Evidence that the effects of phospholipids on the activity of the Ca^{2+} -ATPase do not involve aggregation

Anthony P. STARLING, J. Malcolm EAST and Anthony G. LEE*

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

The Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum, solubilized in monomeric form in C_{12}E_8 , has been reconstituted by dialysis into sealed vesicles of dioleoyl phosphatidylcholine [$\text{di}(\text{C}_{18:1})\text{PC}$], dimyristoleoyl phosphatidylcholine [$\text{di}(\text{C}_{14:1})\text{PC}$], dinervonyl phosphatidylcholine [$\text{di}(\text{C}_{24:1})\text{PC}$] or dipalmitoyl phosphatidylcholine [$\text{di}(\text{C}_{16:0})\text{PC}$] in the gel phase, at a phospholipid/ATPase molar ratio of 10000:1. Cross-linking experiments show that ATPase molecules are present in these reconstituted vesicles as isolated monomeric species. ATPase activities for the reconstituted vesicles are about half of those for the ATPase reconstituted with the same lipid in unsealed membrane fragments, attributed to a close to random orientation for the ATPase molecules in the reconstituted vesicles. ATPase activities for the ATPase in reconstituted vesicles of $\text{di}(\text{C}_{14:1})\text{PC}$ or

$\text{di}(\text{C}_{24:1})\text{PC}$ are less than in vesicles of $\text{di}(\text{C}_{18:1})\text{PC}$, and no activity could be detected for the ATPase in $\text{di}(\text{C}_{16:0})\text{PC}$ in the gel phase. It is concluded that effects of lipids on the activity of the ATPase are independent of any changes in the state of aggregation of the ATPase. Inhibition of ATPase activity by spermine and by the hydrophilic domain of phospholamban are observed both for the unreconstituted ATPase and for the ATPase in reconstituted vesicles, so that inhibition is independent of any aggregation caused by these polycationic species. Stimulation of ATPase activity by jasmone is also observed both for the unreconstituted ATPase and for the ATPase in reconstituted vesicles, so that stimulation of the ATPase also does not follow from any change in the state of aggregation of the ATPase.

INTRODUCTION

An important component of the environment of an integral membrane protein is the phospholipid bilayer in which it is embedded. The fatty-acyl chain length, the headgroup and the physical phase (gel or liquid-crystalline) of the phospholipid are all important determinants of activity [1]. For the Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum (SR) the optimal phospholipid chain length for activity is C_{18} , with shorter or longer chains supporting lower activities [2–5]. In phosphatidylcholines with chains of lengths C_{14} or C_{24} , major changes in the properties of the Ca^{2+} -ATPase include a change in the stoichiometry of Ca^{2+} binding, from the usual 2 Ca^{2+} ions bound per ATPase molecule to 1 Ca^{2+} ion bound per ATPase molecule [4,6]. An optimum chain length of about C_{18} has also been established for the glucose transporter [7] and for the Na^{+} -dependent leucine transport system in *Pseudomonas aeruginosa* [8].

Theoretical models have suggested that one likely response to a mismatch between the thickness of the hydrophobic region of a membrane protein and the thickness of the hydrophobic region of a phospholipid bilayer is aggregation of the membrane protein [9–11]. For bacteriorhodopsin, such aggregation has only been detected in electronmicrographs for chains of length C_{10} and C_{24} [12,13]. However, for rhodopsin, static aggregation has been detected in phosphatidylcholines of chain length C_{12} using ESR [14,15], and transient, concentration-dependent aggregation has been detected in phospholipids containing longer fatty-acyl chains [15]. For the Ca^{2+} -ATPase, the most unhindered rotation was detected in bilayers of dioleoyl phosphatidylcholine

[$\text{di}(\text{C}_{18:1})\text{PC}$] [16]. Reduced rotational mobility was detected for the ATPase in lipids with shorter or longer fatty-acyl chains, the major effect being on the residual anisotropy observed at long times, taken to be an indication of large-scale aggregation of the ATPase; in phosphatidylcholines with C_{14} or C_{24} chains, almost complete immobilization of the ATPase was observed [16].

