Effects of Gel Phase Phospholipid on the Ca^{2+}-ATPase†

A. P. Starling, J. M. East, and A. G. Lee*

Department of Biochemistry and Institute for Biomolecular Sciences, University of Southampton, Southampton, SO9 3TU, U.K.

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ABSTRACT: ATPase activities for the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum reconstituted in dimyristoylphosphatidylcholine [di(C14:0)PC] or dipalmitoylphosphatidylcholine [di(C16:0)PC] are very low at temperatures below 25 and 30 °C, respectively. The stoichiometry of Ca^{2+} binding to the ATPase is 1 Ca^{2+} ion bound per ATPase molecule in di(C14:0)PC in both gel and liquid-crystalline phases; addition of cholesterol at a 1:1 molar ratio with di(C14:0)PC increases Ca^{2+} binding to two Ca^{2+} ions bound per ATPase molecule. The affinity of the ATPase for Ca^{2+} is slightly higher in di(C16:0)PC in the gel phase than in the liquid-crystalline phase, consistent with a shift in the E1/E2 equilibrium toward E1 in gel phase lipid. The rates of dissociation of Ca^{2+} from the ATPase in gel and liquid-crystalline phase lipids are the same in the absence of Mg^{2+}, but whereas addition of Mg^{2+} to the ATPase in liquid-crystalline lipid increases the rate of dissociation in liquid-crystalline phase lipid, Mg^{2+} has no effect in gel phase lipid. The fluorescence intensity of the Ca^{2+}-ATPase labeled with 4-(bromomethyl)-6,7-dimethoxycoumarin decreases on addition of Mg^{2+} in liquid-crystalline phase lipid, but is unaffected by Mg^{2+} in gel phase lipid. The rate of phosphorylation of the ATPase in gel phase lipid is very slow, and rates of dephosphorylation of the phosphorylated ATPase are also very slow. *p*-Nitrophenolphosphatase activity is also very low in gel phase lipid. Binding of ATP results in the same changes in the fluorescence of the ATPase labeled with IAEDANS in gel and liquid-crystalline phase lipids, but changes in tryptophan fluorescence intensity are different. It is concluded that the conformational change following binding of ATP to the ATPase ($E1Ca_2ATP \rightleftarrows E1'Ca_2ATP$) is unaffected by the phase of the lipid, but the rate of phosphate transfer ($E1'Ca_2ATP \rightleftarrows E1PCa_2ADP$) is decreased.

The effects of phospholipids on the activity of the Ca^{2+}-ATPase purified from skeletal muscle sarcoplasmic reticulum (SR)[1] can be studied by reconstituting the ATPase into phospholipid bilayers of defined composition. The activity of the ATPase in phosphatidylcholine bilayers in the liquid-crystalline phase varies little with fatty acyl chain length between C16 and C20, but longer or shorter fatty acyl chains support lower ATPase activities (Lee & East, 1993). In bilayers of dimyristoleoylphosphatidylcholine [di(C14:1)PC] or dinervonylphosphatidylcholine [di(C24:1)PC] the stoichiometry of Ca^{2+} binding to the ATPase changes from the usual 2 Ca^{2+} ions bound per ATPase molecule to 1 Ca^{2+} ion bound per ATPase molecule (Michelangeli et al., 1990; Starling et al., 1993), and in bilayers of di(C14:1)PC, the rate of phosphorylation of the ATPase by ATP becomes very slow (Michelangeli et al., 1991).

Phosphatidylcholines undergo a phase transition between gel and liquid-crystalline phases at a temperature defined by the fatty acyl chain length and the degree of unsaturation. For dimyristoylphosphatidylcholine [di(C14:0)PC] and dipalmitoylphosphatidylcholine [di(C16:0)PC], the transition temperatures are 24 and 42 °C, respectively (Lee, 1983). The phase transition results in a marked change in the physical properties of the bilayer, with the fatty acyl chains being packed in a rigid crystalline array in the gel phase, but having considerable freedom of motion in the liquid-crystalline phase (Lee, 1983). It has been shown that phospholipids must be in the liquid-crystalline phase to support activity of the Ca^{2+}-ATPase; in the gel phase, the rate of hydrolysis of ATP becomes very slow (Warren et al., 1974a,b; Nakamura et al., 1976; Hidalgo et al., 1978; Gomez-Fernandez et al., 1985). Here we report a detailed study of the kinetics of the ATPase in di(C16:0)PC in the gel phase, and show that, unexpectedly, Ca^{2+} binding is normal, and that the low steady-state activity of the ATPase follows from a very slow rate of transfer of the γ-phosphate from ATP to the ATPase in the $E1Ca_2MgATP$ complex.

MATERIALS AND METHODS

Dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine were obtained from Sigma and dimyristoleoylphosphatidylcholine and dioleoylphosphatidylcholine from Avanti Polar Lipids.

ATPase was purified from skeletal muscle sarcoplasmic reticulum as described in East and Lee (1982). Concentrations of protein were estimated by using the extinction coefficient (1.2 L g^{-1} cm^{-1} for a solution in 1% SDS) given by Hardwicke and Green (1974). Lipid reconstitutions were performed largely as described in Starling et al. (1993). Typically, 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 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(C14:1)PC and di(C18:1)-

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[1] Abbreviations: di(C14:0)PC, dimyristoylphosphatidylcholine; di(C16:0)PC, dipalmitoylphosphatidylcholine; di(C18:1)PC, dioleoylphosphatidylcholine; AMPPCP, adenosine 5′-(β,γ-methylenetriphosphate); FITC, fluorescein isothiocyanate; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; IAEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid; SR, sarcoplasmic reticulum; Br-DMC, 4-(bromomethyl)-6,7-dimethoxycoumarin.

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PC left for 15 min at room temperature and 45 min at 5 °C to equilibrate before being diluted with buffer (2 mL; 20 mM Hepes/Tris, pH 7.2, 100 mM KCl, and 5 mM $MgSO_4$) and stored on ice until use. For reconstitutions with di(C14:0)PC and di(C16:0)PC, the ATPase was incubated with the lipid in cholate for 15 min at 24 and 42 °C, respectively, followed by a further 45 min at 5 °C.

