Effects of polycations on Ca^{2+} binding to the Ca^{2+} -ATPase

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Spermine and polyarginine have been shown to increase the rate of dissociation of Ca^{2+} from the Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum. They also decrease the affinity of the ATPase for Mg^{2+} as detected by changes in the fluorescence intensity of the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin (DMC). Polyarginine itself also decreases the fluorescence intensity of DMC-labelled ATPase. These results are consistent with binding of spermine and polyarginine to a gating site controlling the rate of access of Ca^{2+} to its binding sites on the ATPase. A basic peptide PLN-(1–25) corresponding to residues 1–25 of phospholamban had no effect on the rate of

dissociation of Ca^{2+} or on the fluorescence of DMC-labelled ATPase. Spermine, polyarginine and PLN-(1–25) all increased the equilibrium constant E1/E2, and spermine and polyarginine increased the rate of Ca^{2+} binding to the ATPase, consistent with an increase in the rate of the E2 → E1 transition. Spermine displaced Tb^{3+} and Ruthenium Red from the ATPase, consistent with binding in the stalk region of the ATPase. Polyarginine and PLN-(1–25), however, had no effect on Tb^{3+} or Ruthenium Red binding, suggesting a greater specificity in binding basic peptides to the ATPase than spermine.

INTRODUCTION

Ca^{2+} binds to and dissociates from the Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum at a relatively low rate, indicating a conformation change associated with the Ca^{2+} -binding process [1]. Residues important in Ca^{2+} binding have been identified using site-directed mutagenesis [2], and, in the ten-helix model for the Ca^{2+} -ATPase [3,4], these residues are located toward the centre of the transmembrane domain of the ATPase. Such a location is consistent with fluorescence studies in which hydrophobic carboxy groups have been labelled with fluorescent carbodi-imides [5]. The access pathway to these sites is unknown.

Binding of Ca^{2+} to the Ca^{2+} -ATPase has been described [6,7] in terms of Scheme 1, in which E1 represents a conformation of the ATPase with two high-affinity binding sites for Ca^{2+} exposed to the cytoplasm, and E2 a state that cannot bind Ca^{2+} from the cytoplasm [6]. It has been suggested that the fluorescence of the ATPase labelled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) is sensitive to the E2–E1 equilibrium, with fluorescence intensity being high in the E1 conformation and low in the E2 conformation [7,8]. Of the two Ca^{2+} -binding sites on E1, it has been proposed that only the first, inner, site of lower affinity is initially available for binding, but that after binding the first Ca^{2+} ion, a second, outer, binding site of higher affinity becomes available after the $\text{E1Ca} \rightleftharpoons \text{E1}'\text{Ca}$ transition (see ref. [7]).

Binding of Mg^{2+} or K^{+} ions to the ATPase increase the rate of dissociation of Ca^{2+} ions from the ATPase, and binding of H^{+} decreases the rate of Ca^{2+} dissociation [1,9,10], suggesting that these ions bind at a 'gating site' on the ATPase which controls access to the Ca^{2+} -binding sites [1]. Changes in the fluorescence

of the Ca^{2+} -ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin (DMC) on addition of Mg^{2+} as a function of pH match effects of pH on the Mg^{2+} -dependence of the rate of dissociation of Ca^{2+} from the ATPase, and it has therefore been suggested that the fluorescence of DMC-labelled ATPase is sensitive to binding at the gating site [1,7]. It was shown [7] that effects of Mg^{2+} at the gating site were consistent with Scheme 2, with equal binding constants for Mg^{2+} to all forms, but with different H^{+} -binding constants in the absence and presence of Ca^{2+} ($K_{\text{H}2} = 5 \times 10^8$ and $K_{\text{H}3} = 1.25 \times 10^7$). The same scheme accounted for the effects of Mg^{2+} and pH on the rate of dissociation of Ca^{2+} from the ATPase, with fast dissociation of Ca^{2+} from the E1Ca and E1CaMg forms [1]. Lee et al. [10] showed that the effects of K^{+} on the rate of dissociation of Ca^{2+}

Scheme 2 Gating site on the ATPase

Binding constants for E1'Ca and E1'Ca₂ forms of the ATPase (Scheme 1) are the same as for the E1Ca forms and for E2 are the same as for E1 [1,7].

Scheme 1 Binding of Ca^{2+} to the ATPase

and on the Mg^{2+} -dependence of DMC fluorescence could be interpreted in terms of the same scheme if K^+ can bind to E1 in competition with H^+ .

