Effects of phospholipid fatty acyl chain length on phosphorylation and dephosphorylation of the Ca^{2+}-ATPase

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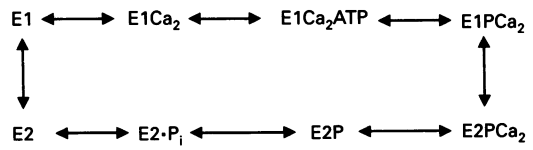
The kinetics of the Ca^{2+}-ATPase purified from sarcoplasmic reticulum have been studied after reconstitution into bilayers of dimyristoleoylphosphatidylcholine [di($C_{14:1}$)PC], dioleoylphosphatidylcholine [di($C_{18:1}$)PC] and dinervonylphosphatidylcholine [di($C_{24:1}$)PC]. In di($C_{24:1}$)PC the rate of phosphorylation of the ATPase by ATP was comparable with that in di($C_{18:1}$)PC (about 70 s^{-1}), but in di($C_{14:1}$)PC the rate was much lower (21 s^{-1}). Fluorescence responses of the ATPase suggest changes in the phosphoryl-transfer step rather than in the preceding conformational change $E1Ca_2ATP \rightleftarrows E1'Ca_2ATP$. The rate of dephosphorylation of the phosphorylated ATPase was found to decrease in the order di($C_{24:1}$)PC < di($C_{14:1}$)PC < di($C_{18:1}$)PC. For the ATPase in di($C_{24:1}$)PC the rate of dephosphorylation (3.3 s^{-1}) was slow enough to be the rate-limiting step for ATP hydrolysis; in di($C_{14:1}$)PC, it is suggested that both phosphorylation and dephosphorylation contribute to rate limitation. Phosphorylation of the ATPase in di($C_{24:1}$)PC by P_i was normal, but no phosphoenzyme could be detected in di($C_{14:1}$)PC. The rate of the Ca^{2+}-transport step was normal in di($C_{24:1}$)PC, suggesting that the single Ca^{2+} ion bound to the ATPase in di($C_{24:1}$)PC could be transported.

INTRODUCTION

The cell, by controlling the structures of its membrane phospholipids, exerts a degree of control over the environment of membrane proteins which is not possible for proteins in the cytoplasm. It is difficult from studies of intact membranes to define what are the most important features of the phospholipids because of the complexity of the lipid composition of the native membrane. The problem can be simplified by using reconstituted membrane systems containing a purified membrane protein and one species of phospholipid [1]. Effects of phospholipids on the function of the Ca^{2+}-ATPase purified from skeletal-muscle sarcoplasmic reticulum (SR) have been studied in this way. It has been shown that phospholipids need to be in the liquid-crystalline phase to support ATPase activity [2–7] and that hydrolysis of ATP by the Ca^{2+}-ATPase in bilayers of dimyristoleoylphosphatidylcholine [di($C_{14:1}$)PC] or dinervonylphosphatidylcholine [di($C_{24:1}$)PC] is much slower than in bilayers of dioleoylphosphatidylcholine [di($C_{18:1}$)PC] [1,8,9].

In both di($C_{14:1}$)PC and di($C_{24:1}$)PC the stoicheiometry of Ca^{2+} binding to the ATPase changes from the usual two Ca^{2+} ions bound per ATPase molecule in the native SR membrane [or in di($C_{18:1}$)PC] to one Ca^{2+} ion bound per ATPase molecule [10–12], and in both it appears that the single bound Ca^{2+} ion occupies the outer of the two normal Ca^{2+}-binding sites [12]. Nevertheless, there are significant differences between the properties of the ATPase in di($C_{14:1}$)PC and di($C_{24:1}$)PC. Reconstitution in di($C_{14:1}$)PC results in a shift in the E1/E2 equilibrium for the ATPase towards E1, which is not observed in di($C_{24:1}$)PC [12]. The response to the binding of Mg^{2+} at the gating site on the ATPase is normal in di($C_{24:1}$)PC but no response is seen in di($C_{14:1}$)PC, as detected by changes in the fluorescence of the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin [12]. Lastly, we have shown that effects of di($C_{14:1}$)PC can be reversed by addition of a variety of hydrophobic molecules (including cholesterol) whereas effects of di($C_{24:1}$)PC cannot be reversed in this way [11,13–18]. We have shown that the effects of cholesterol follow from binding to sites on the ATPase other than those accessible to phosphatidylcholines [13,19].

