Effects of phosphatidylethanolamines on the activity of the Ca^{2+} -ATPase of sarcoplasmic reticulum

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ATPase activities for the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum reconstituted into dioleoylphosphatidylethanolamine [$\text{di}(\text{C}_{18:1})\text{PE}$] are, at temperatures higher than 20 °C, lower than in dioleoylphosphatidylcholine [$\text{di}(\text{C}_{18:1})\text{PC}$], whereas in egg yolk phosphatidylethanolamine the activities are the same as in $\text{di}(\text{C}_{18:1})\text{PC}$ up to 25 °C, suggesting that low ATPase activities occur when the phosphatidylethanolamine species is in the hexagonal H_{II} phase. ATPase activities measured in mixtures of $\text{di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{18:1})\text{PE}$ do not change with changing $\text{di}(\text{C}_{18:1})\text{PE}$ content up to 80 %. It is concluded that curvature frustration in bilayers containing $\text{di}(\text{C}_{18:1})\text{PE}$ has no effect on ATPase activity. The rates of phosphorylation and of Ca^{2+} transport are identical for the native ATPase and for the ATPase in $\text{di}(\text{C}_{18:1})\text{PE}$. Dephosphorylation of the phosphorylated ATPase in $\text{di}(\text{C}_{18:1})\text{PE}$ at 25 °C is, however,

slower than for the native ATPase, explaining the lower steady-state rate of ATP hydrolysis; in egg yolk phosphatidylethanolamine at 25 °C the rate of dephosphorylation is equal to that for the unreconstituted ATPase. Phosphorylation of the ATPase by P_i in the absence of Ca^{2+} is unaffected by reconstitution in $\text{di}(\text{C}_{18:1})\text{PE}$. The stoichiometry of Ca^{2+} binding to the ATPase is also unaltered. Studies of the effect of $\text{di}(\text{C}_{18:1})\text{PE}$ on the fluorescence intensity of the ATPase labelled with 7-chloro-4-nitro-2,1,3-benzoxadiazole are consistent with an increase in the E1/E2 equilibrium constant, where E1 is the conformation of the ATPase with two high-affinity binding sites for Ca^{2+} exposed to the cytoplasm, and E2 is a conformation unable to bind cytoplasmic Ca^{2+} . A slight increase in affinity for Ca^{2+} can be attributed to the observed increase in the E1/E2 equilibrium constant.

INTRODUCTION

The major species of phospholipids found in mammalian membranes is phosphatidylcholine, with smaller amounts of phosphatidylethanolamine, another zwitterionic lipid, and anionic lipids such as phosphatidylserine and phosphatidylinositol. In the sarcoplasmic reticulum (SR) of rabbit skeletal muscle, phosphatidylcholine and phosphatidylethanolamine make up about 70 % and 20 % respectively of the total phospholipid [1]. It might be expected that the activity of the Ca^{2+} -ATPase of SR is particularly sensitive to the chemical structure and physical properties of the lipids surrounding it in the membrane, as the function of the ATPase is to transport Ca^{2+} ions across the membrane, and this is likely to involve conformational changes in the region of the ATPase spanning the membrane.

Studies in which the Ca^{2+} -ATPase has been purified from the SR membrane and reconstituted into bilayers of defined composition have shown that the ATPase has a slightly higher activity in bilayers of dioleoylphosphatidylcholine [$\text{di}(\text{C}_{18:1})\text{PC}$] than in the native membrane [2,3]. The activity of the Ca^{2+} -ATPase is strongly dependent on the chain length of the phosphatidylcholine, the optimal chain length being C_{18} , with lower activities for lipids with shorter (C_{14} or less) or longer (C_{24}) fatty acyl chains [2,4,5]. Reconstitution into bilayers with sub-optimal chain lengths results in distinct changes in the properties of the ATPase, including a change in the stoichiometry of Ca^{2+} binding [6–8], a decrease in the rate of dephosphorylation of the phosphorylated ATPase, and, in the short-chain phosphatidyl-

