Effects of K^+ on the binding of Ca^{2+} to the Ca^{2+}-ATPase of sarcoplasmic reticulum

Anthony G. LEE, Keren BAKER, Yamin M. KHAN and J. Malcolm EAST
Department of Biochemistry and Institute of Biomolecular Sciences, University of Southampton, Southampton SO9 3TU, Hants. U.K.

Equilibrium and kinetic fluorescence methods have been used to characterize the interactions between K^+ and the Ca^{2+}-ATPase of skeletal-muscle sarcoplasmic reticulum. K^+ shifts the E2–E1 equilibrium of the ATPase towards E1 and increases the rate of Ca^{2+} binding to the ATPase, as detected by changes in tryptophan fluorescence intensity, suggesting that K^+ increases the rate of the E2–E1 transition. The data are consistent with binding of K^+ at the inner Ca^{2+}-binding site on the ATPase in competition with H^+ and Mg^{2+}, with a higher affinity in the E1 than in the E2 conformation. K^+ has no effect on the affinity for Mg^{2+}, as detected by changes in tryptophan fluorescence intensity; since it has been proposed that the changes in tryptophan fluorescence follow from binding to Mg^{2+} at the outer Ca^{2+}-binding site, this suggests that K^+ is unable to bind at the outer Ca^{2+}-binding site. K^+ increases the rate of dissociation of Ca^{2+} from the Ca^{2+}-bound ATPase and reduces the effect of Mg^{2+} on the fluorescence intensity of the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin. It is suggested that these effects of K^+ are the result of binding at a 'gating' site on the ATPase, in competition with binding of H^+. Binding of K^+ at the inner Ca^{2+}-binding site and at the gating site account for the observed effects of K^+ on the affinity of the ATPase for Ca^{2+}.

INTRODUCTION

The concentration of K^+ in the cytoplasmic space of muscle has been estimated to be 160–175 mM (Sreter 1963). The presence of K^+ has a marked effect on the rate of accumulation of Ca^{2+} into the lumen of the sarcoplasmic reticulum (SR) of skeletal muscle (Duggan, 1977). At saturating concentrations of Ca^{2+}, K^+ in the millimolar range increases the rate of hydrolysis of ATP by Ca^{2+}-ATPase (Shigekawa and Pearl, 1976) whereas at less than saturating concentrations of Ca^{2+}, K^+ can inhibit the ATPase (Ribeiro and Vianna, 1978).

A major effect of K^+ on the ATPase is to increase the rate of dephosphorylation of the phosphorylated form E2P, which largely accounts for the observed stimulation of the steady-state rate of ATP hydrolysis at saturating Ca^{2+} concentrations (Shigekawa and Pearl, 1976; Chaloub and de Meis, 1980). K^+ has also been reported to increase the rate of the $E1PCa_2 \rightarrow E2PCa_2$ transition (Shigekawa et al., 1978; Medda et al., 1987). Medda et al. (1987) reported that K^+ produced a small increase in the rate of phosphorylation of the ATPase on simultaneous addition of Ca^{2+} and ATP to the Ca^{2+}-free ATPase, which they attributed to an increase in the rate of the E2 → E1 transition. However, Wang et al. (1981) saw no effect of K^+ in such experiments. Moutin and Dupont (1991) observed that K^+ increases the rates of change in tryptophan fluorescence intensity observed on addition of Ca^{2+} to ATPase incubated with EGTA or on addition of EGTA to ATPase incubated with Ca^{2+}, suggesting an effect of K^+ on the rates of the Ca^{2+}-binding steps. Orlowski and Champeil (1991) have shown that K^+ increases the rate of dissociation of $^{45}Ca^{2+}$ from the ATPase.

A study of Na^+ binding to SR using ^{23}Na n.m.r. indicated a number of binding sites for univalent ions on the Ca^{2+}-ATPase (Timonin et al., 1991). Na^+ binding was detected at sites with high affinity for Ca^{2+} (presumably the Ca^{2+}-binding sites on the ATPase), at Mg^{2+}-sensitive sites and at Mg^{2+}-insensitive sites; affinities of these sites for Na^+ were about 10 mM (Timonin et al., 1991). Kinetic studies have also identified a site at which K^+ and Mg^{2+} appear to compete (Ribeiro and Vianna, 1978) and K^+ binding at the Ca^{2+}-binding site(s) is suggested by the observed reduction in Ca^{2+} affinity observed at high K^+ concentrations (Scofano et al., 1985).

In previous papers (Henderson et al., 1994a,b), we have used a combination of equilibrium and stopped-flow fluorescence methods to characterize the E2 ⇄ E1 transition for the ATPase and the process of Ca^{2+} binding. The fluorescence of the ATPase labelled with 4-nitrobenzo-2-oxa-1,3-diazole (NBD) is sensitive to the E2–E1 transition (Wakabayashi et al., 1990a,b; Wictome et al., 1992; Henderson et al., 1994a). The E2–E1 transition can also be detected by observing changes in the fluorescence of the ATPase labelled with fluorescein isothiocyanate (FITC) (Pick, 1982; Pick and Karlish, 1982; Froud and Lee, 1986), but the intrinsic pH sensitivity of the fluorescence of the fluorescein group (Froud and Lee, 1986) makes this a less convenient probe. The equilibrium has been found to be pH sensitive, low pH favouring the E2 state because of binding of H^+ at a site with higher affinity in the E2 than in the E1 conformation (Henderson et al. 1994a) (see Figure 1).

