Binding Sites for Cholesterol on Ca^{2+}-ATPase Studied by Using a Cholesterol-Containing Phospholipid†

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

Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

Received December 1, 1993; Revised Manuscript Received February 24, 1994®

ABSTRACT: Phosphatidylcholines have been synthesized containing a cholesterol moiety at the 2-position of the glycerol backbone. Fluorescence quenching studies show that cholesterol-containing phosphatidylcholines can bind at the lipid–protein interface of the Ca^{2+}-ATPase from skeletal muscle sarcoplasmic reticulum, with an affinity half that of dioleoylphosphatidylcholine. The ATPase activity measured for the ATPase reconstituted with the cholesterol-containing phosphatidylcholine containing an oleoyl fatty acyl chain, (C18:1,CHS)PC, is less than that measured for the ATPase reconstituted with dioleoylphosphatidylcholine. The activity measured for the ATPase reconstituted with the cholesterol-containing phosphatidylcholine containing a myristoleoyl fatty acyl chain, (C14:1,CHS)PC, is less than that measured in (C18:1,CHS)PC and is comparable to that measured in dimyristoleoylphosphatidylcholine (di(C14:1)PC. The stoichiometry of Ca^{2+} binding to the ATPase is two Ca^{2+} ions bound per ATPase molecule in the native membrane or in (C18:1,CHS)PC, but one bound per ATPase molecule in di(C14:1)PC or (C14:1,CHS)PC. Addition of cholesterol to the ATPase in di(C14:1)PC or (C14:1,CHS)PC increases the Ca^{2+} binding stoichiometry to the usual 2:1, but the binding stoichiometry remains 1:1 in mixtures of di(C14:1)PC and (C14:1,CHS)PC. Removal of Ca^{2+} from the Ca^{2+}-bound ATPase results in a decrease in tryptophan fluorescence intensity for the ATPase in the native membrane, but an increase in fluorescence intensity for the ATPase in di(C14:1)PC or (C14:1,CHS)PC. Addition of cholesterol to the ATPase in di(C14:1)PC or (C14:1,CHS)PC reverses this change. It is concluded that cholesterol linked to a phospholipid molecule can interact with the ATPase only at the lipid–protein interface. Free cholesterol, although largely excluded from the lipid–protein interface, can bind at other hydrophobic sites on the ATPase. It is suggested that these sites could be located between transmembrane α-helices.

The activity of the Ca^{2+}-ATPase is dependent on the structure of the phospholipids surrounding it in the membrane. The phospholipid supporting the highest ATPase activity is dioleoylphosphatidylcholine (di(C18:1)PC),[1] and phosphatidylcholines with longer or shorter fatty acyl chains support lower activity (Johannsson et al., 1981; Caffrey & Feigenson, 1981; East & Lee, 1982; Starling et al., 1993). On reconstitution into 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 two Ca^{2+} ions bound per ATPase molecule to one Ca^{2+} ion bound per ATPase molecule (Michelangeli et al., 1990b; Starling et al., 1993). For the ATPase reconstituted with di(C14:1)PC, this effect can be reversed by addition of a variety of hydrophobic molecules, including sterols, alcohols, and esters, whereas for the ATPase reconstituted with di(C24:1)PC, addition of sterols has no effect on Ca^{2+} binding (Starling et al., 1993). Similarly, addition of sterols and other hydrophobic molecules increases the ATPase activity of the ATPase in di(C14:1)PC but not in di(C24:1)PC (Starling et al., 1993).

