A kinetic model for Ca^{2+} efflux mediated by the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -activated ATPase of sarcoplasmic reticulum

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We present a model for Ca^{2+} efflux from vesicles of sarcoplasmic reticulum (SR). It is proposed that efflux is mediated by the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -activated ATPase that is responsible for Ca^{2+} uptake in this system. In the normal ATPase cycle of the ATPase, phosphorylation of the ATPase is followed by a conformational change in which the Ca^{2+} -binding sites change from being outward-facing and of high affinity to being inward-facing and of low affinity. To mediate Ca^{2+} efflux, it is proposed that the ATPase can adopt a conformation in which the Ca^{2+} -binding sites are of low affinity but still outward-facing. It is shown that experimental data on the rates of Ca^{2+} efflux can be simulated in terms of this model, with Ca^{2+} -binding-site affinities previously proposed to explain ATPase activity [Gould, East, Froud, McWhirter, Stefanova & Lee (1986) *Biochem. J.* 237, 217–227]. Effects of Mg^{2+} and adenine nucleotides on efflux rates are explained. It is suggested that Ca^{2+} efflux from SR mediated by the ATPase could be important in excitation–contraction coupling in skeletal muscle.

INTRODUCTION

The initial step in the activation of fast-twitch skeletal muscle is a depolarization of the sarcolemma, which is conducted into the muscle fibre by membrane invaginations, the T tubules. The T tubules are closely apposed to the sarcoplasmic reticulum (SR), an internal membrane system, and signal transmission from the T tubule initiates Ca^{2+} release from SR into the myofilament space. When the Ca^{2+} concentration in the myofilament space increases from approx. $0.1 \mu\text{M}$ to $5 \mu\text{M}$, interaction of Ca^{2+} with regulatory proteins on the myofilaments permit actin–myosin interaction and force generation. Active transport of Ca^{2+} back into SR then lowers the Ca^{2+} concentration in the myofilament space to $0.1 \mu\text{M}$, and the muscle relaxes (Martonosi & Beeler, 1983).

The protein responsible for Ca^{2+} uptake in sarcoplasmic reticulum, the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -activated ATPase (ATP phosphohydrolase, EC 3.6.1.3), has been well characterized, and a kinetic model for the ATPase has been presented (Gould *et al.*, 1986). The process of Ca^{2+} release is, however, much less well characterized. It has been suggested that charge movement in the T tubule or ionic current flow from the T tubule to the SR could open Ca^{2+} channels in the junctional region of the SR, causing the release of accumulated Ca^{2+} . Alternatively, it has been suggested that a diffusible chemical transmitter could be released following depolarization of the T tubule, this transmitter then triggering release of Ca^{2+} from the SR. One possibility for this transmitter is Ca^{2+} itself, in a process referred to as ‘ Ca^{2+} -induced Ca^{2+} -release’ (reviewed in Martonosi, 1984).

Vesicular fragments of SR can be isolated from skeletal muscle that actively accumulate Ca^{2+} . A number of studies have appeared of Ca^{2+} release from these SR vesicles. Under conditions where the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -activated ATPase can run backwards, forming ATP from

ADP and P_i , Ca^{2+} is released (de Meis, 1981), but this is unlikely to be of importance physiologically. More interestingly, it has also been found that addition of Ca^{2+} to SR vesicles loaded with Ca^{2+} can induce release of Ca^{2+} , as can changing anion concentrations to cause a change in membrane potential (Martonosi, 1984). Spontaneous release of Ca^{2+} from vesicles has also been studied and, in the presence of micromolar external Ca^{2+} and high concentrations of adenine nucleotides, has been found to be fast enough to account for Ca^{2+} release *in vivo* (Meissner *et al.*, 1986). Although it has been suggested that this Ca^{2+} release could be mediated by the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -activated ATPase (Chiu & Haynes, 1980; Katz *et al.*, 1980), more commonly it has been proposed that release is mediated via a Ca^{2+} channel within the SR membrane (Bindoli & Fleischer, 1983; Gerdes *et al.*, 1983; Morii & Tonomura, 1983; Kim *et al.*, 1983; Martonosi, 1984; Salama & Abramson, 1984; Chamberlain *et al.*, 1984; Smith *et al.*, 1985; Endo, 1985). However, it is notable that agents that modify Ca^{2+} release through this proposed Ca^{2+} channel also modify the activity of the $\text{Ca}^{2+} + \text{Mg}^{2+}$ -activated ATPase. Thus Ca^{2+} release is stimulated by micromolar external Ca^{2+} and inhibited by millimolar external Ca^{2+} (Kim *et al.*, 1983; Nagasaki & Kasai, 1983; Meissner, 1984; Meissner *et al.*, 1986), as is ATPase activity (de Meis, 1981; Gould *et al.*, 1986). Ca^{2+} release is stimulated by adenine nucleotides (Morii & Tonomura, 1983; Nagasaki & Kasai, 1983; Meissner, 1984; Smith *et al.*, 1985; Meissner *et al.*, 1986), which also bind to the ATPase (de Meis, 1981). Mg^{2+} inhibits Ca^{2+} release (Nagasaki & Kasai, 1983; Meissner, 1984; Meissner *et al.*, 1986), and Mg^{2+} is also known to bind to the ATPase (see Gould *et al.*, 1986; Stefanova *et al.*, 1987). Ca^{2+} release is inhibited by carbodi-imides (Yamamoto & Kasai, 1982), as is the ATPase (Murphy, 1981; Chadwick & Thomas, 1985). Ca^{2+} release is inhibited by tetracaine, procaine,

Abbreviations used: ATPase, $\text{Ca}^{2+} + \text{Mg}^{2+}$ -activated ATPase; SR, sarcoplasmic reticulum; [$\beta\gamma$ -methylene]ATP or [$\beta\gamma\text{-CH}_2$]ATP, adenosine 5'-[$\beta\gamma$ -methylene]triphosphate.