A sequential mechanism for Ca^{2+} binding to the ATPase has been established by studies of the rate of dissociation of $^{45}Ca^{2+}$ from the Ca^{2+}-bound ATPase; washing the Ca^{2+}-bound ATPase with $^{40}Ca^{2+}$ allowed a distinction to be made between the first, inner, Ca^{2+} bound to the ATPase and the second, outer, Ca^{2+}. Dissociation of the inner Ca^{2+} was inhibited by the presence of $^{40}Ca^{2+}$ in the medium whereas dissociation of the outer Ca^{2+} was not (Dupont, 1982; Inesi, 1987; Orlowski and Champeil, 1991; Henderson et al., 1994b), consistent with Scheme 1. Binding of Ca^{2+} to the ATPase can be studied by following changes in tryptophan fluorescence of the ATPase (Dupont and Leigh, 1978; Guillain et al., 1980, 1981; Verjovski-Almeida and Silva, 1981; Champeil et al., 1983; Fernandez-Belda et al., 1984; Scofano et al., 1985; Moutin and Dupont, 1991). The observed changes fit Scheme 1, with the change in tryptophan fluorescence

Abbreviations used: DMC, 4-(bromomethyl)-6,7-dimethoxycoumarin; FITC, fluorescein isothiocyanate; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; SR, sarcoplasmic reticulum.

$$E2 \rightleftarrows E1 \rightleftarrows E1Ca \rightleftarrows E1'Ca \rightleftarrows E1'Ca_2$$

Scheme 1

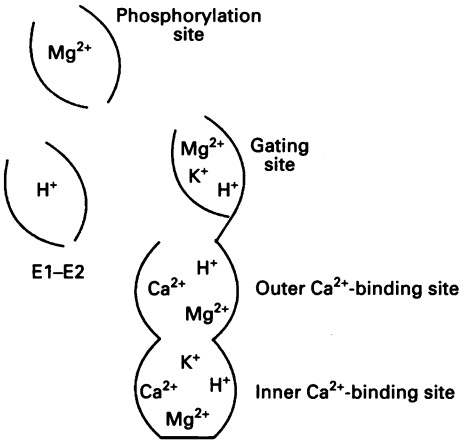


Figure 1 Schematic diagram of the Ca^{2+}-, Mg^{2+}- and K^+-binding sites on Ca^{2+}-ATPase

It is proposed that H^+, K^+, Ca^{2+} and Mg^{2+} compete for binding at the inner of the two Ca^{2+}-binding sites, whereas K^+ does not bind at the outer Ca^{2+}-binding site. K^+ can also bind at the 'gating' site in competition with binding of Mg^{2+} and H^+; binding at the gating site affects the affinity of the ATPase for Ca^{2+} and the rate of binding and dissociation of Ca^{2+} (Henderson et al., 1994a). Protonation of a further site on the ATPase affects the E2–E1 equilibrium (Henderson et al., 1994a).

occurring at the $E1Ca \rightleftarrows E1'Ca$ transition (Henderson et al., 1994a).

Fluorescence of the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin (DMC) is sensitive to the binding of Mg^{2+}, in a pH-dependent manner (Stefanova et al., 1992; Henderson et al., 1994a). The pH- and Mg^{2+}-dependence of fluorescence matches the effects of pH and Mg^{2+} on the rate of dissociation of Ca^{2+} from the ATPase, and thus it was suggested that the binding site affecting DMC fluorescence was the site affecting Ca^{2+} dissociation, referred to as the gating site (Henderson et al., 1994b) (Figure 1).

Here we show that the effects of K^+ on the E2–E1 equilibrium and on the rates of binding and dissociation of Ca^{2+} can be readily interpreted in terms of the model for Ca^{2+} binding shown in Figure 1.

MATERIALS AND METHODS

SR from rabbit skeletal muscle and purified Ca^{2+}-ATPase were prepared as described in Stefanova et al. (1992). The ATPase was labelled with Br-DMC or NBD as described by Henderson et al. (1994a). Equilibrium fluorescence measurements were performed at 25 °C using an SLM Aminco 8000C fluorimeter (Henderson et al., 1994a). All measured fluorescence-intensity changes were corrected for dilution. Rapid-kinetic fluorescence measurements were performed using a Biologic SFM-3 fluorimeter with BioKine V3.20a software for data acquisition and analysis (Henderson et al., 1994a). 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).

Simulations of Ca^{2+} dissociation were performed on an IBM-compatible PC using either the program M30 of Zhang et al. (1989) or FACSIMILE v 3.0 (AEA Technology, Harwell, Oxfordshire, U.K.). Simulations were analysed as single or double exponentials using the BioKine software also used for analysis of the experimental data. In all simulations it was assumed that H^+ and K^+ binding were fast compared with the rates of Ca^{2+} binding and dissociation so that binding of H^+ and K^+ could be treated by the quasiequilibrium approach (Cha, 1968). Free concentrations of Ca^{2+} were calculated using the binding constants for Ca^{2+}, Mg^{2+} and H^+ to EGTA given by Godt (1974).

RESULTS AND DISCUSSION

NBD fluorescence

It has been shown that the fluorescence of NBD-labelled ATPase is high in the E1 conformation and low in the E2 conformation (Wakabayashi et al., 1990a; Henderson et al., 1994a). As shown in Figure 2(a), the fluorescence intensity of the NBD-labelled ATPase increases with increasing pH (Henderson et al., 1994a). The data are 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

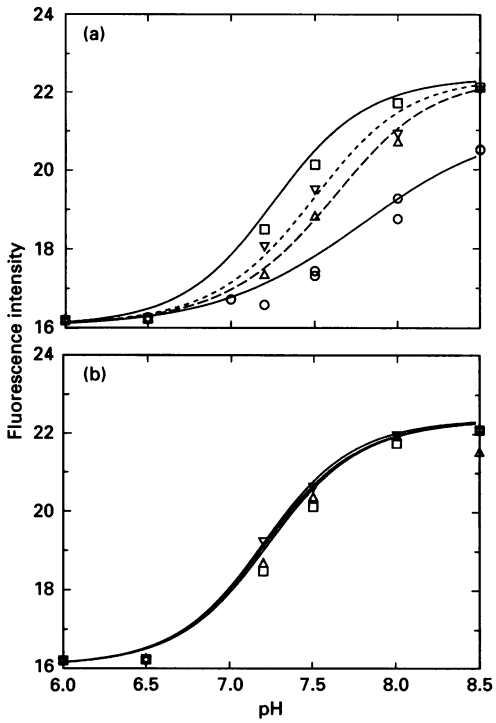


Figure 2 Effect of K^+ on the fluorescence intensity of NBD-labelled ATPase, as a function of pH and Mg^{2+} concentration