The effects of sterols on the activity of the ATPase in bilayers of di(C14:1)PC could, in principle, follow from changes in the properties of the lipid component of the membrane (e.g., fluidity or membrane thickness) or could follow directly from conformational changes on the ATPase induced by binding of the sterol to the ATPase. Binding to the ATPase can be studied using fluorescence quenching methods (Caffrey & Feigenson, 1981; East & Lee, 1982). It has been shown that reconstitution of the ATPase into bilayers of di(dibromostearoyl)phosphatidylcholine (di(Br_2C18:0)PC) results in quenching of approx. 60% of the tryptophan fluorescence of the ATPase (East & Lee, 1982). Quenching of fluorescence by bromine occurs by a process of heavy atom quenching which involves an intimate collision between the fluorophore and the quencher. Quenching of the fluorescence of a tryptophan residue in the ATPase by a bromine-containing phospholipid then requires either that the tryptophan residue be at the lipid–protein interface or that it be connected by energy transfer to a tryptophan residue which is at the interface. Since the rate of exchange of phospholipids between the surface of the ATPase (annular sites) and the bulk phospholipid phase is slow compared to the fluorescence lifetime for tryptophan (Simmonds et al., 1982; East et al., 1985), the degree of fluorescence quenching in mixtures of brominated and nonbrominated lipids is related to the fractional occupation of the lipid–protein interface by the bromine-containing lipid (East & Lee, 1982). Using this approach, it was shown that the ATPase exhibits little selectivity in binding phospholipids at the lipid–protein interface (East & Lee, 1982), but since addition of sterols to the ATPase reconstituted with di(Br_2-C18:0)PC was found to have little effect on the fluorescence intensity of the ATPase, sterols can bind only weakly at the interface. However, addition of bromine-containing derivatives of the sterols to the ATPase reconstituted with di(C18:

† The Wellcome Trust, the SERC, and the Wessex Medical Trust are thanked for financial support.

® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: di(C14:1)PC, dimyristoleoylphosphatidylcholine; di(C18:1)PC, dioleoylphosphatidylcholine; di(Br_2C18:0)PC, dibromostearoylphosphatidylcholine; CHS, cholesteryl hemisuccinate; Br_2CHS, 5,6-dibromocholestan-3β-ol hemisuccinate; DPH, 1,6-diphenyl-1,3,5-hexatriene; SR, sarcoplasmic reticulum.

0006-2960/94/0433-4974$04.50/0

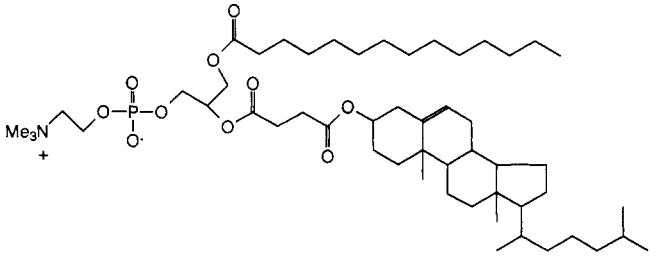

FIGURE 1: Structure of the cholesterol-containing phosphatidylcholine (C14:0,CHS)PC.

1)PC resulted in fluorescence quenching, suggesting that they could bind to sites on the ATPase other than those at the lipid–protein interface, possibly between transmembrane α-helices (Simmonds et al., 1982, 1984; Froud et al., 1986b; Michelangeli et al., 1990a). It was suggested that this binding was responsible for the effects of sterols on the function of the ATPase in bilayers of di(C14:1)PC (Simmonds et al., 1982; Froud et al., 1986a; Michelangeli et al., 1990a).

To help distinguish between direct effects of sterols on the ATPase and effects mediated via the lipid phase, we have synthesized phosphatidylcholines containing cholesterol at the 2-position (Figure 1) and report here on their effects on the properties of the ATPase.

MATERIALS AND METHODS

1-Oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, 1-myristoyl-2-hydroxy-*sn*-glycero-3-phosphocholine, dioleoylphosphatidylcholine, and dimyristoleoylphosphatidylcholine were obtained from Avanti Polar Lipids, and egg yolk lysophosphatidylcholine, cholesterol, and cholesteryl hemisuccinate were obtained from Sigma.

ATPase was purified from skeletal muscle sarcoplasmic reticulum as described in East and Lee (1982). ATPase activities were measured at 25 °C in 40 mM Hepes/KOH, pH 7.2, 100 mM KCl, 5 mM $MgSO_4$, and 2.1 mM ATP and maximally stimulating Ca^{2+} as described in Starling et al. (1993). Reconstitutions were performed 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 $MgSO_4$, 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 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 studies with sterols, sterols were added to solutions of the phospholipid in chloroform/methanol to the required molar ratio, dried, and then dispersed in cholate as described.

Binding of $^{45}Ca^{2+}$ to the ATPase was measured using the double-labeling method described in Starling et al. (1993). 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 was quenched after incubation at 25 °C for 10 s, 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 was then collected by filtration through Whatman GF/C glass fiber filters. Each filter was washed three times with 15 mL of cold 25% trichloroacetic acid in 0.13 M potassium phosphate and was finally counted in OptiPhase HighSafe 3 .