The mechanism of the ATPase can be described in terms of the E2–E1 scheme [20] (Scheme 1). It is proposed that in the E1 conformation in the native SR membrane the ATPase has two outward-facing Ca^{2+}-binding sites of high affinity. After the binding of MgATP, the ATPase is phosphorylated to form $E1PCa_2$, which can undergo a change in conformation to the state $E2PCa_2$ from which Ca^{2+} dissociates to the lumen of the SR, followed by dephosphorylation of E2P and recycling to E1. Here we show that the slow kinetics for the ATPase in di($C_{14:1}$)PC and di($C_{24:1}$)PC follow from decreases in the rates of phosphorylation and dephosphorylation.



Scheme 1 Mechanism of action of ATPase

MATERIALS AND METHODS

Di($C_{14:1}$)PC, di($C_{18:1}$)PC and di($C_{24:1}$)PC were obtained from Avanti Polar Lipids. The Ca^{2+}-ATPase was purified from skeletal-muscle SR as described in East and Lee [21]. Concentrations of ATPase were estimated by using the absorption coefficient (1.2 litre·g^{-1}·cm^{-1} for a solution in 1% SDS) given by Hardwicke and Green [22]. Lipid reconstitutions were performed largely as described in Starling et al. [11]. Phospholipid (10 μmol) in buffer (400 μl; 10 mM Hepes/Tris, pH 8.0, containing 15% sucrose, 5 mM $MgSO_4$, 5 mM ATP and 12 mg/ml potassium cholate) was sonicated to clarity in a bath sonicator. ATPase (1.25 mg) in a volume of 20–30 μl was then added and, for di($C_{14:1}$)PC and di($C_{18:1}$)PC, left for 15 min at room temperature

Abbreviations used: di($C_{14:1}$)PC, dimyristoleoylphospyhatidylcholine; di($C_{18:1}$)PC, dioleoylphosphatidylcholine; di($C_{24:1}$)PC, dinervonylphosphatidylcholine; p[CH_2]ppA, adenosine 5′-[β, γ-methylene]triphosphate; IAEDANS, 5-({2-[(iodoacetyl)amino]ethyl}amino)naphthalene-1-sulphonic acid; SR, sarcoplasmic reticulum.

and 45 min at 5 °C to equilibrate. For di($C_{24:1}$)PC, samples were equilibrated for 1 h at room temperature. After equilibration, the samples were added to precooled Oakridge tubes containing ice-cold buffer (10 mM Hepes/Tris, pH 8.0, 2 mM dithiothreitol) and centrifuged at 200000 *g* for 1 h at 4 °C. Samples were re-homogenized, and suspended in buffer (10 mM Hepes/Tris, 15% sucrose) to a concentration of 3–8 mg/ml, and stored at −20 °C until use.

Measurements of tryptophan fluorescence intensity were made by diluting aliquots of the reconstitution mixture into 2.5 ml of buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl, 5 mM $MgSO_4$, 100 μM Ca^{2+}) recording tryptophan fluorescence using an SLM-Aminco 8000C fluorimeter, with excitation and emission wavelengths of 295 and 330 nm respectively. Effects of ATP were determined by addition of aliquots of a concentrated stock solution of ATP (10 mM).

ATPase (36 μM) was labelled with 5-({2-[iodoacetyl)amino]-ethyl}amino)naphthalene-1-sulphonic acid (IAEDANS) by incubation with IAEDANS (72 μM) in buffer (50 mM Tris/HCl, pH 7.0, 0.2 M sucrose) at 25 °C in the dark for 3 h. Unbound label was removed by centrifugation through a column of Sephadex G-50. The molar ratio of bound IAEDANS to ATPase was about 1:1 under these conditions [23]. IAEDANS fluorescence was recorded with excitation and emission wavelengths of 380 and 475 nm respectively.