NBD-labelled ATPase was incubated in buffer containing 0.3 mM EGTA: (**a**) in buffer alone (○), or in the presence of 5 mM Mg^{2+} (□), 100 mM KCl (△) or 200 mM KCl (▽); (**b**) in 5 mM Mg^{2+} and 0 (□), 100 (△) or 200 (▽) mM KCl. The curves are simulations calculated using the parameters in the text, assuming fluorescence intensities of 16.0 and 22.5 for E2 and E1 forms respectively. Buffers were Mes/Tris for pH 6.0–6.5, Mops/Tris for pH 7.0–7.5 and Tris/Mes for pH 8.0–8.5.

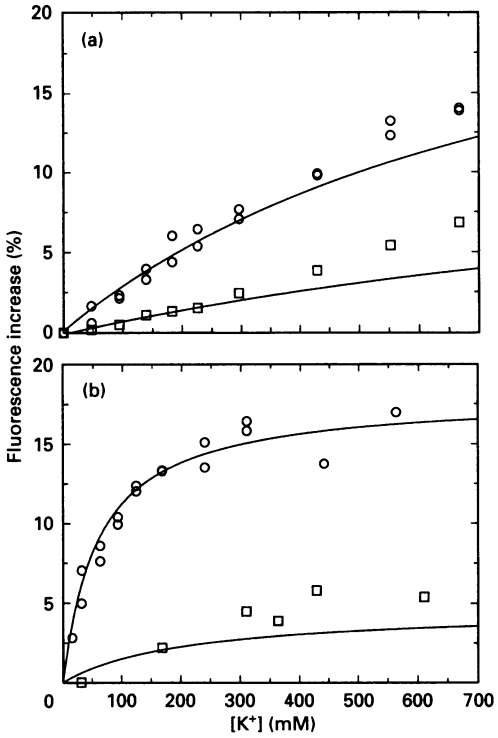


Figure 3 Effect of KCl on the fluorescence intensity of NBD-labelled ATPase

NBD-labelled ATPase was incubated in 150 mM Mops/Tris, pH 7.2 (**a**) or 50 mM Tris/Mes, pH 8.0 (**b**), containing 0.3 mM EGTA. The percentage increases in fluorescence intensity on addition of KCl to the given concentration are shown, in the absence (○) or presence (□) of Mg^{2+} at (**a**) 5 mM or (**b**) 1 mM. The curves are simulations calculated using the parameters in the text.

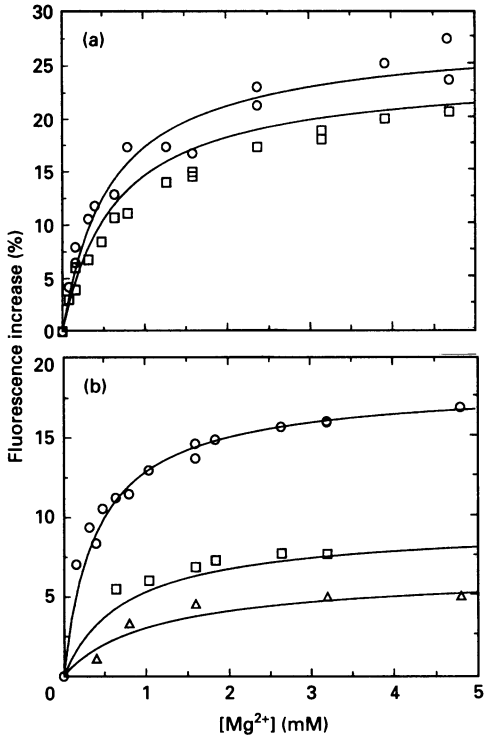


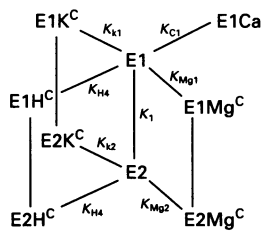
Figure 4 Effect of Mg^{2+} on the fluorescence intensity of NBD-labelled ATPase as a function of KCl concentration

NBD-labelled ATPase was incubated in 150 mM Mops/Tris, pH 7.2 (**a**) or 50 mM Tris/Mes, pH 8.0 (**b**), containing 0.3 mM EGTA. The percentage increases in fluorescence intensity on addition of Mg^{2+} to the given concentration are shown in the absence of KCl (○) or in the presence of (**a**) 100 mM KCl (□), (**b**) 50 mM KCl (□) or 100 mM KCl (△). The curves are simulations calculated using the parameters in the text.

M^{-1} in the E1 and E2 conformations respectively, and an equilibrium constant for the unprotonated forms E1/E2 of 4.0 (Henderson et al., 1994a). Addition of Mg^{2+} at acid pH values has little effect on NBD fluorescence, but results in a marked increase in fluorescence intensity at more alkaline pH values (Figure 2a). Addition of K^+ at pH values above 7.0 also results in a significant increase in NBD fluorescence, whereas at more acid pH values, any effects are very small (Figure 2a). The observed effects of K^+ are much smaller in the presence of Mg^{2+} than in its absence (Figure 2b), so that the effects of K^+ and Mg^{2+} are not simply additive.