ATPase activities were measured in 40 mM Hepes/KOH, pH 7.2, 100 mM KCl, 5 mM $MgSO_4$, 2.1 mM ATP, and maximally stimulating Ca^{2+} (East & Lee, 1982). *p*-Nitrophenylphosphatase activity was measured in 40 mM Hepes/KOH, pH 7.4, 100 mM KCl, 20 mM Mg^{2+}, 1.01 mM EGTA, and 0.91 mM Ca^{2+}, at *p*-nitrophenyl phosphate concentrations between 5 and 20 mM. The liberation of *p*-nitrophenol was monitored at 410 nm, and activities were calculated using an extinction coefficient of 60 000 M^{-1} cm^{-1} for *p*-nitrophenol.

Measurements of tryptophan fluorescence intensity were made by diluting 10 μL aliquots of the reconstitution mixture into 2.5 mL of buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl, 5 mM $MgSO_4$, and 100 μM Ca^{2+}) and recording the tryptophan fluorescence using an SLM-Aminco 8000C fluorometer, with excitation and emission wavelengths of 295 and 330 nm, respectively. Ca^{2+} titrations were performed by addition of aliquots of a stock solution of EGTA (500 mM) to vary the free Ca^{2+} concentrations. Effects of ATP were determined by addition of aliquots of a concentrated stock solution of ATP (10 mM). All measurements were corrected for dilution. Free concentrations of Ca^{2+} were calculated using the binding constants for Ca^{2+}, Mg^{2+} and H^+ to EGTA given by Godt (1974).

ATPase (36 μM) was labeled with 5-[[2-[(iodoacetyl)-amino]ethyl]amino]naphthalene-1-sulfonic acid (IAEDANS) by incubation with 72 μM IAEDANS in buffer (50 mM Tris/HCl, pH 7.0, and 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 ca. 1:1 under these conditions (Baker et al., 1994). IAEDANS fluorescence was excited at 380 nm and observed at 475 nm.

The ATPase was labeled with NBD as described (Wictome et al., 1992a). Fluorescence measurements were performed with excitation and emission wavelengths of 430 and 520 nm, respectively. To reduce the signal due to scattered light, the excitation beam was passed through a 450 nm long-wavelength cutoff filter (450FLO T-50, Andover Corp.), and the emission was passed through a Hoya Y50 500 nm short-wavelength cutoff filter. The ATPase was labeled with 4-(bromomethyl)-6,7-dimethoxycoumarin (Br-DMC) as described in Stefanova et al. (1992). DMC fluorescence was excited at 350 nm and observed at 425 nm. Binding of $^{45}Ca^{2+}$ to the ATPase was measured using the double-labeling method described in Starling et al. (1993). For experiments with the ATPase reconstituted with di(C16:0)PC in the liquid-crystalline phase, the filtration manifold was warmed to 40 °C by filtration of a large volume of buffer at 40 °C, followed by rapid filtration of the reconstituted ATPase diluted into the $^{45}Ca^{2+}$-containing buffer, also at 40 °C.

Binding of ATP to the ATPase was determined as described by Dupont (1977). ATPase (10 mg/mL) was incubated for 5 min in buffer (150 mM Mops/Tris, pH 7.2, 100 mM KCl, 5 mM Mg^{2+}, and 0.5 mM EGTA). Fifty microliters (equivalent to 0.5 mg of ATPase) was then filtered through a Whatman GF/C filter and washed under vacuum with 1 mL of the same buffer containing [^{14}C]ATP. The filter was then counted. Blanks were determined by filtering the same solution in the absence of ATPase.

The maximum observable levels of phosphorylation of the ATPase were determined by incubating the ATPase (100 μg) in 0.5 mL of a medium containing 20 mM Hepes/Tris, pH 7.2, 5 mM $MgSO_4$, 100 mM KCl, and 1 mM $CaCl_2$. The reaction was started by addition of 100 μM [γ-^{32}P]ATP and, after incubation at the required temperature for 10 s, was quenched by addition of 5 mL of an ice-cold mixture of 25% trichloroacetic acid in 0.13 M potassium phosphate. The quenched protein was allowed to stand on ice for 15 min and then collected by filtration through Whatman GF/C glass fibre filters. The filter was washed 3 times with 15 mL of cold 25% trichloroacetic acid in 0.13 M potassium phosphate, and finally counted in OptiPhase "HighSafe" 3.

The time course of phosphoenzyme formation for the ATPase reconstituted in di(C16:0)PC in the gel phase was determined by incubation for the required time with 100 μM [γ-^{32}P]ATP in 20 mM Hepes/Tris, pH 7.2, 5 mM $MgSO_4$, 100 mM KCl, and 1 mM $CaCl_2$. The rate of dephosphorylation was determined by addition of excess ATP (2 mM) or ADP (2 mM), followed by removal of aliquots at the chosen times and quenching as described above.

The ATPase was phosphorylated with [^{32}P]P_i as described by Henao et al. (1991). ATPase (0.2 mg) was incubated in buffer (0.5 mL; 12.5 mM MES/Tris, pH 6.0) containing 10 mM EGTA, 20 mM $MgSO_4$, 14% (v/v) dimethyl sulfoxide, and 1 mM [^{32}P]P_i at 25 °C for 15 s and then quenched by addition of 5 mL of an ice-cold mixture of 25% trichloroacetic acid in 0.13 M potassium phosphate. The mixture stood on ice for 15 min and then was filtered through a Whatman GF/B glass fibre filter. The filter was washed and counted in OptiPhase "HighSafe" 3.

The time dependence of Ca^{2+} dissociation from the ATPase was determined using a Biologic rapid filtration system (Starling et al., 1993). Reconstituted ATPase, prepared as described above, corresponding to 50 μg of ATPase, was loaded onto a Millipore HAWP filter and then rapidly perfused with buffer (20 mM Hepes/Tris, pH 7.2, and 100 mM KCl), containing 2 mM EGTA, in the absence or presence of 5 mM $MgSO_4$. The filter was then counted, and the $^{45}Ca^{2+}$ bound to the ATPase was calculated as described above.

Ca^{2+} binding to the ATPase was simulated according to the sequence:

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

Parameters giving the equilibrium and binding constants for these steps and their pH dependence are presented in Henderson et al. (1994a) and Lee et al. (1995). At pH 7.2, calculated binding constants for the first and second Ca^{2+} ions were respectively 5.0×10^5 and 1.28×10^7 M^{-1} in the absence of Mg^{2+} and 1.05×10^5 and 4.1×10^6 M^{-1} in the presence of 5 mM Mg^{2+}. The value of the equilibrium constant E1/E2 at pH 7.2 was calculated to be 0.21 in the absence of Mg^{2+} and 0.99 in the presence of 5 mM Mg^{2+}.