The time-dependence of phosphorylation-induced Ca^{2+} release from the ATPase was determined using a Biologic Rapid filtration system as described in Starling et al. [24]. Reconstituted ATPase, prepared as above, was diluted with buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl, 5 mM $MgSO_4$) containing 100 μM $^{45}CaCl_2$ and 500 μM [^{3}H]sucrose to a concentration of 0.1 mg of protein/ml. Then 50 μg of the ATPase was loaded on to a Millipore HAWP filter (0.45 μm) and perfused for the given time with the same buffer containing 100 μM $^{40}CaCl_2$ and 2 mM ATP. The filters were dried overnight and then counted in OptiPhase HiSafe 3. The amount of [^{3}H]sucrose on the filter was used to calculate the wetting volume of the filter, from which the amount of $^{45}Ca^{2+}$ bound to the ATPase on the filter was calculated [11].

Steady-state measurements of phosphorylation by [^{32}P]P_i were carried out in 150 mM Mes/Tris, pH 6.2, containing 5 mM EGTA and the required concentrations of Mg^{2+} and P_i at 25 °C and a protein concentration of 0.2 mg/ml. After 15 s the reaction was quenched by addition of 10 vol. of quenching solution (25% trichloroacetic acid, 0.13 M phosphoric acid). The samples were stood on ice for 15 min and then the precipitate was collected by filtration through Whatman GF/B glass-fibre filters, and finally counted in Optiphase HiSafe 3.

The time-dependence of phosphorylation of the ATPase by [γ-^{32}P]ATP at 25 °C was determined using a Biologic QFM-5 system. ATPase (0.2 mg/ml) was incubated in 20 mM Hepes/Tris, pH 7.2, containing 5 mM $MgSO_4$, 100 mM KCl and 100 μM $CaCl_2$. This was mixed with an equal volume of the same buffer containing 100 μM [γ-^{32}P]ATP, followed by quenching with 25% trichloroacetic acid/0.13 M phosphoric acid. The precipitated protein was filtered, washed and counted as described above.

The time-dependence of dephosphorylation of the ATPase phosphorylated with [^{32}P]P_i was determined as described by Henao et al. [25]. The ATPase (4 mg/ml) was incubated in 12.5 mM Mes/Tris, pH 6.0, containing 10 mM EGTA, 1 mM [^{32}P]P_i, 20 mM $MgSO_4$ and 14% DMSO. Then 1 vol. of this suspension was mixed in the Biologic QFM-5 system with 16 vol. of 100 mM Mes/Tris, pH 7.5, containing 100 mM KCl, 4 mM $MgSO_4$ and 5.3 mM ATP, followed by quenching as described above.

The time-dependence of dephosphorylation of the ATPase phosphorylated with [γ-^{32}P]ATP was determined using the triple-mixing capability of the Biologic QFM-5 system. The ATPase (0.2 mg/ml) was incubated in 20 mM Mes/Tris, pH 7.2, containing 5 mM $MgSO_4$, 100 mM KCl and 100 μM $CaCl_2$. This was mixed with an equal volume of the same buffer containing 100 μM [γ-^{32}P]ATP. After 100 ms, the mixture was mixed with an equal volume of 2.5 mM unlabelled ATP, followed by quenching as described above.

RESULTS AND DISCUSSION

Phosphorylation of the ATPase by ATP

Mixing the ATPase incubated in the presence of Ca^{2+} with [γ-^{32}P]ATP leads to the very rapid formation of phosphoenzyme (Figure 1). For the ATPase reconstituted with di($C_{18:1}$)PC, the rate of phosphorylation observed with 50 μM ATP fits to a single exponential process with a rate of 78.7 ± 10.0 s^{-1} and an amplitude of 2.7 ± 0.1 nmol of EP/mg of protein. A very similar rate (60.7 ± 3.0 s^{-1}) was obtained for the ATPase in di($C_{24:1}$)PC, but in di($C_{14:1}$)PC the rate was reduced to 21.6 ± 4.8 s^{-1}. The rate obtained for the ATPase in di($C_{14:1}$)PC did not increase on increasing the concentration of ATP to 100 μM (rate of 20.4 ± 3.4 s^{-1}), suggesting that the maximum possible rate for the phosphorylation step is about 21 s^{-1} in di($C_{14:1}$)PC.