It has been suggested that the low ATPase activity observed for the ATPase in phosphatidylcholines with C_{14} or C_{24} fatty-acyl chains can be attributed to a low activity for the ATPase in large aggregates, with optimal functioning of the ATPase requiring free rotation of the ATPase [16]. It is important to emphasize that the aggregation here referred to is much more extensive than that which is commonly referred to as ‘oligomerization’ [16,17]. The state of oligomerization of the Ca -ATPase in SR membranes is unclear; electron-microscopic studies suggest that both monomeric and dimeric species are possible, depending on conditions [18]. Studies of the rate of rotation of the Ca -ATPase using saturation-transfer ESR are consistent with a dimeric species [19]. The rate of rotation of the ATPase observed using phosphorescence depolarization is consistent with either a monomeric or dimeric species [17]. In the presence of melittin, progressive aggregation of the ATPase is observed [17]. The initial monomeric or dimeric species first aggregates to a slower-rotating species which is thought to correspond not to a well-defined oligomer but to a distribution of different oligomers (for which the average size is larger than a dimer, perhaps a tetramer [20]), and, finally, large-scale protein aggregation occurs to give large immobile aggregates [17]. The number of ATPase molecules in the very large aggregates cannot

Abbreviations used: DSP, dithiobis(succinimidylpropionate); SR, sarcoplasmic reticulum; $\text{di}(\text{C}_{14:1})\text{PC}$, dimyristoleoyl phosphatidylcholine; $\text{di}(\text{C}_{18:1})\text{PC}$, dioleoyl phosphatidylcholine; $\text{di}(\text{C}_{24:1})\text{PC}$, dinervonyl phosphatidylcholine; $\text{di}(\text{C}_{16:0})\text{PC}$, dipalmitoyl phosphatidylcholine, C_{12}E_8 , octa(ethylene glycol) mono-n-dodecyl ether.

* To whom correspondence should be addressed.

be defined [17], but must be greater than about 10 [21], and may be very many more than this.

A test of the proposal that large-scale aggregation of the ATPase in phospholipids with C_{14} or C_{24} fatty-acyl chains is responsible for the low ATPase activity would be to study the effects of fatty-acyl chain length on the activity of the ATPase reconstituted into vesicles each containing only one or two ATPases molecules so that large-scale aggregation is impossible. Andersen et al. [22] and Heegaard et al. [23] have published a procedure for reconstituting the ATPase in which the ATPase is first dissolved in monomeric form in octa(ethylene glycol) monon-dodecyl ether ($C_{12}E_8$) and then mixed with a large excess of lipid in cholate solution, followed by extensive dialysis to remove detergent. A uniform population of sealed unilamellar vesicles is formed, containing about 2 ATPase molecules per vesicle [23]. The small number of ATPase molecules per vesicle, and their monomeric nature in the vesicles, was confirmed by the lack of cross-linking observed with dithiobis(succinimidylpropionate) (DSP) [23], a cross-linker which causes extensive cross-linking of ATPase molecules in the native SR [23,24]. Here we report on the effects of phospholipid structure on the activity of the ATPase in such reconstituted preparations.

MATERIALS AND METHODS

Phospholipids were obtained from Avanti Polar Lipids. SR was prepared from rabbit skeletal muscle, and the Ca^{2+} -ATPase was purified from SR using cholate as detergent, as described by East and Lee [3]. ATPase activities were determined at 25 °C by using a coupled enzyme assay in a medium containing 40 mM Hepes/KOH, pH 7.2, 100 mM KCl, 5 mM $MgSO_4$, 2.1 mM ATP, 1.1 mM EGTA, 0.53 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 units) and lactate dehydrogenase (18 units) in a total volume of 2.5 ml. The reaction was initiated by addition of an aliquot of a 25 mM $CaCl_2$ solution to a cuvette containing the ATPase and the other reagents to give a maximally stimulating concentration of Ca^{2+} (the free Ca^{2+} concentration was about 10 μ M).

Reconstitutions of the ATPase into sealed vesicles was performed as described by Andersen et al. [22]. SR (0.3 mg) was solubilized with $C_{12}E_8$ (0.6 mg) in buffer [75 μ l; 30 mM Tris/HCl (pH 7.1)/0.4 M KCl/0.4 M sucrose/4 mM $MgCl_2$ /1 mM EDTA/5 mM dithiothreitol/1 mM sodium azide]. For reconstitution with di($C_{18:1}$)PC, di($C_{18:1}$)PC (21 mg) was suspended in the same buffer (300 μ l) containing 10.8 mg of cholate and sonicated to clarity in a bath sonicator; these amounts correspond to a final phospholipid/ATPase molar ratio of 10000:1 and 0.5 mg of cholate/mg of phospholipid plus 1.0 mg of cholate/mg of ATPase. Samples of the other phospholipids were prepared in the same way. The phospholipid and ATPase samples were then mixed and dialysed for 60 h at 5 °C against 0.1 M Na_2HPO_4 (pH 7.1)/4 mM $MgCl_2$ /1 mM EDTA/5 mM dithiothreitol/1 mM sodium azide, the dialysis buffer being changed every 10 h.