RESULTS AND DISCUSSION

Effect of the Phospholipid Phase Transition on ATPase Activity. The ATPase activities of the Ca^{2+}-ATPase reconstituted with the saturated phospholipids di(C14:0)PC and di(C16:0)PC are very low below 25 and 30 °C, respectively

Table 1: Effects of Temperature on the ATPase Activity of the Reconstituted ATPase[a]

lipid	ATPase act. (IU/mg of protein) at temp (°C)				
	15	25	30	35	45
di(16:0)PC	0	0.1	0.2	3.2	8.9
di(16:0)PC + cholesterol[b]	0	0.1	0.2	3.5	8.6
di(14:0)PC	0	0.4	1.1	1.5	5.2
di(14:0)PC + cholesterol[b]	0	0.3	1.2	2.9	8.2

[a] Activities measured at pH 7.2 in 100 mM KCl, 2.1 mM ATP, and maximally saturating concentrations of Ca^{2+}. [b] Molar ratio of phospholipid to cholesterol of 1:1.

(Table 1), as reported previously (Warren et al., 1974a). For the Ca^{2+}-ATPase in di(C14:0)PC, activity appears above 25 °C (Table 1), a temperature very close to that of the gel to liquid-crystalline phase transition temperature for the pure lipid [24 °C; see Lee (1977)]. In contrast, the ATPase is active in di(C16:0)PC at temperatures considerably below the phase transition temperature of pure di(C16:0)PC [42 °C; see Lee (1977)]. It has been suggested that the phase transition for di(C16:0)PC bound to the Ca^{2+}-ATPase occurs over a wide temperature range, starting at ca. 30 °C (Hesketh et al., 1976). Thus, the Ca^{2+}-ATPase is inactive when the phospholipid molecules surrounding it in the membrane are in the gel phase. The observed effects of temperature on the activity of the ATPase were fully reversible.

It has been shown that the low activity observed for the Ca^{2+}-ATPase reconstituted in di(C14:1)PC in the liquid-crystalline phase can be increased by addition of cholesterol (Simmonds et al., 1982; Starling et al., 1993). As shown in Table 1, addition of cholesterol increases the activity of the ATPase in di(C14:0)PC at temperatures of 25 °C or above when di(C14:0)PC will be in the liquid-crystalline phase, but cholesterol has no effect on activities below this temperature. Cholesterol has been shown to have no effect on the activity of the Ca^{2+}-ATPase reconstituted in di(C16:1)PC in the liquid-crystalline phase (Starling et al., 1993). Similarly, cholesterol has no effect on the activity of the Ca^{2+}-ATPase in di(C16:0)PC in either the liquid-crystalline or the gel phase (Table 1). The effect of cholesterol on phospholipid bilayers in the gel phase is to induce a state of "intermediate fluidity", with fluorescence and ESR probes showing a lower order in the presence of cholesterol than in its absence (Lee, 1977). The lack of effect of cholesterol on the activity of the ATPase in gel phase lipid shows that this state of intermediate fluidity is not sufficient to support the activity of the ATPase.

Comparison of *p*-nitrophenolphosphatase activity for the native ATPase and for the ATPase reconstituted in di(C16:0)PC at 25 and 37 °C shows that this activity also requires that the phospholipids surrounding the ATPase be in the liquid-crystalline phase (Table 2).

Effect of the Phospholipid Phase Transition on Ca^{2+} Binding. The stoichiometry of Ca^{2+} binding to the ATPase was determined by comparison of the level of Ca^{2+} binding with the maximum level of phosphorylation of the ATPase, determined by incubation with 100 μM [γ-^{32}P]ATP in the presence of 1 mM Ca^{2+} to reduce the rate of dephosphorylation of the phosphorylated ATPase. The levels of phosphoenzyme formation obtained at 42 °C for the ATPase reconstituted with di(C14:0)PC or di(C16:0)PC were 2.9 and 3.1 nmol/mg of protein, respectively (Table 3), comparable to those observed for the ATPase in SR vesicles (3.5 nmol of EP/mg of protein). The measured levels of Ca^{2+} binding for the ATPase in di(C16:0)PC at either 20 or 42 °C, in the gel and liquid-crystalline phases, respectively, then correspond to a stoichiometry of 2 Ca^{2+} ions bound per molecule of phosphorylatable ATPase (Table 3). For the ATPase reconstituted with di(C14:0)PC, Ca^{2+} binding was again independent of temperature, within experimental error, and the stoichiometry of Ca^{2+} binding was 1 Ca^{2+} ion bound per molecule of phosphorylatable ATPase (Table 3), as reported previously for the ATPase reconstituted with di(C14:1)PC in the liquid-crystalline phase (Michelangeli et al., 1990; Starling et al., 1993).

Table 2: *p*-Nitrophenolphosphatase Activity (IU/mg of Protein) for the Ca^{2+}-ATPase[a]

system	*p*-nitrophenol phosphate concn (mM)		
	5	10	20
di(C16:0)PC, 25 °C	0	0	0
di(C16:0)PC, 37 °C	0.10	0.16	0.17
SR, 25 °C	0.05	0.06	0.07
SR, 37 °C	0.11	0.16	0.16

[a] Measured in 40 mM Hepes/KOH, pH 7.4, 100 mM KCl, 20 mM Mg^{2+}, 1.01 mM EGTA, and 0.91 mM Ca^{2+}.

Table 3: $^{45}Ca^{2+}$ Binding to the Reconstituted Ca^{2+}-ATPase[a]

lipid	$^{45}Ca^{2+}$ bound (nmol/mg of protein)		$[EP]_{max}$[b] (nmol/mg of protein)
	20 °C	42 °C	42 °C
di(C14:0)PC	3.8	2.5	2.9
di(C14:0)PC + cholesterol (1:1)	7.4	7.8	
di(C16:0)PC	6.2	7.0	3.1

[a] $^{45}Ca^{2+}$ binding was determined at pH 7.2. [b] Maximal level of phosphorylation observed with 100 μM [γ-^{32}P]ATP in the presence of 1 mM, as described in the text.

Addition of cholesterol to the ATPase in di(C14:1)PC in the liquid-crystalline phase has been reported to increase the Ca^{2+}-binding stoichiometry from 1 Ca^{2+} ion bound per ATPase molecule to 2 (Starling et al., 1993). Addition of cholesterol to the ATPase in di(C14:0)PC, in either the gel or the liquid-crystalline phases, also increases the stoichiometry to 2 Ca^{2+} ions bound per ATPase molecule (Table 3). It has been suggested that this effect of cholesterol follows from binding at "non-annular" sites on the ATPase, possibly located between transmembrane α-helices (Simmonds et al., 1982; Ding et al., 1994).