Fluorescence measurements 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. Fluorescence polarizations of DPH were measured at a molar ratio of DPH to phosphatidylcholine of 1:100, with excitation and emission wavelengths of 348 and 426 nm, respectively, and corrected for instrument polarization.

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 pure non-brominated 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 (East & Lee, 1982). 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 (Simmonds et al., 1982).

Synthesis of Cholesterol-Containing Phospholipids. 1-Oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine ((C18:1,CHS)PC) was synthesized by condensation of cholesteryl hemisuccinate with lysophosphatidylcholine. 1-Oleoyl-2-hydroxyphosphatidylcholine (38 mg, 73 μmol) and cholesteryl hemisuccinate (100 mg, 205 μmol) were dissolved in chloroform (10 mL) purified by passage through a column packed with activated, basic aluminium oxide powder. 1,3-Dicyclohexylcarbodiimide (150 mg, 726 μmol) and 4-(dimethylamino)pyridine (45 mg, 370 μmol) were added. The mixture was stirred at room temperature for 48 h under dry nitrogen in the dark, and the reaction was then stopped by addition of methanol (10 mL). After evaporation of the chloroform and methanol, the solid residuals were redissolved in chloroform and the insoluable dicyclohexylurea was filtered off. The crude product was purified by flash column chromotography: a column (22 mm i.d.) was packed with 32 g of silica gel (E. Merck; particle size, 0.040–0.063 mm), and the eluant used was chloroform/methanol/concentrated ammonium/water, 5:3:0.3:0.15 (v/v). Pure (C18:1,CHS)PC (62 mg) was obtained, running as a single spot on TLC with $R_f \sim 0.3$, with a yield of 86% based on the lysophosphatidylcholine. The product was characterized by mass spectroscopy and NMR to confirm the assigned chemical structure. 1-Myristoleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine (C14:1,CHS)-PC was prepared in the same way from 1 myristoleoyl –2-hydroxy-phosphatidylcholine.

Synthesis of Brominated Phospholipids. Dioleoylphosphatidylcholine (di(C18:1)PC) and 1-oleoyl-2-hydroxyphosphatidylcholine were brominated to give 1,2-bis(9,10-dibromostearoyl)phosphatidylcholine (di(Br_2C18:0)PC)) and 1-(9,10-dibromostearoyl)-2-hydroxyphosphatidylcholine, respectively, by the method described in East and Lee (1982). Cholesteryl hemisuccinate was brominated to give 5,6-dibromocholestan-3β-ol hemisuccinate (Br_2CHS) as described

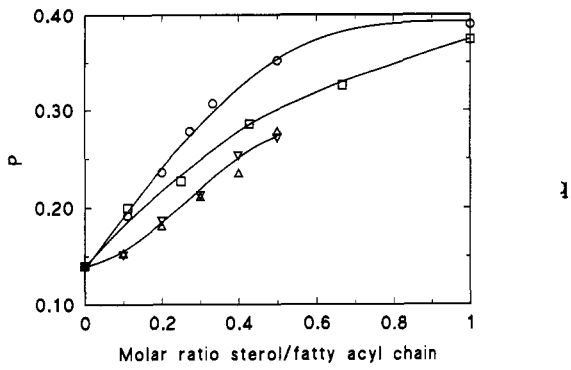

FIGURE 2: Fluorescence polarization of DPH in di(C18:1)PC at 25 °C and the effects of (C16:0,CHS)PC (○), (C18:1,CHS)PC (□), cholesterol (▽), and CHS (△) at the given molar ratios of sterol to fatty acyl chain.

Table 1: ATPase Activities at 25 °C for Reconstituted Ca^{2+}-ATPase[a]

lipid	activity [IU/(mg of protein)]	[EP][b] [nmol/(mg of protein)]
di(C14:1)PC	0.4	3.5
di(C18:1)PC	4.3	3.5
(C14:1,CHS)PC	0.3	3.3
(C18:1,CHS)PC	1.0	3.3
(C16:0,CHS)PC	0.04	3.3

[a] Measured at pH 7.2, 100 mM KCl, 2.1 mM ATP, and 10 μM free Ca^{2+}. [b] Maximal level of phosphorylation observed with 100 μM [γ-^{32}P]ATP in the presence of 1 mM Ca^{2+}.

by Simmonds et al. (1984). Brominated sterol-containing phospholipids were then synthesized as described above.