Reconstitution of the ATPase into membrane fragments was performed as described in Starling et al. [4]. Phospholipid (10 μ mol) was mixed with buffer [400 μ l; 10 mM Hepes/Tris/15 % (w/v) sucrose, pH 8.0] containing $MgSO_4$ (5 mM) and potassium cholate (12 mg/ml) and sonicated to clarity in a bath sonicator. Purified ATPase (1.25 mg) in a volume of 20–30 μ l was then added and, for di($C_{14:1}$)PC and di($C_{18:1}$)PC, left for 15 min at room temperature and 45 min at 5 °C to equilibrate before being diluted with buffer (2 ml) and stored on ice until use; for di($C_{24:1}$)PC, samples were equilibrated for 1 h at room temperature, and for di($C_{16:0}$)PC, samples were equilibrated at 42 °C for 15 min and then at 5 °C for 45 min.

For cross-linking with DSP (Pierce), the ATPase was suspended in buffer [20 mM Mes (pH 7.0)/0.3 M sucrose/1.0 mM EGTA/1.01 mM Ca^{2+} /0.1 mM ATP] at 0.8 mg of protein/ml. DSP was added from a stock solution (100 mM) in dimethyl sulphoxide to a final concentration of 1.0 mM. After incubation for 20 min at 22 °C, the reaction was terminated by addition of glycine and SDS to final concentrations of 50 mM and 2 % respectively. Samples were analysed by SDS/PAGE on 5 % polyacrylamide using 4 % polyacrylamide as stacking gel with 40 μ g of protein per lane. Gels were stained with Coomassie Blue and, after destaining, were scanned on a Joyce–Loebl Chromoscan 3 instrument.

For cross-linking with glutaraldehyde, the ATPase was suspended in buffer [50 mM Mops (pH 6.8)/20 mM KCl/5 mM $MgSO_4$ /50 μ M Ca^{2+}] at 1.6 mg of protein/ml. Glutaraldehyde was added to a final concentration of 20 mM. After incubation for 10 min at 22 °C, the reaction was terminated by addition of hydrazine to a final concentration of 100 mM. Samples were analysed by SDS/PAGE as described above.

RESULTS AND DISCUSSION

The ATPase is dispersed in monomeric form in reconstituted vesicles

If SR vesicles are dissolved in $C_{12}E_8$ to give monomeric Ca^{2+} -ATPase, mixed with a solution of egg-yolk phosphatidylcholine in cholate at a phospholipid/ATPase molar ratio of 10000:1 and reconstituted by dialysis, sealed vesicles are formed containing about 2 ATPase molecules per vesicle [22,23]. From an analysis of freeze–fracture electron micrographs, Heegaard et al. [23] concluded that the Ca^{2+} -ATPase in these reconstituted vesicles was present in a monomeric form. This was confirmed by cross-linking experiments which showed no depletion of monomeric ATPase species and no formation of dimeric or other polymeric species [22,23]. Cross-linking of the ATPase in native SR vesicles could follow either from the presence of pre-existing aggregates or from diffusional collisions between initially separated ATPase molecules [24]. The diameter of the reconstituted vesicles has been estimated to be about 70 nm (700 Å) [23]. For a vesicle containing two ATPases molecules, the maximum distance between ATPase molecules would then be about 100 nm (1000 Å), and, given a diffusion coefficient of about 1×10^{-8} cm²·s⁻¹ for a membrane protein [25], this would correspond to an average time between collisions of about 20 s. Thus ATPase molecules are effectively isolated as monomeric species in the reconstituted vesicles [16].