$^{45}Ca^{2+}$ binding to the ATPase reconstituted with di(C16:0)PC is shown as a function of pCa value at 20 and 42 °C in Figure 1A. The maximum amount of the reconstituted ATPase that could be adsorbed onto Millipore filters was ca. 100 μg, which limited the accuracy with which the binding curves could be determined. Nevertheless, the data showed that the affinity of the ATPase for Ca^{2+} was similar in the gel and liquid-crystalline phases. A more accurate determination of Ca^{2+} affinity could be made by observing the change in tryptophan fluorescence intensity when EGTA was added to the ATPase incubated in the presence of Ca^{2+}. As shown in Figure 2, removal of Ca^{2+} from the ATPase reconstituted with di(C16:0)PC resulted in a decrease in fluorescence intensity of the same magnitude at 20 °C (gel phase) and 37 °C (liquid-crystalline phase). Measuring the magnitude of the change in tryptophan fluorescence intensity as a function of final pCa value gives pCa values for 50%

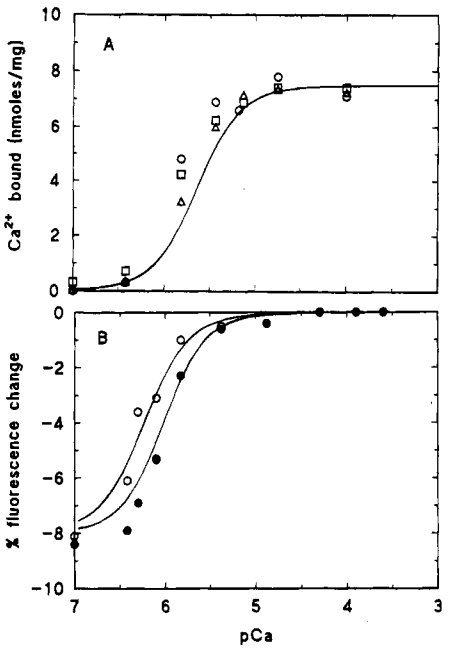


FIGURE 1: Effect of temperature on Ca^{2+} binding to the ATPase. (A) Binding of $^{45}Ca^{2+}$ (nanomoles per milligram of protein) to the ATPase reconstituted with di(C16:0)PC: (□) 20 °C; (○) 42 °C; (△) 20 °C in the presence of 2 mM ATP. Binding was measured at pH 7.2, with 5 mM Mg^{2+} in 150 mM Mops/80 mM Tris with EGTA added to give the required Ca^{2+} concentration. The solid line shows a simulation of Ca^{2+} binding calculated using the parameters in Henderson et al. (1994a) and Lee et al. (1995). (B) Ca^{2+} dependence of the tryptophan fluorescence intensity for the ATPase reconstituted with di(C16:0)PC: (○) 20 °C; (●) 37 °C. The figure shows the decrease in tryptophan fluorescence intensity for the ATPase in 150 mM Mops/80 mM Tris, pH 7.2, 1.0 mM Ca^{2+}, on addition of EGTA to the given pCa value. The solid lines show simulations calculated using the parameters in Henderson et al. (1994a) and Lee et al. (1995) for the liquid-crystalline phase and the modified value for K_{H6} in the gel phase, as described in the text.

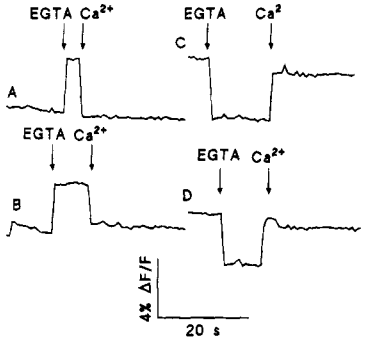


FIGURE 2: Effects of EGTA and Ca^{2+} on the intensity of tryptophan fluorescence for the ATPase reconstituted with di(C14:0)PC at 15 °C (A) or 30 °C (B) or with di(C16:0)PC at 20 °C (C) or 37 °C (D). The ATPase was incubated in 2.5 mL of buffer (20 mM Mops/Tris, pH 7.2, 5 mM Mg^{2+}, and 100 mM KCl) containing 100 μM Ca^{2+}, and then EGTA (12.5 μL) and Ca^{2+} (6 μL) were added to give final concentrations of 1.2 and 2.4 mM, respectively.

effects of 6.24 and 6.04 at 20 °C (gel phase) and 37 °C (liquid-crystalline phase), respectively (Figure 1B).

Ca^{2+} binding to the ATPase has been described in terms of the scheme:

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

where the ATPase can bind Ca^{2+} at two cytoplasmically exposed sites in the E1 conformation. The measured tryptophan fluorescence intensity changes for the ATPase in di(C16:0)PC in the liquid-crystalline phase can be

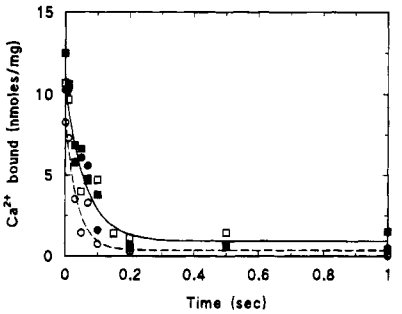


FIGURE 3: Rapid filtration measurement of the rate of $^{45}Ca^{2+}$ dissociation from the ATPase reconstituted in di(C16:0)PC (■, ●) or di(C18:1)PC (□, ○). The ATPase was incubated with $^{45}Ca^{2+}$ in buffer containing 100 μM Ca^{2+}, pH 7.2, 20 °C, and then adsorbed on a filter and perfused with buffer (150 mM Mops/80 mM Tris) containing 2 mM EGTA and either no Mg^{2+} (□, ■) or 20 mM Mg^{2+} (○, ●). The level of $^{45}Ca^{2+}$ bound to the ATPase (nanomoles per milligram of protein) is plotted against the perfusion time (in seconds). Curves represent fits to single-exponential decays, with the parameters given in the text.

simulated using the Ca^{2+}-binding parameters given in Henderson et al. (1994a) and Lee et al. (1995) for the Ca^{2+}-ATPase in the native SR membrane (Figure 1B). The slightly higher affinity for Ca^{2+} observed in gel phase di(C16:0)PC (Figure 1B) is consistent with a small increase in the equilibrium constant E1/E2. As shown (Figure 1B), the data in gel phase di(C16:0)PC can be simulated assuming an increase in the equilibrium constant E1/E2 by a factor of 4, in agreement with measurements of the E1/E2 equilibrium constant using NBD-labeled ATPase, as described below.

