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Mechanism of inhibition of the calcium pump of sarcoplasmic reticulum by thapsigargin

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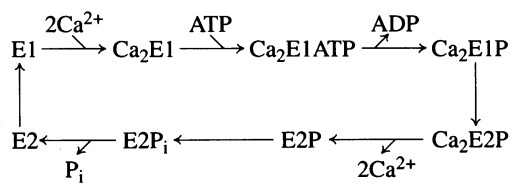
The steady-state ATPase activity of sarcoplasmic-reticulum (Ca^{2+}–Mg^{2+})-ATPase is inhibited by thapsigargin at a molar ratio of 1:1, with a dissociation constant for thapsigargin estimated to be in the sub-nanomolar range. In the presence of thapsigargin, only a single Ca^{2+} ion binds to the ATPase. Similarly, addition of thapsigargin to the ATPase incubated in the presence of Ca^{2+} results in the release of one of the two originally bound Ca^{2+} ions. As monitored by the fluorescence of nitrobenzo-2-oxa-1,3-diazole-labelled ATPase, thapsigargin appears to shift the transition between E1 and E2 conformations towards E2. Addition of thapsigargin prevents phosphorylation of the ATPase by P_i and results in a very low steady-state level of phosphorylation of the ATPase by ATP, as observed previously for nonylphenol.

INTRODUCTION

The tumour promoter thapsigargin, a sesquiterpene lactone obtained from the plant *Thapsia garganica*, is thought to exert its effects by the release of Ca^{2+} from intracellular stores (Jackson *et al.*, 1988; Scharff *et al.*, 1988). This Ca^{2+} release is believed to be the result of specific inhibition by thapsigargin of the (Ca^{2+}–Mg^{2+})-ATPase, which loads intracellular Ca^{2+} stores (Thastrup *et al.*, 1990), and it has also been reported that thapsigargin inhibits the (Ca^{2+}–Mg^{2+})-ATPase of skeletal-muscle sarcoplasmic reticulum (Sagara & Inesi, 1991).

In previous papers we have studied the effects of hydrophobic inhibitors such as nonylphenol (Michelangeli *et al.*, 1990*a*) and abnormal phospholipids such as dimyristoleoylphosphatidylcholine [(C14:1)PC] (Michelangeli *et al.*, 1990*b*, 1991) on the activity of the (Ca^{2+}–Mg^{2+})-ATPase purified from skeletal-muscle sarcoplasmic reticulum. We have shown that these effects can be understood in terms of the model for ATPase activity shown in simplified form in Scheme 1, based on that of de Meis & Vianna (1979).

The model postulates two major conformational states of the enzyme, E1 and E2. These two states differ in that the affinity for Ca^{2+} is high in the E1 conformation but low in the E2 conformation, and in that the Ca^{2+}-binding sites are exposed on the outer (cytoplasmic) side of the sarcoplasmic reticulum in E1 but exposed to the inside in E2. After binding of two Ca^{2+} ions and MgATP to E1, the enzyme is phosphorylated and undergoes a conformation change to E2P(Ca_2) from which Ca^{2+} is lost to the inside. After dephosphorylation, the ATPase is recycled. The binding of two Ca^{2+} ions to the native ATPase is normally a critical step, since only in the Ca^{2+}-bound form can the ATPase be phosphorylated by MgATP. The two Ca^{2+} ions bind in a co-operative manner, in a channel-like structure (Inesi *et al.*, 1990). However, after reconstitution of the (Ca^{2+}–Mg^{2+})-ATPase into bilayers of (C14:1)PC, we found that only a single Ca^{2+} ion bound to the ATPase (Michelangeli *et al.*, 1990*b*) and that in this form the ATPase could still be phosphorylated by MgATP, although at a slower rate than normal (Michelangeli *et al.*, 1991). Here we show that binding of thapsigargin to the ATPase also results in a change in extent of Ca^{2+} binding, from the usual two Ca^{2+} ions per ATPase molecule to one at 50 μM-Ca^{2+}.