RESULTS

Cholesteryl hemisuccinate (CHS) can be linked to lysophosphatidylcholines using 1,3-dicyclohexylcarbodiimide to produce cholesterol-containing phosphatidylcholines of the general structure shown in Figure 1, with myristoleic ((C14:1,CHS)PC), palmitic ((C16:0,CHS)PC), or oleic acid ((C18:1,CHS)PC) as the fatty acyl chain.

Figure 2 compares the effects of the cholesterol-containing phosphatidylcholines on the fluorescence polarization of diphenylhexatriene (DPH) in mixtures with di(C18:1)PC with the effects of cholesterol and cholesteryl hemisuccinate. For ease of comparison the data is expressed in terms of the molar ratio of sterol to fatty acyl chain so that, for example, pure (C16:0,CHS)PC corresponds to a molar ratio of sterol to fatty acyl chain of 1. The cholesterol-containing phosphatidylcholines have a somewhat greater effect on the fluorescence polarization of DPH than cholesterol.

Effects on the Function of the Ca^{2+}-ATPase. As shown in Table 1, ATPase activity for the ATPase reconstituted with (C14:1,CHS)PC is comparable to that observed in di(C14:1)PC, whereas that observed in (C18:1,CHS)PC is much less than in di(C18:1)PC. Only very low levels of activity are observed in (C16:0,CHS)PC. As shown in Table 1, maximal levels of phosphorylation observed with [γ-^{32}P]ATP are the same in all reconstituted systems, and they are the same as observed for sarcoplasmic reticulum (Starling et al., 1993).

Figure 3A shows that the activity of the ATPase reconstituted in mixtures of di(C18:1)PC and (C18:1,CHS)PC decreases smoothly with increasing proportions of (C18:1,CHS)PC. In contrast, as shown in Figure 4, in mixtures of (C18:1,CHS)PC with either di(C14:1)PC or (C14:1,CHS)-

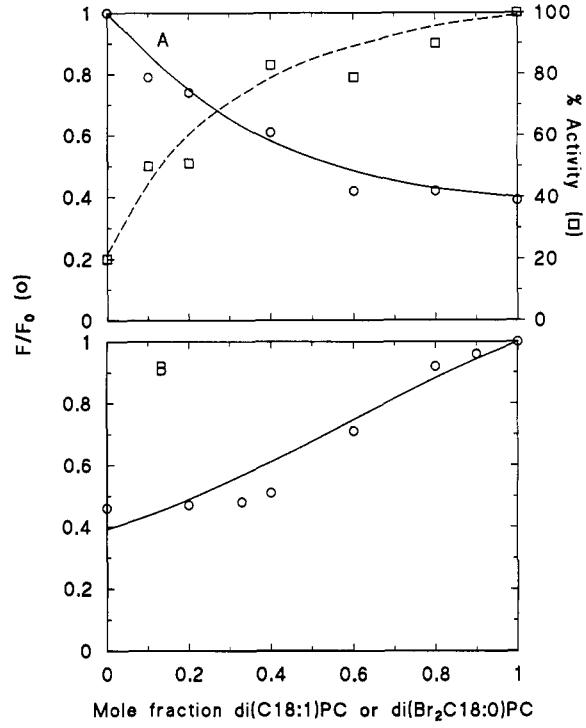

FIGURE 3: Effects of cholesterol-containing phosphatidylcholines on the fluorescence intensity and ATPase activity of the reconstituted ATPase at 25 °C. (A) Effects of (C18:1,CHS)PC on the fluorescence intensity (○) and activity (□) of the ATPase reconstituted with di(Br_2C18:0)PC. (B) Effects of (Br_2C18:0,Br_2CHS)PC on the fluorescence intensity (○) of the ATPase reconstituted with di(C18:1)PC. Concentrations are expressed as mole fractions of di(Br_2C18:0)PC (A) or di(C18:1)PC (B). The solid lines show simulations (eq 2) with $K = 0.6$.

