Interaction of phosphatidic acid and phosphatidylserine with the Ca^{2+}-ATPase of sarcoplasmic reticulum and the mechanism of inhibition

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The sarcoplasmic reticulum of skeletal muscle contains anionic phospholipids as well as the zwitterionic phosphatidylcholine and phosphatidylethanolamine. Here we study the effects of anionic phospholipids on the activity of the Ca^{2+}-ATPase purified from the membrane. Reconstitution of the Ca^{2+}-ATPase into dioleoylphosphatidylserine [di($C_{18:1}$)PS] or dioleoylphosphatidic acid [di($C_{18:1}$)PA] leads to a decrease in ATPase activity. Measurements of the quenching of the tryptophan fluorescence of the ATPase by brominated phospholipids give a relative binding constant for the anionic lipids compared with dioleoylphosphatidylcholine close to 1 and suggest that phosphatidic acid only binds to the ATPase at the bulk lipid sites around the ATPase. Addition of di($C_{18:1}$)PS or di($C_{18:1}$)PA to the ATPase in the short-chain dimyristoleoylphosphatidylcholine [di($C_{14:1}$)PC] reverse the effects of the short-chain lipid on ATPase activity and on Ca^{2+} binding, as revealed by the response of tryptophan fluorescence intensity to Ca^{2+} binding. It is concluded that the lipid headgroup and lipid fatty acyl chains have separate effects on the function of the ATPase. The anionic phospholipids have no significant effect on Ca^{2+} binding to the ATPase; the level of Ca^{2+} binding to the ATPase, the affinity of binding and the rate of dissociation of Ca^{2+} are unchanged by reconstitution into di($C_{18:1}$)PA. The major effect of the anionic lipids is a reduction in the maximal level of binding of MgATP. This is attributed to the formation of oligomers of the Ca^{2+}-ATPase, in which only one molecule of the ATPase can bind MgATP dimers in di($C_{18:1}$)PS and trimers or tetramers in di($C_{18:1}$)PA. The rates of phosphorylation and dephosphorylation for the proportion of the ATPase still able to bind ATP are unaffected by reconstitution. Larger changes were observed in the level of phosphorylation of the ATPase by P_i, which became very low in the anionic phospholipids. The fluorescence response to Mg^{2+} for the ATPase labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin was also changed in di($C_{18:1}$)PS and di($C_{18:1}$)PA, so that effects of Mg^{2+} became comparable with those seen on phosphorylation for the unreconstituted ATPase. The anionic phospholipids could induce a conformational change in the ATPase on binding Mg^{2+} equivalent to that normally induced by phosphorylation or by binding inhibitors such as thapsigargin.

INTRODUCTION

The transmembrane domain of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR) makes contact with about 30 lipid molecules in the membrane [1]. The interaction with these surrounding lipids, variously referred to as boundary, bulk or annular lipids, is relatively non-specific, and the interaction between the Ca^{2+}-ATPase and any particular lipid molecule is short lived [1–3]. Nevertheless, the activity of the ATPase is dependent on the chemical structure and physical phase of these surrounding phospholipids. This is important, since the native SR membrane contains a wide variety of different species of phospholipid molecule, differing in fatty acyl chains and headgroup. We have been studying the effects of these different phospholipid structures on the activity of the Ca^{2+}-ATPase. The major lipid of the SR membrane is the zwitterionic phosphatidylcholine. For the Ca^{2+}-ATPase reconstituted into a series of phosphatidylcholines in the liquid crystalline phase, ATPase activity is highest when the fatty acyl chain length is about C_{18}, with low activities for shorter (C_{14}) or longer (C_{24}) fatty acyl chains [4,5]. The molecular basis for the observed changes in activity is complex, since a number of distinct changes in the properties of the ATPase are observed in the short- and long-chain lipids, including decreases in the rates of phosphorylation and dephosphorylation and a change in the stoichiometry of Ca^{2+}-binding from the normal two to just one Ca^{2+} ion bound per ATPase molecule [5–7].

As well as zwitterionic phospholipids, the SR membrane contains three species of negatively charged phospholipid, phosphatidylserine, phosphatidylinositol and Ptd-Ins4*P* [8]. The plasma membrane Ca^{2+}-ATPase has been shown to be activated by phosphatidylserine, phosphatidic acid and Ptd-Ins4*P* [9]. The Ca^{2+}-ATPase of SR has also been shown to be activated by Ptd-Ins4*P* [10], but the effects of other anionic phospholipids have not yet been studied in any detail. Since the effect of Ptd-Ins4*P* was observed at low concentrations of Ptd-Ins4*P* in the membrane (10 mol-% Ptd-Ins4*P*), it was suggested that the effect could follow from binding to a small number of 'special' sites on the ATPase, distinct from the regions of the ATPase that interact with the boundary lipids [10]. Here we investigate the effects of phosphatidylserine and phosphatidic acid on the activity of the SR Ca^{2+}-ATPase. We interpret the observed effects in terms of a modified form of the E1–E2 model, as shown in Scheme 1. The scheme proposes that two Ca^{2+} ions bind in a cooperative fashion to the E1 conformation of the ATPase, from the cytoplasmic side of the membrane [11,12]. After binding of MgATP to the ATPase, the ATPase is phosphorylated and undergoes a change in conformation to a state in which the two Ca^{2+}-binding sites are of low affinity and inward facing ($E2PCa_2$). Ca^{2+} is lost from this phosphorylated intermediate to the lumen

Abbreviations used: di($C_{14:1}$)PC, dimyristoleoylphosphatidylcholine; di($C_{18:1}$)PC, dioleoylphosphatidylcholine; di($C_{18:1}$)PA, dioleoylphosphatidic acid; di($C_{18:1}$)PS, dioleoylphosphatidylserine; di($Br_2C_{18:0}$)PC, di(9,10-dibromostearoyl)phosphatidylcholine; di($Br_2C_{18:0}$)PA, di(9,10-dibromostearoyl)-phosphatidic acid; di($C_{22:1}$)PC, dierucylphosphatidylcholine; SR, sarcoplasmic reticulum; Br-DMC, 4-(bromomethyl)-6,7-dimethoxycoumarin.

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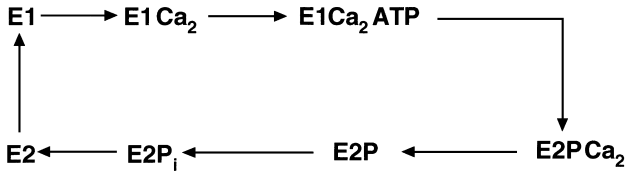

Scheme 1

of the SR. Dephosphorylation of E2P then allows recycling to E1.