choline, a decrease in the rate of phosphorylation [9,10]. Lipid headgroup structure has also been shown to have significant effects on ATPase activity: the activity in dioleoylphosphatidylethanolamine [$\text{di}(\text{C}_{18:1})\text{PE}$] has been shown to be about half that in $\text{di}(\text{C}_{18:1})\text{PC}$ [11]. Unexpectedly, however, for the ATPase reconstituted into vesicles containing mixtures of phosphatidylcholine and phosphatidylethanolamine, levels of accumulation of Ca^{2+} are observed to increase with increasing phosphatidylethanolamine content up to 80 % [12,13]. Racker [14] suggested that the effect of phosphatidylethanolamine was to prevent hydrolysis of the phosphorylated intermediate of the ATPase before transport of Ca^{2+} had occurred, so that the ratio of the number of Ca^{2+} ions transported to the number of ATP molecules hydrolysed would be higher in the presence of phosphatidylethanolamine than in its absence. However, this ratio has been shown to be 2:1 for the Ca^{2+} -ATPase in the native SR membrane (in which the phosphatidylethanolamine content is only 20 %) [15], and the fact that the kinetics of the ATPase in the native SR membrane is similar to that in $\text{di}(\text{C}_{18:1})\text{PC}$ argues against any new reaction pathway in the absence of phosphatidylethanolamine [2,9]. Further, the presence of phosphatidylethanolamine has been shown to decrease the rate of passive Ca^{2+} leakage from the reconstituted vesicles, and the reduced rate of leak provides a ready explanation for the increased level of accumulation of Ca^{2+} [13,16].

Phosphatidylethanolamines are of particular interest because many, in isolation, exist in a non-bilayer, hexagonal H_{II} phase under physiological conditions, and this might have important effects on the properties of biological membranes [17,18]. Here

Abbreviations used: $\text{di}(\text{C}_{18:1})\text{PC}$, dioleoylphosphatidylcholine; $\text{di}(\text{C}_{18:1})\text{PE}$, dioleoylphosphatidylethanolamine; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; SR, sarcoplasmic reticulum.

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Scheme 1 A simplified reaction scheme for the Ca^{2+} -ATPase

we use rapid kinetic and fluorescence techniques to study the effects of phosphatidylethanolamine on the kinetics of the Ca^{2+} -ATPase. These studies are interpreted in terms of the E1/E2 reaction scheme shown in Scheme 1 with a single Ca^{2+} -bound, phosphorylated form of the ATPase, consistent with recent experiments [19].

MATERIALS AND METHODS

$\text{Di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{18:1})\text{PE}$ were obtained from Avanti Polar Lipids, and hen egg yolk phosphatidylethanolamine from Sigma. The Ca^{2+} -ATPase was purified from skeletal muscle SR as described in East and Lee [11] and concentrations of ATPase were estimated by using an extinction coefficient of $1.2 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ for a solution in 1% (w/v) SDS [20]. Reconstitutions were performed as described in Starling et al. [7]. The ATPase was labelled with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) as described [21]. Measurements of NBD and tryptophan fluorescence were performed at 25 °C with an SLM Aminco 8000C fluorimeter with excitation and emission wavelengths of 430 and 520 nm respectively for NBD fluorescence and 290 and 340 nm respectively for tryptophan fluorescence. Measurements of ATPase activities were performed at 25 °C by using a coupled enzyme assay at pH 7.2 in 100 mM KCl/5 mM MgSO_4 /2.1 mM ATP and the required concentration of Ca^{2+} [7]. Free concentrations of Ca^{2+} were calculated from the binding constants for Ca^{2+} , Mg^{2+} and H^+ to EGTA given by Godt [22].

The time dependence of Ca^{2+} release from the ATPase was determined with a Biologic rapid filtration system. A suspension of the ATPase in buffer [20 mM Hepes/Tris (pH 7.2)/100 mM KCl/5 mM MgSO_4] containing 100 μM $^{45}\text{Ca}^{2+}$ and 500 μM [^3H]sucrose, corresponding to 50 μg of ATPase, was loaded on a Millipore HAWP filter and then perfused rapidly with the same buffer containing 2 mM EGTA. The filters were 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. The time-dependence of phosphorylation-induced Ca^{2+} release from the ATPase was determined in a similar way, by perfusing with buffer containing 100 μM $^{40}\text{CaCl}_2$ and 2 mM ATP.