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quercetin and other amine-based drugs (Nash-Adler *et al.*, 1980; Volpe *et al.*, 1983; Kim *et al.*, 1983; Kurebayashi & Ogawa, 1984; Antonia *et al.*, 1985), and these same compounds inhibit ATPase activity (Shoshan & MacLennan, 1981; Rooney & Lee, 1983; Kurebayashi & Ogawa, 1984). Thiol-blocking reagents cause Ca^{2+} release (Bindoli & Fleischer, 1983; Salama & Abramson, 1984) and affect the activity of the ATPase (de Meis, 1981). Ca^{2+} release (Meissner, 1984) and ATPase activity (Gould *et al.*, 1986) are affected by pH over similar ranges. Finally, typical Ca^{2+} -channel blockers such as dantrolene or nifedipine do not affect the release of Ca^{2+} from SR vesicles, although they do bind to the sarcolemma and T tubule (Volpe *et al.*, 1983; Danko *et al.*, 1985; J. M. McWhirter & A. G. Lee, unpublished work).

These many similarities between the properties of the Ca^{2+} + Mg^{2+} -activated ATPase and the putative Ca^{2+} channel strongly suggest that Ca^{2+} release from SR may, in fact, be explainable in terms solely of the ATPase, with no requirement for a separate Ca^{2+} channel. This conclusion is further strengthened by the observation of a fast Ca^{2+} efflux from reconstituted phospholipid vesicles containing the ATPase as the sole protein, with this efflux showing many of the properties reported for efflux of Ca^{2+} from SR vesicles (G. W. Gould & A. G. Lee, unpublished work).

In previous publications we have presented a complete kinetic model for the ATPase activity of the ATPase (Froud & Lee, 1986a,b; Gould *et al.*, 1986). Here we show that this model can be extended to account for fast efflux of Ca^{2+} from SR vesicles, mediated by the ATPase. We stress that here we are attempting only to interpret the Ca^{2+} release that has been observed from isolated SR vesicles: the relevance of this release to the Ca^{2+} release in muscle leading to contraction must remain uncertain at the present time.

RESULTS AND DISCUSSION

It has been shown (Froud & Lee, 1986a,b; Gould *et al.*, 1986) that the activity of the Ca^{2+} + Mg^{2+} -activated ATPase can be fitted to a reaction scheme very similar to that suggested by de Meis & Vianna (1979). The ATPase is proposed to exist in one of two conformations E1 or E2. In the E1 conformation the two Ca^{2+} -binding sites per ATPase monomer are of high affinity and are exposed to the outer (cytoplasmic) side of the SR, whereas in the E2 conformation the two Ca^{2+} -binding sites are of low affinity and face inwards into the sarcoplasmic reticulum. The E1 conformation has a high-affinity binding site for MgATP , and the binding of MgATP and Ca^{2+} to E1 leads to phosphorylation of the ATPase. Following phosphorylation, the ATPase undergoes a conformation change to the E2 form in which the Ca^{2+} -binding sites become of low affinity and exposed to the inside of the SR. Ca^{2+} is then lost from these sites and, after hydrolytic cleavage of the phosphorylated intermediate, the enzyme recycles.

If SR vesicles are passively loaded with $^{45}\text{Ca}^{2+}$ and are then diluted into media containing no labelled Ca^{2+} there is a gradual loss of $^{45}\text{Ca}^{2+}$ from the SR vesicles (Meissner, 1984). The rate of loss of Ca^{2+} depends on the ionic composition of the external medium. Thus efflux is inhibited by Mg^{2+} (Fig. 1; Meissner, 1984), but is stimulated by increasing concentrations of Ca^{2+} up to about $10\ \mu\text{M}$ and is inhibited by higher concentrations (see

Fig. 1. Ca^{2+} efflux from SR vesicles

The experimental data from Meissner (1984) show the Ca^{2+} content (nmol/mg of protein) of SR vesicles loaded with $^{45}\text{Ca}^{2+}$ as a function of time after dilution into release media containing unlabelled Ca^{2+} (20 nM) and either 5 mM- Mg^{2+} (O) or zero Mg^{2+} (Δ), at pH 6.8. The initial Ca^{2+} content was 80 nmol/mg of protein, of which 8 nmol/mg was non-releasable (Meissner, 1984). The curves are simulations with the parameters in Table 2 assuming an initial Ca^{2+} load of 72 nmol/mg of protein and adding 8 nmol/mg of protein to the calculated Ca^{2+} load to account for the non-releasable fraction.

Fig. 2). The stimulation of release by low concentrations of Ca^{2+} occurs over a concentration range similar to that observed to cause stimulation of ATPase activity, and inhibition of release occurs at concentrations comparable with those that cause inhibition of ATPase activity. Stimulation of ATPase activity by low concentrations of Ca^{2+} has been postulated to result from binding to the Ca^{2+} -binding sites of high affinity on E1, whereas inhibition by high concentrations of Ca^{2+} has been attributed to binding of Ca^{2+} to the sites of low affinity present on the E2 conformation of the ATPase (Gould *et al.*, 1986). If these same Ca^{2+} -binding sites on the ATPase are to be involved in Ca^{2+} release, then it is necessary to postulate a new conformational state for the ATPase in which the Ca^{2+} -binding sites are both of low affinity and outward-facing: only in terms of such a conformation is it possible to explain Ca^{2+} release from the SR into a medium where the Ca^{2+} concentration is millimolar. The proposed scheme is shown in Scheme 1. In the presence of micromolar Ca^{2+} , both Ca^{2+} -binding sites on E1 will be occupied by Ca^{2+} to give $\text{E1}(\text{Ca}_2)$. It is proposed that $\text{E1}(\text{Ca}_2)$ can undergo a slow conformation change to

Fig. 2. Effect of external Ca^{2+} concentration on the rate of Ca^{2+} efflux from SR vesicles

The experimental data from Meissner *et al.* (1986) show the fractional rate of Ca^{2+} release from SR vesicles as a function of the pCa of the external medium at pH 7.0 with Mg^{2+} at 1 nM, expressed relative to the value observed at pCa = 5. The curve is a simulation with the parameters in Table 2 assuming an initial Ca^{2+} load of 120 nmol/mg of protein.