The effect of K^+ on NBD fluorescence is shown as a function of K^+ concentration at pH 7.2 and 8.0 in Figure 3. The data at pH 7.2 and 8.0 in the absence of Mg^{2+} fit a simple binding equation for K^+, with apparent K_d values of 1100 ± 300 and 64 ± 15 mM respectively. As shown, the effect of K^+ is much reduced in the presence of Mg^{2+}, particularly at pH 8.0. Figure 4 shows the effects of Mg^{2+} on NBD fluorescence at pH 7.2 and 8.0 in the absence or presence of K^+. At pH 7.2, the data fit a simple binding equation for Mg^{2+} with apparent K_d values of 6.3 ± 0.8 and 8.7 ± 1.4 mM in the absence of K^+ and in the presence of 100 mM KCl respectively. At pH 8.0, the data in the absence of K^+ fit a K_d of 0.48 ± 0.02 mM; the effect of Mg^{2+} is much reduced in the presence of K^+, and changes are too small to give reliable estimates for the Mg^{2+}-binding constant.

The effect of Mg^{2+} on the E2–E1 equilibrium has been shown to be consistent with binding of Mg^{2+} in competition with H^+ at the inner Ca^{2+}-binding site, with a higher affinity for the E1 conformation than for the E2 conformation, according to Scheme 2, with values of K_{H4}, K_{Mg1} and K_{Mg2} of 5.0×10^9 M^{-1}, 2.4×10^5 M^{-1} and 1.5×10^4 M^{-1} respectively (Henderson et al., 1994a). The observed competition between K^+ and H^+ and the reduced effect of K^+ in the presence of Mg^{2+} suggests that the effect of K^+ could also follow from binding at this site (Scheme 2). As shown in Figures 2–4, the data are consistent with this Scheme, with binding constants for K^+ to E1 and E2 of 1600 and 100 M^{-1} respectively (Table 1). The binding constant for E2 must be less than for E1 to account for the shift to E1 on binding K^+; the exact value has little effect on these calculations, but was



Scheme 2

Superscripts c and g denote binding at the inner Ca^{2+}-binding site and at the gating site respectively.

Table 1 Equilibrium constants describing the effects of K^+ on the Ca^{2+}-ATPase

The superscripts c and g denote binding of K^+ at the inner Ca^{2+}-binding site and at the 'gating' site respectively (see Figure 1).

Reaction step	Equilibrium constant symbol	Value (m^{-1})
$E1 + K \rightarrow E1K^c$	K_{K1}	1600
$E2 + K \rightarrow E2K^c$	K_{K2}	100
$E1 + K \rightarrow E1K^g$	K_{K3}	60

* The same value applies for K binding to the 'gating' site on E2 and to Ca^{2+}-bound and Ca^{2+}-free forms of E1 (see Scheme 3).

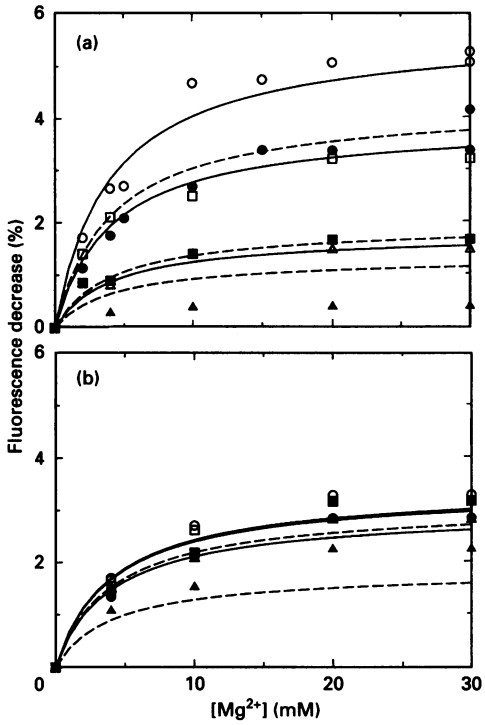


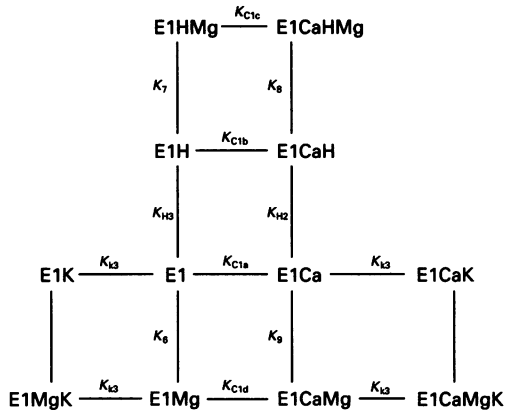
Figure 5 Effect of K^+ on the fluorescence response of DMC-labelled ATPase to Mg^{2+} as a function of pH, in the absence and presence of Ca^{2+}

DMC-labelled ATPase was incubated in 40 mM Tris/maleate containing 1 mM EGTA, at pH 6.0 (○, ●), 7.0 (□, ■) or 8.0 (△, ▲) in the absence (open symbols) or presence (filled symbols) of 100 mM KCl, in the presence of 1 mM EGTA (**a**) or 1 mM Ca^{2+} (**b**). The solid and broken lines are simulations in the absence and presence of 100 mM KCl respectively calculated by using parameters in Table 1 and Henderson et al. (1994a).

chosen to be consistent with the observed effects of K^+ on the rate of the E2–E1 transition (see below).

Identical effects of K^+ on the E2–E1 equilibrium are observed for the purified ATPase and for SR vesicles (results not shown), consistent with exposure of the binding site to the cytoplasm in both the E1 and E2 conformations, although in this case the presence of K^+ channels in the SR membrane (Gray et al, 1988) could allow access of K^+ to the lumenal face of the ATPase.

Attempts to study the effect of K^+ on the E2–E1 transition by following changes in the fluorescence intensity of the ATPase labelled with FITC were not successful. Addition of 100 mM



Scheme 3

choline chloride to FITC-labelled ATPase at pH 7.2 led to a 6% increase in fluorescence intensity, in the presence or absence of Ca^{2+} (results not shown). This change, attributed to the change in ionic strength, was of comparable magnitude to that seen on addition of Ca^{2+} to the ATPase, and any effects of K^+ and Mg^{2+} were considerably smaller than this (results not shown).