Here we use the cross-linking method to confirm the presence of isolated monomeric ATPase molecules in vesicles of di($C_{14:1}$)PC and di($C_{24:1}$)PC in the liquid-crystalline phase and in vesicles of di($C_{16:0}$)PC in the gel phase. As shown in Figure 1, cross-linking of the purified ATPase with 1 mM DSP for 20 min leads to almost complete loss of the band in SDS/polyacrylamide gels attributable to monomeric Ca^{2+} -ATPase (Figure 1). As reported previously, only slight traces of dimeric species are formed, the major effect of cross-linking being the formation of aggregated species too large to enter the stacking gel [24]. Similarly for the ATPase reconstituted into membrane fragments of di($C_{18:1}$)PC by dilution from cholate solution at a di($C_{18:1}$)PC/ATPase molar ratio of 1000:1, cross-linking with DSP led to almost complete loss of monomeric ATPase species (Figure 1). However, for the ATPase reconstituted into sealed vesicles of di($C_{18:1}$)PC, di($C_{14:1}$)PC or di($C_{24:1}$)PC at a phospholipid/ATPase molar ratio of 10000:1, addition of DSP resulted in no significant decrease in intensity for the monomeric ATPase

Figure 1 Cross-linking of the reconstituted Ca²⁺-ATPase by DSP: SDS/PAGE of the reconstituted ATPase.

ATPase was reconstituted by dialysis into sealed vesicles of: di(C_{14:1})PC (lanes 1 and 2); di(C_{16:0})PC (lanes 3 and 4); di(C_{18:1})PC (lanes 5 and 6); di(C_{24:1})PC (lanes 7 and 8). Lanes 9 and 10 contained unreconstituted ATPase; lanes 11 and 12 contained ATPase reconstituted into membrane fragments of di(C_{18:1})PC by dilution from cholate solution at a lipid/protein molar ratio of 1,000:1. Lane 13 shows the molecular-mass (*M*) markers. Odd-numbered lanes, absence of cross-linking; even-numbered lanes, after cross-linking with 1 mM DSP for 20 min at 22 °C.

Figure 2 Cross-linking of the reconstituted Ca²⁺-ATPase by glutaraldehyde: SDS/PAGE of the reconstituted ATPase

ATPase was reconstituted by dialysis into sealed vesicles of: di(C_{14:1})PC (lanes 1 and 2); di(C_{16:0})PC (lanes 3 and 4); di(C_{18:1})PC (lanes 5 and 6); di(C_{24:1})PC (lanes 7 and 8). Lanes 9 and 10 contained unreconstituted ATPase; lanes 11 and 12 contained ATPase reconstituted into membrane fragments of di(C_{18:1})PC by dilution from cholate solution at a lipid/protein molar ratio of 1000:1. Odd-numbered lanes, absence of cross-linking; even-numbered lanes, after cross-linking with 20 mM glutaraldehyde for 10 min at 22 °C. Abbreviation: *M*, molecular mass.

species and no significant increase in dimeric species (Figure 1); densitometer scans showed that any loss of monomeric ATPase was less than 4%. Similarly, DSP resulted in no significant cross-linking of the ATPase reconstituted in di(C_{16:0})PC and cross-linked at 22 °C, when the phospholipid will be in the gel phase [26] (Figure 1).

Very similar results are obtained on cross-linking with glutaraldehyde (Figure 2). Whereas cross-linking of the purified ATPase or the ATPase reconstituted into membrane fragments of di(C_{18:1})PC leads to almost complete loss of monomeric ATPase, glutaraldehyde has very little effect on the ATPase reconstituted into sealed vesicles of di(C_{18:1})PC, di(C_{14:1})PC, or di(C_{24:1})PC or di(C_{16:0})PC in the gel phase (Figure 2).

We conclude that, in the reconstituted vesicles, the ATPase is present in a dispersed, monomeric state, probably with 1 or 2 ATPase molecules per vesicle, as for the ATPase reconstituted with egg-yolk phosphatidylcholine [22,23].

Table 1 Effects of phosphatidylcholines on the activity of the Ca²⁺-ATPase

Phospholipid	Activity (units/mg of protein)*		
	Fragments†	Reconstituted vesicles‡	
		–C ₁₂ E ₈	+C ₁₂ E ₈ §
di(C _{18:1})PC	3.4	1.5	3.1
di(C _{14:1})PC	0.3	0.1	0.3
di(C _{24:1})PC	0.7	0.2	0.6
di(C _{16:0})PC	0	0	0

* Activities measured at pH 7.2, with 2.1 mM ATP and 5 mM Mg²⁺ at 25 °C.

† Reconstituted by dilution from cholate solution at a lipid/protein molar ratio of 1000:1.

‡ Reconstituted by dialysis as sealed vesicles at a lipid/protein molar ratio of 10000:1.