For the Ca^{2+}-ATPase reconstituted with di(C14:1)PC in the liquid-crystalline phase, it was observed that addition of EGTA to the ATPase incubated in the presence of Ca^{2+} and Mg^{2+} resulted in an increase in fluorescence intensity (Michelangeli et al., 1990; Starling et al., 1993). As shown in Figure 2, this is also observed for the ATPase reconstituted with di(C14:0)PC, either at 15 °C (gel phase) or at 30 °C (liquid-crystalline phase).

The rate of dissociation of Ca^{2+} from the Ca^{2+}-bound ATPase can be determined by adsorbing the ATPase onto Millipore filters and then washing for defined times with EGTA. The rate of dissociation of Ca^{2+} from the ATPase in the native SR membrane has been shown to increase on addition of Mg^{2+} (Moutin & Dupont, 1991; Henderson et al., 1994b). As shown in Figure 3, this effect is also observed for the ATPase in di(C18:1)PC at 20 °C (liquid-crystalline phase), the rate of dissociation of $^{45}Ca^{2+}$ increasing from 15.8 ± 3.9 s^{-1} in the absence of Mg^{2+} to 26.3 ± 6.7 s^{-1} in the presence of 20 mM Mg^{2+}, at pH 7.2. These rates compare with values of 14.2 and 26.4 s^{-1} in the absence and presence of 20 mM Mg^{2+}, respectively, calculated from the parameters given in Lee et al. (1995) for the native ATPase. However, for the Ca^{2+}-ATPase in di(C16:0)PC in the gel phase, Mg^{2+} had no effect on the rates of dissociation of Ca^{2+}, which were 13.3 ± 3.0 and 15.7 ± 2.0 s^{-1} in the absence and presence of 20 mM Mg^{2+}, respectively (Figure 3).

It was suggested in Henderson et al. (1994b) that effects of Mg^{2+} on the rate of Ca^{2+} dissociation followed from binding at a "gating" site, and that the fluorescence of the ATPase labeled with 4-(bromomethyl)-6,7-dimethoxycoumarin (DMC) was also sensitive to binding of Mg^{2+} at this site. Addition of Mg^{2+} to the ATPase in di(C18:1)PC in the liquid-crystalline phase resulted in decreases in fluorescence intensity identical to those observed for the native

Table 4: Response of DMC-Labeled ATPase to the Addition of Mg^{2+}

system	% fluorescence change[a] at [Mg] (mM)		
	1	10	30
SR	−1.1	−4.0	−5.0
di(C18:1)PC	−1.3	−4.0	−5.0
di(C16:0)PC	0	0	0

[a] Determined in 40 mM Tris/maleate and 1 mM EGTA, pH 6.0, at 20 °C.

ATPase (Table 4). In contrast, addition of Mg^{2+} to the ATPase in di(C16:0)PC in the gel phase had no effect on fluorescence intensity (Table 4), in agreement with the observed lack of effect of Mg^{2+} on the rate of dissociation of Ca^{2+} from the ATPase in gel phase lipid (Figure 3). Thus, either Mg^{2+} fails to bind to the ATPase in gel phase lipid, or a conformational change following binding of Mg^{2+}, responsible for the change in fluorescence of DMC-labeled ATPase and for the increased rate of dissociation of Ca^{2+}, fails to occur. This experiment provides further evidence for a link between the effects of Mg^{2+} on the fluorescence of DMC-labeled ATPase and on the rate of Ca^{2+} dissociation.

Effect of the Phospholipid Phase Transition on ATP Binding. ATP binding to the ATPase was determined by filtration assay as described by Lacapere et al. (1990). Maximal levels of ATP binding of 3.0 and 3.3 nmol/mg of protein were determined at 20 °C for the native ATPase and for the ATPase in gel phase di(C16:0)PC, respectively, with concentrations of ATP giving half-maximal binding of 6.8 and 4.0 μM for the native and reconstituted ATPase, respectively, at pH 7.2, 5 mM Mg^{2+} (data not shown). The slightly higher affinity for ATP in gel phase lipid than in liquid-crystalline phase lipid is consistent with a small increase in the equilibrium constant E1/E2 (see below) with stronger binding of ATP to the E1 conformation.

Phosphorylation of the Ca^{2+}-ATPase in Gel Phase Lipid by ATP. Incubation of the Ca^{2+}-ATPase in di(C14:0)PC or di(C16:0)PC with [γ-^{32}P]ATP for 10 s at 42 °C in the liquid-crystalline phase gave normal levels of phosphoenzyme formation (Table 3), consistent with a rapid rate of phosphorylation of the ATPase by ATP (Stahl & Jencks, 1987). However, in gel phase di(C16:0)PC at 25 °C, no significant level of phosphorylation was observed if the incubation with ATP was for only 10 s, but phosphorylation was observed if the incubation was continued for longer times. A maximum level of phosphorylation of 1 nmol of EP/mg of protein was observed at 20 min, after which a slight decrease in level was observed (Figure 4). Phosphorylation was Ca^{2+}-sensitive; no phosphoenzyme was formed under these conditions in the absence of Ca^{2+} (Figure 4). Phosphorylation had no significant effect on the level of $^{45}Ca^{2+}$ bound to the ATPase (Figure 4), suggesting that the phosphorylated ATPase was in a Ca^{2+}-bound state ($E1PCa_2$ or $E2PCa_2$).

Dephosphorylation of the phosphorylated ATPase is also very slow in gel phase phospholipid. Addition of 2 mM unlabeled ATP to the incubation medium to prevent further formation of radiolabeled ATPase lead to only a very slow decrease in the level of ^{32}P-labeled ATPase (Figure 4). Similarly, addition of 2 mM ADP after 20 min phosphorylation lead to a very slow decrease in the level of ^{32}P-labeled ATPase (Figure 4).