a new conformational state $\text{E3}'\text{Ca}_2$, in which the Ca^{2+} -binding sites, although still outward-facing, are of low affinity. Following loss of Ca^{2+} from $\text{E3}'\text{Ca}_2$, E3 can undergo a conformational change to E4 , in which the Ca^{2+} -binding sites are still of low affinity but are now inward-facing. Ca^{2+} on the inside of the SR will bind to E4 to give $\text{E4}'\text{Ca}_2$, which undergoes a conformational change back to $\text{E3}'\text{Ca}_2$. The rate of transition between $\text{E2}'\text{Ca}_2$ and $\text{E4}'\text{Ca}_2$ is proposed to be negligible. The Ca^{2+} -release pathway thus involves a slow conformational change to a conformation $\text{E3}'\text{Ca}_2$ of low Ca^{2+} affinity, with cycling between $\text{E4}'\text{Ca}_2$ and $\text{E3}'\text{Ca}_2$ being responsible for movement of Ca^{2+} from inside to outside the SR. Importantly, it is shown below that such a scheme will fit the experimental data assuming that the Ca^{2+} affinities of the forms E3 and E4 are equal to that of E2 . The proposed slow conformational change for the ATPase is somewhat analogous to the desensitization of the acetylcholine receptor (Anholt *et al.*, 1985).

Ca^{2+} leak has been shown to be inhibited by binding of Mg^{2+} and stimulated by the binding of ATP and other nucleotides (Meissner, 1984; Meissner *et al.*, 1986). As

shown below, these effects can be simulated assuming binding of Mg^{2+} and ATP to E3 and E4 , with this binding affecting the rates of the transitions between the E3 and E4 conformations (Scheme 1).

As described previously, binding of Ca^{2+} to the E1 conformation of the ATPase is consistent with sequential binding of the type $\text{E1}-\text{E1Ca}-\text{E1}'\text{Ca}-\text{E1}'\text{Ca}_2$, where the $\text{E1Ca}-\text{E1}'\text{Ca}$ transition is relatively slow and is accelerated by the binding of MgATP (Froud & Lee, 1986a). A very similar scheme was suggested for binding of Ca^{2+} to E2 (Gould *et al.*, 1986).

As shown in Scheme 1, Ca^{2+} efflux was simulated assuming that transitions are only possible between E1 and E2 , between $\text{E1}'\text{Ca}_2$ and $\text{E2}'\text{Ca}_2$, between $\text{E3}'\text{Ca}_2$ and $\text{E4}'\text{Ca}_2$ and between E3 and E4 , and not between the intermediate forms containing a single bound Ca^{2+} . Although it seems more likely that transitions between these intermediate forms will also occur, the introduction of such transitions results in a considerable increase in the time required for computation, and sufficient data are not available to fix accurately the rates of such transitions. Further, simulations suggest that their introduction would have only minor effects, since the most important transitions for efflux are those between $\text{E4}'\text{Ca}_2$ and $\text{E3}'\text{Ca}_2$ and between E4 and E3 , and, as described below, the rates for Ca^{2+} dissociation from $\text{E3}'\text{Ca}_2$ and $\text{E4}'\text{Ca}_2$ have been put fast.

The equilibrium between the E1 and E2 conformations of the ATPase in the absence of Ca^{2+} is known to be pH-sensitive, as are the rates of the transitions between the two conformations, and it has been shown that these data can be fitted quantitatively assuming that the rates are affected by protonation, with the rate of the conformational transition being greatest for the non-protonated form (Froud & Lee, 1986a).

Simulations of Ca^{2+} efflux were carried out by using the FACSIMILE program (Chance *et al.*, 1977) running on an ICL 2976 computer.

In fitting Scheme 1 to the experimental data on the rates of Ca^{2+} efflux, an important parameter is the internal Ca^{2+} concentration. This, however, is not generally known, because much of the Ca^{2+} in the SR will be bound to soluble Ca^{2+} -binding proteins within the SR. The major Ca^{2+} -binding proteins of SR, apart from the ATPase itself, are a high-affinity binding protein,

Scheme 1. Proposed scheme for the Ca^{2+} -release pathway

The scheme for Ca^{2+} binding to E1 is given in Froud & Lee (1986a), and that for Ca^{2+} binding to E3 and E4 is the same as to E2 given in Gould *et al.* (1986).

Table 1. Ca^{2+} -binding characteristics of proteins in heavy SR

Ca^{2+} -binding capacities and dissociation constants for Ca^{2+} binding at pH 7.5 and 0.1 M-KCl are taken from Ostwald & MacLennan (1974) and the protein content of heavy SR from McWhirter *et al.* (1987).

Protein component	Dissociation constant, K_d (μM)	Ca^{2+} -binding capacity (nmol/mg of protein)	Protein content* (% of total)	Ca^{2+} -binding capacity (nmol/mg of SR protein)
High-affinity Ca^{2+} -binding protein	3	18	4	0.7
Calsequestrin	5000	460		18
Acidic protein (> 30000 Da)	800	850	24	204
	1400	1000	~ 1.5	15
	55	45		0.7
Acidic protein (< 30000 Da)	780	580	~ 1.5	9.0
	26	23		0.3

* ATPase content 69%.

calsequestrin and two acidic proteins of molecular masses greater and less than 30000 Da (Ostwald & MacLennan, 1974; Ikemoto, 1975; Miyamoto & Kasai, 1979). The relative proportions of these various proteins within a sample of SR vesicles depend on the exact method of SR preparation. The vesicle population of an SR preparation can be analysed by fractionation on sucrose gradients to give heavy, light and intermediate SR fractions. In the heavy SR fraction about 25% and 70% of the total protein are calsequestrin and ATPase respectively, whereas in light SR the ATPase content is 90% with little calsequestrin being present (Meissner, 1975; McWhirter *et al.*, 1987). Meissner (1984) found the most rapid Ca^{2+} efflux from an SR fraction corresponding to the heavy SR fraction, with little release from SR fractions corresponding to light SR fractions.