DMC fluorescence

The fluorescence of DMC-labelled ATPase decreases on addition of Mg^{2+} (Stefanova et al., 1992; Henderson et al., 1994a). The magnitude of the effect decreases with increasing pH but the concentration of Mg^{2+} causing half-maximal changes in fluorescence intensity (2–4 mM) remains constant (Figure 5a). The effects of Mg^{2+} are consistent with Scheme 3, with the decrease in intensity of fluorescence on binding Mg^{2+} to the unprotonated form (E1) being 20% of that resulting from binding to the protonated form (E1H). The effects of pH are less marked in the presence of Ca^{2+}, consistent with a stronger binding of H^+ to the Ca^{2+}-bound form of the ATPase ($K_{H2} > K_{H3}$ in Scheme 3) (Henderson et al., 1994a). As shown in Figure 5, the effects of Mg^{2+} are consistent with binding constants for $K_6 = K_7 = K_8 = K_9$ of 250 M^{-1} and K_{H2} and K_{H3} of 5×10^8 and 1.25×10^7 M^{-1} respectively.

The effect of K^+ in the absence of Ca^{2+} is to reduce the magnitude of the response to the addition of Mg^{2+}, with no significant effect on the concentration-dependence of the effect (Figure 5a). In the presence of Ca^{2+}, the effect of K^+ is reduced, but addition of K^+ results in no observable change in the affinity of Mg^{2+}. The effects of K^+ are therefore comparable with those of H^+. The data are consistent with Scheme 3, with a value for K_{K3} of 60 M^{-1} (Table 1). Measurements of the decrease in fluorescence intensity caused by 20 mM Mg^{2+} at pH 6.0 in the absence of Ca^{2+} as a function of K^+ concentration were also consistent with a value for K_{K3} of 60 M^{-1} (results not shown).

Rates of Ca^{2+} binding and dissociation

Binding of Ca^{2+} to the ATPase results in an increase in tryptophan fluorescence intensity when fluorescence is excited at 290 nm and observed at 340 nm (Dupont et al., 1988). The fluorescence response closely follows occupancy of the Ca^{2+}-binding sites on the ATPase, and it has been shown that this is consistent with Scheme 1, with the tryptophan fluorescence response corresponding to the conformational change $E1Ca \rightleftarrows E1'Ca$ (Henderson et al., 1994a).

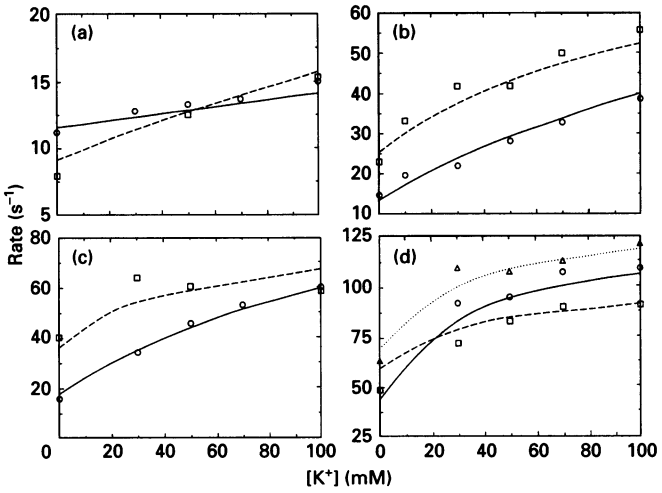


Figure 6 Effect of K^+ on the rates of fluorescence change caused by Ca^{2+} dissociation from the ATPase

In the stopped-flow experiment, the enzyme syringe contained 0.4 mg/ml ATPase in buffer containing the required concentrations of KCl and Mg^{2+} and 100 μM Ca^{2+}, and was mixed with an equal volume of the same buffer but containing 5 mM EGTA: (**a**) pH 6.0 (130 mM Mes, 50 mM Tris); (**b**), pH 7.2 (150 mM Mops, 80 mM Tris); (**c**), pH 7.5 (150 mM Mops, 124 mM Tris); (**d**), pH 8.5 (27 mM Mes, 100 mM Tris). Buffers contained KCl at the given concentrations, and 0 (○), 1 (△) or 10 (□) mM Mg^{2+}. Data were fitted to single exponentials and the calculated rates are plotted as a function of K^+ concentration. The lines show variation in rate with K^+ obtained when theoretical simulations calculated using the parameters in Tables 1 and 2 are fitted in the same way to single exponentials. Solid dotted and broken lines correspond to 0, 1 and 10 mM Mg^{2+} respectively.

Table 2 Effects of K^+ on rate constants affecting Ca^{2+} dissociation from the ATPase

The effects of binding of H^+, Mg^{2+} and K^+ at the gating site on the rates of dissociation of Ca^{2+} from the ATPase are shown. The superscript c denotes binding of K^+ at the inner Ca^{2+} binding site; k_{1f}, the pH-dependent effective rate for the transition between E2 and E1, is calculated as described in Henderson et al. (1994b).