Equilibrium measurements of phosphorylation by [^{32}P]P_i were performed 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 [9]. The time-dependence of phosphorylation of the ATPase by [γ - ^{32}P]ATP at 25 °C was determined with 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 50 μM [γ - ^{32}P]ATP, followed by quenching with 25% (w/v) 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. [23]. 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% (v/v) DMSO. One volume 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 with 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 50 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 after the required time as described above.

RESULTS

Effect on steady-state ATPase activity

As shown in Figure 1, at 25 °C the rate of hydrolysis of ATP by the Ca^{2+} -ATPase reconstituted in $\text{di}(\text{C}_{18:1})\text{PE}$ is half that in $\text{di}(\text{C}_{18:1})\text{PC}$. In mixtures of $\text{di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{18:1})\text{PE}$, activity varies little with $\text{di}(\text{C}_{18:1})\text{PE}$ content up to 80%. At temperatures above about 18 °C, $\text{di}(\text{C}_{18:1})\text{PE}$ transforms from a bilayer to a hexagonal H_{II} phase [24]. As shown in Figure 2, at 20 °C and below, ATPase activities in $\text{di}(\text{C}_{18:1})\text{PE}$ are very similar to those in $\text{di}(\text{C}_{18:1})\text{PC}$, but above this temperature activities are less in $\text{di}(\text{C}_{18:1})\text{PE}$ than in $\text{di}(\text{C}_{18:1})\text{PC}$, the difference between the activities in the two lipids increasing with increasing temperature. The temperature for the bilayer/hexagonal H_{II} phase transition for egg yolk phosphatidylethanolamine (28 °C) is higher than that for $\text{di}(\text{C}_{18:1})\text{PE}$ [24]. In egg yolk phosphatidylethanolamine, ATPase activities are comparable to those in $\text{di}(\text{C}_{18:1})\text{PC}$ at temperatures up to 25 °C, activities again being less than in $\text{di}(\text{C}_{18:1})\text{PC}$ at temperatures higher than this (Figure 2). We conclude that low activities are observed in phosphatidylethanolamines at temperatures where the phosphatidylethanolamine is in the hexagonal H_{II} phase.

Figure 1 Effect of $\text{di}(\text{C}_{18:1})\text{PE}$ on ATPase activity

Shown are ATPase activities (IU, units) for the purified Ca^{2+} -ATPase reconstituted into mixtures of $\text{di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{18:1})\text{PE}$ containing the given mol% $\text{di}(\text{C}_{18:1})\text{PE}$. Activities were measured at pH 7.2 and 25 °C in 100 mM KCl/2.1 mM ATP/10 μM Ca^{2+} .

Figure 2 Effects of temperature on ATPase activities

ATPase activities were determined at the given temperatures for the Ca^{2+} -ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ (○), $\text{di}(\text{C}_{18:1})\text{PE}$ (□) or egg yolk phosphatidylethanolamine (△).

Figure 3 Effect of $\text{di}(\text{C}_{18:1})\text{PE}$ on Ca^{2+} -dependent changes in tryptophan fluorescence intensity

Shown are the changes in the tryptophan fluorescence intensity of the ATPase in 20 mM Hepes/KOH (pH 7.2)/1 mM Ca^{2+} after the addition of EGTA to give the final free pCa values shown for the native ATPase (○) and for the ATPase reconstituted in $\text{di}(\text{C}_{18:1})\text{PE}$ (□) or a 4:1 mixture of $\text{di}(\text{C}_{18:1})\text{PE}:\text{di}(\text{C}_{18:1})\text{PC}$ (△). The solid lines are simulations calculated from the parameters given in Lee et al. [28] with a proton-binding constant K_{H6} of $5 \times 10^5 \text{ M}^{-1}$ for the native ATPase and $3 \times 10^7 \text{ M}^{-1}$ for the ATPase reconstituted in $\text{di}(\text{C}_{18:1})\text{PE}$; a pCa shift of -0.4 was applied to both curves to match the Ca^{2+} -binding curve for this preparation of the ATPase to that used to derive the parameters in Lee et al. [28] (see text). The simulated Ca^{2+} -binding curves were scaled to the experimental measurements of the changes in tryptophan fluorescence intensity on the assumption that Ca^{2+} binding resulted in 7% and 5% changes in tryptophan fluorescence intensity for the ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{18:1})\text{PE}$ respectively.