Phosphorylation of the ATPase has been shown to involve a conformation change which, it has been suggested, could relocate the γ-phosphate of ATP bound to the nucleotide-binding domain of the ATPase close to Asp-351, the residue in the phosphorylation domain that is phosphorylated by ATP [26]:

$$E1Ca_2ATP \rightleftarrows E1'Ca_2ATP \rightleftarrows E1PCa_2ADP$$

The decrease in fluorescence intensity for IAEDANS-labelled ATPase observed on binding ATP to the ATPase in the presence of Ca^{2+} has been attributed largely to the $E1Ca_2ATP \rightleftarrows E1'Ca_2ATP$ conformation change, with a smaller additional decrease due to phosphorylation [27–30]. Binding of the non-hydrolysable adenosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppA) to IAEDANS-labelled ATPase in the presence of Ca^{2+} results in a fluorescence change slightly smaller than that

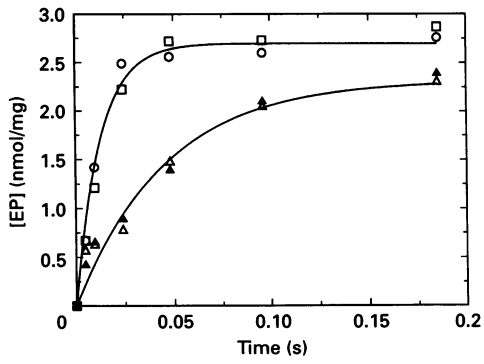


Figure 1 Rates of phosphorylation of the reconstituted ATPase

The ATPase (0.2 mg of protein/ml), reconstituted in di($C_{18:1}$)PC (○), di($C_{24:1}$)PC (□) or di($C_{14:1}$)PC (△, ▲), was incubated in buffer (20 mM Hepes/Tris, pH 7.2, 5 mM Mg^{2+}, 100 mM KCl, 100 μM Ca^{2+}) and mixed in a 1:1 ratio with the same buffer containing [γ-^{32}P]ATP to give final concentrations of 50 μM (open symbols) or 100 μM ATP (closed symbols). The solid lines show fits to single exponential processes with the rates given in the text for phosphorylation of the ATPase in di($C_{18:1}$)PC or di($C_{14:1}$)PC with 50 μM ATP.

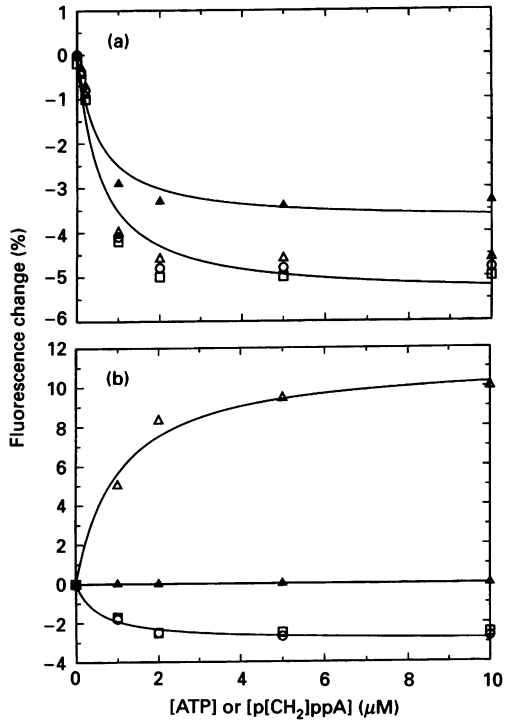


Figure 2 Changes in IAEDANS or tryptophan fluorescence on addition of ATP or p[CH_2]ppA

Effects are shown of ATP (open symbols) or p[CH_2]ppA (closed symbols) on the fluorescence of the ATPase in di($C_{18:1}$)PC (○), di($C_{24:1}$)PC (□) or di($C_{14:1}$)PC (△, ▲). (**a**) Effects on the fluorescence intensity of IAEDANS-labelled ATPase. The solid lines show best fits to a single binding site with K_d values of 0.56 ± 0.20 and 0.51 ± 0.18 μM for ATP and p[CH_2]ppA respectively. (**b**) Effects on the tryptophan fluorescence intensity of unlabelled ATPase. The buffer was 20 mM Mops/Tris, pH 7.0, containing 5 mM Mg^{2+}, 0.51 mM Ca^{2+}, 0.5 mM EGTA and 100 mM KCl, at 25 °C. The solid lines show fits to single binding sites with the parameters in the text.