Scheme 1. Simplified model of the reaction cycle of (Ca^{2+}–Mg^{2+})-ATPase based on that of de Meis & Vianna (1979)

MATERIALS AND METHODS

Thapsigargin was a gift from Dr. O. Thastrup (Rigshospitalet, Copenhagen, Denmark) and was also obtained from L.C. Services Corp. (Woburn, MA, U.S.A.), and stored as a concentrated stock at 1 mg/ml in dimethyl sulphoxide (DMSO). All reagents were of analytical-grade quality.

(Ca^{2+}–Mg^{2+})-ATPase was purified from sarcoplasmic reticulum of rabbit skeletal muscle as described by Michelangeli *et al.* (1990*b*). ATPase activities were determined by the coupled enzyme assay of Froud *et al.* (1986) in a buffer containing 40 mM-Hepes/KOH, pH 7.2, 5 mM-$MgSO_4$, 2.1 mM-ATP, 1.0 mM-EGTA, 0.52 mM-phosphoenolpyruvate, 0.15 mM-NADH, pyruvate kinase (7.5 units) and lactate dehydrogenase (18 units) in a final volume of 2.5 ml. The reaction was initiated by addition of 90 μl of 25 mM-$CaCl_2$ to give a final free concentration of 10 μM-Ca^{2+}. Typically 13 μg of ATPase was added per assay, and thapsigargin was added as a solution in DMSO immediately before assaying. Neither DMSO nor thapsigargin affected the activity of the coupling enzymes in the assay.

(Ca^{2+}–Mg^{2+})-ATPase was labelled with nitrobenzo-2-oxa-1,3-diazole (NBD) by a modification of the method described by Wakabayashi *et al.* (1990). Purified ATPase (20 mg) was incubated in the dark at 25 °C for 1 h with 0.3 mM-NBD-Cl in 10 ml of 0.2 M-KCl/13 mM-Mops/KOH/1 mM-adenosine 5′-[$\beta\gamma$-imido]triphosphate/1 mM-$CaCl_2$, pH 7.0. The incubation was stopped with 40 ml of ice-cold 0.1 M-KCl/0.3 M-sucrose/10 mM-histidine, pH 6.8. The reaction mixture was then centrifuged at 70000 *g* for 30 min to remove excess NBD-Cl. The labelled enzyme was then resuspended in 4.5 ml of 20 mM-Mops/NaOH/0.1 M-NaCl/0.3 M-sucrose, pH 7.0; 0.5 ml of 100 mM-dithiothreitol was then added in the same buffer, and the reaction

Abbreviations used: (C14:1)PC, dimyristoleoylphosphatidylcholine; DMSO, dimethyl sulphoxide; NBD, nitrobenzo-2-oxa-1,3-diazole.

mixture was incubated at 25 °C in the dark for 7 min. The reaction was stopped by adding 40 ml of ice-cold Mops/NaCl/sucrose buffer, and excess dithiothreitol was removed by centrifugation as above. The pellet was then washed twice with the same ice-cold solution by centrifugation. The amount of NBD label incorporated into the ATPase was determined by its A_{420} in 1% SDS, by using an absorption coefficient of 1.3×10^4 M^{-1}·cm^{-1} for NBD-cysteine (Birkett *et al.*, 1970) and one of 1.2 l·g^{-1}·cm^{-1} for the ATPase (Hardwicke & Green, 1974). The fluorescence of the NBD-labelled ATPase was recorded with an SLM 8000 spectrofluorimeter at 25 °C. Excitation was set at 430 nm and emission was measured at 510 nm. Ammonium vanadate (B.D.H., Poole, Dorset, U.K.) was dissolved in 100 mM-KOH to give a 100 mM stock solution and was added to the fluorescence samples to a final concentration of 1.0 mM.