Effect on Ca^{2+} binding

Ca^{2+} binding to the ATPase can be conveniently followed through changes in the fluorescence intensity of tryptophan [25]. Reconstitution of the ATPase into $\text{di}(\text{C}_{18:1})\text{PE}$ results in a small decrease in the magnitude of the fluorescence response to Ca^{2+} binding and a small increase in the affinity for Ca^{2+} as seen by the 0.2 decrease in the pCa value for half-maximal fluorescence response (Figure 3). In a 4:1 mixture of $\text{di}(\text{C}_{18:1})\text{PE}$ and $\text{di}(\text{C}_{18:1})\text{PC}$ the Ca^{2+} affinity is the same as in pure $\text{di}(\text{C}_{18:1})\text{PC}$ (Figure 3).

The increase in affinity for Ca^{2+} in $\text{di}(\text{C}_{18:1})\text{PE}$ is consistent with the shift in the E1/E2 equilibrium for the ATPase (Scheme

Figure 4 Effect of lipid structure on the fluorescence intensity of NBD-labelled ATPase as a function of pH

Shown are the fluorescence intensities for NBD-labelled ATPase reconstituted in $\text{di}(\text{C}_{18:1})\text{PC}$ (○) or $\text{di}(\text{C}_{18:1})\text{PE}$ (□) as a function of pH. The solid lines are simulations with the parameters given in Lee et al. [28] except for a value of K_{H6} of $3 \times 10^7 \text{ M}^{-1}$ for the ATPase in $\text{di}(\text{C}_{18:1})\text{PE}$, with fluorescence intensities for the E1 and E2 states of 25.0 and 14.5 respectively.

Scheme 2 The E1–E2 equilibrium

1) revealed in studies of the fluorescence intensity of the NBD-labelled ATPase (Figure 4). It has been shown that the fluorescence intensity of NBD-labelled ATPase is sensitive to conformation state, with E2 and E1 being states of low and high fluorescence intensity respectively [26,27]. The fluorescence emission intensity of NBD-labelled ATPase increases with increasing pH, consistent with a pH-dependent equilibrium constant E1/E2 (Scheme 2), with proton binding constants $K_{\text{H6}} = 5 \times 10^5 \text{ M}^{-1}$ and $K_{\text{H7}} = 3.0 \times 10^8 \text{ M}^{-1}$ for E1 and E2 respectively, and an equilibrium constant E1/E2 (K_1) of 4.0 for the non-protonated forms [25]. The fluorescence intensity for NBD-labelled ATPase in $\text{di}(\text{C}_{18:1})\text{PE}$ is higher at all pH values than in $\text{di}(\text{C}_{18:1})\text{PC}$, the effect being particularly marked at acid pH. The results can be simulated with a value of $3 \times 10^7 \text{ M}^{-1}$ for the proton-binding constant in the E1 state (K_{H6}), the other constants being unchanged (Figure 4). By using the binding constants for Ca^{2+} and H^+ at the Ca^{2+} -binding sites on the ATPase given in Lee et al. [28] combined with the constants defining the E1/E2 equilibrium constant, it is possible to calculate Ca^{2+} -binding curves for the ATPase [28]. These calculations give a pCa for 50% Ca^{2+} binding of 6.03 in $\text{di}(\text{C}_{18:1})\text{PC}$. With a modified K_{H6} of $3 \times 10^7 \text{ M}^{-1}$, all the other constants being unchanged, the pCa for 50% binding becomes 6.18, the 0.15 shift in pCa value being comparable to that observed experimentally (Figure 3). As reported previously, the affinity of the Ca^{2+} -ATPase for Ca^{2+} varies between preparations [29]. The Ca^{2+} -binding curve in $\text{di}(\text{C}_{18:1})\text{PC}$ for the preparation of the ATPase used here can be matched to the simulated curve by applying a shift in pCa value of -0.4 to the simulated curve, corresponding to a 2.5-fold

