Luminal dissociation of Ca^{2+} from the phosphorylated Ca^{2+} -ATPase is sequential and gated by Mg^{2+}

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Transport of Ca^{2+} across the membrane by the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum involves the transfer of two Ca^{2+} ions from a pair of cytoplasmic sites to a pair of luminal sites, driven by phosphorylation of the ATPase. The ATPase is inhibited by Mg^{2+} at alkaline pH values. Inhibition follows from a decrease in the rate of release of Ca^{2+} from the phosphorylated ATPase. Phosphorylation-induced release of Ca^{2+} from the ATPase is biphasic at alkaline pH, which is consistent with sequential release of Ca^{2+} from the phosphorylated ATPase; the rates of both components decrease with increasing Mg con-

centration. The effect of Mg^{2+} on the slow phase of release follows from the binding of Mg^{2+} at the empty outer luminal site, vacated by the release of the first Ca^{2+} ion. The effect of Mg^{2+} on the rate of release of the first Ca^{2+} ion could follow from binding to a gating site also affecting the binding of Ca^{2+} to the cytoplasmic sites.

Key words: Ca^{2+} transport, mechanism, rapid kinetics, sarcoplasmic reticulum, skeletal muscle.

INTRODUCTION

The Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum (SR) transports two Ca^{2+} ions across the SR membrane for each ATP molecule hydrolysed [1]. The E1–E2 model for the Ca^{2+} -ATPase developed by de Meis and Vianna [1] proposed a single pair of Ca^{2+} -binding sites on the Ca^{2+} -ATPase that changed from being high-affinity and exposed to the cytoplasm in the E1 conformation to being low-affinity and exposed to the lumen of the SR in the E2 conformation. This alternating site model predicts that binding of Ca^{2+} to the Ca^{2+} -ATPase from the luminal side of the membrane (to E2) will be competitive with binding of Ca^{2+} from the cytoplasmic side of the membrane (to E1). However, a variety of kinetic and equilibrium experiments failed to observe any such effect; instead they were consistent with a model in which two pairs of sites for Ca^{2+} exist on the Ca^{2+} -ATPase, transport of Ca^{2+} by the Ca^{2+} -ATPase corresponding to transfer of Ca^{2+} from a cytoplasmic pair of sites to a luminal pair of sites, driven by phosphorylation of the ATPase by ATP [2–4].

Binding of Ca^{2+} to the pair of cytoplasmic sites shows positive co-operativity and, when the rate of dissociation of $^{45}\text{Ca}^{2+}$ from the Ca^{2+} -bound ATPase was measured, it was found that the rate of dissociation of half of the bound $^{45}\text{Ca}^{2+}$ was markedly decreased in the presence of a high concentration of $^{40}\text{Ca}^{2+}$ in the medium [5–8]. These observations are consistent with the two Ca^{2+} ions binding one above the other in a channel-like structure. Ca^{2+} binds to and dissociates from these sites at a relatively low rate, indicating a conformation change associated with the Ca^{2+} -binding process [6–11]. Binding of Mg^{2+} , K^{+} or a variety of polycations to the ATPase increases the rate of dissociation of Ca^{2+} from the ATPase [8–10,12], suggesting that these ions bind at a ‘gating site’ on the ATPase that controls access to the Ca^{2+} -binding sites [8].

The kinetics of release of the two bound Ca^{2+} ions after transfer to the pair of luminal sites will depend on how the two sites are organized; if the sites are organized as an extension of