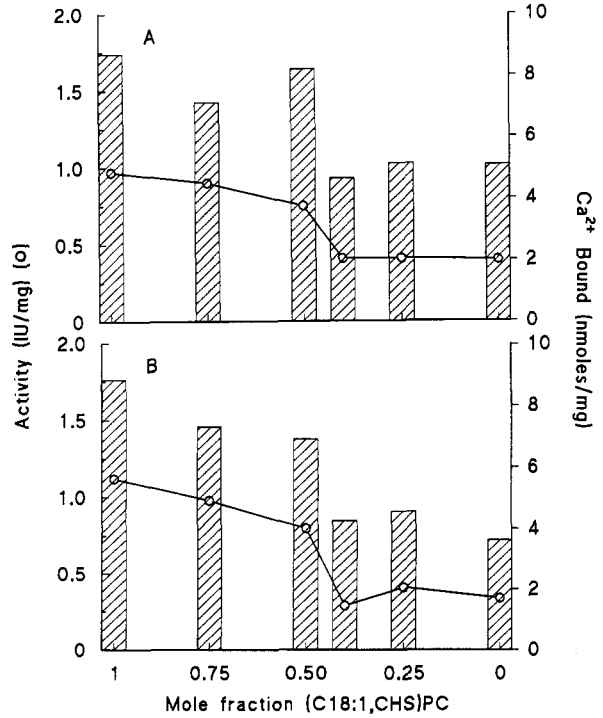

FIGURE 4: Effects on the Ca^{2+}-ATPase of mixtures of (C18:1,CHS)-PC with (A) di(C14:1)PC and (B) (C14:1,CHS)PC at the given mole fractions of (C18:1,CHS)PC; (○) ATPase activities measured at 25 °C. Hatched bars, Ca^{2+} bound (nanomoles per milligram of protein).

PC activity changes little with increasing concentrations of the short-chain lipid until the mole fraction of (C18:1,CHS)-

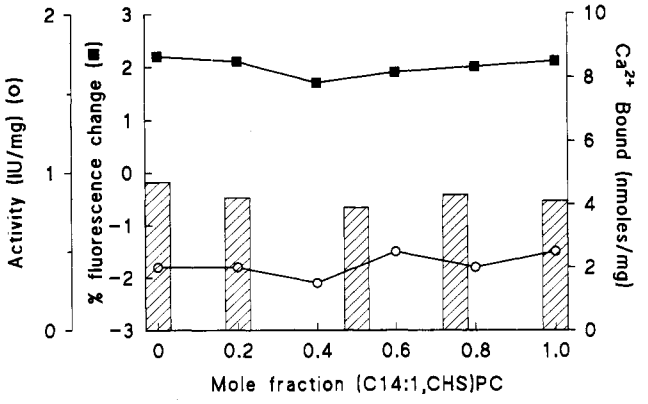

FIGURE 5: Effects on the Ca^{2+}-ATPase of mixtures of (C14:1,CHS)-PC and di(C14:1)PC at the given mole fractions of (C14:1,CHS)-PC. Symbols: (O) ATPase activities measured at 25 °C; (■) % change in tryptophan fluorescence intensity on addition of EGTA to the ATPase initially in the presence of Ca^{2+}, in 5 mM Mg^{2+}, pH 7.2; hatched bars, Ca^{2+} bound (nanomoles per milligram of protein).

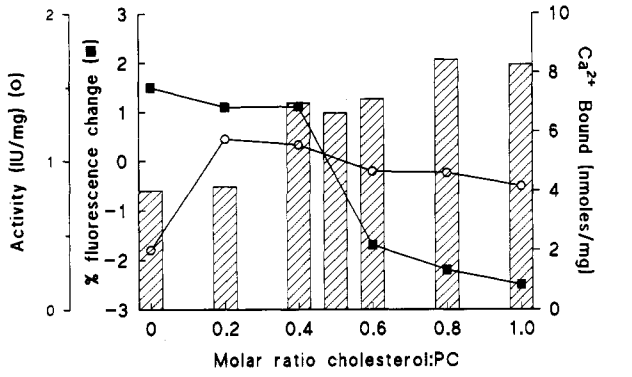

FIGURE 6: Effects on the Ca^{2+}-ATPase of mixtures of cholesterol and (C14:1,CHS)PC at the given molar ratios of cholesterol. Symbols: (O) ATPase activities measured at 25 °C; (■) % change in tryptophan fluorescence intensity on addition of EGTA to the ATPase initially in the presence of Ca^{2+}, in 5 mM Mg^{2+}, pH 7.2; hatched bars, Ca^{2+} bound (nanomoles per milligram of protein).