A number of polycationic species are known to bind to Ca^{2+} -ATPase, and these could potentially have significant effects at the proposed gating site. Ruthenium Red $\{[(\text{NH}_3)_5\text{Ru}-\text{O}-\text{Ru}(\text{NH}_3)_4-\text{O}-\text{Ru}(\text{NH}_3)_5]^{6+}\}$ has been shown to reduce the affinity of Ca^{2+} -ATPase for Ca^{2+} [11,12] and to bind to low-affinity cation-binding sites in competition with Mg^{2+} and Ca^{2+} ; it has been shown that these low-affinity sites are not at the ATP-binding site on the ATPase [11]. Dissociation of Ca^{2+} from the ATPase in the presence of Ruthenium Red has been reported to be biphasic, with one-half of the Ca^{2+} dissociating at a rate comparable with that seen on addition of EGTA, the other half dissociating at a lower rate [12]. A similar result was obtained in the presence of Pr^{3+} [1,13,14]. Pr^{3+} has also been shown to displace Ca^{2+} from the Ca^{2+} -bound ATPase, probably as a result of binding to sites on the ATPase other than the Ca^{2+} -binding sites [7,13,14]. X-ray-diffraction studies have located binding sites for lanthanides about 1.2 nm (12 Å) above the lipid bilayer surface [15], in the 'stalk' region of the ATPase, where a large number of negatively charged residues are located [3,5].

We have shown that spermine, polyarginine and a peptide MEKVQYLTRSAIRRASTIEMPQQARC [PLN(1–25)] corresponding to residues 1–25 of phospholamban all inhibit the Ca^{2+} -transport step of the ATPase ($\text{E1PCa}_2 \rightarrow \text{E2P}$) [16,17]. Here we report on the effects of these compounds on Ca^{2+} binding to the ATPase.

MATERIALS AND METHODS

Sarcoplasmic reticulum from rabbit skeletal muscle and purified Ca^{2+} -ATPase were prepared as described by East and Lee [18]. ATPase activity was measured using a coupled enzyme assay as described [18]. Ca^{2+} concentrations were calculated using the binding constants for EGTA, Ca^{2+} , Mg^{2+} and H^+ given by Godt [19]. Poly-L-arginine (M_r range 5000–15000) and Ruthenium Red were obtained from Sigma. Concentrations of poly-L-arginine were calculated assuming an average M_r of 10000. Terbium chloride was obtained from Aldrich, and concentrations of solutions in 10 mM HCl were checked using the absorption coefficient, ϵ_{350} , $0.31 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [13]. The peptide MEKVQYLTRSAIRRASTIEMPQQARC [PLN(1–25)] was synthesized as described by Hughes et al. [17].

DMC was obtained from Molecular Probes. The ATPase was labelled with NDB or DMC as described [20,21]. NBD and DMC fluorescence was measured at 25 °C using an SLM Aminco 8000C fluorimeter with excitation and emission wavelengths of 430 and 520 nm for NBD fluorescence and 350 and 425 nm for DMC fluorescence.

Rapid-kinetic fluorescence measurements were performed using a Biologic SFM-3 fluorimeter with BioKine V3.20a software for data acquisition and analysis [1]. Tryptophan fluorescence was excited through a monochromator set at 290 nm, and emitted light was collected at 90° and filtered by a high-pass cut-off filter with 50 % transmission at 320 nm (UV 32; Andover Corporation).

The time-dependence of $^{45}\text{Ca}^{2+}$ release from the ATPase was determined using a Biologic RFS-04 rapid filtration system, at room temperature (typically 20 °C). A suspension of the ATPase (0.2 mg) in buffer containing $100 \mu\text{M}$ $^{45}\text{Ca}^{2+}$ and $500 \mu\text{M}$ [^3H]-sucrose was loaded on to a Millipore HAWP filter (0.45 μm) and then rapidly perfused with buffer. The filter was dried overnight in air and counted in OptiPhase 'HiSafe' 3. The amount of [^3H]-sucrose trapped on the filter was used to calculate the wetting

volume of the filter, and the amount of $^{45}\text{Ca}^{2+}$ calculated to be in this volume was subtracted from the total $^{45}\text{Ca}^{2+}$ on the filter to give that bound to the ATPase.

Simulations of Ca^{2+} dissociation were performed on an IBM-compatible PC using the program M30 of Zhang et al. [22] which uses the method of Laplace transforms to solve differential equations, with the parameters given in Henderson et al. [1] and Lee et al. [10].