One approach to the problem of calculating the internal free Ca^{2+} concentration is to use the Ca^{2+} -binding parameters for the purified protein components determined by Ostwald & MacLennan (1974) at pH 7.5 and 0.1 M-KCl. By using the protein composition of heavy SR reported in McWhirter *et al.* (1987) it is then possible to calculate the expected Ca^{2+} -binding capacity of these sites within the SR (Table 1). An internal volume of 5 μl /mg of protein was assumed (Weber *et al.*, 1963; Duggan & Martonosi, 1970; Miyamoto & Kasai, 1976; Malan *et al.*, 1975; Campbell *et al.*, 1980). This procedure predicts a large maximal internal Ca^{2+} -binding capacity (Table 1) of approx. 200 nmol/mg of protein. However, the exact binding parameters are very dependent on pH, ionic strength and Mg^{2+} concentration (MacLennan & Wong, 1971; Ostwald & MacLennan, 1974), so that the calculation of binding-site concentration is very uncertain. A number of direct measurements of Ca^{2+} binding to SR vesicles have been reported, and estimates of internal Ca^{2+} -binding capacity have been made by relating bound Ca^{2+} to free external Ca^{2+} concentrations (Meissner, 1975; Prager *et al.*, 1979; Miyamoto & Kasai, 1979; Hasselbach & Koenig, 1980; Feher & Briggs, 1982; Gerdes *et al.*, 1983; Sorenson, 1983). Unfortunately, these estimates of maximal internal Ca^{2+} binding capacity differ very considerably. Thus Meissner (1975) and Miyamoto & Kasai (1979) have estimated maximal Ca^{2+} -binding capacities for heavy SR close to the value of 200 nmol/mg calculated in Table 1. Hasselbach & Koenig (1980) have reported a

maximal Ca^{2+} -binding capacity of 100 nmol/mg, as has Meissner (1975) for light SR. A number of other reports, however, suggest a much lower maximal Ca^{2+} -binding capacity of the order of 30 nmol/mg of protein (Prager *et al.*, 1979; Feher & Briggs, 1982; Gerdes *et al.*, 1983; Sorenson, 1983). The reasons for these very different estimates of maximal binding capacity are unclear. However, one possible complication is that Miyamoto & Kasai (1979) reported large changes in the internal volume of SR when it was incubated with solutions containing high concentrations of Ca^{2+} . Further, the final extent of Ca^{2+} loading achieved by passive loading is highly variable. Thus incubation of SR vesicles with 5 mM external Ca^{2+} has been reported to produce Ca^{2+} loads of 10 (Sorenson, 1983), 20 (Gerdes *et al.*, 1983), 30 (Prager *et al.*, 1979), 50 (Waas & Hasselbach, 1981), 60 (Meissner, 1984) and 80–120 nmol/mg of protein (Meissner *et al.* 1986) under apparently similar conditions.

As is shown below, Ca^{2+} release from heavy SR fractions can be simulated assuming a range of possible values for the extent of maximal internal Ca^{2+} binding. The calculations illustrated here were performed assuming a single internal Ca^{2+} -binding site of affinity 1.16 mM and a maximal capacity of 24 nmol/mg, as determined by Gerdes *et al.* (1983). Equally good simulations (not shown) were, however, also obtained by using the binding parameters given in Table 1. In this latter case, for a given total Ca^{2+} content, the distribution of Ca^{2+} between its various possible binding sites was calculated numerically by using a programme modified from that given by O'Sullivan & Smithers (1979).

Although Feher & Briggs (1982) have reported an exponential time course for Ca^{2+} efflux from SR vesicles, those reported by Kasai *et al.* (1981), Meissner (1984) and Smith *et al.* (1985) are markedly non-exponential (see Fig. 1). Sorenson (1983) also observed a non-exponential time course for Ca^{2+} efflux, and attributed this to the presence of a mixed population of SR vesicles, with some showing fast efflux and some slow efflux. However, the scheme presented here for Ca^{2+} efflux (Scheme 1) with a slow conformational change to the E3'Ca_2 form leads directly to a non-exponential rate of efflux (Fig. 1). The experimentally determined time course of efflux can be simulated reasonably well with

Table 2. Kinetic parameters for Ca^{2+} leak mediated by the ATPase calculated for a maximal Ca^{2+} load of 34 nmol/mg of protein

Parameters were obtained by simulation as described in the text. Parameters describing binding of Ca^{2+} and H^+ to E1 and E2 are as given in Froud & Lee (1986a,b) and Gould *et al.* (1986), and to E3 and E4 are assumed to be equal to those for E2 (Gould *et al.*, 1986). Parameters describing binding of Mg^{2+} and MgATP to E1 and E2 are as given in Stefanova *et al.* (1987). All unassigned rate constants are assumed to be fast.