§ The C₁₂E₈ concentration was 0.4 mg/ml.

Table 2 Effects of additives on the activity of the Ca²⁺-ATPase

Additive	Activity (% of control)*	
	Unreconstituted	Reconstituted vesicles†
10 mM Spermine	43	42
25 μM PLN(1–25)‡	62	67
10 μM Jasmone	190	190

* Activities expressed relative to those measured in the absence of additive at pH 7.2, with 2.1 mM ATP and 5 mM Mg²⁺ at 25 °C.

† Reconstituted by dialysis as sealed vesicles at a lipid/protein molar ratio of 10000:1.

‡ The peptide MEKVQLTRSAIRRASTIEMPPQAR-Cys corresponding to residues 1–25 of phospholamban [29].

Effects of lipid fatty-acyl chain length on ATPase activity do not involve aggregation of the ATPase

ATPase activities for the reconstituted ATPase are shown in Table 1. For the ATPase reconstituted into membrane fragments by dilution from cholate solution, activities are lower in di(C_{14:1})PC or di(C_{24:1})PC than in di(C_{18:1})PC, and no activity could be detected in di(C_{16:0})PC at 25 °C, as reported previously [4,26,35]. For the ATPase reconstituted into sealed vesicles by dialysis from C₁₂E₈ and cholate, the ATPase activity in di(C_{18:1})PC is about half that observed for membrane fragments (Table 1). This has been attributed to the formation of sealed vesicles in which about 50% of the ATPase molecules are oriented with their ATP-binding sites facing the lumen of the vesicles [27]. Addition of 0.4 mg/ml C₁₂E₈ to the reconstituted vesicles to make them leaky to ATP leads to a doubling of activity (Table 1); the concentration of C₁₂E₈ used here causes no significant displacement of phospholipids from around the ATPase [6,28]. For the ATPase reconstituted into sealed vesicles of di(C_{14:1})PC or di(C_{24:1})PC, ATPase activities are also about half of those recorded for membrane fragments, and activities again double on addition of 0.4 mg/ml C₁₂E₈. No activity could be detected in di(C_{16:0})PC (Table 1).

Effects of phospholamban, spermine and jasmone on the activity of the Ca²⁺-ATPase

A number of other interactions affecting the activity of the ATPase do not involve aggregation (Table 2). Binding of phospholamban to the cardiac Ca²⁺-ATPase has been shown to result in reduced motional freedom, and it has been suggested

that this is responsible for the corresponding decrease in ATPase activity [21]. It has been suggested that inhibition of the ATPase by phospholamban follows from binding of the hydrophilic N-terminal domain of phospholamban to the ATPase, and it has been shown that binding of a peptide corresponding to residues 1–25 of phospholamban to the skeletal-muscle Ca^{2+} -ATPase inhibits activity [29]. As shown in Table 2, the level of inhibition of ATPase activity observed with the peptide is identical for the unreconstituted ATPase and for the ATPase reconstituted in the sealed vesicles. Similarly, inhibition by spermine [30] is identical in the two systems (Table 2). It has been shown that binding of jasmone to the ATPase increases ATPase activity [31]. Effects of jasmone are also identical for the unreconstituted ATPase and for the ATPase reconstituted in monomeric form (Table 2).

Conclusion

The observation that ATPase activities are lower for the monomeric ATPase in $\text{di}(\text{C}_{14:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ than in $\text{di}(\text{C}_{18:1})\text{PC}$ indicates that effects of the short- and long-chain phospholipids on activity follow directly from effects of phospholipid–protein interactions on the conformation of the ATPase. The aggregation of the ATPase observed when the ATPase is reconstituted with $\text{di}(\text{C}_{14:1})\text{PC}$ or $\text{di}(\text{C}_{24:1})\text{PC}$ in membrane fragments at phospholipid/ATPase molar ratios of 1000:1 [16] is evidently not required to produce the low activity observed for the ATPase in these lipids. Similarly, no activity could be detected for the ATPase in gel phase $\text{di}(\text{C}_{16:0})\text{PC}$ (Table 1) under conditions where cross-linking experiments show a lack of aggregation (Figures 1 and 2). Thus although it is known that the ATPase tends to segregate into protein-rich patches in gel-phase phospholipid [32–34], this segregation is not necessary to produce a low ATPase activity. Inhibition of the ATPase by phospholamban and spermine, and stimulation by jasmone, also do not involve changes in the aggregation state of the ATPase.

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