Measurements of enzyme phosphorylation were carried out essentially as described by Michelangeli *et al.* (1991). ATPase (0.2 mg) was incubated in 1 ml of 150 mM-Mes/Tris, pH 6.2, containing 10 mM-$MgCl_2$, 5 mM-EGTA and 10 mM-[^{32}P]P_i (5 Ci/mol) at 25 °C for 20 s, after which the reaction was quenched with 5 ml of 12% (w/v) trichloroacetic acid/0.2 M-phosphate. The precipitate was collected by filtration through Whatman GF/B glass-fibre filters washed with 3 × 5 ml of 12% trichloroacetic acid/0.2 M-phosphate and counted for radioactivity in 10 ml of Optiphase HiSafe 3 scintillant for ^{32}P. Background non-specifically bound radioactivity was assessed by quenching the ATPase before addition of the radiolabel and was typically 20% of total counts. A similar procedure was followed to study the phosphorylation of the ATPase by ATP, except that ATPase (0.1 mg) was incubated in 0.5 ml of 20 mM-Hepes/Tris, pH 7.2, containing 5 mM-$MgSO_4$, 100 mM-KCl, 1 mM-$CaCl_2$ and 100 μM-[γ-^{32}P]ATP (10–100 Ci/mol). After 10 s at 25 °C the reaction was quenched and counted for radioactivity as described above.

Ca^{2+} release from the ATPase was monitored by using the metallochromic indicator Antipyrylazo III (Champeil & Guillain, 1986). ATPase (2 mg) was incubated in 3 ml of 20 mM-Mes/Tris/100 mM-KCl/5 mM-$MgSO_4$/0.1 mM-Antipyrylazo III, pH 6.0 at 25 °C. Release of Ca^{2+} after thapsigargin treatment was monitored by measuring the change in $A_{720} - A_{790}$ in an SLM-Aminco DW 2000 dual-wavelength spectrophotometer at 25 °C. Portions of a concentrated solution of $CaCl_2$ were added to a final concentration of 10 μM to calibrate the signal. Thapsigargin (2.55 nmol in 1 μl of DMSO) was added and the Ca^{2+} release was monitored. Measurements of Ca^{2+} release were corrected for the small dilution artefact observed on addition of 1 μl of DMSO.

Ca^{2+} binding to the ATPase was measured by the dual-labelling method of Yamaguchi & Watanabe (1989). ATPase (1 mg) was incubated in 5 ml of medium containing 20 mM-Hepes/Tris, 100 mM-KCl, 5 mM-$MgSO_4$, 100 μM-EGTA, 150 μM-$^{45}CaCl_2$ (3 Ci/mol) and 500 μM-[^{3}H]glucose (0.2 Ci/mol), pH 7.2. Samples (1 ml) were rapidly filtered through Millipore HAWP 0.45 μm-pore-size filters. After drying, 10 ml of Optiphase HiSafe 3 scintillant was added and the filters were counted for both ^{3}H and $^{45}Ca^{2+}$ radioactivity in a liquid-scintillation counter. The amount of [^{3}H]glucose trapped on the filter was used to calculate the wetting volume for the filter, and the amount of Ca^{2+} trapped in this volume was subtracted from the total Ca^{2+} on the filter to give that bound to the ATPase. A correction was also applied for Ca^{2+} bound to the filter alone.

RESULTS

Fig. 1 shows the inhibitory effect of thapsigargin on steady-state ATPase activity. In the experiment shown, the ATPase concentration was 50 nM, so that inhibition is complete at a 1:1 molar ratio of thapsigargin:ATPase. The plot of ATPase activity against thapsigargin concentration is essentially linear between 5 and 55 nM, and the lack of inhibition observed at 5 nM can probably be attributed to the binding of thapsigargin to the glass vessels used in this experiment. As shown in Table 1, addition of thapsigargin at a 1:1 molar ratio to the ATPase completely inhibits phosphorylation of the ATPase by P_i. The steady-state level of phosphorylation of the ATPase by [γ-^{32}P]ATP measured in the presence of 1 mM-Ca^{2+} is also very low when thapsigargin is added to the ATPase before addition of Ca^{2+} (Table 1). These results are in agreement with those of Sagara & Inesi (1991).

Wakabayashi *et al.* (1990) have studied the fluorescence

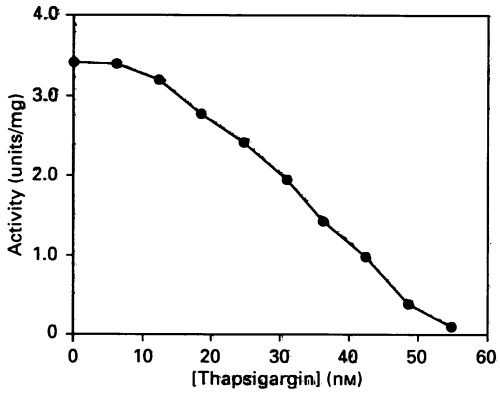


Fig. 1. Inhibition of the steady-state ATPase activity of (Ca^{2+}–Mg^{2+})-ATPase by thapsigargin

(Ca^{2+}–Mg^{2+})-ATPase activity was determined at 25 °C by a coupled enzyme assay (see the Materials and methods section). The ATPase (50 nM) was incubated with the given concentration of thapsigargin for 30 s, and then assayed for activity at 25 °C, pH 7.2, with ATP = 2.1 mM and optimal free Ca^{2+} (10 μM).