Reaction step	Off rate (s^{-1})
E1′Ca_2HMg → E1′CaHMg	13
E1′Ca_2H → E1′CaH	29
E1′Ca_2 → E1′Ca	300
E1′Ca_2Mg → E1′CaMg	2700
E1′Ca_2K → E1′CaK	450
E1′Ca_2KMg → E1′CaKMg	675
E1′CaHMg → E1CaHMg	60
E1′CaH → E1CaH	150
E1′Ca → E1Ca	150
E1′CaMg → E1CaMg	0
E1′CaK → E1CaK	225
E1′CaKMg → E1CaKMg	75
E1CaHMg → E1HMg	13
E1CaH → E1H	20
E1Ca → E1	225
E1CaMg → E1Mg	2000
E1CaK → E1K	600
E1CaKMg → E1KMg	4000
E2 → E1	80
E2H → E1H	4.0
E2K → E1K^c	20 × k_{1f}

Moutin and Dupont (1991) have shown that K^+ markedly increases the rate of Ca^{2+} dissociation from the ATPase, as monitored by the rate of change in tryptophan fluorescence intensity observed when the Ca^{2+}-bound ATPase is mixed with excess EGTA. The rates of change in tryptophan fluorescence intensity for the preparation of ATPase used here in the absence of K^+ are significantly faster than those reported previously (Henderson et al. 1994b), and, whereas the fluorescence curves reported by Moutin and Dupont (1991) and Henderson et al. (1994b) at pH 7.2 were not single exponential, for the preparation used here the data fitted well to single exponentials under all conditions tested (Figure 6). Orlowski and Champeil (1991) also have reported significant differences between preparations of Ca^{2+}-ATPase in the rates of $^{45}Ca^{2+}$ dissociation and in the rates of tryptophan fluorescence change.

The non-exponential character of the decays observed in Henderson et al. (1994b) was attributed to a significantly lower rate for the E1Ca → E1 transition that for the E1′Ca_2 → E1′Ca transition. However, if the rates controlling the E1Ca → E1 transition (Table 2) are increased by a factor of 1.5–2.0 compared with those given in Henderson et al. (1994b), then single exponential decays are obtained, with rates that agree well with the experimentally determined rates at pH values between 6 and 8.5, in the presence or absence of Mg^{2+} (Figure 6).

In Henderson et al. (1994b) it was shown that the effects of Mg^{2+} and pH on the rates of Ca^{2+} dissociation were consistent with binding to a single site, referred to as the 'gating' site (Scheme 3). Binding constants for H^+ and Mg^{2+} at this site were obtained from measurements of fluorescence changes for DMC-labelled ATPase (Henderson et al., 1994a). In Henderson et al. (1994b) it was shown that the rate of dissociation of Ca^{2+} from the Ca^{2+}-bound ATPase increased with increasing pH and increased on binding Mg^{2+}, consistent with a faster rate of dissociation from species of the ATPase without H^+ and with Mg^{2+} at the gating site (Table 2). The effects of K^+ on rates (Figure 6) are also consistent with Scheme 3, with the binding constants determined as described above from experiments with DMC-labelled ATPase (Table 1), and with faster rates of Ca^{2+} dissociation from K^+-bound forms than from protonated forms (Table 2). In general, the rates proposed for the K^+-bound forms are within a factor of 4 of those for the corresponding unprotonated forms (Table 2).

The rate of the tryptophan fluorescence change on binding Ca^{2+} at pH 7.2 is increased by K^+, both in the presence and absence of Mg^{2+}. At pH 7.2, the fluorescence curves fit to the sum of two exponentials (Henderson et al. 1994b). In the absence of K^+, the magnitude and rate of the fast component stay constant at about 17 % and 100 s^{-1} (the rates are at the limit of detection), but the rate of the slow component increases with increasing K^+ concentration (Figure 7). In the presence of 10 mM Mg^{2+} at pH 7.2, the magnitude and rates of the fast component are constant at about 42 % and 80 s^{-1} respectively, and again the rate of the slow component increases with increasing K^+ concentration (Figure 7).

The slow phase is largely attributable to the E2 → E1 transition and the fast phase to rapid binding of Ca^{2+} to that fraction of the ATPase initially present in the E1 conformation (Henderson et al., 1994b). The observed increase in the rate of the slow component (Figure 7) then implies that K^+ increases the rate of the E2 → E1 transition. The magnitude of the effect will depend on the binding constant for K^+ to E2 and on the rate of the E2K → E1K transition compared with that of the E2 → E1 transition in the absence of K^+. The dependence of the rate of the slow component of Ca^{2+} binding on K^+ concentration (Figure 7) is consistent with a binding constant of 100 M^{-1} for E2 (Table 1)

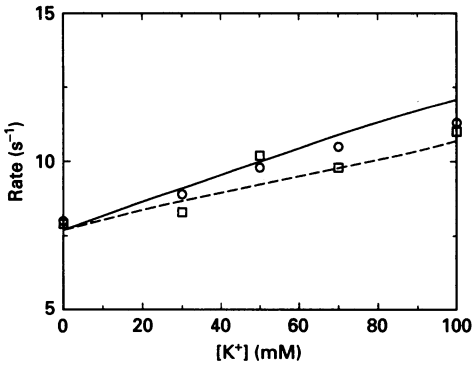


Figure 7 Effect of K^+ on the rate of the slow component of the increase in tryptophan fluorescence caused by Ca^{2+} binding

The ATPase (0.4 mg/ml) in buffer at pH 7.2 (150 mM Mops, 80 mM Tris) containing 0.2 mM EGTA was mixed in a 1:1 ratio with the same buffer containing 1 mM Ca^{2+} and the increase in tryptophan fluorescence recorded. Data were fitted to the sum of two exponentials. The rates of the slow component obtained in the presence of 0 (○) or 10 (□) mM Mg^{2+} are plotted. The solid and broken lines show variation in rate of the slow component as a function of K^+ in the absence and presence respectively of 10 mM Mg^{2+}, obtained when theoretical simulations calculated using the parameters in Tables 1 and 2 are fitted in the same way to the sum of two exponentials.

Table 3 Rates describing tryptophan fluorescence changes on Ca^{2+} binding

Rates were obtained by fitting the tryptophan fluorescence curves and the simulations of Ca^{2+} binding at pH 6.0 at the given KCl concentrations in the absence or presence of 10 mM Mg^{2+} to a single exponential.