In studies of the effects of negatively charged phospholipids on the activity of the Ca^{2+}-ATPase special attention has to be paid to interactions between the phospholipid headgroups and divalent metal ions such as Ca^{2+} and Mg^{2+}, both necessary for the activity of the ATPase. Phosphatidic acid is doubly ionizable, with apparent pK values of 3.5 and 9.5 in 100 mM salt [13]. Dioleoylphosphatidic acid [di($C_{18:1}$)PA] adopts a bilayer structure at room temperature in the pH range 4–9 in the absence of divalent metal ions [14] but, in the presence of Ca^{2+} or Mg^{2+}, structures are very pH dependent, rather ill-defined, and different for saturated and unsaturated lipids [14–16]. At pH 8.0, when di($C_{18:1}$)PA is present as a mixture of the singly and doubly charged forms, addition of Ca^{2+} or Mg^{2+} to vesicles of di($C_{18:1}$)PA causes flocculation due to vesicle aggregation, but the lipid remains in a bilayer structure. At pH 7.4 or pH 6.0, when di($C_{18:1}$)PA is present largely in the singly ionized form, addition of Ca^{2+} or Mg^{2+} results in the formation of an hexagonal H_{II} phase or a unique phase, which is neither lamellar nor hexagonal, with the fatty acyl chains being highly ordered [17]. Mixtures of phosphatidylcholine and phosphatidic acid in the liquid crystalline phase, in the absence of divalent metal ions, mix almost ideally [18,19], but addition of Ca^{2+} leads to the formation of clusters enriched in phosphatidic acid; Mg^{2+} is much less effective than Ca^{2+} in inducing cluster formation [17,19–22]. Unsaturated phosphatidylserines also adopt a liquid crystalline, bilayer structure in the absence of divalent metal ions over the pH range 4–8 [23–25]. Addition of Ca^{2+} to vesicles of phosphatidylserine results in precipitation of the lipid with the formation of cochleate cylinders [26]; in these structures the bilayer is maintained but the temperature of the gel to liquid crystalline phase-transition is much increased so that the fatty acyl chains become rigidly packed [27,28]. Addition of Mg^{2+} also precipitates vesicles of phosphatidylserine, but at higher concentrations than Ca^{2+}, and no change in chain packing is seen with dioleoylphosphatidylserine [di($C_{18:1}$)PS] [23,29]. Dioleoylphosphatidylcholine [di($C_{18:1}$)PC] and di($C_{18:1}$)PS mix almost ideally at high mole fractions of phosphatidylserine but non-ideally at low phosphatidylserine, the non-ideality being such as to favour interactions between unlike molecules [30]. Addition of Ca^{2+} to mixtures of phosphatidylserine and phosphatidylcholine has been shown to result in phase separation of solid aggregates of phosphatidylserine, but again with Mg^{2+} having little effect [22,31].

The reported changes in phase of the anionic lipids on binding divalent metal ions could be important, since the activity of the Ca^{2+}-ATPase is known to be sensitive to the physical phase of the lipid surrounding it in the membrane. Thus the activity of the Ca^{2+}-ATPase is very low in bilayers of phosphatidylcholine in the gel phase because of a very slow rate of phosphorylation by ATP [32], and activity in phosphatidylethanolamine at temperatures when the lipid is in the hexagonal H_{II} phase is also low because of a decrease in the rate of dephosphorylation of the phosphorylated ATPase [33].

MATERIALS AND METHODS

Di($C_{18:1}$)PA, di($C_{18:1}$)PS, di($C_{18:1}$)PC and dimyristoleoylphosphatidylcholine [di($C_{14:1}$)PC] were obtained from Avanti Polar Lipids. Di($C_{18:1}$)PC and di($C_{18:1}$)PA were brominated to give di(9,10-dibromostearoyl)phosphatidylcholine [di($Br_2C_{18:0}$)PC] and di(9,10-dibromostearoyl)phosphatidic acid [di($Br_2C_{18:0}$)PA] respectively, by the method described by East and Lee [2]. SR was prepared from rabbit skeletal muscle largely as described by Daiho et al. [34], all procedures being carried out at 4 °C. Fast twitch muscle (500 g) was blended with buffer (2 litres; 10 mM Mops/Tris, pH 7.0/100 mM NaCl) in a Waring blender. The homogenate was spun at 8300 ***g*** for 35 min, and the supernatant was filtered through muslin and then recentrifuged at 12000 ***g*** for 30 min. The resulting supernatant was spun at 53000 ***g*** for 40 min and the pellet was resuspended in buffer [5 mM Tris/maleate (pH 6.5)/0.6 M KCl] at 25 mg of protein/ml. The suspension was stirred for 40 min and then spun at 125000 ***g*** for 45 min. The pellet was resuspended in buffer [30 mM Mops/Tris (pH 7.0)/0.1 M sucrose], spun at 125000 ***g*** for 45 min, and the pellet resuspended in a minimum volume of buffer and frozen in liquid nitrogen. Purified ATPase was prepared from SR as described by East and Lee [2] and concentrations of ATPase were estimated by using a specific absorption coefficient of 1.2 litre·g^{-1}·cm^{-1} for a solution in 1% (v/v) SDS [35]. Reconstitutions were performed as described by Starling et al. [5]. Measurements of ATPase activity, equilibrium levels of phosphorylation by [^{32}P]P_i and [γ-^{32}P]ATP and binding of $^{45}Ca^{2+}$ and [^{14}C]ATP, and of the time dependencies of phosphorylation by [γ-^{32}P]ATP, dephosphorylation and $^{45}Ca^{2+}$ dissociation were performed as described in previous papers [5,7].

The ATPase was labelled with 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) before reconstitution, as described in Stefanova et al. [36]. Fluorescence spectra were recorded at 25 °C using an SLM 8000C spectrofluorimeter. DMC fluorescence was excited at 350 nm and observed at 425 nm. Measurements of tryptophan fluorescence were performed with excitation and emission wavelengths of 290 and 340 nm respectively. Fluorescence quenching in mixtures of brominated and non-brominated phospholipids was fitted to the equation:

$$F/F_o = F_{min} + (F_o - F_{min})(1 - f_{Br})^n \quad (1)$$

where F_o and F_{min} are the fluorescence intensities for the ATPase reconstituted with di($C_{18:1}$)PC and brominated phospholipid respectively, F is the fluorescence intensity in the phospholipid mixture when the mole fraction of brominated lipid is x_{Br} and the fraction of sites at the lipid–protein interface occupied by brominated lipid is f_{Br}, and n, representing the number of lipid sites around an average tryptophan residue, has the value 1.6 [2]. The fraction of sites occupied by brominated lipid is related to x_{Br} by:

$$f_{Br} = x_{Br}/[x_{Br} + K(1 - x_{Br})] \quad (2)$$

where K is the relative binding constant of the brominated phospholipid with respect to the non-brominated phospholipid [37].

RESULTS

Effects of anionic lipids on ATPase activity

The ATPase was reconstituted into anionic phospholipid by mixing the purified ATPase with excess phospholipid (molar ratio lipid:protein = 1000:1) in cholate solution, followed by a

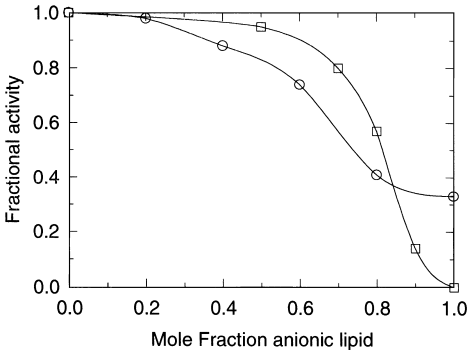

Figure 1 Effects of di($C_{18:1}$)PS and di($C_{18:1}$)PA on ATPase activity

ATPase was reconstituted into mixtures of di($C_{18:1}$)PC and either di($C_{18:1}$)PS (○) or di($C_{18:1}$)PA (□) at the given mole fraction of anionic lipid. ATPase activities were measured at 25 °C, pH 7.2, with 2.1 mM ATP and 10 mM Mg^{2+}, and are expressed as a fraction of the activity observed in di($C_{18:1}$)PC, typically about 8 units/mg of protein for this preparation of ATPase. The ATPase concentration was 0.05 μM.

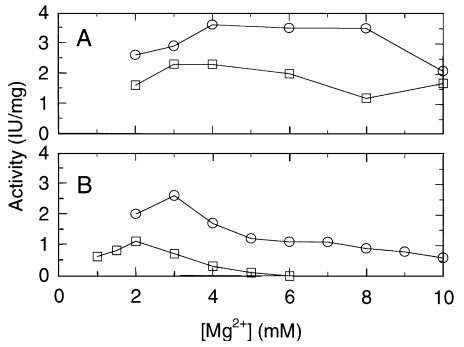

Figure 2 Effects of Mg^{2+} concentration on ATPase activities in the presence of di($C_{18:1}$)PS or di($C_{18:1}$)PA

ATPase was reconstituted into a 1:4 mixture of di($C_{18:1}$)PC and anionic lipid (○) or anionic lipid alone (□) and ATPase activities measured in the presence of 2.1 mM ATP, at the given concentrations of Mg^{2+}: (**A**) di($C_{18:1}$)PS; (**B**) di($C_{18:1}$)PA.