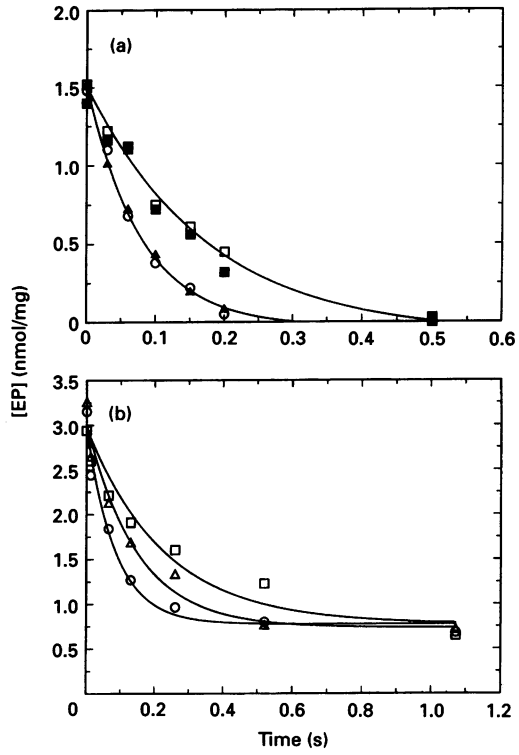


Figure 3 Rates of dephosphorylation of the reconstituted ATPase

The effects of lipid bilayer composition are shown on the rate of dephosphorylation of the ATPase reconstituted with di($C_{18:1}$)PC (○), di($C_{24:1}$)PC (□, ■) or di($C_{14:1}$)PC (△, ▲) in the absence (open symbols) or presence (closed symbols) of a 1:1 molar ratio of cholesterol to phospholipid. (**a**) The enzyme syringe contained ATPase (4 mg of protein/ml) in 12.5 mM Mes/Tris, pH 6.0, containing 10 mM EGTA, 1 mM [^{32}P]P_i, 20 mM Mg^{2+} and 14% (v/v) DMSO. The second syringe contained 100 mM Mes/Tris, pH 7.5, 100 mM KCl, 4 mM Mg^{2+} and 5.3 mM ATP. The contents of the enzyme syringe were mixed in a 1:16 (v/v) ratio with the dephosphorylation mixture and the reaction quenched at the given times with 25% trichloroacetic acid. (**b**) The enzyme syringe contained ATPase (0.2 mg of protein/ml) in 20 mM Mes/Tris, pH 7.2, containing 5 mM Mg^{2+}, 100 mM KCl and 100 μM Ca^{2+}. This was mixed in a 1:1 ratio with a solution containing 50 μM [γ-^{32}P]ATP in the same buffer. The mixture was incubated for 20 ms, then mixed in a 1:1 ratio with the same buffer containing 2.5 mM unlabelled ATP. The reaction was quenched at the given time with 25% trichloroacetic acid. The solid lines show fits to single exponential processes.

seen with ATP, consistent with this interpretation [27–30]. As shown in Figure 2(a), addition of ATP to IAEDANS-labelled ATPase reconstituted in di($C_{14:1}$)PC or di($C_{24:1}$)PC results in a decrease in fluorescence intensity comparable with that seen for the ATPase reconstituted in di($C_{18:1}$)PC. Addition of p[CH_2]ppA to the ATPase in di($C_{14:1}$)PC results in a change in fluorescence intensity smaller than that observed on addition of ATP, but with a similar concentration-dependence (Figure 2a). These results suggest that the conformation change detected by IAEDANS fluorescence after ATP binding can occur for the ATPase in both di($C_{14:1}$)PC and di($C_{24:1}$)PC although, of course, the experiments would not detect any alteration in the rate of the conformation change.