It has been shown (Stahl & Jencks, 1987) that following binding of Ca^{2+} and ATP to the ATPase, a conformational

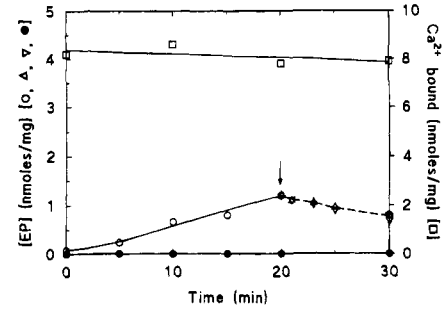


FIGURE 4: Rate of phosphorylation of the ATPase reconstituted with di(C16:0)PC, at 25 °C. The ATPase was incubated in 150 mM Mops/Tris, pH 7.2, 100 mM KCl, 5 mM Mg^{2+}, and 100 μM [γ-^{32}P]ATP either in 1 mM EGTA (●) or in 100 μM Ca^{2+} (○). Samples were taken at the given times and the level of phosphoenzyme formation was determined. For the points (△, ▽), the ATPase was incubated as above, and then at 20 min, either 2 mM ATP (△) or 2 mM ADP (▽) was added, and the level of phosphoenzyme formation was determined. The level of $^{45}Ca^{2+}$ bound to the ATPase (□) was determined by incubation in the above media except that the Ca^{2+} was labeled ($^{45}Ca^{2+}$) and the ATP was unlabeled, and the media contained 500 μM [^{3}H]sucrose. $^{45}Ca^{2+}$ binding was determined by filtration as described under Materials and Methods.

change must occur before the ATPase can be phosphorylated:

$$E1Ca_2ATP \rightleftharpoons E1'Ca_2ATP \rightleftharpoons E1PCa_2ADP$$

It has been suggested that the conformational change 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 (Stahl & Jencks, 1987). A conformational change preceding phosphorylation has been detected spectroscopically. Binding of ATP to IAEDANS-labeled ATPase in the presence of Ca^{2+} results in a decrease in fluorescence intensity (Suzuki et al., 1987, 1994; Obara et al., 1988; Kubo et al., 1990). A decrease in fluorescence intensity was also observed on addition of the nonhydrolyzable analogue of ATP, adenosine 5′-(β,γ-methylenetriphosphate) (AMPPCP) (Suzuki et al., 1994). In rapid kinetic studies, it was observed that the change in fluorescence preceded phosphoenzyme formation, and it was suggested that the fluorescence change could correspond to a $E1Ca_2ATP \rightleftharpoons E1'Ca_2ATP$ conformational change (Suzuki et al., 1987).

Addition of ATP to the Ca^{2+}-ATPase reconstituted with di(C16:0) at either 20 or 37 °C in the presence of Ca^{2+} resulted in a decrease in fluorescence intensity, comparable in magnitude to that observed on addition of AMPPCP (Figure 5A). The conformational change detected by IAEDANS fluorescence following ATP binding can therefore occur for the ATPase in gel phase lipid.

Changes in tryptophan fluorescence intensity have also been observed on addition of ATP to the Ca^{2+}-ATPase in the presence of Ca^{2+} (Dupont & Leigh, 1978; Fernandez-Belda et al., 1984; Andersen et al., 1985; Champeil et al., 1986; Nakamura et al., 1994). In kinetic studies, a slow decrease in tryptophan fluorescence intensity has been observed, as has a mixture of fast and slow changes, depending on the wavelengths of tryptophan fluorescence detected (Dupont & Leigh, 1978; Fernandez-Belda et al., 1984; Andersen et al., 1985; Champeil et al., 1986). The fast phase could correspond to phosphoenzyme formation (Champeil et al., 1986; Nakamura et al., 1994), and the slow phase was suggested to correspond to the formation of E2P by the slow $E1'PCa_2 \rightarrow E2P$ step (Fernandez-Belda et al.,

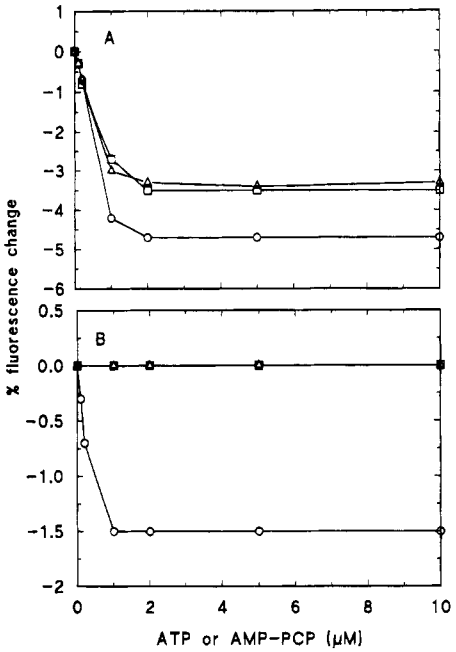


FIGURE 5: Effect of ATP and AMPPCP on the ATPase reconstituted with di(C16:0)PC. (A) Effects of ATP on the fluorescence intensity of IAEDANS-labeled ATPase at 20 (□) or 37 (○) °C, and the effect of AMPPCP at 37 °C (△). (B) Effects of ATP on the tryptophan fluorescence intensity of the ATPase at 20 (□) or 37 (○) °C, and the effect of AMPPCP at 37 °C (△). 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.

1984; Andersen et al., 1985; Champeil et al., 1986). Addition of AMPPCP to the ATPase reconstituted with di(C16:0)PC at 37 °C resulted in no change in fluorescence intensity (Figure 5B), consistent with tryptophan and IAEDANS fluorescence being sensitive to different changes on the ATPase. Addition of ATP to the ATPase in di(C16:0)PC at 37 °C in the liquid-crystalline phase resulted in a decrease in fluorescence intensity, but at 20 °C in the gel phase, ATP had no effect (Figure 5B). These results are consistent with the slow step in phosphoenzyme formation in gel phase lipid being the $E1'Ca_2ATP \rightleftharpoons E1PCa_2ADP$ step. A slow rate for phosphate transfer from ATP to the ATPase is also consistent with the observed low rate of hydrolysis of *p*-nitrophenol phosphate in the gel phase (Table 2) since it has been suggested that hydrolysis of acetyl phosphate by the ATPase does not involve the conformational change seen with ATP before phosphorylation (Bodley & Jencks, 1987).

Phosphorylation by P_i. No phosphorylation of the ATPase in di(C16:0)PC in the gel phase at 20 °C was observed following incubation at pH 6.0 with 20 mM Mg^{2+} and 1 mM P_i for 15 s or 30 min, or on incubation in the presence of 14% dimethyl sulfoxide (data not shown). Since for the native ATPase the presence of dimethyl sulfoxide leads to high levels of phosphorylation (de Meis, 1981), this implies a very significant destabilization of the phosphoenzyme in gel phase phospholipid.