RESULTS

Rate of dissociation of Ca^{2+} from Ca^{2+} -ATPase

The rate of dissociation of Ca^{2+} from the ATPase can be determined by incubating the enzyme with $^{45}\text{Ca}^{2+}$, adsorbing it on to a cellulose filter, and then washing it for various times with buffer containing EGTA. At pH 7.2, in the absence of Mg^{2+} , the observed loss of $^{45}\text{Ca}^{2+}$ fits to a single exponential with a rate constant of $11.7 \pm 1.1 \text{ s}^{-1}$ (Figure 1a). On addition of 10 mM spermine, the rate of dissociation of $^{45}\text{Ca}^{2+}$ increased to $27.3 \pm 4.4 \text{ s}^{-1}$ (Figure 1a).

The rate of dissociation of Ca^{2+} from the ATPase can also be determined by measuring the corresponding change in the intensity of the tryptophan fluorescence of the ATPase; under a wide variety of conditions, tryptophan fluorescence intensity has been shown to monitor closely the level of Ca^{2+} binding to the ATPase [1,9,10,23–25]. Figure 1(b) shows the rate of decrease in tryptophan fluorescence intensity observed when the ATPase

Figure 1 Kinetics of Ca^{2+} dissociation from the ATPase

(a) Rates of dissociation of $^{45}\text{Ca}^{2+}$ from the ATPase when the Ca^{2+} -bound ATPase, adsorbed on cellulose filters, was washed for the given times with buffer (150 mM Mops/Tris, pH 7.2) containing 2 mM EGTA in the absence (○) or presence (□) of 10 mM spermine. The solid lines are best fits to single exponentials, with the rates given in the text. (b) The dotted lines show the decrease in tryptophan fluorescence observed when the ATPase (0.4 mg of protein/ml) in buffer (150 mM Mops/Tris, pH 7.2) containing $100 \mu\text{M}$ Ca^{2+} is mixed with an equal volume of the same buffer containing 2 mM EGTA, in the absence (A) or presence (B) of 20 mM spermine. The solid lines show simulations calculated as described in the text.

Figure 2 Effects of spermine on the rates of Ca²⁺ binding and dissociation

The decrease in tryptophan fluorescence on mixing the Ca²⁺-bound ATPase with EGTA was determined as described in the legend to Figure 1(b) and fitted to a single exponential. The rate of increase in tryptophan fluorescence associated with binding Ca²⁺ to the ATPase was determined similarly by mixing the ATPase in EGTA with buffer containing Ca²⁺ to give a final pCa value of 3.7, and fitting the data to a single exponential. (a) Rate of Ca²⁺ dissociation at pH 7.2 in the absence (○) or presence (□) of 20 mM Mg²⁺, and at pH 6.0 in the absence of Mg²⁺ (△). (b) Rate of Ca²⁺ binding in the absence of Mg²⁺ at pH 7.2 (○) or pH 6.0 (□). The lines show simulations calculated as described in the text.

incubated in 100 μ M Ca²⁺ at pH 7.2 is mixed in a stopped-flow fluorimeter with an equal volume of 2 mM EGTA. The data fit well to a single exponential with a rate of 13.5 ± 0.1 s⁻¹. The rate of change in tryptophan fluorescence intensity increased in the presence of 10 mM spermine, again fitting to a single exponential, but with a rate of 29.9 ± 0.3 s⁻¹ (Figure 1b). The rates of Ca²⁺ dissociation from the ATPase vary between preparations [10,25], and, whereas for some preparations, for example that used here, the time-dependence of the measured changes in tryptophan fluorescence intensity fit to single exponentials, for others they do not [1,10,26]. The rate of dissociation of Ca²⁺ in the absence of spermine is the same as that reported by Lee et al. [10].

Rate constants observed in the presence of spermine are given in Figure 2(a) as a function of spermine concentration. Measured rates were faster in the presence of 20 mM Mg²⁺, but effects of spermine were then very small (Figure 2a). At pH 6.0, rates were small and little affected by spermine (Figure 2a).

Polyarginine also increased the rate of the change in tryptophan fluorescence intensity associated with Ca²⁺ dissociation from the ATPase at pH 7.2, but with a half-maximal effect at 0.35 μ M, compared with 2.3 mM for spermine (Figure 3a). Again, the effects of polyarginine were very small in the presence of 20 mM Mg²⁺ at pH 7.2 (Figure 3a) or at pH 6.0 (results not shown). PLN-(1–25) at concentrations up to 25 μ M had no effect on the rate of Ca²⁺ dissociation at pH 7.2 in the absence of Mg²⁺ (results not shown).