Addition of ATP to the ATPase in SR vesicles in the presence of Ca^{2+} results in a decrease in tryptophan fluorescence intensity which has been associated with the formation of phosphoenzyme; addition of p[CH_2]ppA results in no change in tryptophan fluorescence [7,31]. For the ATPase in di($C_{18:1}$)PC or di($C_{24:1}$)PC, a 3% decrease in fluorescence intensity is seen on addition of ATP, the data fitting to a K_d value of 0.53 ± 0.14 μM (Figure 2b). In marked contrast, addition of ATP to the ATPase in di($C_{14:1}$)PC results in an 11% increase in fluorescence intensity, with a K_d value of 1.0 ± 0.32 μM (Figure 2b). Addition of p[CH_2]ppA to the ATPase in di($C_{14:1}$)PC results in no change in fluorescence intensity (Figure 2b). Although no definitive explanation can be given for the altered response to phosphorylation in di($C_{14:1}$)PC, these experiments do suggest a change in the $E1'Ca_2ATP \rightleftarrows E1PCa_2ADP$ step for the ATPase reconstituted in di($C_{14:1}$)PC. This same step has been suggested to be altered for the ATPase reconstituted in gel-phase phospholipid, conditions where the phosphorylation of the ATPase by ATP is also slow [7].

For the ATPase in the SR membrane the rate of the conformational change $E1Ca_2ATP \rightleftarrows E1'Ca_2ATP$ has been estimated to be between 70 and 220 s^{-1}, dependent on conditions, with a rate of phosphoryl transfer of higher than 1000 s^{-1} [26]. If indeed the slow step in di($C_{14:1}$)PC is the phosphoryl-transfer step, then the rate of the step has been markedly reduced.

Rate of dephosphorylation of the phosphorylated ATPase

The rate of dephosphorylation can be determined either by first phosphorylating the ATPase with [γ-^{32}P]ATP in the presence of Ca^{2+} and then mixing with an excess of unlabelled ATP, or by phosphorylating with [^{32}P]P_i at pH 6.0 in the absence of Ca^{2+}

Table 1 Rates of dephosphorylation of the phosphorylated ATPase

Phosphorylation conditions are given in the legend to Figure 3. Where applicable, the molar ratio of cholesterol/phosphatidylcholine was 1:1.

Lipid	Rate of dephosphorylation (s^{-1})	
	P_i	ATP + Ca^{2+}
$Di(C_{18:1})PC$	12.5 ± 1.1	11.6 ± 2.4
$Di(C_{14:1})PC$	–	6.7 ± 1.5
$Di(C_{24:1})PC$	5.6 ± 0.7	3.3 ± 0.9
$Di(C_{14:1})PC$ + cholesterol	12.7 ± 0.6	–
$Di(C_{24:1})PC$ + cholesterol	5.9 ± 1.0	–

Table 2 Phosphorylation of the ATPase by P_i

The ATPase (0.2 mg of protein/ml) was incubated in 150 mM Mes/Tris, pH 6.2, containing 5 mM EGTA and the given concentrations of P_i and Mg^{2+}

	[EP] (nmol/mg of protein)	
	$Di(C_{18:1})PC$	$Di(C_{24:1})PC$
P_i (mM)*		
1	1.3	1.4
2	2.0	1.6
3	2.2	2.0
5	2.6	2.4
10	3.0	2.9
Mg^{2+} (mM)†		
1	0.5	0.8
2	1.2	1.2
3	1.6	1.6
5	1.6	1.6
10	1.6	1.8

*Mg^{2+} = 10 mM.
†P_i = 1 mM.

and presence of DMSO followed by mixing with an excess of a pH 7.5 buffer containing KCl and ATP to induce dephosphorylation. Dephosphorylation of the reconstituted ATPase phosphorylated with ATP fits to a single exponential process with a rate that decreases in the order $di(C_{18:1})PC > di(C_{14:1})PC > di(C_{24:1})PC$ (Figure 3, Table 1). The ATPase reconstituted in $di(C_{18:1})PC$ or $di(C_{24:1})PC$ can be phosphorylated with P_i (see below), and the rate of dephosphorylation of the ATPase phosphorylated in this way also decreases in the order $di(C_{18:1})PC > di(C_{24:1})PC$. The ATPase reconstituted in $di(C_{14:1})PC$ cannot be phosphorylated with P_i, but addition of cholesterol to a 1:1 molar ratio of cholesterol/$di(C_{14:1})PC$ restores phosphorylation to near normal levels [16]. The rate of dephosphorylation of the ATPase reconstituted with a 1:1 mixture of $di(C_{14:1})PC$ and cholesterol is comparable with that seen for the ATPase in $di(C_{18:1})PC$; cholesterol has no effect on the rate of dephosphorylation of the ATPase in $di(C_{24:1})PC$ (Figure 3, Table 1).