Figure 5 Rates of dissociation of $^{45}\text{Ca}^{2+}$ from the ATPase

(A) Dissociation of $^{45}\text{Ca}^{2+}$ from the unphosphorylated ATPase. Native ATPase ($\circ$) or the ATPase reconstituted in $\text{di}(\text{C}_{18:1})\text{PC}$ ($\square$) were incubated with $100\ \mu\text{M}$ $^{45}\text{Ca}^{2+}$ and $0.5\ \text{mM}$ $[\text{H}]\text{sucrose}$ and adsorbed on Millipore filters. The filters were perfused for the given times with buffer ($20\ \text{mM}$ HEPES/KOH, pH 7.2) containing $100\ \text{mM}$ KCl, $20\ \text{mM}$ Mg^{2+} and $2\ \text{mM}$ EGTA, and the amount of $^{45}\text{Ca}^{2+}$ bound to the ATPase was determined. The line is the best single-exponential fit to the data for the native ATPase, with a rate constant ($\pm$ S.E.) of $29.3 \pm 8.2\ \text{s}^{-1}$. (B) ATP-induced release of $^{45}\text{Ca}^{2+}$ from the ATPase. The native ATPase ($\circ$) or the ATPase reconstituted in $\text{di}(\text{C}_{18:1})\text{PE}$ ($\square$) was equilibrated in buffer [$20\ \text{mM}$ HEPES/Tris (pH 7.2)/ $100\ \text{mM}$ KCl/ $20\ \text{mM}$ Mg^{2+}] containing $100\ \mu\text{M}$ $^{45}\text{Ca}^{2+}$ and $0.5\ \text{mM}$ $[\text{H}]\text{sucrose}$ and then adsorbed on filters as above. The loaded filter was perfused for the given times with the same buffer containing $100\ \mu\text{M}$ unlabelled Ca^{2+} and $2\ \text{mM}$ ATP. The solid line shows a single-exponential fit to the data for the native ATPase, with a rate constant ($\pm$ S.E.) of $17.5 \pm 3.8\ \text{s}^{-1}$.

decrease in Ca^{2+} affinity for this preparation of the ATPase compared with that used to define the parameters in Lee et al. [28]. Applying the same shift to the curve simulated with a K_{H7} value of $3 \times 10^7\ \text{M}^{-1}$ gives a reasonable fit to the experimental data for the ATPase in $\text{di}(\text{C}_{18:1})\text{PE}$ (Figure 3), implying that the shift in E1/E2 equilibrium constant is sufficient to explain the observed small shift in Ca^{2+} affinity.

The rate of dissociation of Ca^{2+} from the ATPase can be determined by incubating the ATPase with $^{45}\text{Ca}^{2+}$, adsorbing it on Millipore filters and then washing the filters for defined periods of time with EGTA. As shown in Figure 5(A), the dissociation of Ca^{2+} fits, within experimental error, a single exponential, with rates ($\pm$ S.E.) of 29.3 ± 8.2 and $23.7 \pm 9.4\ \text{s}^{-1}$ for the purified ATPase and for the ATPase reconstituted in $\text{di}(\text{C}_{18:1})\text{PE}$ respectively.

The rate of phosphorylation of the ATPase

Mixing the ATPase incubated in the presence of $100\ \mu\text{M}$ Ca^{2+} with an equal volume of $50\ \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at pH 7.2 leads to a