the channel, release of the two Ca^{2+} ions could be independent, but if the two sites are organized side by side, release will be sequential, the inner Ca^{2+} only being released after the outer Ca^{2+} has been released (Figure 1). Hanel and Jencks [13] and Orlowski and Champeil [14] found that when $^{45}\text{Ca}^{2+}$ was bound to the inner of the cytoplasmic sites and $^{40}\text{Ca}^{2+}$ was bound to the outer of the sites, the rate of phosphorylation-induced release of $^{45}\text{Ca}^{2+}$ was the same as when $^{45}\text{Ca}^{2+}$ was bound to the outer of the cytoplasmic sites with $^{40}\text{Ca}^{2+}$ at the inner site, consistent with either the independent release of Ca^{2+} from the luminal sites or with the randomization of the two Ca^{2+} ions during transfer from the cytoplasmic to the luminal sites, followed by sequential release. Hanel and Jencks [13] and Orlowski and Champeil [14] also found that the rates of internalization of Ca^{2+} at pH 6–7 and room temperature were the same for empty vesicles and for vesicles loaded with 20 mM Ca^{2+} , arguing either that the release of the two Ca^{2+} ions is not sequential or that the affinity of the phosphorylated intermediate with a single bound Ca^{2+} ion for Ca^{2+} is low, so that the presence of luminal Ca^{2+} has no observable effect on the dissociation process. Forge et al. [15] studied phosphorylation-induced release of Ca^{2+} by using a rapid filtration technique in which the Ca^{2+} -ATPase was incubated with $^{45}\text{Ca}^{2+}$ and then adsorbed on Millipore filters before being perfused with buffer for short (millisecond) periods of time, after which the level of $^{45}\text{Ca}^{2+}$ remaining bound to the ATPase was determined; when the buffer contained ATP, rapid phosphorylation of the ATPase meant that loss of $^{45}\text{Ca}^{2+}$ from the ATPase represented release from the luminal sites on the phosphorylated ATPase. Forge et al. [15] found that at pH 8.0 and 5 °C, phosphorylation-induced release of Ca^{2+} was biphasic; the rates of both components were decreased in the presence of high (mM) concentrations of Ca^{2+} , which was interpreted in terms of sequential release of Ca^{2+} .

Here we report on the Mg^{2+} -dependence of phosphorylation-induced release of Ca^{2+} from the ATPase, and suggest both that the release of Ca^{2+} from the phosphorylated ATPase is gated by

Abbreviation used: SR, sarcoplasmic reticulum.

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Figure 1 Models for transport of Ca^{2+} by the Ca^{2+} -ATPase

(A) During transport, two Ca^{2+} ions are transferred from a pair of sites exposed to the cytoplasm to a pair of sites exposed to the lumen. The pair of cytoplasmic sites are believed to be organized as a channel across the membrane. The pair of luminal sites could be organized as an extension of this channel, so that the Ca^{2+} ions could be released independently from the two luminal sites (left). Alternatively, the two luminal sites could be organized 'side by side'; the release of the two Ca^{2+} ions would then be sequential (right). (B) Sequences of loops on the luminal side of the membrane, between putative transmembrane α -helices M1–M10.

Mg^{2+} and that, as suggested by Forge et al. [15], release is sequential.

MATERIALS AND METHODS

SR vesicles were prepared from rabbit skeletal muscle as described in [16]. Concentrations of protein were estimated by using the specific absorption coefficient [$1.2 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ for a solution in 1% (w/v) SDS] given by Hardwicke and Green [17]. ATPase activities were measured with the use of a linked enzyme assay in which ADP production was linked to NADH oxidation, detected through an absorption change at 340 nm [18]. Assays were performed at 25 °C in the presence of 1 mg of A23187/mg of protein. Free concentrations of Ca^{2+} and Mg^{2+} were calculated by using the binding constants given by Godt [19] and Fabiato and Fabiato [20].

The time course of phosphorylation of the ATPase by [γ - ^{32}P]ATP was determined with the use of a Biologic QFM-5 system, as described in [21]. The time dependence of phosphorylation-induced Ca^{2+} release from the ATPase was determined at room temperature (typically 20 °C) with a Biologic rapid-filtration device. Unless stated otherwise, SR was incubated in the appropriate buffer containing 20 mM Mg^{2+} , 50 μM $^{45}\text{Ca}^{2+}$, 500 μM [^3H]sucrose and 40 μg of A23187/mg of protein. An aliquot of the sample, corresponding to 100 μg of protein, was loaded on a Millipore HAWP 0.45 μm filter and then perfused rapidly with the same buffer containing 2.1 mM ATP, 50 μM $^{40}\text{Ca}^{2+}$ and the required concentration of Mg^{2+} . The filter was then counted for radioactivity in Optiphase HiSafe 3. The amount of [^3H]sucrose trapped on the filter was used to calculate the amount of $^{45}\text{Ca}^{2+}$ in the aqueous space of the filter, which was subtracted from the total $^{45}\text{Ca}^{2+}$ on the filter to give that bound