Figure 3 Effects of polyarginine on the rates of Ca²⁺ binding and dissociation

Rates of Ca²⁺ binding and dissociation were determined as described in the legend to Figure 2: (a) rates of Ca²⁺ dissociation at pH 7.2 in the absence (○) or presence (□) of 20 mM Mg²⁺; (b) rates of Ca²⁺ binding at pH 7.2 (○) or pH 6.0 (□). The lines show simulations calculated as described in the text.

Table 1 Binding constants for polycationic species to the ATPase

Cation	Binding constant (M ⁻¹)		
	Gating site	E1	E2
Spermine	250	3226*	806*
Polyarginine	3.3×10^6	8×10^6	2×10^6
PLN-(1–25)	—	3.8×10^5 †	9.7×10^4 †

* Value taken from ref. [16].

† Value taken from ref. [17].

The effects of spermine and polyarginine on the rates of dissociation of Ca²⁺, and the apparent competition with H⁺ and Mg²⁺, can be fitted in terms of Scheme 2, with competition between Mg²⁺ and spermine or polyarginine for binding to the ATPase at the gating site. With rate constants for non-cation-bound species as given in Lee et al. [10], the experimental data for spermine (Figures 1 and 2) can be fitted assuming a binding constant K_A of 250 M⁻¹ for spermine (Table 1) and rates of dissociation for spermine-bound species 1.5 times those observed for the corresponding Mg²⁺-bound species (Table 2). Similarly, effects of polyarginine can be fitted (Figure 3) assuming a binding constant of 3.3×10^6 for polyarginine with rates of dissociation for polyarginine-bound species 0.6 times those observed for the corresponding Mg²⁺-bound species (Table 2).

Table 2 Effects of polycationic species on dissociation rate constants for Ca^{2+}

Calculated rate constants for dissociation of Ca^{2+} from the polycation-bound species of the ATPase shown in Scheme 1 are given, and also those for the rate of the E2–E1 transition for the polycation-bound species. All other rate constants are as given in Lee et al. [10].

Reaction step	Rate constant (s^{-1})	
	Spermine	Polyarginine
$\text{E1'Ca}_2\text{HA} \rightarrow \text{E1'CaHA}$	43	17
$\text{E1'Ca}_2\text{A} \rightarrow \text{E1'CaA}$	4050	1620
$\text{E1'CaHA} \rightarrow \text{E1CaHA}$	225	90
$\text{E1'CaA} \rightarrow \text{E1CaA}$	225	90
$\text{E1'CaHA} \rightarrow \text{E1CaHA}$	29	12
$\text{E1'CaA} \rightarrow \text{E1CaA}$	340	135
$\text{E2A} \rightarrow \text{E1A}$	800	240

Figure 4 Effect of spermine and PLN-(1–25) on the fluorescence response of DMC-labelled ATPase to Mg^{2+} as a function of pH, in the absence and presence of Ca^{2+}

DMC-labelled ATPase was incubated in 40 mM Tris/maleate containing 1 mM EGTA, at pH 6.0 ($\circ, \square, \triangle$) or 7.0 ($\bullet, \blacksquare, \blacktriangle$) in the absence ($\circ, \bullet$) or presence of 10 mM spermine ($\square, \blacksquare$) or 25 μM PLN-(1–25) ($\triangle, \blacktriangle$) in the presence of 1 mM EGTA (a) or 1 mM Ca^{2+} (b). — and — — —, simulations in the absence and presence of 10 mM spermine respectively calculated by using the parameters in Table 1 and Lee et al. [10]; in the presence of Ca^{2+} , simulated curves for pH 6.0 and 7.0 are very similar, and thus, for clarity, only those for pH 6.0 are shown.

DMC fluorescence

The effects of Mg^{2+} on the fluorescence of DMC-labelled ATPase are consistent with Scheme 2, with the fluorescence decrease on binding Mg^{2+} to the unprotonated form (E1) being 20% of that resulting from binding to the protonated form (E1H), and with a more marked effect of pH in the absence of Ca^{2+} than in the

Figure 5 Effect of polyarginine on the fluorescence intensity of DMC-labelled ATPase

The decrease in fluorescence intensity for DMC-labelled ATPase is shown on addition of polyarginine at pH 6.0 ($\circ, \bullet$) or 7.0 ($\square, \blacksquare$) in the presence of 1 mM EGTA ($\circ, \square$) or 1 mM Ca^{2+} ($\bullet, \blacksquare$). The buffer was 40 mM Tris/maleate. —, Simulation at pH 6.0; — — — and — · — · —, simulations at pH 7.0 in the absence or presence of 1 mM Ca^{2+} respectively calculated as described in the text.

presence of Ca^{2+} because of stronger binding of H^+ to the Ca^{2+} -bound form of the ATPase ($K_{\text{H}2} > K_{\text{H}3}$) [7].