Reaction	Equilibrium symbol	Value of constant	Forward rate constant (s^{-1})
$\text{E2}'\text{Ca}_2 \rightleftharpoons \text{E1}'\text{Ca}_2^*$		†	0.008
$\text{E2}'\text{H}\text{Ca}_2 \rightleftharpoons \text{E1}'\text{H}\text{Ca}_2^*$		†	0.008
$\text{E2}'\text{H}_2\text{Ca}_2 \rightleftharpoons \text{E1}'\text{H}_2\text{Ca}_2^*$		†	0.008
$\text{E1}'\text{Ca}_2 \rightleftharpoons \text{E3}'\text{Ca}_2^{\dagger\dagger}$	K_{26}	8.3×10^{-4}	0.5
$\text{E1}'\text{Ca}_2 + \text{ATP} \rightleftharpoons \text{E1}'\text{ATPCa}_2$	K_{27}	1.7×10^4	3×10^5
$\text{E3}'\text{Ca}_2 + \text{ATP} \rightleftharpoons \text{E3}'\text{ATPCa}_2^{\S}$	K_{28}	5×10^2	1×10^7
$\text{E4}'\text{Ca}_2 + \text{ATP} \rightleftharpoons \text{E4}'\text{ATPCa}_2^{\S}$	K_{29}	2×10^3	1×10^7
$\text{E3}' + \text{ATP} \rightleftharpoons \text{E3}'\text{ATP}^{\S}$	K_{28}	5×10^2	
$\text{E4}' + \text{ATP} \rightleftharpoons \text{E4}'\text{ATP}^{\S}$	K_{29}	2×10^3	
$\text{E1}'\text{Ca}_2 + \text{Mg} \rightleftharpoons \text{E1}'\text{MgCa}_2^{\parallel}$	K_{16}	110.0	
$\text{E2}'\text{Ca}_2 + \text{Mg} \rightleftharpoons \text{E2}'\text{MgCa}_2^{\parallel}$	K_{16}	110.0	
$\text{E3}'\text{Ca}_2 + \text{Mg} \rightleftharpoons \text{E3}'\text{MgCa}_2^{\parallel}$	K_{30}	110.0	
$\text{E4}'\text{Ca}_2 + \text{Mg} \rightleftharpoons \text{E4}'\text{MgCa}_2^{\parallel}$	K_{31}	1.3×10^5	
$\text{E1}'\text{ATPCa}_2 \rightleftharpoons \text{E3}'\text{ATPCa}_2^{\ddagger}$		**	10
$\text{E4} \rightleftharpoons \text{E3}$	K_{32}	100.0	$\ddagger\ddagger$
$\text{E4}'\text{Ca}_2 \rightleftharpoons \text{E3}'\text{Ca}_2^*$	K_{32}	100.0	2.3×10^2
$\text{E4}'\text{H}\text{Ca}_2 \rightleftharpoons \text{E3}'\text{H}\text{Ca}_2^*$	K_{32}	100.0	2.3×10^2
$\text{E4}'\text{H}_2\text{Ca}_2 \rightleftharpoons \text{E3}'\text{H}_2\text{Ca}_2^*$	K_{32}	100.0	2.3×10^2
$\text{E4}'\text{ATPCa}_2 \rightleftharpoons \text{E3}'\text{ATPCa}_2$		$\ddagger\ddagger$	2.3×10^5
$\text{E4}'\text{ATPHCa}_2 \rightleftharpoons \text{E3}'\text{ATPHCa}_2$		$\ddagger\ddagger$	2.3×10^5
$\text{E4}'\text{ATPH}_2\text{Ca}_2 \rightleftharpoons \text{E3}'\text{ATPH}_2\text{Ca}_2$		$\ddagger\ddagger$	2.3×10^5
$\text{E3}'\text{Ca}_2 + [\beta\gamma\text{-CH}_2]\text{ATP} \rightleftharpoons \text{E3}'[\beta\gamma\text{-CH}_2]\text{ATPCa}_2$	K_{33}	3.8×10^2	1×10^7
$\text{E4}'\text{Ca}_2 + [\beta\gamma\text{-CH}_2]\text{ATP} \rightleftharpoons \text{E4}'[\beta\gamma\text{-CH}_2]\text{ATPCa}_2$	K_{34}	1.5×10^3	1×10^7
$\text{E3}' + [\beta\gamma\text{-CH}_2]\text{ATP} \rightleftharpoons \text{E3}'[\beta\gamma\text{-CH}_2]\text{ATP}$	K_{33}	3.8×10^2	
$\text{E4}' + [\beta\gamma\text{-CH}_2]\text{ATP} \rightleftharpoons \text{E4}'[\beta\gamma\text{-CH}_2]\text{ATP}$	K_{34}	1.5×10^3	
$\text{E1}'\text{Ca}_2 + [\beta\gamma\text{-CH}_2]\text{ATP} \rightleftharpoons \text{E1}'[\beta\gamma\text{-CH}_2]\text{ATPCa}_2$	K_{35}	1.7×10^4	3×10^5
$\text{E2}'\text{Ca}_2 + \text{ATP} \rightleftharpoons \text{E2}'\text{ATPCa}_2$	K_{36}	10.0	
$\text{E2}' + \text{ATP} \rightleftharpoons \text{E2}'\text{ATP}$	K_{36}	10.0	
$\text{E2}'\text{ATPCa}_2 \rightleftharpoons \text{E1}'\text{ATPCa}_2$		$\S\S$	39
$\text{E2}'\text{ATPHCa}_2 \rightleftharpoons \text{E1}'\text{ATPHCa}_2$		$\S\S$	39
$\text{E2}'\text{ATPH}_2\text{Ca}_2 \rightleftharpoons \text{E1}'\text{ATPH}_2\text{Ca}_2$		$\S\S$	39

* Rate zero for Mg- and MgATP-bound forms.

† Defined by Ca^{2+} -binding parameters given in Froud & Lee (1986a) and Gould *et al.* (1986).

‡ Independent of state of protonation.

§ Same binding parameters assumed for MgATP.

|| Parameters independent of state of protonation and of Ca^{2+} binding.

¶ Same parameters for MgATP-bound form, but rate zero for Mg-bound form.

** Defined by K_{26} , K_{27} and K_{28} , with same rate for $[\beta\gamma\text{-methylene}]\text{ATP}$ bound form.

†† Assumed fast for all forms.

‡‡ Defined by K_{28} , K_{29} and K_{32} , with the same rate for $[\beta\gamma\text{-methylene}]\text{ATP}$ bound form.

§§ Defined by Ca^{2+} -binding parameters given in Froud & Lee (1986a) and Gould *et al.* (1986), K_{27} and K_{36} .

the parameters given in Table 2. Where simulations give non-exponential efflux rates, as illustrated in Fig. 1, rate constants for efflux were estimated by ignoring the first 10% of release beyond which point efflux was generally close to exponential.

Fig. 3 illustrates the changes with time of the relative proportions of the various forms of the ATPase during release, for the experiment shown in Fig. 1 where the external Ca^{2+} and Mg^{2+} concentrations are 20 nM and 1 nM respectively. It is clear that under the conditions of this experiment the ATPase is present predominantly as E2Ca and $\text{E2}'\text{Ca}$. The proportion of E2Ca_2 falls rapidly as that of E2 increases, and a small amount of E3 is present throughout. The relative proportions of all other forms are very low.