	Experimental		Simulation	
KCl (mM)	$[Mg^{2+}] = 0$	$[Mg^{2+}] = 10$ mM	$[Mg^{2+}] = 0$	$[Mg^{2+}] = 10$ mM
0	4.0±0.1	3.2±0.1	3.6	3.4
30	3.3±0.1	–	3.6	3.6
50	3.7±0.1	3.4±0.1	3.6	3.7
70	4.0±0.1	–	3.6	3.7
100	4.0±0.1	3.7±0.1	3.6	3.8

and a rate for the E2K → E1K transition 20 times that for the E2 → E1 transition (Table 2). Simulations using these parameters match the experimental data well (Figure 7), with the fraction of the fast component varying between 0.1 and 0.13 with increasing K^+; the rate of the fast component is calculated as 150 s^{-1}. The data in the presence of 10 mM Mg^{2+} are also matched well by the stimulations, which give values for the fractions of the fast and slow components of 0.47–0.44 and 0.53–0.56 respectively, with a fast component whose rate varies between 81 and 78 s^{-1} with varying K^+, and a K^+-dependent rate for the slow component shown in Figure 7.

At pH 6.0, the rate of tryptophan fluorescence change on binding Ca^{2+} fits to a single exponential (Henderson et al., 1994b). The rate varies only very slightly with K^+ (Table 3). At pH 6.0, the ATPase is present predominantly in the E2 conformation (Henderson et al., 1994a) and the low rate of change in tryptophan fluorescence on binding Ca^{2+} is attributed to a low rate for the E2 → E1 transition (Henderson et al., 1994b). The lack of effect of K^+ at pH 6.0 (Table 3) is consistent with competition between K^+ and H^+ for binding to the ATPase, as in Scheme 2.

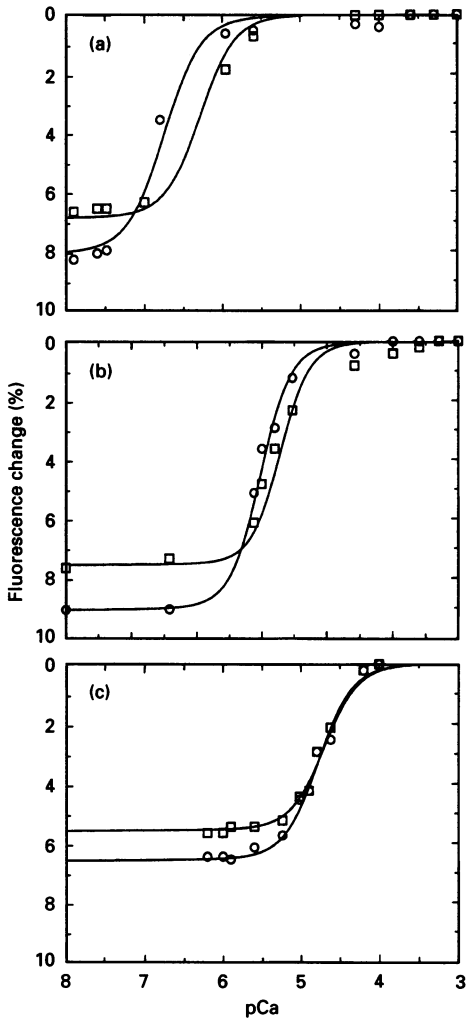


Figure 8 Ca^{2+}-dependence of tryptophan fluorescence intensity in the presence and absence of K^+

The decrease in tryptophan fluorescence intensity of the ATPase (0.7 μM) on addition of EGTA to the given pCa value is shown: (**a**), pH 8.0; (**b**), pH 7.2; (**c**), pH 6.0. ○, absence of KCl; □, presence of 100 mM KCl. The lines are simulations of Ca^{2+} occupancy of the ATPase calculated using the parameters in Henderson et al. (1994a) and the binding constants for K^+ given in Table 1, and scaled to match the observed changes in fluorescence intensity. Buffers are as given in the legend to Figure 2.

Tryptophan fluorescence

Addition of Mg^{2+} to the ATPase results in a small increase in tryptophan fluorescence intensity when fluorescence is excited at 290 nm and observed at 315 nm (Guillain et al., 1982). The data fitted to a K_d for Mg^{2+} of 3.3±0.7 at pH 7.2, and neither KCl nor choline chloride had any significant effect (results not shown). In Henderson et al. (1994b) it was suggested that the increase in tryptophan fluorescence intensity observed on addition of Mg^{2+} to the ATPase resulted from binding at the outer Ca^{2+}-binding site (Figure 1). The lack of effect of KCl therefore implies that K^+ did not bind significantly to the outer Ca^{2+}-binding site. The outer Ca^{2+}-binding site on ATPase has been suggested to have a higher affinity for Ca^{2+} than the inner site, to account for the co-operativity of Ca^{2+} binding (Henderson et al., 1994a). It is therefore of interest that it has been suggested that Na^+ binds competitively with Ca^{2+} at the low-affinity Ca^{2+}-binding sites on

troponin C, but cannot bind at the high-affinity sites (Delville et al., 1980).

Tryptophan fluorescence was excited at 290 nm and observed at 340 nm to monitor Ca^{2+} binding to the ATPase (Dupont et al., 1988). It has been shown that Ca^{2+}-binding curves determined using tryptophan fluorescence are identical with those determined using $^{45}Ca^{2+}$ in the presence or absence of Mg^{2+} or in the presence or absence of K^+ (Scofano et al., 1985; Forge et al., 1993; Henderson et al., 1994a). However, whereas non-specific binding of $^{45}Ca^{2+}$ in the absence of high concentrations of Mg^{2+} makes it difficult to determine the end points of the $^{45}Ca^{2+}$ titrations, the tryptophan fluorescence of the ATPase is unaffected by non-specific binding (Forge et al., 1993) and is therefore a more convenient method of determining Ca^{2+}-binding curves.