250-fold dilution into buffer to decrease the concentration of cholate below its critical micelle concentration. The procedure results in the formation of membrane fragments unable to accumulate Ca^{2+} and which thus show full (uncoupled) ATPase activity, as demonstrated by a lack of stimulation of activity on addition of the Ca^{2+} ionophore A23187 [38].

The ATPase activity of the Ca^{2+}-ATPase reconstituted into di($C_{18:1}$)PS is about a third of that in di($C_{18:1}$)PC when measured in the presence of 2.1 mM ATP and 10 mM Mg^{2+} at pH 7.2, whereas no activity could be detected for the ATPase in di($C_{18:1}$)PA under these conditions (Figure 1). In mixtures of di($C_{18:1}$)PC, and either di($C_{18:1}$)PS or di($C_{18:1}$)PA, activity decreases with increasing content of anionic lipid, effects of the anionic lipid being relatively small at mole fractions less than 0.5 (Figure 1). For the ATPase in di($C_{18:1}$)PS, activities vary little with Mg^{2+} concentration (Figure 2). In contrast, the activity of the ATPase in di($C_{18:1}$)PA decreases markedly with increasing Mg^{2+} concentration; maximal activity is observed at a total Mg^{2+} concentration of 2.0 mM which, at pH 7.2 in the presence of 2.1 mM ATP, corresponds to a free Mg^{2+} concentration of 0.3 mM (Figure 2). The decrease in activity observed at higher concentrations of Mg^{2+} is reversible (Table 1). Addition of EDTA to reduce the free Mg^{2+} concentration from 2.0 to 0.3 mM results in an increase in ATPase activity from 0.3 to 0.8 units/mg of protein; addition of further EDTA results in a decrease in activity, attributable to a large decrease in the concentration of MgATP, the true substrate for the ATPase.

To establish the mechanism of inhibition of the ATPase we measured the binding of Ca^{2+} and ATP to the ATPase and the rates of phosphorylation and dephosphorylation.

Ca^{2+} binding to the ATPase

The affinity of the Ca^{2+}-ATPase for Ca^{2+} was determined by incubating the Ca^{2+}-ATPase adsorbed onto Millipore filters with $^{45}Ca^{2+}$. For the native ATPase at pH 6.0 in the presence of 100 mM KCl and 20 mM Mg^{2+}, Ca^{2+}-binding occurs to a maximum level of about 12.5 nmol of Ca^{2+} bound/mg of protein, with 50 % of maximal binding being observed at a pCa value of 4.6 (Figure 3), in agreement with the binding parameters given by Lee et al. [12]. Binding data recorded for the ATPase reconstituted in di($C_{18:1}$)PA are very similar to those for the native ATPase (Figure 3). Reliable binding data could not be obtained at pH 7.2, even in the presence of 20 mM Mg^{2+}, because of high levels of non-specific binding of Ca^{2+} to the anionic lipid.

Ca^{2+}-binding to the ATPase can also be studied from the associated change in the tryptophan fluorescence intensity of the ATPase [11]. Removal of Ca^{2+} from the Ca^{2+}-bound ATPase after addition of EGTA results in a decrease in fluorescence intensity (Figure 4). At pH 6.0 similar responses are observed for the ATPase in di($C_{18:1}$)PS and di($C_{18:1}$)PA, with 50 % responses being observed at pCa values of about 5.5 and 5.4 respectively, compared with a value of 4.8 for the native ATPase [11]. Similar responses are observed for the ATPase in di($C_{18:1}$)PA at pH values up to 6.7; the pCa value corresponding to 50 % responses increasing with increasing pH. However, at pH values greater than 6.7, fluorescence intensity increases on lowering the concentration of Ca^{2+}, and the response occurs at a much higher concentration of Ca^{2+} (Figure 4). The interpretation of the changed response to Ca^{2+} is unclear, but presumably relates to binding of Ca^{2+} to di($C_{18:1}$)PA. The fluorescence signal from the ATPase in di($C_{18:1}$)PS was too unstable to allow titrations with Ca^{2+} at higher pH values and, in the presence of Mg^{2+}, aggregation of the sample prevented any useful measurements. Thus, under conditions where it can be measured, reconstitution with negatively charged lipid has little effect on the affinity of the Ca^{2+}-ATPase for Ca^{2+} or on the stoichiometry of Ca^{2+} binding.

The rate of dissociation of Ca^{2+} from the Ca^{2+}-bound ATPase is relatively slow, consistent with a gated process [11]. We therefore measured the rate of dissociation of Ca^{2+} for the ATPase in di($C_{18:1}$)PA, looking for any possible effects of negatively charged lipids on this process. The Ca^{2+}-bound ATPase, reconstituted in di($C_{18:1}$)PA, was incubated with 100 μM $^{45}Ca^{2+}$, followed by adsorption onto Millipore filters and washing with buffer containing 100 mM KCl/20 mM Mg^{2+} for a predetermined length of time. A relatively high initial concentration of Ca^{2+} was used to ensure that the two Ca^{2+}-binding sites on the ATPase were fully occupied, and this resulted in a high level of non-specific binding. Nevertheless, at both pH 6 and pH 7.2, the magnitude of Ca^{2+} loss over a time period of 1 s washing was very similar for the native ATPase and for the ATPase reconstituted in di($C_{18:1}$)PA, suggesting that over this time period little of the non-specifically bound Ca^{2+} was lost from the reconstituted system. The measured rates of dissociation at pH 6.0 were 5.6 ± 0.8 s^{-1} for the native ATPase and 7.2 ± 2.9 s^{-1} for the reconstituted ATPase, with rates at pH 7.2 of 29.4 ± 8.2 s^{-1}

Table 1 Reversibility of the effect of Mg^{2+} on ATPase activity in di($C_{18:1}$)PA

ATPase was reconstituted in di($C_{18:1}$)PA and the ATPase activity was measured at pH 7.2 with 2.1 mM ATP and 4 mM Mg^{2+}. EDTA was then added to the concentration given and activity was re-measured.

EDTA concentration (mM)	Free Mg^{2+} concentration (mM)*	MgATP concentration (mM)*	ATPase activity (units/mg protein)
0	2.0	2.1	0.3
1	1.0	2.0	0.5
2	0.3	1.7	0.8
4	0.02	0.4	0.3

* Calculated using the binding constants given in [56].

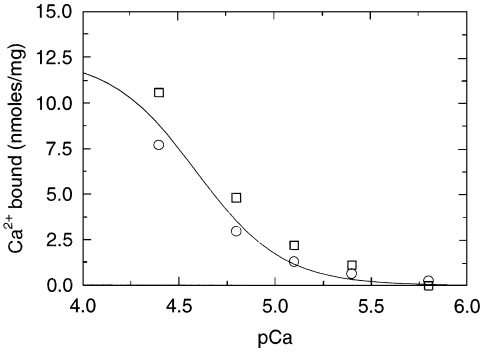

Figure 3 Binding of $^{45}Ca^{2+}$ to the ATPase

Native ATPase (○) or ATPase reconstituted in di($C_{18:1}$)PA (□) was incubated with 0.1 mM $^{45}Ca^{2+}$ in 130 mM Mes/Tris, pH 6.0/100 mM KCl/20 mM Mg^{2+}, containing EGTA, to give the required free concentration of Ca^{2+}. The solid line shows a simulation using the binding parameters given by Lee et al. [12].