to the ATPase. Under each set of conditions, the level of $^{45}\text{Ca}^{2+}$ bound to the ATPase was determined at ten time points in the range 0–1 s. Typically, duplicates were taken at four time points; these showed that the average error in the determination of bound $^{45}\text{Ca}^{2+}$ was $\pm 0.1 \text{ nmol of Ca}^{2+}/\text{mg of protein}$ (see, for example, Figure 3; values here and throughout the text are presented as $\pm \text{S.E.M.}$).

Data were fitted to single- or double-exponential processes by using the Marquardt–Levenberg algorithm in SigmaPlot (SPSS); for the double-exponential fits, the two components were constrained to have equal amplitudes, each equal to half the total Ca^{2+} released. A measure of the goodness of fit is the χ^2 value; for the data shown in Figure 3 at 2.5 mM Mg^{2+} , χ^2 values of 0.946 and 0.986 were obtained for single- and double-exponential fits respectively. The reliability of the double-exponential fit procedure was tested by fitting sets of simulated data, chosen to represent the range of the experimental data. Data were simulated by assuming a double-exponential process with amplitudes of the two components of the decay equal to 2.5 nmol of Ca^{2+}/mg of protein with a residual binding of 2.0 nmol of Ca^{2+}/mg of protein; rates for the slow and fast components were chosen as 17 and 6 s^{-1} , 10 and 3 s^{-1} , and 5 and 3 s^{-1} to represent the range of values observed in the experiments (see Figure 4). Levels of bound Ca^{2+} were then calculated at the time intervals between 0.03 and 1 s corresponding to the experimental measurements; a random error of $\pm 0.1 \text{ nmol of Ca}^{2+}/\text{mg of protein}$ was then applied to match the experimental accuracy. When these simulated data were free-fitted to double-exponential processes, the returned parameters were not, in general, in good agreement with the parameters used to calculate the simulated data. However, if the amplitudes of the two components of the double-exponential fit were constrained to be equal, then the returned

amplitude, residual binding levels and rates of the two components were in excellent agreement with the original parameters, agreeing within 5%.

The ability to distinguish between single- and double-exponential processes was determined by simulating single-exponential processes with amplitudes of 5 nmol of Ca^{2+} /mg of protein, residual binding of 2 nmol of Ca^{2+} /mg of protein, and rates of 10, 6 and 4 s^{-1} ; the simulated curves were then fitted to double-exponential processes with the amplitudes of the two components constrained to be equal. For the single-exponential data simulated with rates of 10 and 4 s^{-1} , the two returned rates were equal within the estimated error, both being equal, within the estimated error, to the true single-exponential rate constant. For the single exponential data simulated with a rate of 6 s^{-1} , although the two returned rates of 7.3 and 4.6 s^{-1} were not equal within the estimated error ($\pm 0.3 \text{ s}^{-1}$), they differed by only 35%. Thus it seems that the fitting procedure is able to distinguish between single- and double-exponential processes and, as expected, fitting a single-exponential process to a double-exponential process with two components of equal amplitude gives two components with very similar rates.

RESULTS

Inhibition of ATPase activity by Mg^{2+} at pH 8.0

ATPase activities were measured for SR vesicles in the presence of A23187, a bivalent-cation ionophore [22]. At pH 8.0, activities decreased with increasing free Mg^{2+} concentration in the medium (Figure 2). The data can be fitted to Scheme 1 with Mg^{2+} binding

Figure 2 Effect of Mg^{2+} concentration on ATPase activity at pH 8.0

ATPase activity, measured at pH 8.0 and 25 °C in 50 mM Tris/Hepes containing 2.1 mM ATP, 50 μM Ca^{2+} , 1 mg of A23187/mg of ATPase and 0.03 μM ATPase, is shown as a function of the calculated free concentration of Mg^{2+} . Symbols: $\circ$, experimental data; solid line, best fit to a single inhibitory binding site for Mg^{2+} , with the parameters given in the text.