As shown in Figure 4(a), in the presence of 10 mM spermine, the K_d for Mg^{2+} , as detected from changes in DMC fluorescence at pH 6.0, increased from 3.8 ± 0.2 mM to 16.7 ± 3.0 mM, with an unchanged maximal fluorescence change of -5.6% . At pH 7.0, K_d values of 4.5 ± 1.3 mM and 13.2 ± 3.6 mM were obtained in the absence and presence of 10 mM spermine respectively (Figure 4a). In the presence of Ca^{2+} , determined K_d values were 3.2 ± 0.5 mM and 4.2 ± 0.7 mM at pH 6.0 and pH 7.0 respectively in the absence of spermine and 14.9 ± 4.0 mM and 18.7 ± 5.9 mM at pH 6.0 and pH 7.0 respectively in the presence of 10 mM spermine (Figure 4b). The increased K_d value for Mg^{2+} observed in the presence of spermine is consistent with competition between spermine and Mg^{2+} for binding to the ATPase, as in Scheme 2. As shown, a binding constant for spermine of 250 M^{-1} (Table 1), combined with a Mg^{2+} -binding constant ($K_6 = K_7 = K_8 = K_9$) of 250 M^{-1} [7], gives a good fit to the experimental data.

The addition of 10 mM spermine to DMC-labelled ATPase resulted in a 2% decrease in fluorescence intensity, a change that is too small to allow for an accurate determination of a binding constant. Binding of polyarginine to DMC-labelled ATPase, however, resulted in a decrease in fluorescence intensity slightly greater than that seen with Mg^{2+} , and at pH 6.0 in the absence of Ca^{2+} , the data fit to a K_d value of $0.3 \pm 0.07 \mu\text{M}$ for polyarginine, with a maximum fluorescence change of -7.2% (Figure 5). At pH 7.0, polyarginine had a smaller effect on DMC fluorescence than at pH 6.0, but with the same K_d value ($0.24 \pm 0.09 \mu\text{M}$), an effect comparable with that seen with Mg^{2+} (Figure 4). In the presence of Ca^{2+} , the effects of polyarginine were very similar at pH 6.0 and 7.0 (Figure 5), again as seen with Mg^{2+} (Figure 4), the data fitting to K_d values of $0.34 \pm 0.07 \mu\text{M}$ and $0.40 \pm 0.14 \mu\text{M}$ at pH 6.0 and 7.0 respectively. The effects of polyarginine can be simulated in terms of Scheme 2 as for Mg^{2+} , with a value of K_A of $3.3 \times 10^6 \text{ M}^{-1}$ (Table 1) and an effect of polyarginine on DMC fluorescence 1.3 times that seen with Mg^{2+} (Figure 5).

In the presence of 0.1 μM polyarginine, the effect of Mg^{2+} on the fluorescence of DMC-labelled ATPase fits a K_d value of 5.3 ± 0.4 mM at pH 6.0 (results not shown). The K_d value for Mg^{2+} calculated at 0.1 μM polyarginine assuming competition

Figure 6 Effects of polyarginine on the fluorescence intensity of NBD-labelled ATPase, as a function of pH

The fluorescence intensity of NBD-labelled ATPase was determined in buffer containing 0.3 mM EGTA at the given pH in the absence (○) or presence of 2 μ M polyarginine (□). The lines are simulations calculated using the parameters in the text, assuming fluorescence intensities of 6.5 and 12.5 for E2 and E1 forms respectively. The insert shows a plot of percentage increase in fluorescence intensity (vertical axis) on addition of the given concentrations (μ M) of polyarginine (horizontal axis) at pH 7.2.

between polyarginine and Mg²⁺ for binding to the ATPase, with a K_d value of 4 mM for Mg²⁺ and 0.3 μ M for polyarginine is 5.3 mM.

PLN-(1–25) at concentrations up to 25 μ M had no effect on the Mg²⁺-dependence of the fluorescence of DMC-labelled ATPase (Figure 4) and itself had no effect on the fluorescence of DMC-labelled ATPase at concentrations up to 25 μ M.

NBD fluorescence

The fluorescence intensity of NBD-labelled ATPase increased with increasing pH (Figure 6), consistent with binding of H⁺ at a single site on the ATPase, with affinities K_{H6} and K_{H7} of 5.0×10^5 M⁻¹ and 3.0×10^8 M⁻¹ in the E1 and E2 conformations respectively, and an equilibrium constant for the unprotonated forms E1/E2 of 4.0 [7]. The addition of 2 μ M polyarginine resulted in a marked increase in fluorescence intensity, which is consistent with a 4-fold increase in the equilibrium constant E1/E2 (Figure 6). A titration of NBD-labelled ATPase with polyarginine at pH 7.2 gave an apparent K_d of 0.33 μ M (Figure 6).