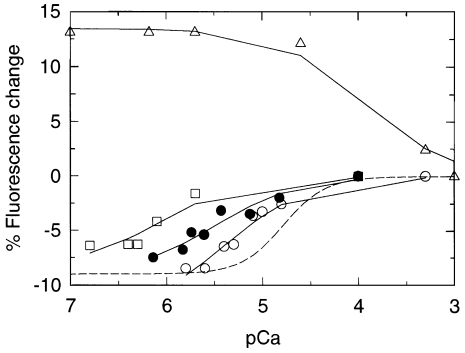

Figure 4 Ca^{2+} dependence of tryptophan fluorescence intensity as a function of pH

The change in tryptophan fluorescence intensity on addition of EGTA to the given pCa value is shown for the ATPase in di($C_{18:1}$)PA at pH 6.0 (○), pH 6.5 (□) and pH 7.2 (△) and for the ATPase in di($C_{18:1}$)PS at pH 6.0 (●). The solid lines show fits to a simple one-site binding equation. The broken line shows the binding curve for the native ATPase at pH 6.0 calculated using the binding parameters given by Lee et al. [12], assuming a maximum fluorescence change of −9%.

and $20.2 \pm 3.6\ s^{-1}$ for the native and reconstituted enzyme respectively. We conclude that reconstitution in di($C_{18:1}$)PA has no significant effect on the rate of dissociation of Ca^{2+} from the ATPase.

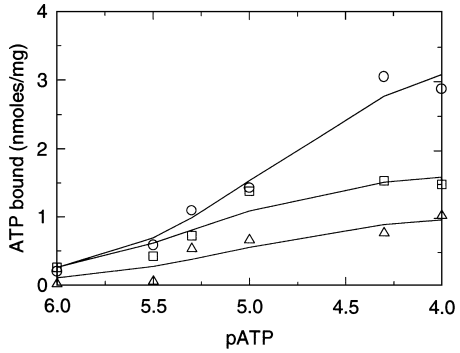

Figure 5 ATP binding to the ATPase

ATPase was incubated with [^{14}C]ATP in 150 mM Mops/Tris, pH 7.2/100 mM KCl/5 mM $MgSO_4$/0.5 mM EGTA, and the level of bound ATP was determined by filtration: ○, native ATPase; □, ATPase in di($C_{18:1}$)PS; △, ATPase in di($C_{18:1}$)PA. The solid lines show fits to a simple one-site binding equation with the parameters given in the text.

ATP binding to the ATPase

In contrast to the lack of effect of reconstitution on Ca^{2+} binding, reconstitution had a marked effect on binding of MgATP to the ATPase (Figure 5). For the native ATPase the binding data fitted to a single binding site with a maximum binding of 3.5 ± 0.2 nmol MgATP bound/mg of protein, and an affinity of $13 \pm 2\ \mu$M. Reconstitution in di($C_{18:1}$)PS or di($C_{18:1}$)PA had little effect on the affinity for MgATP (8.8 ± 4.8 and $5.5 \pm 2.0\ \mu$M respectively), but reduced the level of maximum binding to 1.7 ± 0.2 and 1.1 ± 0.2 nmol MgATP bound/mg of protein in di($C_{18:1}$)PS and di($C_{18:1}$)PA respectively (Figure 5).

Phosphorylation and dephosphorylation of the ATPase

A decrease in the level of binding of MgATP to the ATPase would be expected to lead to a reduced level of phosphorylation of the ATPase. Steady-state levels of phosphorylation of the ATPase by ATP were determined by incubation of the ATPase with [γ-^{32}P]ATP in the presence of 100 μM Ca^{2+} for 5 s, followed by quenching of the reaction. For the native ATPase the maximal levels of phosphorylation observed at 7 mM Mg^{2+} was 2.35 nmol [EP]/mg of protein (Table 2). Levels of phosphorylation observed for the ATPase reconstituted in di($C_{18:1}$)PS and di($C_{18:1}$)PA were 52% and 27% respectively of that observed for the native ATPase. These decreases in the level of phosphorylation were comparable with the observed decreases in the level of ATP binding to the reconstituted ATPase (Figure 5), corresponding to 49% and 31% respectively in di($C_{18:1}$)PS and di($C_{18:1}$)PA. The

Table 2 Steady-state level of phosphorylation of the ATPase by ATP

ATPase (0.2 mg of protein/ml) was incubated in 20 mM Hepes, pH 7.2/100 mM KCl/100 μM Ca^{2+}, containing 100 μM ATP and the given concentration of Mg^{2+} for 15 s before quenching the reaction and determining the level of phosphorylation of the ATPase.

	EP (nmol/mg of protein)		
Mg^{2+} (mM)	Native ATPase	ATPase in di($C_{18:1}$)PS	ATPase in di($C_{18:1}$)PA
0.5	0.95	–	0.38
1.0	1.23	0.79	0.50
3.0	1.74	1.12	0.66
5.0	2.18	1.21	0.65
7.0	2.35	1.23	0.63

steady-state level of phosphorylation by ATP of the native ATPase decreased with decreasing concentration of Mg^{2+}, as reported previously [39]. The fractional decrease in the level of phosphorylation observed in di($C_{18:1}$)PS and di($C_{18:1}$)PA is independent of the concentration of Mg^{2+} (Table 2).

The parallel decreases in levels of MgATP binding and maximal levels of phosphorylation observed on reconstitution in negatively charged lipid suggest that the portion of the ATPase which does bind MgATP, phosphorylates and dephosphorylates normally. The rate of phosphorylation of the ATPase by ATP at pH 7.2 and 25 °C was determined by rapidly mixing the ATPase, incubated in the presence of Ca^{2+}, with 50 μM ATP (final concentration), and quenching the reaction after predetermined lengths of time (results not shown). The time course of phosphorylation of the native ATPase fitted to a single exponential process with a rate constant of $132 \pm 14\ s^{-1}$. For the ATPase in di($C_{18:1}$)PS or di($C_{18:1}$)PA, maximal levels of phosphorylation were lower, in agreement with the steady-state data given in Table 2. Again the time courses of phosphorylation fitted to single exponential processes with rate constants of 84 ± 33 and $77 \pm 23\ s^{-1}$ for the ATPase in di($C_{18:1}$)PS and di($C_{18:1}$)PA respectively, the errors on the determinations being relatively large because of the low levels of maximal phosphorylation. Thus within experimental error we conclude that reconstitution has no effect on rates of phosphorylation.

Table 3 Rates of dephosphorylation of the phosphorylated ATPase

ATPase (0.2 mg/ml) was incubated in 150 mM Mops/Tris, pH 7.2, containing 100 mM KCl and 100 μM $CaCl_2$ and then mixed with an equal volume of the same buffer containing 10 mM Mg^{2+} and 100 μM [γ-^{32}P]ATP. After 200 ms, the mixture was mixed with an equal volume of 5 mM Mg^{2+} and 5.0 mM unlabelled ATP and quenched at the times given. The data were fitted to single-exponential processes to give the rate constants for dephosphorylation shown.

System	Rate of dephosphorylation (s^{-1})
Native ATPase	12.9 ± 0.2
ATPase in di($C_{18:1}$)PS	9.0 ± 1.8
ATPase in di($C_{18:1}$)PA	21.0 ± 7.3

The rate of dephosphorylation of the phosphorylated ATPase was determined by incubating the ATPase with [γ-^{32}P]ATP and Ca^{2+} for 15 s, followed by mixing with a high concentration of unlabelled ATP to initiate dephosphorylation. Dephosphorylation fitted to a single exponential process with the rates given in Table 3. Again the error in the determination of the rate of dephosphorylation of the phosphorylated ATPase in di($C_{18:1}$)PA is relatively large because of the low initial level of phosphorylation, but it is clear that any effects of the anionic lipids on the rate of dephosphorylation are small.