Figure 6 The rate of dephosphorylation of the phosphorylated ATPase

(A) The enzyme syringe contained $0.2\ \text{mg/ml}$ ATPase in $20\ \text{mM}$ Mes/Tris (pH 7.2)/ $5\ \text{mM}$ Mg^{2+} / $100\ \text{mM}$ KCl/ $100\ \mu\text{M}$ Ca^{2+} . This was mixed in a 1:1 ratio with a solution containing $50\ \mu\text{M}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the same buffer. The mixture was incubated for $200\ \text{ms}$ then mixed in a 1:1 ratio with the same buffer containing $2.5\ \text{mM}$ unlabelled ATP. The reaction was quenched at the given times with 25% (w/v) trichloroacetic acid, and the level of phosphorylated ATPase (nmol of EP/mg of protein) determined: native ATPase ($\circ$) or ATPase in $\text{di}(\text{C}_{18:1})\text{PE}$ ($\square$). (B) The enzyme syringe contained $4\ \text{mg/ml}$ ATPase in $12.5\ \text{mM}$ Mes/Tris, pH 6.0, containing $10\ \text{mM}$ EGTA, $1\ \text{mM}$ $[\text{H}]\text{P}_i$, $20\ \text{mM}$ Mg^{2+} and 14% (v/v) DMSO. The second syringe contained $100\ \text{mM}$ Mes/Tris, pH 7.5, containing $100\ \text{mM}$ KCl, $4\ \text{mM}$ Mg^{2+} and $5.3\ \text{mM}$ ATP. The contents of the enzyme syringe were mixed 1:16 (v/v) with the dephosphorylation mixture and the reaction was quenched at the given times with 25% (w/v) trichloroacetic acid: native ATPase ($\circ$) or ATPase in $\text{di}(\text{C}_{18:1})\text{PE}$ ($\square$). (C) The experiment described in (A) was repeated for a different preparation of the Ca^{2+} -ATPase ($\circ$) and for this preparation of the ATPase reconstituted in egg yolk phosphatidylethanolamine ($\square$). The solid lines show fits to single-exponential processes with the rate constants given in the text.

rapid formation of phosphoenzyme, which fits a single exponential process with rates ($\pm$ S.E.) of 48.7 ± 2.8 and $52.9 \pm 4.9\ \text{s}^{-1}$ for the ATPase reconstituted in $\text{di}(\text{C}_{18:1})\text{PC}$ and $\text{di}(\text{C}_{18:1})\text{PE}$ respectively. The maximal level of phosphorylation ($2.4\ \text{nmol}$ of EP/mg of protein) was the same for the ATPase in $\text{di}(\text{C}_{18:1})\text{PC}$ or $\text{di}(\text{C}_{18:1})\text{PE}$ (results not shown).

The Ca^{2+} transport step

When the rate of phosphorylation of the ATPase by ATP is faster than the rate of dissociation of Ca^{2+} from the

Table 1 Effect of di(C_{18:1})PE on the phosphorylation of the ATPase by P_i

The ATPase was incubated in 150 mM Mes/Tris, pH 6.2, in the presence of 5.0 mM EGTA and the given concentrations of Mg²⁺ and P_i, and the level of phosphorylation (nmol of EP/mg protein) was determined.

		Phosphorylation (nmol of EP/mg of protein)	
[P _i] (mM)	[Mg ²⁺] (mM)	Native ATPase	ATPase in di(C _{18:1})PE
Experiment A			
1	1	0.7	0.8
1	3	1.2	1.3
1	5	1.3	1.5
1	10	1.3	1.5
Experiment B			
1	10	1.4	1.2
3	10	1.9	1.5
5	10	2.1	1.9
10	10	2.2	2.0

unphosphorylated ATPase, the rate of the Ca²⁺ transport step (the E2PCa₂ → E2P step in Scheme 1) can be measured by pre-equilibrating the ATPase with ⁴⁵Ca²⁺ and then perfusing it on Millipore filters with ⁴⁰Ca²⁺ and ATP [30]. As shown in Figure 5(B), phosphorylation-induced Ca²⁺ release fits a single exponential with rates (± S.E.) of 17.5 ± 3.8 and 15.5 ± 3.5 s⁻¹ for the native ATPase and the ATPase reconstituted in di(C_{18:1})PE respectively.