Table 1. Effect of thapsigargin on the phosphorylation of (Ca^{2+}–Mg^{2+})-ATPase by ATP and P_i

Phosphorylation of 2.0 μM-ATPase from P_i was carried out in 1 ml of 150 mM-Mes/Tris, pH 6.2, containing 10 mM-$MgCl_2$, 5 mM-EGTA, 10 mM-P_i and [^{32}P]P_i for 20 s in the absence or presence of thapsigargin (2.0 μM) at 25 °C. The reaction was quenched with 12% trichloroacetic acid/0.2 M-phosphoric acid and the precipitate collected and washed on Whatman GF/B filters with 3 × 5 ml of quenching solution. The [^{32}P]P_i on the filters was determined by liquid-scintillation counting. Non-specific binding was assessed by quenching the ATPase before addition of label, and was the same in the absence or presence of thapsigargin and was unaffected by DMSO. A similar procedure was used to study the phosphorylation by [γ-^{32}P]ATP; ATPase (2.0 μM) was incubated in 0.5 ml of 20 mM-Hepes/Tris, pH 7.2, containing 5 mM-$MgSO_4$, 100 mM-KCl, 1 mM-$CaCl_2$, 100 μM-ATP and [γ-^{32}P]ATP, in the absence or presence of thapsigargin (2.0 μM). After 10 s at 25 °C the reaction was quenched as described above. In these experiments the effect of thapsigargin added before and after the addition of Ca^{2+} on phosphorylation by [γ-^{32}P]ATP was investigated.

	E–P produced (nmol/mg of ATPase)
ATPase + [^{32}P]P_i	3.6
ATPase + thapsigargin + [^{32}P]P_i	0.2
ATPase + Ca^{2+} + [γ-^{32}P]ATP	2.7
ATPase + thapsigargin + Ca^{2+} + [γ-^{32}P]ATP	0.1
ATPase + Ca^{2+} + thapsigargin + [γ-^{32}P]ATP	0.1