From Scheme 3, the effective dissociation constant K^{eff} for binding of polyarginine to the ATPase is given by:

$$(1/K^{eff}) = [K^{E2} + K_1 K^{E1}]/(1 + K_1)$$

where K^{E2} and K^{E1} are binding constants for polyarginine for E2 and E1 respectively and K_1 is the equilibrium constant E1/E2 (0.21 at pH 7.2, calculated using the parameters in Henderson et al. [7]). In terms of Scheme 3, a 4-fold increase in the equilibrium constant E1/E2 in the presence of excess polyarginine puts $K^{E1} = 4.0 \times K^{E2}$, and with $K^{eff} = 0.33$ μ M, K^{E2} and K^{E1} become 2.0×10^6 and 8.0×10^6 respectively (Table 1).

Ca²⁺ affinity

Binding of Ca²⁺ to high-affinity sites on the ATPase can be monitored by observation of the resulting changes in tryptophan fluorescence intensity [7,25,27,28]. As shown in Figure 7, the

Scheme 3 Effect of cations on the E1–E2 equilibrium

Figure 7 Effect of polyarginine on Ca²⁺ binding to the ATPase

Changes are shown in the tryptophan fluorescence intensity of the ATPase in 20 mM Hepes/KOH, pH 7.2, measured after the addition of EGTA to give the final free pCa values in the absence (○) or presence (□) of 2 μ M polyarginine. Fluorescence was excited at 295 nm and observed at 325 nm. The lines are simulations of Ca²⁺ occupancy of the ATPase, calculated as described in the text, and scaled to match the maximum observed decrease in fluorescence intensity.

change in fluorescence intensity on dissociation of Ca²⁺ from the ATPase was slightly smaller in the presence of 2 μ M polyarginine than in its absence, and there was a slight decrease in the concentration of Ca²⁺ resulting in a half-maximal change in fluorescence, by about 0.2 pCa unit.

Binding of Ca²⁺ to the ATPase can be calculated from Scheme 1 as described in Henderson et al. [7] and Lee et al. [10]. The increase in the equilibrium constant E1/E2 in the presence of polyarginine described above will result in an increase in affinity for Ca²⁺, as the E1 conformation of the ATPase has high-affinity binding sites for Ca²⁺ whereas E2 does not [7]. As shown in Figure 7, the calculated shift in Ca²⁺ affinity (0.23 pCa unit) is in good agreement with the experimentally determined shift.

Rate of Ca²⁺ binding

The time-dependence of the change in tryptophan fluorescence intensity observed when the ATPase incubated in EGTA is mixed with excess Ca²⁺ at pH 7.2 fits the sum of two exponentials, the slow phase, of larger amplitude than the fast phase, being attributable to the E2 $\rightarrow$ E1 transition and the fast phase to rapid binding of Ca²⁺ to that fraction of the ATPase initially present in the E1 conformation [1,10]. In the presence of spermine or polyarginine the rate of binding of Ca²⁺ is observed to increase, with the data giving a good fit to a single exponential (Figures 2b and 3b). For ease of comparison, the rate constant for the best single exponential fit for the data in the absence of spermine or polyarginine is also plotted in Figures 2(b) and 3(b). Rates of Ca²⁺ binding at pH 6.0 were lower than those at pH 7.2, and the

Figure 8 Displacement of Tb^{3+} from the Ca^{2+} -ATPase by Mg^{2+} and polycationic species

The fluorescence intensity for Tb^{3+} observed at 546 nm with excitation at 294 nm in the presence of sarcoplasmic reticulum (0.1 mg of protein/ml) is shown, corrected for the intensity observed in the absence of sarcoplasmic reticulum: $\circ$, in buffer; $\square$, +100 mM Mg^{2+} ; $\triangle$, +10 mM spermine; ∇ , +5 μM polyarginine; $\diamond$, +25 μM PLN-(1-25). The buffer was 2 mM Mops/KOH, pH 7.2, containing 100 mM KCl. The lines are simulations in terms of two binding sites present in the proportions 2:1, as described in the text.