Phosphorylation of the ATPase by P_i

At acid pH values in the absence of Ca^{2+} and the presence of Mg^{2+}, the ATPase can be phosphorylated by P_i [20]. Comparable levels of phosphorylation are observed for the ATPase in $di(C_{18:1})PC$ and $di(C_{24:1})PC$ with very similar dependencies on

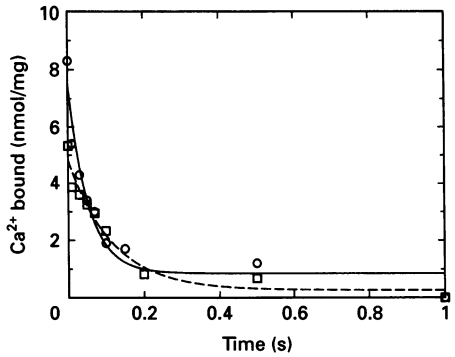


Figure 4 ATP-induced release of Ca^{2+} from the purified ATPase

Purified ATPase (○) or the ATPase reconstituted with $di(C_{24:1})PC$ (□) was first equilibrated at 0.4 mg/ml in pH 7.2 buffer (20 mM Hepes/Tris, 100 mM KCl, 20 mM Mg^{2+}) containing 100 μM $^{45}Ca^{2+}$ and 0.5 mM [^{3}H]sucrose and then 0.1 mg of ATPase was adsorbed on to Millipore filters. The loaded filter was perfused for the given times with the same buffer containing 100 μM unlabelled Ca^{2+} and 2 mM ATP. The solid and broken lines show fits to single exponential processes for the purified ATPase and reconstituted ATPase respectively; the rate constants are given in the text.

the concentrations of Mg^{2+} and P_i (Table 2). These results suggest that the equilibrium constant for the phosphorylation reaction ($E2 \cdot P_i \rightleftarrows E2P$) is very similar in $di(C_{24:1})PC$ and $di(C_{18:1})PC$, implying that, since the rate of dephosphorylation is lower in $di(C_{24:1})PC$ than in $di(C_{18:1})PC$ (Table 1), the rate of phosphorylation must also be lower.

As reported previously [16], no phosphorylation of the ATPase by P_i was observed in $di(C_{14:1})PC$. Increased levels of phosphorylation of the ATPase in SR vesicles by P_i are observed in the presence of DMSO [20]. No measurable level of phosphoenzyme was obtained on incubating the ATPase in $di(C_{14:1})PC$ with 20 mM Mg^{2+} and 1 mM P_i at pH 6.0 in the presence of 14% (v/v) DMSO (results not shown).

Rate of the Ca^{2+}-transport step

For the ATPase in the native SR membrane, the rate of phosphorylation of the ATPase by ATP is much faster than the rate of dissociation of $^{45}Ca^{2+}$ from the unphosphorylated ATPase [32]. Under these conditions Orlowski and Champeil [32] have shown that the rate of Ca^{2+} dissociation from the phosphorylated ATPase (the $E1PCa_2 \rightarrow E2P$ step in Scheme 1) can be measured by pre-equilibrating the ATPase with $^{45}Ca^{2+}$ and then perfusing it on Millipore filters with $^{40}Ca^{2+}$ and ATP. When the ATPase was incubated with 100 μM $^{45}Ca^{2+}$ in buffer containing 20 mM Mg^{2+} and 100 mM KCl, at pH 7.2, and then perfused with the same medium containing 100 μM unlabelled Ca^{2+} and 2 mM ATP, an initial level of Ca^{2+} binding of 8 nmol/mg of protein was observed. Dissociation of $^{45}Ca^{2+}$ from ATPase fitted to a single exponential process with a rate constant of 20.0 ± 4.9 s^{-1} (Figure 4). As the rate of phosphorylation of the ATPase in $di(C_{24:1})PC$ is comparable with that observed for the ATPase in SR vesicles (see Figure 1), the same protocol can be used to measure the rate of dissociation of Ca^{2+} from the ATPase in $di(C_{24:1})PC$. As shown previously [11], the initial level of Ca^{2+} binding to the ATPase in $di(C_{24:1})PC$ is about half that in the native membrane (Figure 4). Dissociation of Ca^{2+} again fits to a single exponential with a rate of 16.3 ± 3.6 s^{-1}. The similarity of the rate to that observed for the ATPase in SR vesicles suggests that the Ca^{2+}-transport step occurs normally in $di(C_{24:1})PC$ despite the fact