By using the Ca^{2+} -binding parameters for E1 and E2

given in Froud & Lee (1986a) and Gould *et al.* (1986) and assuming that the same parameters can be used to describe binding of protons and Ca^{2+} to E3 and E4 as were used to describe binding to E2, it is possible to simulate the dependence of Ca^{2+} -efflux rate on external Ca^{2+} concentration reported by Meissner *et al.* (1986) (Fig. 2). The rate of Ca^{2+} efflux is particularly sensitive to the rates of the $\text{E2}'\text{Ca}_2$ - $\text{E1}'\text{Ca}_2$ and $\text{E4}'\text{Ca}_2$ - $\text{E3}'\text{Ca}_2$ transitions. It is possible to fit the Ca^{2+} -release data equally well assuming different maximal extents of Ca^{2+} binding within the SR (and thus different internal free concentrations of Ca^{2+} at any given extent of Ca^{2+} loading) by changing the rates of these two steps with only minor changes in the other parameters (Table 3). The close agreement between the experimental data and the simulations based on Ca^{2+} -binding data determined

Fig. 3. Changes with time of the intermediates of the Ca^{2+} -release process

Shown are the results of simulations of the relative proportions of the ATPase present in its various forms under the conditions of the release experiment illustrated in Fig. 1 where external Ca^{2+} and Mg^{2+} concentrations are 20 nM and 1 nM respectively: $\text{E2Ca} + \text{E2}'\text{Ca}$ (curve A); E2 (curve B); E1 (curve C); $\text{E2}'\text{Ca}_2$ (curve D); E3 (curve E); all other intermediates are present in very small amounts.

previously from kinetic studies of the ATPase (Gould *et al.* 1986) provides strong support that the ATPase and the ' Ca^{2+} channel' are one and the same protein.

Meissner *et al.* (1986) have shown that the release of Ca^{2+} is markedly inhibited by Mg^{2+} (Fig. 4 and Table 4).

Fig. 4. Effect of Mg^{2+} on Ca^{2+} efflux from SR vesicles

The experimental data from Meissner *et al.* (1986) show the fractional rate of Ca^{2+} release from SR vesicles as a function of the Mg^{2+} concentration in the external medium at pH 7.0 with an external Ca^{2+} concentration of 1 nM, expressed relative to the value observed with zero external Mg^{2+} . The curve is a simulation with the parameters in Table 2 assuming an initial Ca^{2+} load of 120 nmol/mg of protein.

From an analysis of the effect of Mg^{2+} on phosphorylation of the ATPase by P_i , it was suggested that Mg^{2+} bound equally to the E1 and E2 conformations of the ATPase, with an association constant of 110.0 (Froud & Lee, 1986b). Inhibition of Ca^{2+} release by Mg^{2+} can be simulated assuming that the affinity of Mg^{2+} for E3 is the same as for E1 and E2 but that its affinity for E4 is stronger (Table 2 and Fig. 4) and that the rates of the transitions between the E3 and E4, the $\text{E2}'\text{Ca}_2$ and

Table 3. Kinetic parameters for Ca^{2+} leak mediated by the ATPase calculated for the Ca^{2+} -binding parameters in Table 1

Parameters were obtained by simulation as described in the text. Values for all parameters not listed were assigned the values given in Table 2.

Reaction	Equilibrium symbol	Value of constant	Forward rate constant (s^{-1})
$\text{E2}'\text{Ca}_2 \rightleftharpoons \text{E1}'\text{Ca}_2$			0.31
$\text{E2}'\text{H}_2\text{Ca}_2 \rightleftharpoons \text{E1}'\text{H}_2\text{Ca}_2$			0.31
$\text{E2}'\text{H}_2\text{Ca}_2 \rightleftharpoons \text{E1}'\text{H}_2\text{Ca}_2$			0.31
$\text{E3}'\text{Ca}_2 + \text{ATP} \rightleftharpoons \text{E3}'\text{ATPCa}_2$	K_{28}	5×10^2	1×10^7
$\text{E4}'\text{Ca}_2 + \text{ATP} \rightleftharpoons \text{E4}'\text{ATPCa}_2$	K_{29}	1×10^5	1×10^7
$\text{E3}' + \text{ATP} \rightleftharpoons \text{E3}'\text{ATP}$	K_{28}	5×10^2	
$\text{E4}' + \text{ATP} \rightleftharpoons \text{E4}'\text{ATP}$	K_{29}	1×10^5	
$\text{E3}'\text{Ca}_2 + \text{Mg} \rightleftharpoons \text{E3}'\text{MgCa}_2$	K_{30}	5×10^4	
$\text{E4}'\text{Ca}_2 + \text{Mg} \rightleftharpoons \text{E4}'\text{MgCa}_2$	K_{31}	6×10^6	
$\text{E1}'\text{ATPCa}_2 \rightleftharpoons \text{E3}'\text{ATPCa}_2$			2.5
$\text{E4}'\text{Ca}_2 \rightleftharpoons \text{E3}'\text{Ca}_2$			3.9×10^4
$\text{E4}'\text{H}_2\text{Ca}_2 \rightleftharpoons \text{E3}'\text{H}_2\text{Ca}_2$			3.9×10^4
$\text{E4}'\text{H}_2\text{Ca}_2 \rightleftharpoons \text{E3}'\text{H}_2\text{Ca}_2$			3.9×10^4
$\text{E4}'\text{ATPCa}_2 \rightleftharpoons \text{E3}'\text{ATPCa}_2$			7.8×10^6
$\text{E4}'\text{ATPHCa}_2 \rightleftharpoons \text{E3}'\text{ATPHCa}_2$			7.8×10^6
$\text{E4}'\text{ATPH}_2\text{Ca}_2 \rightleftharpoons \text{E3}'\text{ATPH}_2\text{Ca}_2$			7.8×10^6
$\text{E3}'\text{Ca}_2 + [\beta\gamma\text{-CH}_2]\text{ATP} \rightleftharpoons \text{E3}'[\beta\gamma\text{-CH}_2]\text{ATPCa}^{2+}$	K_{33}	4×10^2	1×10^7
$\text{E4}'\text{Ca}_2 + [\beta\gamma\text{-CH}_2]\text{ATP} \rightleftharpoons \text{E4}'[\beta\gamma\text{-CH}_2]\text{ATPCa}^{2+}$	K_{34}	8×10^4	1×10^7
$\text{E3}' + [\beta\gamma\text{-CH}_2]\text{ATP} \rightleftharpoons \text{E3}'[\beta\gamma\text{-CH}_2]\text{ATP}$	K_{23}	4×10^2	
$\text{E4}' + [\beta\gamma\text{-CH}_2]\text{ATP} \rightleftharpoons \text{E4}'[\beta\gamma\text{-CH}_2]\text{ATP}$	K_{34}	8×10^4	
$\text{E2}'\text{ATPCa}_2 \rightleftharpoons \text{E1}'\text{ATPCa}_2$			3.1×10^2
$\text{E2}'\text{ATPHCa}_2 \rightleftharpoons \text{E1}'\text{ATPHCa}_2$			3.1×10^2
$\text{E2}'\text{ATPH}_2\text{Ca}_2 \rightleftharpoons \text{E1}'\text{ATPH}_2\text{Ca}_2$			3.1×10^2

Table 4. Ca^{2+} -efflux properties of SR vesicles

The experimental data from Meissner *et al.* (1986) describe Ca^{2+} efflux from SR vesicles, loaded to 120 nmol/mg of protein, following dilution into media at pH 7.0. In experiments involving [$\beta\gamma$ -methylene]ATP, external Ca^{2+} concentrations were lowered to the given values in an initial step before the mixing with [$\beta\gamma$ -methylene]ATP. Calculated values were obtained from Scheme 1 by using the parameters listed in Tables 1 and 2.