The ATPase can also be phosphorylated by incubation with millimolar concentrations of P_i and Mg^{2+} at acid pH values. Maximal levels of phosphorylation of the ATPase in di($C_{18:1}$)PS or di($C_{18:1}$)PA were very low compared with those for the native ATPase at both low and high concentrations of Mg^{2+} (Table 4). Thus effects of reconstitution with negatively charged lipid are greater on phosphorylation by P_i than on phosphorylation by ATP.

Reconstitution with mixtures of di($C_{14:1}$)PC and anionic lipid

In previous studies we have shown that the activity of the Ca^{2+}-ATPase is sensitive to the thickness of the lipid bilayer surrounding it, as defined by the lengths of the phospholipid fatty acyl chains [4,5]. Here we have shown that the activity of the Ca^{2+}-ATPase is also sensitive to the structure of the lipid headgroup. By studying the effects of mixtures of negatively charged lipid with oleoyl ($C_{18:1}$) chains and a phosphatidylcholine with a short myristoleoyl ($C_{14:1}$) chain we can determine whether the effects of chain length and headgroup are linked or independent.

The Ca^{2+}-ATPase shows an activity of 0.4 units/mg of protein in bilayers of di($C_{14:1}$)PC [5]. Figure 6 shows ATPase activities measured for the ATPase in mixtures of di($C_{14:1}$)PC and either

Table 4 Phosphorylation of the ATPase by P_i

ATPase (0.2 mg of protein/ml) was incubated in 150 mM Mes/Tris, pH 6.2, containing 5 mM EGTA and the required concentrations of Mg^{2+} and [^{32}P]P_i at 25 °C for 15 s, followed by quenching and determination of the level of phosphorylated ATPase.

	EP (nmol/mg of protein)		
Mg^{2+} (mM) at $P_i = 1$ mM	Native ATPase	ATPase in di($C_{18:1}$)PS	ATPase in di($C_{18:1}$)PA
1	0.33	0.15	0.03
5	1.47	0.27	0.07
10	1.50	0.25	0.05
[^{32}P]P_i (mM) at $Mg^{2+} = 10$ mM			
1	1.50	0.25	0.05
2	1.90	0.32	0.21
5	2.62	0.22	0.24
10	2.70	0.42	0.13

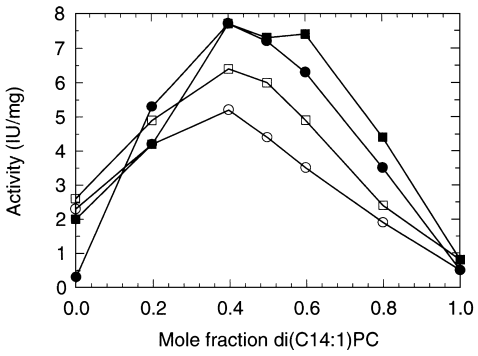

Figure 6 ATPase activities in mixtures of di($C_{14:1}$)PC and di($C_{18:1}$)PS or di($C_{18:1}$)PA

ATPase was reconstituted into mixtures of di($C_{14:1}$)PC and either di($C_{18:1}$)PS (○,□) or di($C_{18:1}$)PA (●,■) and ATPase activities were measured with 2.1 mM ATP and either 2.1 mM Mg^{2+} (■,□) or 5 mM Mg^{2+} (●,○).

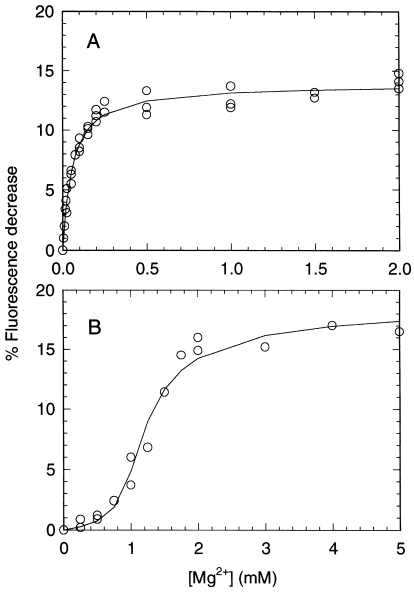

Figure 7 Fluorescence response of DMC-labelled ATPase to the addition of Mg^{2+}

DMC-labelled ATPase was reconstituted in di($C_{18:1}$)PS (**A**) or di($C_{18:1}$)PA (**B**) and the fluorescence response to the addition of Mg^{2+} was determined in 40 mM Tris, pH 6.0. The solid lines show fits to binding equations as described in the text.

di($C_{18:1}$)PS or di($C_{18:1}$)PA, at high and low concentrations of Mg^{2+}. Starting from the ATPase in di($C_{14:1}$)PC, activity first increases with decreasing mole fraction of di($C_{14:1}$)PC to a maximum at a mole fraction of di($C_{14:1}$)PC of 0.4, activity then decreasing with further decreases in the content of di($C_{14:1}$)PC; the maximum activities observed in these mixtures are comparable with those observed in di($C_{18:1}$)PC for this preparation of ATPase. In mixtures of di($C_{14:1}$)PC and di($C_{18:1}$)PC, activity was also observed to increase with decreasing mole fraction of di($C_{14:1}$)PC up to a value of about 0.5, but, in this case, activity then remained constant with further decreases in the content of di($C_{14:1}$)PC [5]. The initial increase in activity observed with decreasing mole fraction of di($C_{14:1}$)PC in mixtures with di($C_{18:1}$)PS or di($C_{18:1}$)PA is therefore likely to be an effect of fatty acyl chain length. The decrease in activity observed on addition of higher concentrations of di($C_{18:1}$)PS or di($C_{18:1}$)PA would then follow from effects of the lipid headgroup. The greater maximal activity observed in mixtures of di($C_{14:1}$)PC with di($C_{18:1}$)PA than with di($C_{18:1}$)PS would then reflect the higher mole fraction of di($C_{18:1}$)PA required before inhibition of activity is observed (Figure 1). Thus the effects of chain length and headgroup structure on the activity of the Ca^{2+}-ATPase are independent.

Independent effects of chain length and headgroup are also suggested by fluorescence experiments. Reconstitution of the Ca^{2+}-ATPase with di($C_{14:1}$)PC results in a change in the stoichiometry of Ca^{2+} binding to the ATPase and a change in the response of Trp fluorescence to Ca^{2+} binding [5]. For the ATPase in di($C_{18:1}$)PC, removal of Ca^{2+} from the Ca^{2+}-bound ATPase results in a decrease in fluorescence intensity. For the ATPase in di($C_{14:1}$)PC, removal of Ca^{2+} results in an increase in fluorescence intensity in the presence of Mg^{2+}, but no change in fluorescence intensity in the absence of Mg^{2+} [40]. In mixtures of di($C_{14:1}$)PC and either di($C_{18:1}$)PS or di($C_{18:1}$)PA, the fluorescence response to the removal of Ca^{2+} gradually decreases in magnitude with an increase in the content of di($C_{14:1}$)PC (results not shown), consistent with the gradual increase in activity to the maximum observed in Figure 6.