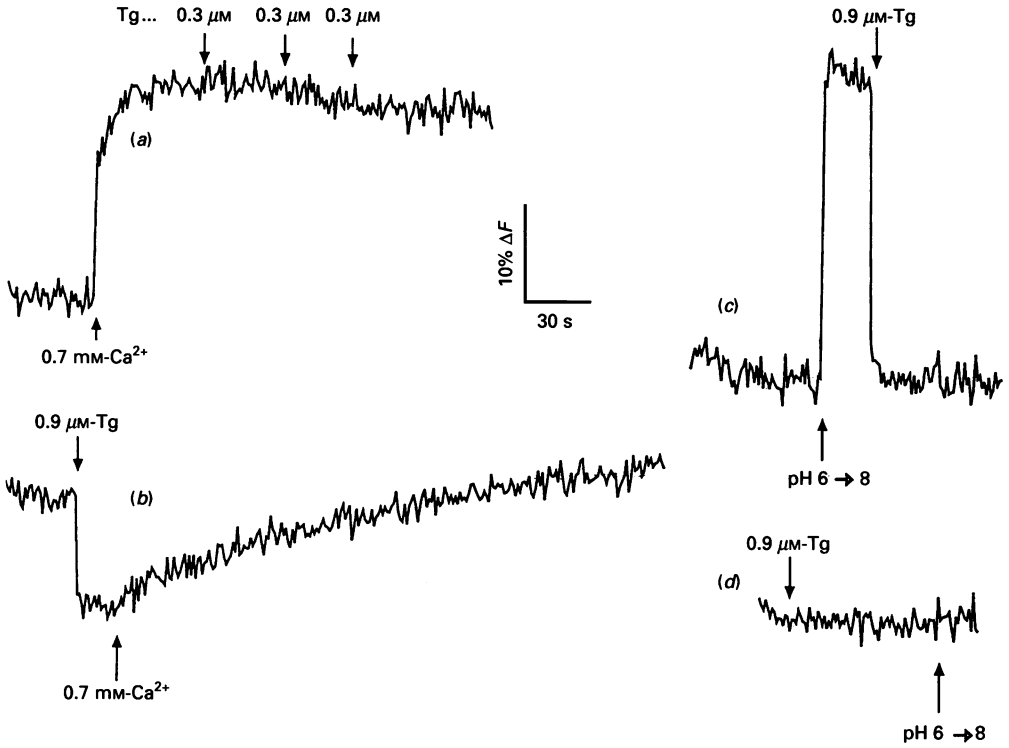


Fig. 2. Effect of thapsigargin (Tg) on the response of NBD-labelled ATPase to pH and Ca^{2+}

The Figure shows the fluorescence intensity of NBD-labelled ATPase (0.9 μM) in 50 mM-Mops/KOH/0.3 mM-EGTA, pH 6.0 (*a*, *c*, *d*) or 50 mM-Hepes/KOH, pH 7.0 (*b*), as a function of time, and the response to the addition of Ca^{2+} (*a*, *b*) or changing pH (*c*, *d*). In traces (*a*) and (*b*) the response to the addition of Ca^{2+} to a final free concentration of 0.4 mM was recorded. In trace (*b*), addition of Ca^{2+} was preceded by addition of thapsigargin to a final concentration of 0.9 μM. In traces (*c*) and (*d*) responses to a pH jump from 6 to 8 were recorded in the presence of 0.5 mM-EGTA, for trace (*d*) after addition of 0.9 μM-thapsigargin.

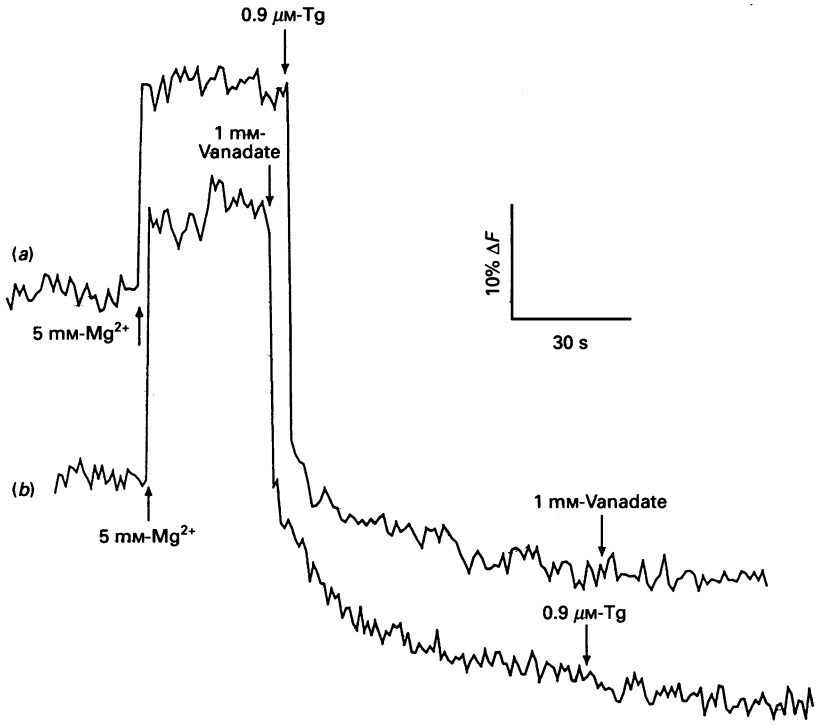


Fig. 3. Effect of thapsigargin (Tg) on the response of NBD-labelled ATPase to Mg^{2+} and vanadate ions

The Figure shows the fluorescence intensity of NBD-labelled ATPase (0.9 μM) in 50 mM-Tris/HCL/0.3 mM-EGTA, pH 8.0, as a function of time, and the response to the addition of Mg^{2+} and vanadate. In trace (*a*) the effects of the sequential additions of Mg^{2+} (final concn. 5 mM), thapsigargin (final concn. 0.9 μM) and vanadate (final concn. 1 mM) are shown. In trace (*b*), addition of vanadate precedes that of thapsigargin.