At pH 8.0 and 7.2 the presence of 100 mM K^+ results in an increase in the concentration of Ca^{2+} required to produce a half-maximal change in tryptophan fluorescence intensity, whereas at pH 6.0 any effects of KCl are very small (Figure 8). Addition of 200 mM choline chloride had no detectable effect on Ca^{2+} affinity at pH 7.2 (results not shown).

The proposed binding of K^+ at the inner Ca^{2+}-binding site and at the gating site predicts a decrease in affinity for Ca^{2+}. Binding of K^+ at the inner Ca^{2+}-binding site is proposed to be competitive with the binding of Ca^{2+} (Figure 1). Binding of K^+ at the gating site will also reduce the affinity for Ca^{2+}. Scheme 3 with $K_{H2} > K_{H3}$ implies stronger binding of Ca^{2+} to the protonated forms of the ATPase (E1H and E1HMg) than to the non-protonated forms (E1 and E1Mg), i.e. $K_{C1c} = K_{C1b} > K_{C1a} = K_{C1d}$. Binding of K^+ to the non-protonated form of the ATPase will therefore shift the equilibrium towards the forms of the ATPase with a lower affinity for Ca^{2+}. As shown in Figure 8 these two effects are sufficient to explain the observed shifts in the Ca^+-dependence of tryptophan fluorescence caused by K^+.

We thank Professor H. D. White for his very generous provision of the source code for the simulation program M30. We thank the SERC and Wessex Medical Trust for financial support, and the SERC for studentships (to K.B. and Y.M.K.)

REFERENCES

Cha, S. (1968) J. Biol. Chem. **243**, 820–825
Chaloub, R. M. and de Meis, L. (1980) J. Biol. Chem. **255**, 6168–6172
Champeil, P., Gingold, M. P., Guillain, F. and Inesi, G. (1983) J. Biol. Chem. **258**, 4453–4458
Delville, A., Grandjean, J., Laszlo, P., Gerday, C., Grabarek, Z. and Drabikowski, W. (1980) Eur. J. Biochem. **105**, 289–295
Duggan, P. F. (1977) J. Biol. Chem. **252**, 1620–1627
Dupont, Y. (1982) Biochim. Biophys. Acta **688**, 75–87
Dupont, Y. and Leigh, J. B. (1978) Nature (London) **273**, 396–398
Dupont, Y., Guillain, F. and Lacapere, J. J. (1988) Methods Enzymol. **157**, 206–219
Fernandez-Belda, F., Kurzmack, M. and Inesi, G. (1984) J. Biol. Chem. **259**, 9687–9698
Forge, V., Mintz, E. and Guillain, F. (1993) J. Biol. Chem. **268**, 10953–10960
Froud, R. J. and Lee, A. G. (1986) Biochem. J. **237**, 197–206
Godt, R. E. (1974) J. Gen. Physiol. **63**, 722–739
Gray, M. A., Tomlins, B., Montgomery, R. A. P. and Williams, A. J. (1988) Biophys. J. **54**, 233–239
Guillain, F., Gingold, M. P., Buschlen, S. and Champeil, P. (1980) J. Biol. Chem. **255**, 2072–2076
Guillain, F., Champeil, P., Lacapere, J. J. and Gingold, M. P. (1981) J. Biol. Chem. **256**, 6140–6147
Guillain, F. Gingold, M. P. and Champeil, P. (1982) J. Biol. Chem. **257**, 7366–7371
Henderson, I. M. J., Khan, Y. M., East, J. M. and Lee, A. G. (1994a) Biochem. J. **297**, 615–624
Henderson, I. M. J., Starling, A. P., Wictome, M., East, J. M. and Lee, A. G. (1994b) Biochem. J. **297**, 625–636
Inesi, G. (1987) J. Biol. Chem. **262**, 16338–16342
Medda, P., Fassold, E. and Hasselbach, W. (1987) Eur. J. Biochem. **165**, 251–259
Moutin, M. J. and Dupont, Y. (1991) J. Biol. Chem. **266**, 5580–5586
Orlowski, S. and Champeil, P. (1991) Biochemistry **30**, 352–361
Pick, U. (1982) J. Biol. Chem. **257**, 6111–6119
Pick, U. and Karlish, S. J. (1982) J. Biol. Chem. **257**, 6120–6126
Ribeiro, J. M. and Vianna, A. L. (1978) J. Biol. Chem. **253**, 3153–3157
Scofano, H., Barrabin, H., Inesi, G. and Cohen, J. A. (1985) Biochim. Biophys. Acta **819**, 93–104
Shigekawa, M. and Pearl, L. J. (1976) J. Biol. Chem. **251**, 6947–6952
Shigekawa, M., Dougherty, J. P. and Katz, A. M. (1978) J. Biol. Chem. **253**, 1442–1450
Sreter, F. A. (1963) Am. J. Physiol. **205**, 1295–1298
Stefanova, H. I., East, J. M., Gore, M. G. and Lee, A. G. (1992) Biochemistry **31**, 6023–6031
Timonin, I. M., Dvoryantsev, S. N., Petrov, V. V., Ruuge, E. K. and Levitsky, D. O. (1991) Biochim. Biophys. Acta **1066**, 43–53
Verjovski-Almeida, S. and Silva, J. L. (1981) J. Biol. Chem. **256**, 2940–2944
Wakabayashi, S., Imagawa, T. and Shigekawa, M. (1990a) J. Biochem. (Tokyo) **107**, 563–571
Wakabayashi, S., Ogurusu, T. and Shigekawa, M. (1990b) Biochemistry **29**, 10613–10620
Wang, T., Grassi de, G.e., Tasi, L. I. and Schwartz, A. (1981) Biochim. Biophys. Acta **637**, 523–529
Wictome, M., Michelangeli, F., Lee, A. G. and East, J. M. (1992) FEBS Lett. **304**, 109–113
Zhang, X. Z., Strand, A. and White, H. D. (1989) Anal. Biochem. **176**, 427–431

Received 11 May 1994/6 July 1994; accepted 10 August 1994