Fluorescence of DMC-labelled ATPase

In previous studies we have shown that changes in the fluorescence intensity for DMC-labelled ATPase can be used to monitor a variety of conformational changes in the ATPase. Addition of Mg^{2+} to DMC-labelled ATPase at pH 6.0, in the absence of Ca^{2+}, results in a 5% decrease in fluorescence intensity, the concentration of Mg^{2+} giving the half-maximal effect being 4 mM [11]. We attributed this change to binding of Mg^{2+} at a 'gating site' on the ATPase, controlling the rate of dissociation of Ca^{2+} from the high-affinity sites on the cytoplasmic side of the ATPase [11]. Phosphorylation of the ATPase by ATP in the presence of Ca^{2+}, addition of a high concentration of vanadate, or addition of the specific inhibitors thapsigargin or thapsivillosin, all result in about a 16% decrease in fluorescence intensity at pH 6.0 [41]. The fluorescence response of DMC-labelled ATPase in di($C_{18:1}$)PS or di($C_{18:1}$)PA to the addition of Mg^{2+} is very different to that seen for the unreconstituted DMC-labelled ATPase (Figure 7); the maximal fluorescence changes on addition of Mg^{2+} are 13.9% and 19% in di($C_{18:1}$)PS and di($C_{18:1}$)PA respectively (fitted values, see below). The data in di($C_{18:1}$)PS fit to a simple binding equation with a K_d value for Mg^{2+} of 57 ± 4 μM (Figure 7). For the ATPase in di($C_{18:1}$)PA, very little change in fluorescence intensity is observed on addition of Mg^{2+} up to a concentration of about 0.5 mM, beyond which the fluorescence intensity decreases as for the ATPase in di($C_{18:1}$)PA. This 'lag phase' can be attributed to binding of Mg^{2+} to the phosphatidic acid in the sample; phosphatidic acid is known to bind Mg^{2+} more strongly than phosphatidylserine [23,29]. The data for the ATPase in di($C_{18:1}$)PA can be fitted to a two-site binding model, with high-affinity binding to the high concentrations of phosphatidic acid present in the sample (1 mM) significantly depleting the free concentration of Mg^{2+}. Binding of Mg^{2+} to di($C_{18:1}$)PA was analysed in terms of the quadratic binding equation

$$[\mathrm{MgPA}] = \{A - (A^2 - 4[\mathrm{PA}]^{\mathrm{total}}[\mathrm{Mg}]^{\mathrm{total}})^{\frac{1}{2}}\}/2.0 \quad (3)$$

where

$$A = K_d + [\mathrm{PA}]^{\mathrm{total}} + [\mathrm{Mg}]^{\mathrm{total}} \quad (4)$$

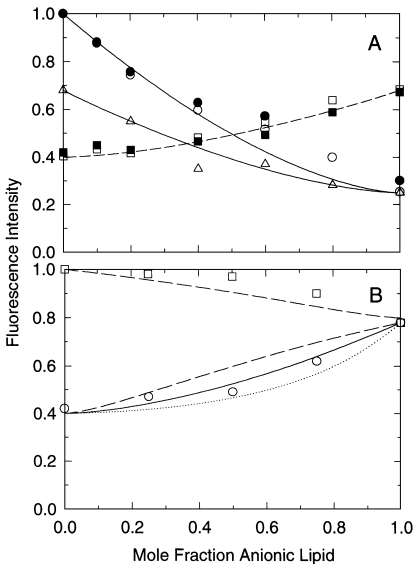

Figure 8 Relative fluorescence intensities for the ATPase in mixtures containing anionic phospholipids

ATPase was reconstituted in mixtures of phospholipids containing either phosphatidic acid (**A**) or phosphatidylserine PS (**B**) at the given molar ratio of anionic lipids, with the fluorescence intensities expressed relative to that in di($C_{18:1}$)PC. (**A**): (○,●), di($C_{18:1}$)PC and di($Br_2C_{18:0}$)PA; (□,■), di($C_{18:1}$)PA and di($Br_2C_{18:0}$)PC; △, di($C_{18:1}$)PA and di($Br_2C_{18:0}$)PA. (○,□,△), in the absence of Mg^{2+}; (●,■), in the presence of 1 mM Mg^{2+}. The lines are simulations, as described in the text, with a relative binding constant K for phosphatidylcholine relative to phosphatidic acid, of 1. (**B**): □, di($C_{18:1}$)PC and di($C_{18:1}$)PS; ○, di($C_{18:1}$)PS and di($Br_2C_{18:0}$)PC. The solid lines represent simulations with a relative binding constant K for phosphatidylcholine relative to phosphatidylserine of 1. The broken and dotted lines are calculated quenching curves with relative binding constants K of 0.5 and 2 respectively. The buffer for these experiments was 20 mM Hepes/Tris, pH 7.2/100 mM KCl and the concentration of ATPase was 0.05 μM.

and [MgPA], $[PA]^{total}$ and $[Mg]^{total}$ are the concentrations of the Mg^{2+}–phosphatidic acid complex and the total concentrations of di($C_{18:1}$)PA and Mg^{2+} in the sample respectively, and K_d is the effective binding constant of di($C_{18:1}$)PA for Mg^{2+} under these conditions. The concentration of Mg^{2+} unbound to di($C_{18:1}$)PA is then given by

$$[Mg]^{free} = [Mg]^{total} - [MgPA] \quad (5)$$

Binding of $[Mg]^{free}$ to the ATPase was then calculated in the usual way. As shown in Figure 8 a good fit can be obtained in this way to the experimental data, with K_d values of 14 ± 1 and 320 ± 80 μM for Mg^{2+} binding to di($C_{18:1}$)PA and the ATPase respectively.

Fluorescence quenching in mixtures with bromine-containing phospholipids

Relative strengths of binding of phospholipids to the Ca^{2+}-ATPase can be established using a fluorescence-quenching assay. Phospholipids containing oleoyl chains can be brominated across the double bond with bromine. The resulting brominated phospholipids behave much like conventional phospholipids with unsaturated fatty acyl chains, because the bulky bromine atoms have similar effects on lipid packing to a *cis* double-bond [2]. In mixtures of brominated and non-brominated phospholipids, the degree of quenching of the fluorescence of the tryptophan residues in the Ca^{2+}-ATPase is related to the fractional occupation of the sites at the lipid–protein interface by the brominated lipid, according to eqn. (1).

The fluorescence intensity for the ATPase in di($Br_2C_{18:O}$)PC is 40% of that in di($C_{18:1}$)PC (Figure 8), as reported previously [2]. Reconstitution of the ATPase into di($C_{18:1}$)PA results in a 30% decrease in fluorescence intensity; a similar decrease in fluorescence intensity has been observed in the presence of oleic acid [42]. In mixtures of di($C_{18:1}$)PA and di($Br_2C_{18:0}$)PA, fluorescence quenching fits to eqn. (1) with a value for n, the number of lipid sites around an average Trp residue, of 1.6 (Figure 8A); the same value of n fitted the data in mixtures of di($C_{18:1}$)PC and di($Br_2C_{18:0}$)PC [2]. In di($Br_2C_{18:0}$)PA the fluorescence intensity was 25% of that in di($C_{18:1}$)PC (Figure 8A). The fluorescence quenching data for the ATPase in mixtures of di($Br_2C_{18:0}$)PC and di($C_{18:1}$)PA fitted to eqn. (1) with a relative binding constant K of 1, indicating equal binding of di($C_{18:1}$)PA and di($C_{18:1}$)PC to the ATPase (Figure 8A). The data for the ATPase reconstituted into mixtures of di($Br_2C_{18:0}$)PA and di($C_{18:1}$)PC also fitted to eqn. (1) with a relative binding constant K of 1. The presence or absence of 1 mM Mg^{2+} had no significant effect on the results (Figure 8A).

Reconstitution of the ATPase into di($C_{18:1}$)PS resulted in a 20% decrease in fluorescence intensity (Figure 8B). Data for mixtures of di($C_{18:1}$)PS and di($Br_2C_{18:0}$)PC were again fitted to eqn. (1) with a relative binding constant K of 1. Figure 8(B) shows calculated quenching curves for relative binding constants of 0.5, 1 and 2, showing that the range of acceptable relative binding constants is between 1 and 2.