The rate of dephosphorylation

The rate of dephosphorylation of the phosphorylated ATPase in the presence of Ca²⁺ and ATP can be determined by first phosphorylating the ATPase with [γ-³²P]ATP in the presence of Ca²⁺ and then mixing with an excess of unlabelled ATP [31]. For the native ATPase the rate (± S.E.) of dephosphorylation determined in this way was 18.1 ± 0.5 s⁻¹ compared with 7.8 ± 0.9 s⁻¹ for the ATPase in di(C_{18:1})PE (Figure 6A). The rate of dephosphorylation in the absence of Ca²⁺ can be determined by first phosphorylating the ATPase with [³²P]P_i at pH 6.0 in the absence of Ca²⁺ and presence of 14 % (v/v) DMSO followed by mixing with an excess of a pH 7.5 buffer containing KCl and ATP to induce dephosphorylation. This gives rates (± S.E.) of dephosphorylation of 19.4 ± 4.6 and 5.7 ± 1.7 s⁻¹ respectively for the native ATPase and for the ATPase in di(C_{18:1})PE (Figure 6B). The 60–70 % decrease in the rate of dephosphorylation explains the 50 % decrease in steady-state ATPase activity.

For another preparation of the ATPase, the rate of dephosphorylation after phosphorylation with ATP in the presence of Ca²⁺ was 13.0 ± 3.0 s⁻¹ (Figure 6C). On reconstitution in egg yolk phosphatidylethanolamine, the rate of dephosphorylation was not changed significantly, the data fitting a rate of 12.3 ± 2.8 s⁻¹ (Figure 6C).

Phosphorylation by P_i

Incubation of the ATPase with P_i at pH 6.2 in the presence of Mg²⁺ and the absence of Ca²⁺ led to phosphorylation of the ATPase. The level of phosphorylation of the ATPase and its dependence on the concentrations of Mg²⁺ and P_i were not

significantly changed on reconstitution of the ATPase into di(C_{18:1})PE (Table 1).

DISCUSSION

Although there is no evidence for the presence of lipids in other than a bilayer organization in biological membranes under physiological conditions, membranes generally contain phospholipids that, in isolation, prefer to adopt non-bilayer structures. The presence, in a bilayer, of lipids favouring the hexagonal H_{II} phase will result in curvature frustration in the bilayer because the non-bilayer-favouring lipid will make unequal contributions to the headgroup and chain areas [32]. This results in an increased surface free energy (increased surface tension) owing to the increased water contact with the hydrophobic region. A higher surface tension will, in turn, result in an increased lateral compression in the acyl chain region. It has been suggested that this strain could modulate the function of some membrane proteins [32]. In particular, the presence of lipids able to form a hexagonal H_{II} phase seems to be required to produce the metarhodopsin II-to-metarhodopsin I ratio found for rhodopsin in the retinal rod membrane [33].

In the rhodopsin system, the metarhodopsin II-to-metarhodopsin I ratio was found to increase steadily with increasing content of di(C_{18:1})PE up to 75 % [33]. For the Ca²⁺-ATPase the result is very different, with di(C_{18:1})PE having little effect on activity up to 80 % di(C_{18:1})PE (Figure 1); in pure di(C_{18:1})PE the ATPase activity is half that in di(C_{18:1})PC. It has been found that approx. 20 % of a bilayer-prefering lipid such as di(C_{18:1})PC is able to maintain a net bilayer organization for mixtures with lipids such as di(C_{18:1})PE [34]. Thus ATPase activity is unaffected by the presence of lipids favouring the hexagonal H_{II} phase, as long as a bilayer structure is maintained; curvature frustration in the bilayer has no effect on ATPase activity. The 20 % phosphatidylethanolamine found in the native SR membrane is unlikely to have any significant effect on ATPase activity.