Scheme 1 Mg^{2+} binding

Figure 3 Phosphorylation-induced release of Ca^{2+} from the Ca^{2+} -ATPase at pH 8.5

SR vesicles (0.2 mg/ml protein) were incubated in pH 8.5 buffer (27 mM Mes/100 mM Tris) containing 20 mM Mg^{2+} , 50 μM $^{45}\text{Ca}^{2+}$ and 40 μg of A23187/mg of protein, followed by adsorption on Millipore filters and washing with buffer containing 50 μM $^{40}\text{Ca}^{2+}$, 2.1 mM ATP, and Mg^{2+} concentrations of 2.5 mM ($\triangle$) and 10.0 mM ($\square$). The solid lines show fits to a double-exponential process with the rate parameters given in Figure 4(A); the broken lines show fits to a single-exponential process.

to a single site on the ATPase with a K_d of 0.2 ± 0.14 mM, with specific activities of 3.35 ± 1.0 units/mg of protein and 0.08 ± 0.14 unit/mg protein for the Mg^{2+} -free and Mg^{2+} -bound species respectively.

Effect of Mg^{2+} on the rate of phosphorylation-induced release of Ca^{2+}

Orlowski and Champeil [14] have shown that the rate of release of Ca^{2+} from the phosphorylated ATPase can be measured by pre-equilibrating SR with $^{45}\text{Ca}^{2+}$ and the bivalent-metal-ion ionophore A23187, and then perfusing the sample on Millipore filters with $^{40}\text{Ca}^{2+}$ and ATP. The experiment requires the rate of phosphorylation of the ATPase by ATP to be much faster than the rate of dissociation of $^{45}\text{Ca}^{2+}$ from the unphosphorylated ATPase [14]. We have found that the dissociation of $^{45}\text{Ca}^{2+}$ from the unphosphorylated ATPase fits to a double-exponential process with rates of 16.4 ± 2 and $3.1 \pm 0.5 \text{ s}^{-1}$ at pH 8.0 and 2.5 mM Mg^{2+} ; the rate of phosphorylation of the ATPase by 50 μM ATP in the presence of 100 μM Ca^{2+} and 5 mM Mg^{2+} at pH 8.0 was $89 \pm 6.3 \text{ s}^{-1}$ (A. P. Starling, R. C. Duggleby, J. M. East and A. G. Lee, unpublished work). The rates of Ca^{2+} dissociation and of phosphorylation by ATP were not significantly different at pH 8.5 so that, at alkaline pH, phosphorylation was much faster than Ca^{2+} dissociation, as required.

Rates of phosphorylation-induced release of Ca^{2+} from the ATPase at pH 8.5 were determined by the incubation of SR with buffer containing 20 mM Mg^{2+} , 50 μM $^{45}\text{Ca}^{2+}$ and 40 μg of A23187/mg of protein, followed by adsorption on Millipore filters and washing with buffer containing 50 μM $^{40}\text{Ca}^{2+}$, 2.1 mM ATP and the required concentration of Mg^{2+} . The rate of release of $^{45}\text{Ca}^{2+}$ from the ATPase was found to decrease with increasing concentration of Mg^{2+} in the wash medium (Figure 3). The

Figure 4 Effects of Mg^{2+} on the rate of phosphorylation-induced release of Ca^{2+} from the ATPase

The rates of the fast ($\circ$) and slow ($\square$) components of phosphorylation-induced release of Ca^{2+} from the ATPase obtained from double-exponential fits to the data at pH 8.0 (A) and 8.0 (B) are plotted as a function of the calculated concentration of free Mg^{2+} . The lines show fits to the model shown in Scheme 2 with the parameters given in Table 1.

release of Ca^{2+} was found not to fit to a single-exponential process but to fit to a double-exponential process with two components of equal magnitude, each equal to half the total Ca^{2+} released. The rate constants of the two components are shown in Figure 4(A) as a function of the calculated free concentration of Mg^{2+} in the wash buffer, for a range of Mg^{2+} concentrations up to 50 mM; as shown, both rate constants decreased with increasing Mg^{2+} concentration. The phosphorylation-induced release of Ca^{2+} at pH 8.0 also fits to a double-exponential process, with the rates of the fast and slow components again decreasing with increasing free Mg^{2+} concentration (Figure 4B).