DISCUSSION

The SR membrane contains negatively charged phospholipids as well as the zwitterionic phospholipids phosphatidylcholine and phosphatidylethanolamine [8]. In previous papers we have studied the effects of the zwitterionic phospholipids on the activity of the Ca^{2+}-ATPase purified from SR [1–5]. Here we have studied the effects of the negatively charged phospholipids phosphatidylserine and phosphatidic acid. Reconstitution of the plasma membrane Ca^{2+}-ATPase into bilayers of phosphatidylserine, phosphatidic acid or Ptd-Ins4*P* has been shown to result in an increase in activity due to both an increase in v_{max} and to an increase in affinity for Ca^{2+} [8]. It has been suggested that anionic phospholipids interact with two sites on the plasma membrane Ca^{2+}-ATPase, the first being the C-terminal calmodulin-binding domain and the second a large positively charged loop just before the third transmembrane α-helix [43]. The SR Ca^{2+}-ATPase contains no region corresponding to the C-terminal calmodulin-binding domain on the plasma membrane Ca^{2+}-ATPase, but, although the N-terminal region of the second binding domain is absent from the SR Ca^{2+}-ATPase, the C-terminal region, corresponding to the sequence ^{245}DKTPLQQKLDEFGE in the SR Ca^{2+}-ATPase, containing two conserved Lys residues, is present, as part of the proposed third stalk region [44].

The presence of phosphatidylserine or phosphatidic acid decreases the activity of the SR Ca^{2+}-ATPase

The effects of phosphatidylserine and phosphatidic acid on the activity of the Ca^{2+}-ATPase of SR are very different to those on the plasma membrane Ca^{2+}-ATPase, both inhibiting activity (Figure 1). The activity of the ATPase in di($C_{18:1}$)PA was found to be very sensitive to the Mg^{2+} concentration, so that at a total Mg^{2+} concentration of 2 mM, corresponding to a free Mg^{2+} concentration of 0.3 mM, an activity of about 1 unit/mg of protein, was measured, decreasing to zero at a total Mg^{2+} concentration of 5 mM (Figure 2). The effect of Mg^{2+} on the

activity of the ATPase in di($C_{18:1}$)PA was reversible (Table 1). No significant effects of Mg^{2+} concentration were observed on the activity of the Ca^{2+}-ATPase in di($C_{18:1}$)PS (Figure 2).

The different effects of Mg^{2+} on the activities of the ATPase in di($C_{18:1}$)PS and di($C_{18:1}$)PA are likely to follow from the reported different effects of Mg^{2+} on the structures of these two lipids. Binding of Mg^{2+} to di($C_{18:1}$)PA gives a non-lamellar structure in which the lipid fatty acyl chains are highly ordered [17], and we have shown that the activity of the Ca^{2+}-ATPase is very low in gel-phase phosphatidylcholine in which the fatty acyl chains are also highly ordered [32]. In contrast, addition of Mg^{2+} to di($C_{18:1}$)PS results in no change in chain packing [23,29]. Therefore in phosphatidic acid the low ATPase activity is likely to follow both from an effect of the anionic lipid headgroup, as seen in phosphatidylserine, and from an effect of increased fatty acyl chain packing induced by Mg^{2+} binding to the headgroup.

In mixtures of di($C_{18:1}$)PC and either di($C_{18:1}$)PS or di($C_{18:1}$)PA, activity measured in the presence of excess Mg^{2+} remains fairly constant up to about 60 mol-% anionic lipid, beyond which inhibition is observed (Figure 1). This is in contrast with experiments with Ptd-Ins4*P*, where activity was seen to increase sharply with increasing Ptd-Ins4*P* content up to 10 mol-%, beyond which no further change was seen [10]. Thus the stimulatory effect of Ptd-Ins4*P* is structurally specific and is not a general effect of anionic phospholipids.

Mechanism of inhibition

Rapid kinetic and equilibrium binding experiments have shown that there is no one unique step in the reaction pathway of the Ca^{2+}-ATPase that is sensitive to lipid structure, but rather that a number of different steps can be affected, with different changes in lipid structure affecting different steps. The Ca^{2+}-binding sites on the ATPase are sensitive to the thickness of the phospholipid bilayer, reconstitution into bilayers of phosphatidylcholines containing short (C_{14}) or long (C_{24}) fatty acyl chains resulting in a change in the stoichiometry of Ca^{2+}-binding to the ATPase [5,40]. However, changing the phase of the phospholipid from liquid crystalline bilayer to gel phase [32] or to hexagonal H_{II} phase [33] has little effect on the Ca^{2+}-binding sites. Here we have shown that Ca^{2+}-binding to the ATPase is also unaffected by reconstitution in di($C_{18:1}$)PS or di($C_{18:1}$)PA (Figures 3 and 4) and that anionic lipids also have no effect on the rate of dissociation of Ca^{2+} from the ATPase. Thus, despite the fact that the Ca^{2+}-binding sites on the ATPase are located in the trans-membrane region of the ATPase [45,46], only changing the thickness of the bilayer has a large effect on the sites.

The decrease in ATPase activity observed on reconstitution of the Ca^{2+}-ATPase in di($C_{18:1}$)PS and di($C_{18:1}$)PA can be attributed very largely to effects on the binding of ATP. As shown in Figure 5 the affinity of the ATPase for MgATP is little affected by reconstitution in anionic phospholipids, but the maximal levels of binding of MgATP decrease to values in di($C_{18:1}$)PS and di($C_{18:1}$)PA which are 49% and 31% of those in the native ATPase respectively. A reduction in the level of binding with little change in affinity is unexpected. In a simple scheme of the type $E^A \rightleftharpoons E^B \rightleftharpoons E^B.ATP$, with an equilibrium between two forms of the ATPase E^A and E^B, where only one form, E^B, can bind ATP, the enzyme will be fully ATP-bound at high ATP concentrations. A reduction in the level of binding could follow if the rate of equilibration between the two forms E^A and E^B were very slow on the time scale of the binding experiments (min). This is a possibility, since phase changes in phospholipid systems can be very slow processes because of the requirement to separate the phospholipid molecules into separate macroscopic phases. However, in the absence of Mg^{2+}, both di($C_{18:1}$)PS and di($C_{18:1}$)PA adopt simple bilayer phases, and although complex changes in phase are seen on addition of high concentrations of Mg^{2+} [14,23,29], the effects of high concentrations of Mg^{2+} on the activity of the ATPase in di($C_{18:1}$)PA are reversed within less than 1 min (Table 1).

An alternative explanation for the reduced level of binding of MgATP in di($C_{18:1}$)PS or di($C_{18:1}$)PA could be oligomer formation. The reduction in binding level observed in di($C_{18:1}$)PS would be consistent with the formation of a dimer in which only one of the ATPase molecules was able to bind MgATP. Similarly, in di($C_{18:1}$)PA the data would be consistent with formation of a trimer or tetramer, with again only one ATPase molecule per oligomer being able to bind MgATP. The ATPase is known under some conditions to adopt a dimer structure with the dimers linked head-to-tail to form a ribbon, the structure changing on phosphorylation of the ATPase [47,48]. In the ribbon structure, the ATPase molecules are linked by a bridge between the tops of the molecules, and the MgATP-binding site has been suggested to be located in a cleft towards the top of the molecule [49].