Concn. in release medium (M)			Ca^{2+} -efflux rate (s^{-1})	
Ca^{2+}	Mg^{2+}	[$\beta\gamma\text{-CH}_2$]ATP	Experimental	Calculated
10^{-8}	5×10^{-3}		0.004	0.002
10^{-8}			0.1	0.2
10^{-8}		5×10^{-3}	24	17
10^{-8}	8×10^{-3}	5×10^{-3}	0.04	0.06
4×10^{-6}		5×10^{-3}	0.004	0.02
4×10^{-6}			1.5	1.7
4×10^{-6}	8×10^{-3}	5×10^{-3}	90 (or 56)	41
4×10^{-6}		5×10^{-3}	1.1	2.0
5×10^{-3}		5×10^{-3}	0.01	0.002

Fig. 5. Effect of intracellular Ca^{2+} concentration on the rate of Ca^{2+} efflux from SR vesicles

The experimental data from Meissner (1984) show the relative rate of Ca^{2+} efflux from SR vesicles as a function of the Ca^{2+} load following dilution into a medium of pH 6.8 with an external Ca^{2+} concentration of $10 \mu\text{M}$ and an external Mg^{2+} concentration of zero (O) or 0.6 mM (□). Ca^{2+} -efflux rates were measured as nmol of Ca^{2+} released/s per mg of protein and are expressed as a fraction of the rate observed following dilution of SR loaded with 160 nmol/mg of protein into medium containing zero Mg^{2+} . The curves are simulations with the parameters in Table 2.

Fig. 6. Effect of pH on Ca^{2+} efflux from SR vesicles

The experimental data from Meissner (1984) show the relative rate of Ca^{2+} efflux from SR vesicles as a function of pH, following dilution into a medium with an external Ca^{2+} concentration of $20 \mu\text{M}$ and an external Mg^{2+} concentration of zero (O) or 0.6 mM (□). Ca^{2+} -efflux rates were measured as nmol of Ca^{2+} released/s per mg of protein and are expressed as a fraction of the rate observed at pH 7.2 following dilution into a medium containing zero Mg^{2+} . The curves are simulations with the parameters given in Table 2 and the Ca^{2+} loads determined by Meissner (1984).

$\text{E1}'\text{Ca}_2$ and the E2 and E1 conformations are zero for the Mg^{2+} -bound forms, assuming a maximal internal binding capacity of 34 nmol/mg (Table 2). For a maximal binding capacity of 200 nmol/mg, the rates of the $\text{E2}'\text{Ca}_2\text{-E1}'\text{Ca}_2$ transitions have to be greater to give the same Ca^{2+} -efflux rates, and the binding constants for Mg^{2+} have to be correspondingly greater to give the same degree of inhibition, with Mg^{2+} now binding more strongly to both E3 and E4 than to E2 and E1 (Table 3). In Stefanova *et al.* (1987) it is shown that an effect of Mg^{2+} on the E2-E1 transition is also consistent with studies on the effect of Mg^{2+} on the rate of the ATPase.

The inhibition caused by Mg^{2+} has been reported to depend on the external Ca^{2+} concentration (Meissner *et al.*, 1986). As shown in Table 4, the experimental data are reproduced reasonably well by Scheme 1 with the parameters listed in Table 2, although agreement is not

so good at high Ca^{2+} and Mg^{2+} concentrations, where the experimentally measured efflux rates are very low.

The dependence of Ca^{2+} -efflux rate on the internal Ca^{2+} concentration has been reported by Meissner (1984). SR vesicles were incubated at external Ca^{2+} concentrations of 2–63 mM, to give total Ca^{2+} loads of between 40 and 161 nmol/mg. For a Ca^{2+} -binding capacity of 34 nmol/mg these Ca^{2+} loads correspond to internal Ca^{2+} concentrations of between 3 and 25.7 mM respectively, whereas for the Ca^{2+} -binding capacities given in Table 1 they correspond to internal Ca^{2+} concentrations of between 0.15 and 1.24 mM respectively. As shown in Fig. 5, the calculated dependence of Ca^{2+} -release rates on total Ca^{2+} load are in reasonable agreement with the experimental data. Meissner (1984) has also reported the pH-dependence of Ca^{2+} efflux. The scheme for Ca^{2+} binding (Gould *et al.*, 1986a) predicts that Ca^{2+} binding to the ATPase will decrease with

Fig. 7. Effect of nucleotides on Ca^{2+} efflux from SR vesicles

The experimental data from Meissner *et al.* (1986) show the rate of Ca^{2+} efflux from SR vesicles as a function of the concentration of ATP ($\circ$) or $[\beta\gamma\text{-methylene}]\text{ATP}$ ($\triangle$), following dilution into a medium containing zero Ca^{2+} and Mg^{2+} , pH 7.0. The curves are simulations with the parameters in Table 2, assuming an initial Ca^{2+} load of 120 nmol/mg of protein.

Table 5. Effect of Mg^{2+} on Ca^{2+} efflux from SR vesicles

The experimental data from Meissner *et al.* (1986) describe Ca^{2+} efflux from SR vesicles, loaded to 120 nmol/mg of protein, following dilution into media at pH 7.0 containing $4\ \mu\text{M}\text{-Ca}^{2+}$ and $5\ \text{mM}\text{-}[\beta\gamma\text{-methylene}]\text{ATP}$ and the given concentrations of Mg^{2+} . Calculated values were obtained from Scheme 1 by using the parameters listed in Tables 1 and 2.