Table 1 Binding and rate parameters describing the effect of Mg^{2+} on the phosphorylation-induced release of Ca^{2+} from the Ca^{2+} -ATPase

Parameters were obtained by fitting the rate data in Figure 4 to eqns. (1) and (2). Results are means $\pm$ S.E.M.

pH	Component	k_1 (s^{-1})	k_2 (s^{-1})	K_d (mM)
8.0	Fast	18.7 ± 1.9	3.5 ± 1.8	4.6 ± 2.9
	Slow	8.5 ± 2.9	2.4 ± 0.6	0.7 ± 0.9
		9.3*	2.4 ± 0.5	0.5 ± 0.3
8.5	Fast	17.6 ± 1.2	7.1 ± 0.6	2.5 ± 1.1
	Slow	8.8*	1.3 ± 0.2	0.16 ± 0.05

* Fixed at half the rate of the fast component (see the text).

Figure 5 Phosphorylation-induced release of Ca^{2+} from the Ca^{2+} -ATPase at pH 7.2

SR vesicles (0.2 mg/ml protein) were incubated in pH 7.2 buffer (150 mM Mops/80 mM Tris) containing 20 mM Mg^{2+} , 50 μM $^{45}\text{Ca}^{2+}$ and 40 μg of A23187/mg of protein, followed by adsorption on Millipore filters and washing with buffer containing 50 μM $^{40}\text{Ca}^{2+}$, 2.1 mM ATP, and Mg^{2+} concentrations of 1.5 mM ($\circ$) and 50 mM ($\square$). The lines show fits to a single-exponential process with the rate parameters given in Figure 6.

The biphasic release of Ca^{2+} is consistent with a sequential release process with an initial fast release of one Ca^{2+} ion followed by a slower release of the second Ca^{2+} ion:

where EP is the phosphorylated ATPase, and k^f and k^s are the rate constants of the fast and slow releases respectively. If it is assumed that the fast phase of release is modified by the binding of Mg^{2+} to a single site with a dissociation constant K_d^1 for Mg^{2+} and that the slow phase of release is similarly modified by the binding of Mg^{2+} to a single site with a dissociation constant K_d^2 for Mg^{2+} , as in Scheme 2, then the Mg^{2+} dependences of the rates k^f and k^s , in conditions under which Mg^{2+} binding is fast, are:

$$k^f = (k_1^f K_d^1 + k_2^f [\text{Mg}]) / (K_d^1 + [\text{Mg}]) \quad (1)$$

and

$$k^s = (k_1^s K_d^2 + k_2^s [\text{Mg}]) / (K_d^2 + [\text{Mg}]) \quad (2)$$

where the rate constants k_1 and k_2 refer to the rates of release for the Mg^{2+} -free and Mg^{2+} -bound species respectively.

The data for the fast and slow components of release at pH 8.0 fit to eqns. (1) and (2) to give the binding and rate parameters listed in Table 1. The rate of the fast component for the Mg^{2+} -

Figure 6 Effects of Mg^{2+} on the rate of phosphorylation-induced release of Ca^{2+} from the ATPase at pH 7.2

The rates of phosphorylation-induced release of Ca^{2+} from the ATPase ($\circ$) obtained from single-exponential fits to the data at pH 7.2 are plotted as a function of the calculated concentration of free Mg^{2+} . The solid line shows a free fit to a model in which the release of Ca^{2+} is controlled by the binding of Mg^{2+} to a single site (analogous to Scheme 2); the broken line shows a fit with the K_d value fixed at 4 mM (see the text).