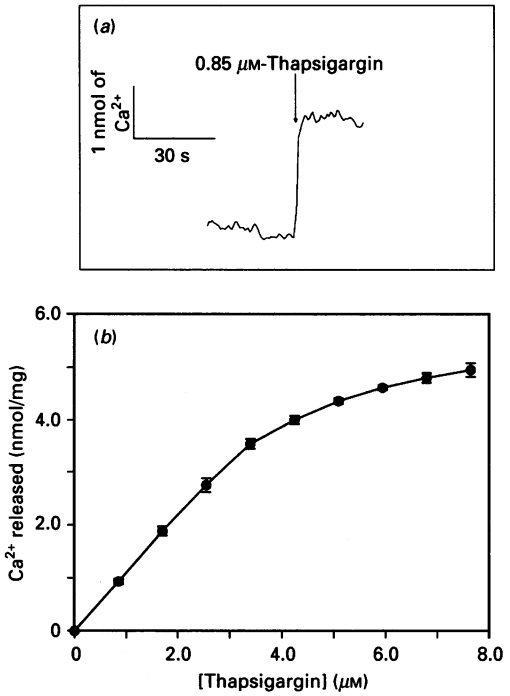


Fig. 4. Effect of thapsigargin on Ca^{2+} binding to (Ca^{2+}–Mg^{2+})-ATPase

Ca^{2+} release from the ATPase on addition of thapsigargin was measured by using Antipyrylazo III. ATPase (6.7 μM) was incubated in 3 ml of 20 mM-Mes/Tris containing 100 mM-KCl, 5 mM-$MgSO_4$, 0.1 mM-Antipyrylazo III and 10 μM-Ca^{2+}, at pH 6.0 at 25 °C. Trace (*a*) shows Ca^{2+} release after addition of 0.85 μM-thapsigargin, and graph (*b*) plots Ca^{2+} released (nmol/mg of protein) as a function of the concentration of thapsigargin.

Table 2. Effect of thapsigargin on Ca^{2+} binding by (Ca^{2+}–Mg^{2+})-ATPase

ATPase (2 μM) was incubated in 5 ml of 20 mM-Hepes/Tris, pH 7.2, containing 100 mM-KCl, 5 mM-$MgSO_4$, 100 μM-EGTA, 150 μM-$^{45}CaCl_2$ (3 Ci/mol) and 500 μM-[^{3}H]glucose (0.2 Ci/mol) for 2 min in the absence or presence of thapsigargin (2 μM). Samples (1 ml) were rapidly filtered through Millipore HAWP 0.45 μm filters. ^{3}H and $^{45}Ca^{2+}$ were determined by liquid-scintillation counting. Ca^{2+} bound to the ATPase was calculated after determining the Ca^{2+} non-specifically trapped on the filter (estimated from the amount of trapped glucose).

	Ca^{2+} bound (nmol/mg of ATPase)
ATPase + $^{45}Ca^{2+}$	7.4
ATPase + thapsigargin + $^{45}Ca^{2+}$	3.6
ATPase + $^{45}Ca^{2+}$ + thapsigargin	3.9

properties of the ATPase labelled with NBD and have suggested that the fluorescence intensity of NBD-labelled ATPase is sensitive to the proposed E2–E1 conformation change (Scheme 1), with a higher fluorescence intensity in the E1 conformation than in the E2 conformation. Thus, as shown in Fig. 2(*a*), addition of Ca^{2+} to NBD-labelled ATPase, initially in the presence of EGTA, results in an increase in fluorescence intensity, attributable to the E2–Ca_2E1 change. Addition of thapsigargin after addition of Ca^{2+} had no effect on fluorescence. However, if thapsigargin was added to the NBD-labelled ATPase before addition of Ca^{2+} at pH 7.0 (Fig. 2*b*), then the response to addition of Ca^{2+} is very slow. The rate of this increase in fluorescence intensity is pH-dependent, the rate increasing as the pH rises from 6.0 to 8.0 (results not shown).