Figure 9 Displacement of Ruthenium Red from the Ca^{2+} -ATPase by Mg^{2+} and polycationic species

The decrease in tryptophan fluorescence intensity on addition of Ruthenium Red to the ATPase (2.5 μg of protein/ml) is shown: $\circ$, in buffer; $\square$, +200 mM Mg^{2+} ; $\triangle$, +10 mM spermine; ∇ , +5 μM polyarginine; $\diamond$, +25 μM PLN-(1-25). The buffer was 75 mM Mops/Tris, pH 7.2, containing 0.3 mM EGTA. Fluorescence was excited at 290 nm and observed at 340 nm.

effects of spermine or polyarginine were small (Figures 2b and 3b). PLN-(1-25), at concentrations up to 25 μM , had no effect on the rate of Ca^{2+} -binding to the ATPase at pH 7.2.

The rate constants for the dissociation of Ca^{2+} given in Table 2, together with the binding constants for Ca^{2+} calculated as given in Henderson et al. [7] and Lee et al. [10], give rate constants that describe binding of Ca^{2+} to the ATPase. The observed increase in rate of binding of pH 7.2 in the presence of spermine or polyarginine with no effect at pH 6.0 can be simulated assuming an increase in the rate constant $\text{E2} \rightarrow \text{E1}$ for the non-protonated species of 10-fold for spermine and 3-fold for polyarginine with no effect on the rate constant for the same transition for the protonated species ($\text{E2H} \rightarrow \text{E1H}$) (Table 2); the simulations in the presence of spermine and polyarginine give good fits to single exponentials, with the rates plotted in Figures 2(b) and 3(b).

Displacement of Tb^{3+} and Ruthenium Red

Girardet et al. [29] have shown that the fluorescence of Tb^{3+} bound to the ATPase can be excited by energy transfer from the tryptophan residues of the ATPase. It was reported that the intensity of Tb^{3+} fluorescence was unaffected by Ca^{2+} in the micromolar range but was reduced by millimolar concentrations of Mg^{2+} [29]. The curve of Tb^{3+} fluorescence against Tb^{3+} concentration (Figure 8) does not fit to a single binding site, but can be stimulated assuming the presence of two sites, one of high and one of low affinity, present in the relative proportions 1:2. In the absence of Mg^{2+} , calculated K_d values for the two sites are 50 and 250 μM (Figure 8). In the presence of 100 mM Mg^{2+} , the K_d values for the two sites increased to 1.25 mM, whereas in the presence of 10 mM spermine, the affinity of the high-affinity Tb^{3+} site was unaffected but that of the low-affinity site increased to 1.25 mM (Figure 8). The presence of 25 μM PLN-(1-25) or 5 μM polyarginine had no significant effect on Tb^{3+} binding (Figure 8).

Moutin et al. [11] have shown that Ruthenium Red binding to the ATPase can be monitored by the decrease in tryptophan fluorescence that follows binding, and that binding is largely prevented in the presence of 200 mM Mg^{2+} . This is confirmed by the data shown in Figure 9, which also shows that 10 mM spermine also largely prevented Ruthenium Red binding to the ATPase, whereas 5 μM polyarginine had only a very small effect and 25 μM PLN-(1-25) had no detectable effect.

DISCUSSION

The rate of dissociation of Ca^{2+} from Ca^{2+} -ATPase has been shown to be increased by the binding of Mg^{2+} or K^{+} and decreased by the binding of H^{+} [1,9,10]. It has been proposed that these effects indicate binding at a gating site on the ATPase, according to Scheme 2 [1,7,10]. It has been further proposed that the fluorescence intensity of DMC-labelled ATPase is sensitive to binding at the gating site, and that binding constants for Mg^{2+} , H^{+} and K^{+} can be obtained from analysis of fluorescence titrations [1,10].

The activity of Ca^{2+} -ATPase is affected by binding of a variety of polycationic species. Spermine, polyarginine and the strongly basic hydrophilic domain of phospholamban, PLN-(1-25), inhibit the ATPase [16,17,30] by decreasing the rate of the Ca^{2+} -transport step ($\text{E1PCa}_2 \rightarrow \text{E2P}$) [16,17]. Inhibition shows some specificity, as polylysine has much less effect than polyarginine [16,30]. Spermine, spermidine and Ruthenium Red also inhibit phosphorylation of Ca^{2+} -ATPase by P_i by competing with the Mg^{2+} essential for the reaction [16,31,32], but Ruthenium Red has no effect on the rate of ATP hydrolysis [11,33]. Polyarginine and PLN-(1-25) have no effect on phosphorylation of the ATPase by P_i ([17]; G. Hughes, Y. M. Khan, J. M. East and A. G. Lee, unpublished work). The inhibition of ATPase activity by spermine cannot involve interaction with negatively charged lipid in the membrane, as inhibition is observed for the ATPase reconstituted into bilayers of dioleoylphosphatidylcholine [34].