free enzyme is approximately twice that of the slow component. This is the expected result for the sequential release of Ca^{2+} from a pair of sites, when the rate constant for dissociation from the outer of the two sites is a constant, and when the second Ca^{2+} ion can equilibrate rapidly between the pair of sites, which have equal affinity for Ca^{2+} , so that the outer site will be occupied half of the time. Data for the fast phase of release of Ca^{2+} measured at pH 8.5 also fit to eqn. (1) with the parameters given in Table 1. For the slow component, stable fits could not be obtained to eqn. (2) because of the steep dependence of the slow rate on the concentration of Mg^{2+} (see Figure 4A). However, at low concentrations of Mg^{2+} , the value for the rate of the slow component (6.2 s^{-1}) was close to half of that for the fast component (16.4 s^{-1}) and if the assumption is made that k_1^* is equal to $k_1^*/2.0$, as at pH 8.0, then a good fit to the data can be obtained (Figure 4A), with the parameters listed in Table 1.

The release of Ca^{2+} at pH 7.2 fits to a single-exponential process (Figure 5), with the rates again decreasing with increasing concentration of Mg^{2+} (Figure 6). The data fit to a scheme analogous to Scheme 2 with a K_d of $14.5 \pm 4.0 \text{ mM}$ with rates of release for the Mg^{2+} -free and Mg^{2+} -bound forms of 18.5 ± 0.5 and $2.7 \pm 1.2 \text{ s}^{-1}$ respectively. Figure 6 also shows a fit to the data where K_d has been fixed at 4 mM, the value that describes the effect of Mg^{2+} on the rate of the fast component of release at pH 8.0; this also gives a reasonable fit to the data for concentrations of Mg^{2+} up to 20 mM.

Champeil and Guillain [23] have shown that the rate of phosphorylation-induced release of Ca^{2+} increases when the concentration of ATP is increased from 0.05 to 5 mM at a constant Mg^{2+} concentration of 20 mM, at pH 6.0. Because, at constant total Mg^{2+} concentration, increasing the ATP concentration will decrease the free Mg^{2+} concentration, we studied the effect of ATP concentration at a fixed free Mg^{2+} concentration of 3 mM, at pH 8.0. As shown in Table 2, the release of Ca^{2+} was biphasic at ATP concentrations between 0.05 and 3 mM, with no significant change in rate constants with changing ATP concentration.

Table 2 Effects of ATP concentration on the rate of phosphorylation-induced release of Ca^{2+} from the Ca^{2+} -ATPase at a fixed concentration of free Mg^{2+} of 3 mM

The phosphorylation-induced release of Ca^{2+} from the ATPase was determined as in Figure 3 but at pH 8.0 and at a fixed concentration of free Mg^{2+} of 3 mM. Results were fitted to a double-exponential process with the amplitudes of the two components fixed at 2.6 nmol of Ca^{2+} /mg of protein. Results are means $\pm$ S.E.M.

[ATP] (mM)	Rate constant (s^{-1})	
	Fast component	Slow component
0.05	20.8 ± 4.5	5.0 ± 1.2
2.1	25.5 ± 4.5	6.0 ± 1.2
3.0	20.0 ± 5.9	4.0 ± 1.6

DISCUSSION

Four acidic residues essential for the high-affinity binding of Ca^{2+} to the Ca^{2+} -ATPase have been identified by using mutagenesis, located one each in predicted transmembrane α -helices M4, M5, M6 and M8 [24]. Measurements of the rate of dissociation of Ca^{2+} from these sites are consistent with the two Ca^{2+} ions binding in a channel-like structure made up of these four transmembrane α -helices [5–8]. In an operational sense, the two sites are located one ‘above’ the other because the binding of Ca^{2+} to the ‘outer’ site prevents the release of Ca^{2+} from the ‘inner’ site [5–8]; this could correspond to a physical location of the outer site above the inner site, as suggested in Figure 1, but would be equally consistent with a side-by-side arrangement, with the translocation of Ca^{2+} across the membrane then taking an angular course [25]. After phosphorylation of the ATPase, the two Ca^{2+} ions are transferred from the pair of cytoplasmic sites to a pair of luminal sites [2–4]. Because the only negatively charged residues in the putative transmembrane α -helices of the Ca^{2+} -ATPase are the four postulated to make up the pair of high-affinity cytoplasmic sites, the pair of low-affinity luminal sites must be made up of residues on the luminal side of the SR membrane. Electron microscopic studies show that the luminal part of the ATPase comprises only approx. 5% of the total mass of the ATPase [26]. This luminal part is predicted to consist of small tight loops between transmembrane α -helices, except for the loop between helices M7 and M8, which is predicted to be 38 residues long, containing 9 acidic residues (Figure 1) [27,28].