The low activity observed for the ATPase in pure di(C_{18:1})PE (Figure 1) could be due either to the tendency of this lipid to form a hexagonal H_{II} phase rather than a bilayer phase or to effects of the phosphatidylethanolamine headgroup structure itself. It is possible to distinguish between these indirect and direct effects of headgroup structure by comparing ATPase activities under conditions where phosphatidylethanolamine would be expected to be in bilayer or hexagonal phases. The phase preference of phospholipids can be understood in terms of the relative areas occupied by the lipid headgroups and the fatty acyl chains, with a conical shape (small headgroup compared with the two chains) favouring the formation of a hexagonal H_{II} or other non-bilayer phase [35]. Thus di(C_{18:1})PE with two unsaturated chains adopts a bilayer structure only below approx. 15 °C whereas egg yolk phosphatidylethanolamine, with predominantly one saturated and one unsaturated chain, adopts a bilayer structure below approx. 28 °C [24]. As shown in Figure 2, temperature effects on ATPase activity in di(C_{18:1})PE and in egg yolk phosphatidylethanolamine are different. Activities for the ATPase in egg yolk phosphatidylethanolamine are comparable to those in di(C_{18:1})PC at temperatures up to 25 °C, whereas in di(C_{18:1})PE activities become less than those in di(C_{18:1})PC above approx. 20 °C. We therefore conclude that effects of phosphatidylethanolamine on activity are observed only at temperatures at which the lipid adopts a hexagonal H_{II} phase, and that the lipid headgroup structure itself has no effect. As described later, this is confirmed in studies of the effects of phosphatidyl-

ethanolamines on the rate of dephosphorylation of the phosphorylated ATPase.

The explanation of the decreased ATPase activity in di(C_{18:1})PE at 25 °C has been established by rapid kinetic experiments. No effects were found on the rate of phosphorylation (results not shown) or on the rate of the Ca²⁺ transport step (Figure 5B). There was, however, a 60–70 % decrease in the rate of dephosphorylation of the phosphorylated ATPase (Figures 6A and 6B) sufficient to explain the 50 % decrease in steady-state ATPase activity (Figure 1). In contrast, the rate of dephosphorylation for the ATPase reconstituted in egg yolk phosphatidylethanolamine at 25 °C was the same as for the unreconstituted ATPase (Figure 6C), consistent with the lack of effect of egg yolk phosphatidylethanolamine on ATPase activity at 25 °C (Figure 2). The level of phosphorylation of the ATPase by P_i in the absence of Ca²⁺ was unaffected by reconstitution in di(C_{18:1})PE (Table 1); this implies that the rate of phosphorylation of the ATPase by P_i must also have decreased in di(C_{18:1})PE so that the equilibrium constant for phosphoenzyme formation was unchanged.

Reconstitution in di(C_{18:1})PE had no effect on the stoichiometry of Ca²⁺ binding to the ATPase or on the rate of dissociation of Ca²⁺ from the ATPase (Figure 3) although the affinity of the ATPase for Ca²⁺ was slightly greater in di(C_{18:1})PE than in di(C_{18:1})PC. The increase in Ca²⁺ affinity could be attributed to a shift in the E1/E2 equilibrium for the ATPase towards E1 (Figure 4). The effects of lipids on the E1/E2 equilibrium for the ATPase have been interpreted in terms of Scheme 2. Reconstitution of the ATPase in di(C_{14:1})PC was found to shift the E1/E2 equilibrium towards E1 with a pH-dependence consistent with an increase in the equilibrium constant K₁; reconstitution in di(C_{24:1})PC had no significant effect [8]. In gel-phase phosphatidylcholine a shift towards E1 was also observed, but in this case it was due to an increase in the H⁺-binding constant K_{H6} [36]. As shown here, the effect of di(C_{18:1})PE is also consistent with an increase in the H⁺-binding constant K_{H6} (Figure 4).

In two cases changes in the phase of the lipid, from liquid crystalline bilayer to gel [36] or to hexagonal H_{II} phase, have now been shown to have little effect on Ca²⁺ binding to the ATPase, despite the location of the Ca²⁺-binding sites within the transmembrane region of the ATPase. These same phase changes have, however, been shown to result in decreases in the rate of dephosphorylation of the phosphorylated ATPase (see [36]). The parallel changes in the rate of dephosphorylation and in the E1/E2 equilibrium constant suggest that changes in the phosphorylation domain of the ATPase might occur during the E2–E1 change and that these changes in the phosphorylation domain are coupled to changes in the transmembrane region of the ATPase for the changes to be lipid-sensitive.

We thank the BBSRC for financial support and for a studentship (to K.A.D.).

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