Ca^{2+} -ATPase consists of two main cytoplasmic regions connected by a narrow stalk to ten transmembrane α -helices [3,35]. The stalk region is predicted to contain a large number of negatively charged residues [3], and X-ray-diffraction studies have located binding sites for lanthanides in this region of the ATPase, as well as in the lipid headgroup region of the two bilayer surfaces [15]. Efficient quenching of the fluorescence of tryptophan residues in the ATPase by Ruthenium Red is consistent with binding of Ruthenium Red in the stalk region of the ATPase [11]. Binding of Ruthenium Red has been shown to be competitive with binding of Mg^{2+} and Tb^{3+} [11,12], and

binding of Tb³⁺ to the ATPase has been shown to be competitive with Mg²⁺ [29,36].

The binding curve for Tb³⁺ is consistent with binding at two sites on the ATPase, with affinities of 50 and 250 μ M (Figure 8). The affinities of both of these sites are reduced in the presence of Mg²⁺, but spermine only decreases the affinity of the low-affinity site; polyarginine and PLN-(1–25) have no effect on Tb³⁺ binding (Figure 8). Binding of Ruthenium Red is competitive with binding of Mg²⁺ or spermine, but again polyarginine and PLN-(1–25) have little effect (Figure 9). These results suggest that binding of spermine to Ca²⁺-ATPase is less specific than binding of polyarginine or PLN-(1–25); the observed competition between spermine and Tb³⁺ and Ruthenium Red suggests that one of the binding sites for spermine on Ca²⁺-ATPase may be the lanthanide-binding site in the stalk region of the ATPase.

Both spermine and polyarginine increase the rate of dissociation of Ca²⁺ from the ATPase (Figures 1–3). The effects of Mg²⁺ on the fluorescence intensity of DMC-labelled ATPase are competitive with spermine and polyarginine (Figure 4 and results not shown), consistent with Scheme 2, with binding constants for spermine and polyarginine given in Table 1. The binding of polyarginine itself to DMC-labelled ATPase results in a decrease in fluorescence intensity, as for Mg²⁺, these data also being consistent with Scheme 2 (Figure 5). Although binding of spermine to DMC-labelled ATPase results in a decrease in fluorescence intensity, the magnitude of the change (2% at 20 mM spermine) is too small to allow an accurate determination of a binding constant for spermine. PLN-(1–25) has no effect on the rate of dissociation of Ca²⁺ from the ATPase (results not shown) and no effect on the Mg²⁺-dependence of the fluorescence of DMC-labelled ATPase (Figure 4). We conclude that both spermine and polyarginine bind to the gating site in competition with Mg²⁺, but that PLN-(1–25) is unable to bind to this site.

Spermine, polyarginine and PLN-(1–25) all bind more strongly to the E1 conformation of ATPase than to the E2 conformation, increasing the equilibrium constant E1/E2 (Figure 5; Table 1). This results in a small increase in affinity for Ca²⁺ (Figure 7). A corresponding increase in the rate of the E2 $\rightarrow$ E1 transition (Table 2) would explain the observed increase in the rate of Ca²⁺ binding to the ATPase which is dependent on the rate of this transition (Figures 2b and 3b). Interaction of phospholamban itself with Ca²⁺-ATPase results in a decrease in apparent affinity for Ca²⁺ [37], presumably involving an interaction of the hydrophobic domain of phospholamban with the ATPase.

As spermine, polyarginine and PLN-(1–25) all decrease the rate of the Ca²⁺-transport step (E1PCa₂ $\rightarrow$ E2P) [16,17] and all increase the equilibrium constant E1/E2 (Table 1), it is possible that the site affecting the E2–E1 equilibrium and the site affecting the Ca²⁺-transport step are the same. This site may therefore be the region around Lys-400 in the phosphorylation domain of Ca²⁺-ATPase which has been suggested to be involved in interaction with phospholamban [38,39]. As PLN-(1–25) does not affect the rate of Ca²⁺ dissociation from the ATPase or the Mg²⁺-dependence of DMC fluorescence, the gating site controlling access to the Ca²⁺-binding sites is probably a separate site on the ATPase, showing more specificity in binding cationic species. The lack of displacement of Tb³⁺ or Ruthenium Red by polyarginine (Figures 8 and 9) means that the gating site cannot be the binding site for lanthanides and Ruthenium Red located in the stalk region. Finally, the ability of spermine to displace Tb³⁺ and Ruthenium Red (Figures 8 and 9) suggests that spermine can bind in the stalk region at the site located by Asturias and Blasie

[15], the selectivity of the site possibly being related to the size of the cationic species.

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