. 1.

constants and, as described, these are rather uncertain. The free Ca^{2+} concentrations calculated here (Table 3) can be compared with indirect estimates made in other studies. Thus Beeler *et al.* (1981) have estimated an internal free Ca^{2+} concentration of 10–50 mM at pH 6.5 from the measured responses of a number of voltage-sensitive dyes, and Haynes & Mandveno (1983) have estimated maximal internal Ca^{2+} concentrations varying from 90 mM to 10 mM with changing pH from 6 to 8 from fluorescence measurements with anilinonaphthalene-sulphonic acid.

It was shown in McWhirter *et al.* (1987b) that the presence of ADP at pH 6.0 significantly diminished the overshoot in Ca^{2+} uptake seen on addition of ATP. As shown in Fig. 17, this effect is also reproduced in the simulations. The importance of ADP in the simulations

is shown in Fig. 18, which compares the patterns of Ca^{2+} uptake and release calculated with the concentration of ATP fixed at its initial concentration of $500\ \mu\text{M}$, with the ADP concentration either allowed to vary or fixed at zero. The result of fixing the ADP concentration at zero is to produce a constantly increasing Ca^{2+} uptake both at pH 6 and at pH 7. The effects of ADP can be further studied experimentally by using a regenerating system consisting of pyruvate kinase and phosphoenolpyruvate to maintain a constant concentration of ATP and a zero concentration of ADP. As shown in Fig. 11, the patterns of Ca^{2+} uptake and release are very different in the presence and in the absence of the regenerating system. As discussed by Lau (1983), this can be attributed both to the removal of ADP and to the formation of high concentrations of P_i in the system, which can in turn lead to the formation of precipitates of $\text{Ca}_3(\text{PO}_4)_2$ within the SR vesicles (see McWhirter *et al.*, 1987b). The relative importance of these two factors is shown by the simulations in Fig. 12. As already described, the simulations show that removal of ADP results in a continued uptake of Ca^{2+} even at low concentrations of Mg^{2+} , where release of Ca^{2+} would normally be relatively fast (Figs. 7 and 8). The production of P_i and the formation of $\text{Ca}_3(\text{PO}_4)_2$ precipitates within the SR leads to further uptake (McWhirter *et al.*, 1987b). Katz *et al.* (1976) determined a solubility product of $7.5 \times 10^{-6}\ \text{M}^2$ for $\text{Ca}_3(\text{PO}_4)_2$. Lau (1983) showed that uptake of P_i into the SR vesicles was slow. Simulations of the effects of P_i are very sensitive to the assumed rate of P_i movement across the membrane. The dependence of uptake of Ca^{2+} on the concentration of Mg^{2+} is best simulated by assuming that the rate of P_i transport is dependent on the concentration of Mg^{2+} , as shown in Fig. 12. Sufficient data are not presently available to take these simulations of the effects of P_i further, but it may be noted that Carley & Racker (1982) have also suggested that the mechanism of P_i transport is not a simple passive movement in response to the Ca^{2+} gradient established by the ATPase: Carley & Racker (1982) suggested that transport of P_i was ATP-dependent.

In the model as presented here, we have ignored any charge imbalances across the SR membrane that might result from the movements of Ca^{2+} that we have been discussing. Charge effects could be introduced in the manner described by Reynolds *et al.* (1985), but this seems to be unnecessary at present. As described in McWhirter *et al.* (1987b), any membrane potentials that might be produced in the SR vesicles appear to be too small and transitory to have any significant effect on Ca^{2+} uptake and release, presumably because of the presence of a variety of anion and cation channels within the SR membrane (Martonosi, 1984).

Although we believe that the model presented here has successfully explained the processes of Ca^{2+} uptake and release observed in SR vesicles, the relevance of these processes to the physiological role of SR in muscle remains unclear. Since we have suggested that the ATPase can provide a pathway for rapid Ca^{2+} release from SR, it is attractive to suggest that this pathway could be involved in Ca^{2+} release from SR in muscle. Of course, the release pathway would need to be controlled efficiently. It has been suggested that Ca^{2+} release is triggered by an increase in the Ca^{2+} concentration in the myofilament space following from depolarization of the

T-tubule membrane, in a process referred to as ' Ca^{2+} -induced Ca^{2+} -release' (reviewed in Martonosi, 1984). Certainly, an increase in external Ca^{2+} concentration from a low resting value to a micromolar level would result in an increase in the rate of Ca^{2+} release by the pathways discussed here (see Fig. 2 in McWhirter *et al.*, 1987a). It has also been suggested that calmodulin and calmodulin-dependent protein kinases could be involved in the Ca^{2+} release process (Chiesi & Carafoli, 1982; Meissner, 1986). If the ATPase is indeed involved in the release of Ca^{2+} in muscle, then perhaps the most likely possibility is that the conformational change to the $\text{E3}^*\text{Ca}_2$ state that is involved in release (see Scheme 1 in McWhirter *et al.*, 1987a) is controlled by interaction with other proteins within the SR membrane, and could then be sensitive to control by phosphorylation of these components.

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