Effect of Gel Phase Lipid on the E2−E1 Equilibrium. The fluorescence of the ATPase labeled with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) is sensitive to the E2−E1 transition of the ATPase, with E2, $E1Ca_2$, and E1 being states of low, intermediate, and high fluorescence intensities, respectively (Wakabayashi et al., 1990; Wictome et al., 1992b; Henderson et al., 1994a). The E2−E1 equilibrium has been shown to be sensitive to pH, low pH favoring the E2 state according to Scheme 1 with values for K_{H6}, K_{H7},

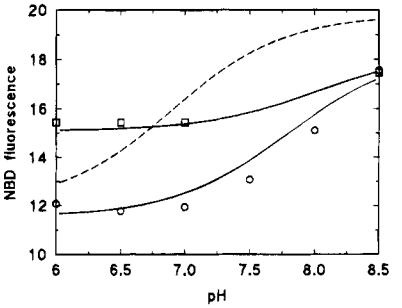


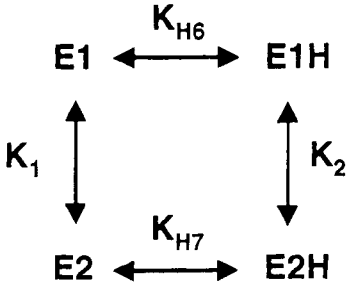
FIGURE 6: Effect of lipid phase on the fluorescence intensity of NBD-labeled ATPase. The fluorescence intensities at 25 °C for NBD-labeled ATPase reconstituted with di(C18:1)PC (○) and di(C16:0)PC (□) are shown. The solid lines are simulations calculated according to Scheme 1 with the parameters in Henderson et al. (1994a) but with a value for K_{H6} of 5.0×10^7 M^{-1} for the ATPase reconstituted with di(C16:0)PC, with values for the fluorescence intensities of the E1 and E2 conformations of 20.0 and 11.5, respectively. The dashed line is a simulation according to Scheme 1 but with a value for K_1 of 40.

Table 5: Fluorescence Response of NBD-Labeled ATPase in Gel Phase di(C16:0)PC to Ligand Addition

addition	% fluorescence change
0.5 mM Ca^{2+}, pH 6.0	0
0.5 mM Ca^{2+}, pH 8.5	−3.8
1 mM vanadate, pH 6.0[a]	−13.8
1 mM vanadate, pH 8.5[a]	−23.0
5 mM Mg^{2+}, pH 7.0	4.4
5 mM Mg^{2+} + 0.2 mM ATP, pH 7.0	4.9

[a] In the presence of 5 mM Mg^{2+} and 0.3 mM EGTA.

Scheme 1



and K_1 of 5×10^5 M^{-1}, 3.0×10^8 M^{-1}, and 4.0, respectively; stronger H^+ binding to E2 than to E1 explains the shift toward E2 with decreasing pH (Henderson et al., 1994a).

The effects of pH on the ATPase in di(C16:0)PC or di(C18:1)PC in the gel and liquid-crystalline phases, respectively, at 25 °C are very different (Figure 6); for the ATPase in di(C16:0)PC, fluorescence intensities at acid pH values are higher, and the pH dependence of the fluorescence intensities is much reduced. The data are consistent with a shift toward E1 in the gel phase.

The ATPase is still able to undergo the E2−E1 transition in gel phase lipid. Thus, addition of vanadate, an analogue of phosphate, results in a decrease in fluorescence intensity, consistent with stronger binding of vanadate to the E2 conformation of the ATPase than to the E1 conformation (Table 5); the larger response seen at pH 8.5 than at pH 6.0 is consistent with a shift toward E1 at high pH (see Figure 6). Addition of ATP to NBD-labeled ATPase in liquid crystalline di(C18:1)PC in the presence of Mg^{2+} results in an increase in fluorescence intensity of 19% (data not shown), consistent with stronger binding of MgATP to the E1 conformation than to the E2 conformation, as for the

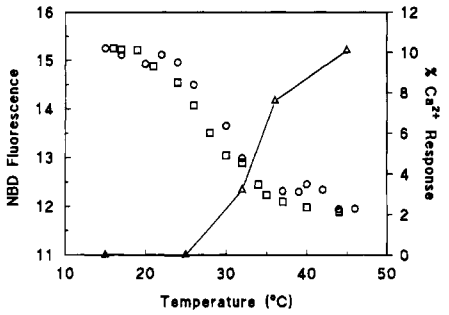


FIGURE 7: Effect of temperature on the fluorescence intensity of NBD-labeled ATPase reconstituted in di(C16:0)PC. Shown are the fluorescence intensities recorded in 150 mM Mops/80 mM Tris, pH 7.0, 0.3 mM EGTA, on increasing (○) or decreasing (□) the temperature. (△) shows the percent change in fluorescence intensity on addition of 0.8 mM Ca^{2+} to the labeled ATPase at the given temperatures.

native ATPase (Henderson, 1994). For the ATPase in gel phase di(C16:0)PC, the response to MgATP is much smaller (Table 5), again consistent with a shift to E1 in gel phase lipid. Addition of Ca^{2+} to NBD-labeled ATPase in the native SR membrane at pH 7.2 results in an increase in fluorescence intensity, attributed to the formation of $E1Ca_2$, a state of intermediate fluorescence intensity, from E2, a state of low fluorescence intensity, whereas addition of Ca^{2+} at pH 8.5 results in a decrease in fluorescence intensity, attributed to formation of $E1Ca_2$ from E1, a state of high fluorescence intensity (Wakabayashi et al., 1990; Wictome et al., 1992b; Henderson et al., 1994a). For the ATPase in gel phase di(C16:0)PC at 25 °C, addition of Ca^{2+} at pH 6.0 resulted in no significant change in fluorescence intensity, but at pH 8.5 resulted in a decrease in fluorescence intensity (Table 5). All these results are consistent with the ATPase being able to undergo an E1−E2 conformational change in gel phase lipid.