The decrease in the fluorescence intensity of NBD-labelled ATPase on addition of thapsigargin in the absence of Ca^{2+} can be attributed to a change in the E2–E1 equilibrium. It has been suggested that the E2–E1 equilibrium is pH-dependent, with low pH favouring the E2 form (Pick & Karlish, 1982; Froud & Lee, 1986; Wakabayashi *et al.*, 1990), so that changing pH can also be used to trigger the E2–E1 transition. As shown in Fig. 2(*c*), increasing pH from 6 to 8 in the presence of thapsigargin results in an increase in fluorescence intensity for NBD-labelled ATPase, this increase being reversed by the addition of thapsigargin. However, if thapsigargin was added to the NBD-labelled ATPase at pH 6.0, and then the pH was jumped to 8.0, no change in fluorescence intensity was detected (Fig. 2*d*). These experiments suggest that thapsigargin favours the E2 state of the ATPase. This is confirmed by the studies shown in Fig. 3. Wakabayashi *et al.* (1990) have shown that addition of Mg^{2+} to NBD-labelled ATPase at pH 8 results in an increase in fluorescence, attributable to a shift in the E1/E2 equilibrium towards E1. As shown in Fig. 3(*a*), this effect can be reversed by addition of thapsigargin. Vanadate, an analogue of P_i, is thought to bind to the ATPase, shifting the equilibrium towards E2 and forming a complex analogous to E2P in Scheme 1 (Pick, 1982). Addition of vanadate to the NBD-labelled ATPase after addition of thapsigargin had no effect on fluorescence intensity (Fig. 3*a*). However, addition of vanadate to the ATPase at pH 8 before addition of thapsigargin resulted in a large decrease in fluorescence intensity, consistent with a shift in the equilibrium towards E2 (Fig. 3*b*). Addition of thapsigargin after addition of vanadate had no effect on fluorescence intensity, as expected (Fig. 3*b*).

The effect of thapsigargin on Ca^{2+} binding to the ATPase was studied by using Antipyrylazo III to monitor the external concentration of Ca^{2+}. As shown in Fig. 4, addition of thapsigargin to the ATPase incubated in the presence of Ca^{2+} results in release of Ca^{2+} into the medium. Fig. 4 also shows the level of Ca^{2+} released from the ATPase as a function of the concentration of thapsigargin. The maximal Ca^{2+} release was 5 nmol/mg of protein. Direct measurement of Ca^{2+} binding to this ATPase preparation using $^{45}Ca^{2+}$ gave a maximal Ca^{2+} binding for this preparation of ATPase of 8 nmol/mg. As shown in Table 2, direct measurements of $^{45}Ca^{2+}$ binding to another preparation of the ATPase, with a slightly lower measured Ca^{2+} binding of 7.4 nmol/mg of protein, also suggest that approximately half the bound Ca^{2+} is released on addition of thapsigargin. Addition of thapsigargin before addition of $^{45}Ca^{2+}$ also results in binding of only half the maximal amount of Ca^{2+} observed in the absence of thapsigargin (Table 2).

DISCUSSION

Our main result is that thapsigargin binds strongly to the (Ca^{2+}–Mg^{2+})-ATPase, holding it in a conformation that can bind only one Ca^{2+} ion, rather than the usual two. This has been shown both by direct measurement of $^{45}Ca^{2+}$ binding to the ATPase (Table 2) and by measuring Ca^{2+} released from the ATPase into the external medium on addition of thapsigargin (Fig. 4). Binding of Ca^{2+} to the ATPase has been interpreted in terms of Scheme 2 (Dupont, 1982; Champeil *et al.*, 1983; Froud & Lee, 1986; Inesi *et al.*, 1990; Michelangeli *et al.*, 1990*b*). In this

$$E2 \longrightarrow E1 \longrightarrow CaE1 \longrightarrow CaE1' \longrightarrow Ca_2E1'$$

Scheme 2. Model of the sequential binding of Ca^{2+} to the (Ca^{2+}–Mg^{2+})-ATPase

model, a site of moderate affinity is immediately available for binding and a second, higher-affinity, site becomes available only after a conformational change CaE1–CaE1′ triggered by binding of the first Ca^{2+} to E1. The results reported here would suggest that this second Ca^{2+} site does not become available when thapsigargin is bound to the ATPase. Thus, on addition of $^{45}Ca^{2+}$ to the ATPase incubated with thapsigargin in the absence of Ca^{2+}, the $^{45}Ca^{2+}$ bound is half that observed in the absence of thapsigargin. Similarly, if thapsigargin is added to the ATPase previously incubated in the presence of $^{45}Ca^{2+}$, again the $^{45}Ca^{2+}$ bound is half that observed in the absence of thapsigargin (Table 2).