The level of phosphorylation of the ATPase in the anionic lipids matches the proportion of the ATPase that can bind ATP (Figure 5 and Table 2). For the ATPase that can bind ATP, the rate of phosphorylation by ATP is normal and any effects on the rate of dephosphorylation are small (Table 3). The activity of the ATPase in di($C_{18:1}$)PS is about 35% of that of the native ATPase (Figure 1), slightly less than the proportion of the ATPase still able to bind MgATP. However, for the ATPase in di($C_{18:1}$)PA, activity is dependent on the concentration of Mg^{2+} and is close to zero at concentrations of Mg^{2+} comparable with those used in the ATP-binding experiments (Figure 2), so that effects of di($C_{18:1}$)PA on activity are more marked than would be expected if the only effects were on the ability to bind MgATP. Thus reconstitution in di($C_{18:1}$)PA must also result in a decrease in the rate of some step following dephosphorylation of the phosphorylated ATPase and before phosphorylation by ATP. Effects of di($C_{18:1}$)PS and particularly di($C_{18:1}$)PA on the level of phosphorylation of the ATPase by P_i are also greater than effects on the level of phosphorylation by ATP (Tables 2 and 4). These effects of the anionic phospholipids resemble the effects of the inhibitor thapsigargin, which both decreases the rate of the E2 $\rightarrow$ E1 transition of the ATPase, thus decreasing ATPase activity, and favours a modified form of the E2 state of the ATPase, thus preventing phosphorylation by P_i [50].

A further effect of the anionic phospholipids is seen in the response of DMC-labelled ATPase to the binding of Mg^{2+} (Figure 7). We have observed two types of fluorescence response for DMC-labelled ATPase [11,33,36]. Addition of Mg^{2+} results in a 5% decrease in fluorescence intensity, with a half-maximal effect being observed at 4 mM Mg^{2+} [11]. We have attributed this effect to binding of Mg^{2+} at a gating site controlling the rate of access of Ca^{2+} to its binding sites on the ATPase. On phosphorylation of the ATPase with ATP or P_i, a larger decrease in fluorescence intensity is seen, with an amplitude of $\approx$ 16% at pH 6.0 [33]; binding of the inhibitors thapsigargin or di-t-butyl hydroquinone to the ATPase [33,50,51] results in a decrease in fluorescence intensity for DMC-labelled ATPase equal to that seen on phosphorylation. We have suggested that the change in fluorescence intensity for DMC-labelled ATPase on phosphorylation or on binding inhibitor could result from closure of a cleft between the phosphorylation and nucleotide-binding domains on the ATPase [50]. The results presented in Figure 7 would then suggest that, for the ATPase in di($C_{18:1}$)PS or

di($C_{18:1}$)PA, binding of Mg^{2+} results in the same cleft closure normally seen on phosphorylation of the ATPase.

For DMC-labelled ATPase in di($C_{18:1}$)PS, the concentration of Mg^{2+} resulting in a half-maximal change in fluorescence intensity is 57 μM (Figure 7), compared with 4 mM for the unreconstituted ATPase. This change in apparent affinity for Mg^{2+} could follow from an increase in the concentration of Mg^{2+} close to the membrane due to the high negative charge on a bilayer of the anionic phosphatidylserine. If the binding site for Mg^{2+} were surface exposed, calculations of the type performed by Lee et al. [52] show that the site would be 7.5 Å above the surface, in the stalk region of the ATPase. X-ray diffraction studies have located a binding site for lanthanide ions 12 Å above the bilayer surface [53], although other studies have suggested that the Mg^{2+}-binding site and the lanthanide-binding site may be separate [54]. Effects of Mg^{2+} on the fluorescence intensity of DMC-labelled ATPase in di($C_{18:1}$)PA are more complex, but can be fitted to binding to di($C_{18:1}$)PA as well as to the ATPase (Figure 7). The smaller effect of di($C_{18:1}$)PA on the effective affinity for Mg^{2+} is consistent with binding of Mg^{2+} to the di($C_{18:1}$)PA, reducing the effective charge on the bilayer surface.

Effects of phospholipid chain length and headgroup structure are separate

To establish whether or not fatty acyl chain length and lipid headgroup structure have separate effects on the activity of the Ca^{2+}-ATPase we studied activities in mixtures of di($C_{14:1}$)PC and either di($C_{18:1}$)PS or di($C_{18:1}$)PA. As shown in Figure 6 incorporation of di($C_{18:1}$)PA or di($C_{18:1}$)PS into bilayers of di($C_{14:1}$)PC, up to a mole fraction of 0.6, led to an increase in ATPase activity, a mole fraction range over which these same anionic lipids had little effect on the activity of the ATPase in di($C_{18:1}$)PC (Figure 1); at higher mole fractions of the anionic lipid, activity then decreased (Figure 6), as in mixtures with di($C_{18:1}$)PC (Figure 1). In mixtures of di($C_{14:1}$)PC and di($C_{18:1}$)PC it has been shown that activity increases with increasing mole fraction of di($C_{18:1}$)PC up to 0.5, beyond which no further change was seen [5]. Thus in mixtures with di($C_{14:1}$)PC, incorporation of lipids containing oleoyl chains can increase activity, even when the lipids themselves inhibit activity.

One of the changes in the ATPase induced by short-chain lipid is a change in the stoichiometry of Ca^{2+} binding to one Ca^{2+} ion bound per ATPase molecule; this change can be reversed by addition of 50 mol-% di($C_{18:1}$)PC [40]. It is not possible to test directly whether the anionic lipids have this same effect, because of extensive non-specific binding of Ca^{2+}. However, the change in stoichiometry of Ca^{2+}-binding for the ATPase in di($C_{14:1}$)PC is accompanied by a change in the response of tryptophan fluorescence to the removal of Ca^{2+} from the Ca^{2+}-bound ATPase: a decrease in fluorescence intensity is observed in di($C_{18:1}$)PC with no change in di($C_{14:1}$)PC [40]. We found that addition of either di($C_{18:1}$)PA or di($C_{18:1}$)PS to the ATPase in di($C_{14:1}$)PC leads to a gradual increase in the magnitude of the fluorescence response to the removal of Ca^{2+}, again consistent with a reversal of the effect of the short fatty acyl chain by the oleoyl-containing di($C_{18:1}$)PS or di($C_{18:1}$)PA. Thus we conclude that effects of lipid fatty acyl chain length on the function of the ATPase are distinct from effects of the lipid headgroup.

Binding sites for anionic phospholipids on the Ca^{2+}-ATPase

Fluorescence quenching experiments measuring the binding constants of phosphatidylserine and phosphatidic acid at the lipid–protein interface of the ATPase suggest that the inhibitory effects of phosphatidic acid and phosphatidylserine are likely to follow from binding to sites of low specificity. The experiments show that di($C_{18:1}$)PC, di($C_{18:1}$)PS and di($C_{18:1}$)PA bind to the ATPase with very similar affinities (Figure 8); the data fit to binding constants for the anionic lipids relative to di($C_{18:1}$)PC of about 1, with a maximum possible value of 2. These results are very similar to those reported for the Na^+,K^+-ATPase using a spin label method, which gave relative binding constants of 1.9 for phosphatidic acid and 1.6 for phosphatidylserine [55]. Fluorescence-quenching plots for mixtures of di($Br_2C_{18:0}$)PC and di($C_{18:1}$)PA and for mixtures of di($C_{18:1}$)PC and di($Br_2C_{18:0}$)PA both fit to a relative binding constant for phosphatidic acid/phosphatidylcholine of 1 (Figure 8), suggesting that there are no high-affinity, specific binding sites on the ATPase for phosphatidic acid to which di($Br_2C_{18:0}$)PA can bind and cause fluorescence quenching, and from which di($Br_2C_{18:0}$)PC is excluded. Thus phosphatidic acid and phosphatidylserine bind only to the non-specific lipid-binding sites on the ATPase, located at the lipid–protein interface.

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

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Received 14 May 1997/15 August 1997; accepted 7 October 1997