that only a single Ca^{2+} ion is bound. Ca^{2+} transport cannot be studied directly by measuring uptake into reconstituted sealed vesicles of phosphatidylcholines because of the rapid efflux of Ca^{2+} observed from such vesicles [33–35]. The low rate of phosphorylation for the ATPase in di($C_{14:1}$)PC (Figure 1) means that the rate of Ca^{2+} dissociation cannot be obtained from such experiments for the ATPase in this lipid.

Conclusions

The reaction step inhibited for the ATPase in di($C_{24:1}$)PC is dephosphorylation of the phosphorylated ATPase, which is markedly slower than for the ATPase in di($C_{18:1}$)PC; it is probably this step that is rate-limiting (Figure 3, Table 1). Measured ATPase activities vary between preparations of ATPase, but typical values are 0.5 unit/mg of protein in di($C_{14:1}$)PC and 0.6 unit/mg of protein in di($C_{24:1}$)PC, at pH 7.2, in reaction mixtures containing 5 mM MgATP and maximally stimulating concentrations of Ca^{2+}, at 25 °C, compared with a value of 3.5 units/mg of protein in di($C_{18:1}$)PC [11,19,24]. Maximal levels of phosphorylation observed for the ATPase in SR vesicles or in the reconstituted systems are about 3 nmol of EP/mg of protein, corresponding to a fraction of active protein of 0.33 [11]. If the rate of dephosphorylation of the ATPase phosphorylated with ATP is taken as the turnover number for ATP hydrolysis, then the rate of 3.3 s^{-1} (Table 1) in di($C_{24:1}$)PC would correspond to an ATPase activity of 0.6 unit/mg of protein, as observed. For the ATPase in di($C_{14:1}$)PC, the rate of dephosphorylation of 6.7 s^{-1} (Table 1) would give an ATPase activity of 1.2 units/mg of protein if dephosphorylation was the single rate-limiting step, significantly higher than the observed ATPase activity. The lower than normal rate of phosphorylation of the ATPase in di($C_{14:1}$)PC therefore probably also contributes to rate limitation in this case.

A proper explanation for the different responses of the ATPase to short- and long-chain phospholipids cannot be given in the absence of detailed structural information about the ATPase. The observed decreases in the rate of phosphorylation and in the extent of phosphorylation from P_i in di($C_{14:1}$)PC suggest that changes in the phosphorylation domain of the ATPase are greater in di($C_{14:1}$)PC than in di($C_{24:1}$)PC. These changes could still be subtle, however, as the extent of the conformational changes occurring on the ATPase during the reaction cycle appear to be small. No changes in the distances between the phosphorylation, nucleotide-binding and hinge domains or in the heights of these domains above the surface of the membrane could be detected between the E1 and E2 conformations in fluorescence energy-transfer measurements [23,36,37] and the height of the hinge domain above the membrane surface appeared to be the same in bilayers of di($C_{14:1}$)PC or di($C_{18:1}$)PC [23].

It has been reported that the ATPase is aggregated in bilayers of both di($C_{14:1}$)PC and di($C_{24:1}$)PC and it has been suggested that this aggregation could be the explanation for the observed low ATPase activities [38]. However, we have shown that aggregation is unconnected with the effects of these lipids on ATPase activity, as we see low activities on reconstitution of the ATPase into vesicles of di($C_{14:1}$)PC or di($C_{24:1}$)PC under conditions where cross-linking studies show that the ATPase is present as isolated monomers within the membrane [39]. The markedly different responses to reconstitution in di($C_{14:1}$)PC and di($C_{24:1}$)PC also argue against a single common mechanism for inhibition in these two lipids.

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