The pair of luminal sites could be organized as an extension of the transmembrane channel or could be arranged side by side, as shown in Figure 1. A side-by-side arrangement of the luminal sites would be expected to result in the sequential release of Ca^{2+} from the phosphorylated ATPase. Forge et al. [15] suggested that the release was indeed sequential, on the basis of the observations that the release of Ca^{2+} at pH 8.0 and 5 °C was biphasic and that the rates of both components of release were decreased in the presence of millimolar concentrations of Ca^{2+} .

Here we make use of the observed inhibition of ATPase activity by Mg^{2+} at alkaline pH to study the release process. ATPase activity is inhibited at pH 8.0 with a K_d for Mg^{2+} of 0.2 mM, calculated from the free concentration of Mg^{2+} in the system (Figure 2). These results (Figure 2) are in good agreement with the data of Bishop and Al-Shawi [29], who showed maximum activity as a function of total Mg^{2+} at a total Mg^{2+} concentration of 4 mM at pH 8.0 and an ATP concentration of 5 mM, with inhibition at higher total Mg^{2+} concentrations. Bishop and Al-

Shawi showed that the effect of Mg^{2+} followed from the binding of Mg^{2+} to the luminal Ca^{2+} -binding sites. The inhibitory effect of Mg^{2+} on ATPase activity follows from a decrease in the rate of release of Ca^{2+} from the phosphorylated ATPase (Figure 3). At high concentrations of Mg^{2+} , at alkaline pH values, the release of Ca^{2+} is biphasic. The relatively small differences in the rates of the two components of release, combined with the limited accuracy of the determinations of the level of Ca^{2+} bound to the ATPase (limited by the amount of ATPase that can be trapped on the Millipore filters) mean that the errors in determining the rates of the two components are relatively large. Nevertheless, two clear results emerge: first, the effects of Mg^{2+} on the rates of the fast and slow components are different, with effects of Mg^{2+} on the slow component being observed at lower concentrations of Mg^{2+} than those affecting the fast component; and secondly, at zero concentration of free Mg^{2+} , the rate of the fast component is about double that of the slow component (Table 1).

A rate for the fast component double that of the slow component is the expected result for the sequential release of Ca^{2+} from a pair of sites, when the rate constant for dissociation from the outer of the two sites is constant and when the second Ca^{2+} ion can equilibrate rapidly between the pair of sites, which have equal affinities for Ca^{2+} , so that the outer site will be occupied half of the time. The same model has been proposed to explain the dissociation of Ca^{2+} from the high-affinity cytoplasmic binding sites for Ca^{2+} [7,30]. The decrease in the rate of the slow component with increasing Mg^{2+} concentration at pH 8.0 and pH 8.5 (Figure 4) then follows from the binding of Mg^{2+} to the outer Ca^{2+} site, made empty by the release of the first Ca^{2+} in the fast phase of release. The affinity of this site is approx. 0.5 mM at pH 8.0 and approx. 0.2 mM at pH 8.5 (Table 1).

It can readily be shown for a sequential process:

where EP is the phosphorylated ATPase, that the release of Ca^{2+} is described by a single-exponential process with a rate of k_2 when $k_2 = k_1/2.0$. At pH 7.2, competition between Mg^{2+} and H^+ for binding to the outer luminal Ca^{2+} -binding site would result in a weak binding of Mg^{2+} , so that $k_2 = k_1/2.0$ at all except the highest Mg^{2+} concentrations, explaining why the release of Ca^{2+} from the phosphorylated ATPase at pH 7.2 fitted to a single-exponential process (Figure 5). However, at alkaline pH values, at non-zero concentrations of free Mg^{2+} , Mg^{2+} binding to the empty outer site on E2PCa reduces the effective value of k_2 so that k_2 will not be equal to $k_1/2.0$ and the release of Ca^{2+} is a double-exponential process.