We have shown previously that the equilibrium constant for E1/E2 is higher for the ATPase in di(C14:1)PC than di(C18:1)PC, both being in the liquid-crystalline phase (Froud et al., 1986; Starling et al., 1994). The shift in equilibrium constant was consistent with Scheme 1 with a value of K_1 of 16 in di(C14:1)PC compared to 4 in di(C18:1)PC, values of K_{H6} and K_{H7} being unaltered (Starling et al., 1994). For the ATPase in gel phase di(C16:0)PC, the pH dependence of fluorescence intensity is not consistent with a change in K_1 (Figure 6), but the data are consistent with an increase in H^+ affinity for E1, this explaining the shift to E1 and the reduced pH dependence of the equilibrium. As shown, the data fit Scheme 1, with a value for K_{H6} of 5×10^7 M^{-1} in gel phase lipid, the other constants being unaltered. Interestingly, the effects of changing phospholipid structure on the metarhodopsin I−metarhodopsin II transition for rhodopsin have been attributed to a change in the pK value for some residue in rhodopsin (Gibson & Brown, 1993).

Figure 7 shows the effect of temperature on the fluorescence intensity of NBD-labeled ATPase reconstituted in di(C16:0)PC. As shown, the steepest change in fluorescence intensity occurs over the temperature range 20−35 °C. Also shown in Figure 7 is the response to the addition of Ca^{2+} at pH 7.0, as a function of temperature. For the ATPase reconstituted in di(C16:0)PC, addition of Ca^{2+} at pH 7.0 at temperatures of 25 °C or below has no effect on fluorescence intensity (Figure 7), whereas at 30 °C or above addition of Ca^{2+} results in an increase in fluorescence intensity, the magnitude of the change increasing with increasing temperature (Figure 7). These results suggest that the ATPase senses a very broad lipid phase transition in di(C16:0)PC, as previously suggested by Hesketh et al. (1976).

Ca^{2+} Binding to the ATPase in the Presence of ATP. Since the rate of phosphorylation of the ATPase by ATP is very slow in the gel phase (Figure 4), it is possible to study effects of ATP on Ca^{2+} binding to the ATPase in pseudoequilibrium experiments. As shown in Figure 1A, the affinity of the ATPase for Ca^{2+} is little affected by addition of 2 mM MgATP. Further, the rate of dissociation of Ca^{2+} from the Ca^{2+}-bound ATPase at pH 7.2 in the presence of 5 mM Mg^{2+} is increased only by 30% on addition of 2 mM ATP (data not shown). If, as suggested by the fluorescence experiments described above, the ATPase in gel phase lipid in the presence of Ca^{2+} and ATP is in the $E1'Ca_2ATP$ form, then these experiments suggest that the Ca^{2+} binding sites are little affected by the binding of ATP.

Orlowski and Champeil (1991) reported a 50% decrease in the rate of dissociation of Ca^{2+} from the ATPase at pH 6.0 in the presence of the nonhydrolyzable analogue of ATP, adenosine 5′-(β,γ-methylenetriphosphate) (AMPPCP), whereas the kinetic analysis of Petithory and Jencks (1986) gives a rate of Ca^{2+} dissociation from the Ca^{2+}- and ATP-bound complex ca. 60% greater than that from the ATP-unbound species. Watanabe et al. (1981) reported that adenyl-5′-yl imidodiphosphate (AMP-PNP) had no significant effect on the affinity of the ATPase for Ca^{2+} whereas Orlowski and Champeil (1991) reported a 2-fold increase in the affinity for Ca^{2+} in the presence of AMPPCP.

The Physical Basis of the Effects of Gel Phase Lipid. The observed effects of gel phase phospholipid on the Ca^{2+}-ATPase could, in principle, follow from the high viscosity expected for gel phase lipid, the high viscosity resulting in a very slow rate for some step or steps in the reaction cycle. A viscosity-dependent mechanism for the effects of gel phase lipid on the ATPase would predict that the ratios of forward to backward rate constants for each step (the equilibrium constant) would be the same in gel and in liquid-crystalline phase lipid, since changes in viscosity cannot result in changes in any equilibrium property of a system (Lee, 1991; Lee & East, 1993). In fact, we show here that a number of equilibrium properties of the ATPase are different in bilayers in the gel and liquid-crystalline phases. Changes in equilibrium properties of the Ca^{2+}-ATPase are likely to follow directly from differences in the energies of interaction of gel and liquid-crystalline phase lipid with the different conformational states of the ATPase; it has been shown that phosphatidylcholines in the gel phase bind less strongly to the ATPase than those in the liquid-crystalline phase (East & Lee, 1982). It is unlikely that aggregation of the ATPase in bilayers in the gel phase (Kleeman & McConnell, 1976; Moore et al., 1981; Gutierrez Merino, 1987) is important in determining ATPase activities, since low activities are also observed for the ATPase reconstituted in monomeric form into sealed vesicles in the gel phase at very high molar ratios of lipid to ATPase, under conditions where no cross-linking of the ATPase could be observed with a variety of cross-linking agents (Starling et al., 1995).

Effects of Bilayer Thickness. The results reported here suggest that the effects of phosphatidylcholines on the ATPase are not related solely to the thickness of the bulk lipid bilayer. It has been shown (Sperotto & Mouritsen, 1988) that the thickness, d (in angstrom), of the hydrophobic region of a bilayer in the liquid-crystalline phase is a linear

function of fatty acyl chain length, n_c, given by

$$d = 1.75(n_c - 1)$$

Thus, the difference in thickness between a bilayer of di(C14:1)PC and di(C16:1)PC would be ca. 3 Å; a Ca^{2+} binding stoichiometry of 1 Ca^{2+} ion per ATPase molecule is found for the former and 2 for the latter (Starling et al., 1993). In the gel phase, the thickness of the bilayer is ca. 30% greater than in the liquid-crystalline phase (Sperotto & Mouritsen, 1988) so that a bilayer of di(C14:0)PC in the gel phase will be ca. 7 Å thicker than the corresponding bilayer in the liquid-crystalline phase, and yet, as shown in Table 3, the stoichiometry of Ca^{2+} binding is independent of lipid phase.

Conclusion. The very low steady-state ATPase activity observed for the Ca^{2+}-ATPase in gel phase phospholipid is attributed to a slow rate of phosphorylation of the ATPase. Spectroscopic evidence suggests that the conformational change that occurs following binding of ATP to the ATPase in the presence of Ca^{2+} is unaffected by the phase of the lipid, but that the rate of phosphate transfer is decreased. Ca^{2+} binding to the ATPase is relatively unaffected by the phase of the lipid, except that binding of Mg^{2+} at a gating site on the ATPase no longer affects the rate of dissociation of Ca^{2+} from the ATPase.

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