Concn. of Mg^{2+} in release medium (M)	Ca^{2+} -efflux rate (s^{-1})	
	Experimental	Calculated
10^{-9}	56	41
3.04×10^{-3}	30	26
3.99×10^{-3}	19	17
4.99×10^{-3}	14	9
7×10^{-3}	1.8	2.7
9×10^{-3}	1.3	1.5

decreasing pH, and simulations show that the decreased binding is sufficient to explain the decrease in Ca^{2+} -efflux rate with decreasing pH, as illustrated in Fig. 6, without any effect of pH on the rates of the $\text{E2}'\text{Ca}_2\text{-E1}'\text{Ca}_2$ and $\text{E4}'\text{Ca}_2\text{-E3}'\text{Ca}_2$ transitions. The lower rate of Ca^{2+} efflux at pH 7.6 than at pH 7.2 shown in Fig. 6 is a result of the lower extent of Ca^{2+} loading achieved at pH 7.6 than at pH 7.2.

Finally, the rate of Ca^{2+} efflux has been shown to be

stimulated markedly by the binding of ATP and other nucleotides in the absence of Mg^{2+} (Meissner 1984; Meissner *et al.*, 1986). Although the appropriate binding constants for ATP are not well defined, reasonable simulations to the experimental data (Fig. 7 and Tables 4 and 5) can be obtained using the parameters given in Table 2. There is a considerable variation in the reported binding constants of MgATP to the ATPase. Values of between 2 and $8\ \mu\text{M}$ have been reported from direct binding studies (Meissner, 1973; Dupont, 1977; Dupont *et al.*, 1982; Møller *et al.*, 1980; Nakamura & Tonomura, 1982; Andersen *et al.*, 1982; Andersen & Møller, 1985), and from indirect measurements Pickart & Jencks (1984) estimated a binding constant of $20\ \mu\text{M}$. The best fit to the ATPase data was obtained by assuming that the dissociation constant for MgATP depended on Cl^- concentration, with a value of $10\ \mu\text{M}$ at $100\ \text{mM}\text{-Cl}^-$ (Gould *et al.*, 1986; Stefanova *et al.*, 1987). Møller *et al.* (1980) report a dissociation constant of $15\ \mu\text{M}$ for ATP compared with a value of $2\text{--}3\ \mu\text{M}$ for MgATP . A dissociation constant of $60\ \mu\text{M}$ for ATP was therefore assumed here (Table 2). Fortunately, the calculated efflux rates are insensitive to the values assumed for the binding of ATP to E1 and $\text{E1}'\text{Ca}_2$. Calculated efflux rates in the absence of external Ca^{2+} (Table 4) are, however, more sensitive to the binding constant assumed for ATP to $\text{E2}'\text{Ca}_2$: since this is unknown, it was assigned a low value (Table 2), consistent with data for ATPase activity (Stefanova *et al.*, 1987). It has been shown that the effects caused by ATP and other nucleotides are diminished in the presence of Mg^{2+} (Meissner *et al.*, 1986; see Table 5). In the presence of Mg^{2+} and ATP, both ATP and MgATP will bind to the ATPase. Sufficient data are not yet available for a full analysis, and so, for simplicity, equal binding constants have been assumed for MgATP and ATP to E3 and E4 . It has been suggested elsewhere (Gould *et al.*, 1986; Stefanova *et al.*, 1987) that binding of Mg^{2+} and MgATP to the ATPase are competitive. Again, for simplicity, it is assumed here that binding of ATP to the ATPase is also competitive with Mg^{2+} . With the parameters in Table 2 it is then possible to simulate the experimental data on the effect of ATP on Ca^{2+} efflux (Fig. 7). Assuming binding constants for $[\beta\gamma\text{-methylene}]\text{ATP}$ 80% of those for ATP (Table 2), it is also possible to fit the experimental data on the effect of $[\beta\gamma\text{-methylene}]\text{ATP}$ on efflux (Fig. 7) and on the effect of Mg^{2+} on efflux in the presence of $[\beta\gamma\text{-methylene}]\text{ATP}$ (Tables 4 and 5). The inhibitory effects of Mg^{2+} are best fitted by assuming that both Mg^{2+} and $\text{Mg}[\beta\gamma\text{-methylene}]\text{ATP}$ prevent the $\text{E2}'\text{Ca}_2\text{-E1}'\text{Ca}_2$ and $\text{E4}'\text{Ca}_2\text{-E3}'\text{Ca}_2$ transitions and that $[\beta\gamma\text{-methylene}]\text{ATP}$ accelerates them, and that, whereas $[\beta\gamma\text{-methylene}]\text{ATP}$ also accelerates the $\text{E1}'\text{Ca}_2\text{-E3}'\text{Ca}_2$ transition and Mg^{2+} prevents it, the rate of the transition in the presence of $\text{Mg}[\beta\gamma\text{-methylene}]\text{ATP}$ is equal to that in its absence (Table 2).

In summary, we have developed a kinetic model that is consistent with the available data on Ca^{2+} efflux from SR vesicles. The model proposes that efflux is mediated by the $\text{Ca}^{2+}\text{-Mg}^{2+}$ -activated ATPase, which is the major protein present in the SR membrane. The Ca^{2+} -efflux pathway involves the same Ca^{2+} -binding sites that are involved in Ca^{2+} uptake. In ATP-driven uptake, a concerted change in the Ca^{2+} -binding sites occurs following phosphorylation, in which the Ca^{2+} -binding

sites change from being outward-facing and of high affinity to being inward-facing and of low affinity. Ca^{2+} efflux is proposed to follow from a slow conformation change in the presence of Ca^{2+} in which the Ca^{2+} -binding sites change from high to low affinity without changing their orientation. This conformation change also results in a change in affinity for nucleotides such as ATP. The suggested conformation change is somewhat analogous to the process of desensitization observed for the acetylcholine receptor, which occurs in the presence of acetylcholine and which also leads to a change in affinity for agonists (Anholt *et al.*, 1985).

The possible control of the proposed release pathway and the possible role of the release pathway in the release of Ca^{2+} in muscle is discussed in Gould *et al.* (1987).

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