The rate of the fast component of release of Ca^{2+} , corresponding to the release of the outer Ca^{2+} ion in the sequential model, also decreases with increasing concentration of Mg^{2+} , the effect of Mg^{2+} being consistent with binding to a site of K_d 2–5 mM at alkaline pH values. Effects of Mg^{2+} on the release of Ca^{2+} at pH 7.2 fit to a site with a K_d of 14 mM, although reasonable fits can also be obtained to a K_d of 4 mM for the lower concentrations of Mg^{2+} (less than 20 mM) (Figure 6). The presence of a gating site on the ATPase with an affinity for Mg^{2+} in the range 2–4 mM has been suggested [8], binding of Mg^{2+} to this site increasing the rate of dissociation of Ca^{2+} ions from the cytoplasmic sites on the ATPase [8–10]. It is possible that the Mg^{2+} -binding site affecting the fast phase of release of Ca^{2+} from the phosphorylated ATPase is the gating site controlling access to the cytoplasmic pair of sites.

The single-exponential release of Ca^{2+} observed here at pH 7.2 is consistent with the experiments of Hanel and Jencks [13]

and Orlowski and Champeil [14], who found that rates of internalization of Ca^{2+} at pH 6–7 at room temperature were the same for empty vesicles and for vesicles loaded with 20 mM Ca^{2+} , suggesting that the affinity of the phosphorylated intermediate for Ca^{2+} is low at neutral or acid pH values so that the presence of luminal Ca^{2+} has no significant effect on the rate of release of bound Ca^{2+} .

It has been suggested that the binding of ATP to the phosphorylated ATPase increases the rate of release of Ca^{2+} [23]. However, at constant concentrations of free Mg^{2+} , the rate of release of Ca^{2+} is independent of ATP concentration in the range 0.05–3 mM (Table 2); it is therefore possible that the reported effect of increasing ATP concentration follows from a decrease in the concentration of free Mg^{2+} .

In the experiments of Forge et al. [15] measuring the rate of release of Ca^{2+} from the phosphorylated ATPase at 5 °C, the release of Ca^{2+} was biphasic at pH 8.0 and, in apparent disagreement with the results shown in Figure 4(B), was not detectably affected by Mg^{2+} in the concentration range 0–5 mM. However, the experiments of Forge et al. [15] were performed in the presence of 1 mM Ca^{2+} and it is therefore possible that Ca^{2+} binds to the same sites as Mg^{2+} , mimicking the effects of Mg^{2+} observed in Figure 4(B).

A side-by-side arrangement of the pair of luminal Ca^{2+} -binding sites is consistent with what is known about the structure of the ATPase on the luminal side of the membrane. The M7–M8 loop seems to be the only large surface-exposed loop on the luminal side because, of all the anti-peptide antibodies raised against luminal loops, only that against the M7–M8 loop bound to the native ATPase [31]. The M7–M8 loop is predicted to contain nine acidic residues, including two ED pairs and the very acidic sequence EGLDCEIFEAP. Despite the low affinity of the luminal sites for Ca^{2+} (millimolar affinity), the rate of dissociation of Ca^{2+} from the phosphorylated ATPase into the lumen of the SR is relatively slow (approx. 20 s^{-1}), suggesting that the dissociation of Ca^{2+} involves a conformation change on the ATPase. Thus the M7–M8 loop could act as a gate covering the luminal end of the channel between helices M4, M5, M6 and M8; as the two Ca^{2+} ions emerge from the channel they could bind to two sites on the loop, possibly between the M7–M8 loop and the other smaller loops located at the lipid/water interface. Movement of the loop would allow the sequential release of Ca^{2+} into the lumen as shown in Figure 1. Helix M7 is believed to be long and tilted [26] and could serve to link changes at the phosphorylation site on the ATPase (in the main cytoplasmic domain between helices M4 and M5) to changes in the M7–M8 loop.

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