The E2–E1 transition of the ATPase has been studied by using changes in the fluorescence of the ATPase labelled with fluorescein isothiocyanate (Pick, 1982; Pick & Karlish, 1982; Froud & Lee, 1986). More recently, however, Wakayabashi *et al.* (1990) have shown that the ATPase labelled with NBD-Cl exhibits fluorescence changes similar to those observed for the fluorescein-labelled ATPase, but of larger magnitude. Wakayabashi *et al.* (1990) suggested that the fluorescence intensity of NBD-labelled ATPase was relatively low in the E2 state and higher in the E1 state, and that addition of Mg^{2+} or increasing pH shifted the ATPase towards the E1 state. We find that addition of thapsigargin to NBD-labelled ATPase results in a decrease in fluorescence to the level observed on addition of vanadate (Fig. 3); since vanadate binds preferentially to the E2 form of the ATPase (Pick, 1982), this argues that binding of thapsigargin also favours the E2 state of the ATPase.

At pH 6, the ATPase is predominantly in the E2 form (Pick & Karlish, 1982; Froud & Lee, 1986; Wakabayashi *et al.*, 1990). In the absence of thapsigargin, addition of Ca^{2+} to NBD-labelled ATPase at pH 6 results in an increase in fluorescence intensity, attributable to the E2–Ca_2E1 transition (Fig. 2*a*). In the presence of thapsigargin at pH 7.0, addition of Ca^{2+} causes a slow increase in fluorescence intensity: presumably the result of a slow transition from E2 to CaE1 (Scheme 2), as the $^{45}Ca^{2+}$-binding experiments show that only a single $^{45}Ca^{2+}$ ion can bind per ATPase molecule under these conditions (Table 2). Addition of thapsigargin to NBD-labelled ATPase in the presence of Ca^{2+} results in no change in fluorescence intensity (Fig. 2*a*), despite the loss of 1 bound Ca^{2+} ion (Fig. 4, Table 2); this implies equal fluorescence intensity for the CaE1 and Ca_2E1 states for NBD-labelled ATPase.

We have previously reported that the Ca^{2+} binding stoichiometry also changes from the normal 2:1 to 1:1 on reconstitution of the ATPase into bilayers of the short-chain phospholipid (C14:1)PC (Michelangeli *et al.*, 1990*b*). It is not possible to change the binding stoichiometry for Ca^{2+} without considerable changes elsewhere in the reaction cycle for the ATPase (Scheme 1), since the product of the equilibrium constants around the reaction cycle must be a constant (being equal to the equilibrium constant for the hydrolysis of MgATP). Thus, on reconstitution with (C14:1)PC we showed that there was also a change in the equilibrium constant E1/E2 towards E1 and a decrease in the equilibrium constant for phosphorylation by P_i (Froud & Lee, 1986; Michelangeli *et al.*, 1991). Binding of thapsigargin results in the opposite change in the ratio E1/E2, favouring E2, but also results in a decrease in the equilibrium constant for phosphorylation by P_i (Table 1). The observed decrease in the steady-state level of phosphorylation by ATP (Table 1) could be due to a decrease in the rate of phosphorylation of the ATPase by ATP, or to an increase in the rate of dephosphorylation, or a combination of these. Previously, we have shown that the decrease in the steady-state level of phosphorylation of the ATPase caused by nonylphenol could be attributed to a large increase in the rate of dephosphorylation, with little effect on the rate of phosphorylation (Michelangeli *et al.*, 1990*a*). We also showed that binding of nonylphenol changed the equilibrium constant E1/E2 towards E2 (Michelangeli *et al.*, 1990*a*).

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