PC drops below 0.5, when a marked decrease in activity is observed. Figure 5 shows that ATPase activities are fairly constant in all mixtures of di(C14:1)PC and (C14:1,CHS)-PC. However, Figure 6 shows that addition of cholesterol to the ATPase reconstituted with (C14:1,CHS)PC results in a significant increase in ATPase activity.

The levels of Ca^{2+} binding to the ATPase reconstituted with (C18:1,CHS)PC and (C14:1,CHS)PC are ca. 8 and 4 nmol/(mg of protein), respectively (Figures 4 and 5) which, combined with the maximal levels of phosphorylation of ca. 3.5 nmol of [EP]/(mg of protein) (Table 1), indicates a Ca^{2+} binding stoichiometry of two Ca^{2+} ions bound per ATPase molecule in (C18:1,CHS)PC and one Ca^{2+} ion bound per ATPase molecule in (C14:1,CHS)PC. As shown in Figure 5, and as reported previously (Michelangeli et al., 1990b; Starling et al., 1993), the Ca^{2+} binding stoichiometry is also one Ca^{2+} ion bound per ATPase molecule in di(C14:1)PC and remains at one in mixtures of (C14:1,CHS)PC and di(C14:1)PC. In mixtures of (C14:1,CHS)PC and (C18:1,CHS)PC the stoichiometry of Ca^{2+} binding changes sharply from two Ca^{2+} ions bound per ATPase molecule for mixtures containing 50% or more (C18:1,CHS)PC to one Ca^{2+} ion bound per ATPase molecule for mixtures containing 40% or less (C18:1,CHS)PC (Figure 4). A very similar result is obtained in mixtures of di(C14:1)PC and (C18:1,CHS)PC (Figure 4). As shown in Figure 6, addition of cholesterol to the ATPase in (C14:1,CHS)PC increases the stoichiometry of Ca^{2+} binding from one Ca^{2+} ion bound per ATPase molecule to two Ca^{2+} ions bound.

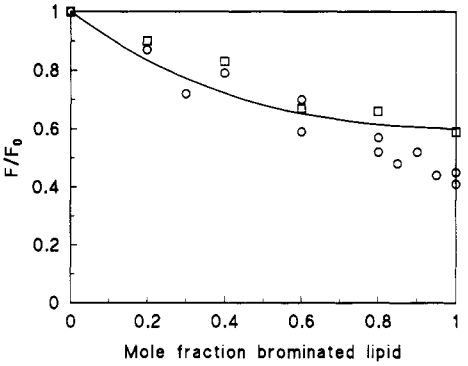

FIGURE 7: Effects of (C18:1,Br_2CHS)PC (O) and (Br_2C18:0,CHS)-PC (□) on the fluorescence intensity of the ATPase at 25 °C reconstituted with di(C18:1)PC at the given mole fractions of the cholesterol-containing phosphatidylcholine. The solid line shows a simulation (eq 2) with $K = 0.6$, assuming a maximum quenching of 40%.

Binding of Ca^{2+} to the ATPase can also be followed from changes in the tryptophan fluorescence of the ATPase (Dupont et al., 1988). Removal of Ca^{2+} from the native ATPase by addition of EGTA results in a decrease in fluorescence intensity, but removal of Ca^{2+} from the ATPase reconstituted with di(C14:1)PC results in an increase in intensity (Michelangeli et al., 1990b). As shown in Figure 6, removal of Ca^{2+} from the ATPase reconstituted with (C14:1,CHS)PC also results in an increase in fluorescence intensity, but on addition of cholesterol the fluorescence change reverts to being negative. As shown in Figure 5, the fluorescence change is constant and positive in mixtures of di(C14:1)PC and (C14:1,CHS)PC.

Effects of Bromine-Containing Phosphatidylcholines on the Fluorescence of Ca^{2+}-ATPase. Interaction of the cholesterol-containing phosphatidylcholines with the ATPase has been studied by comparing the intensities of tryptophan fluorescence for the ATPase reconstituted into bilayers of di(C18:1)PC, di(Br_2C18:0)PC, and mixtures with the cholesterol-containing phosphatidylcholine and its brominated analogues. Figure 3 shows that, as reported previously, the fluorescence intensity for the ATPase in di(Br_2C18:0)PC is ca. 40% of that in di(C18:1)PC. Reconstitution of the ATPase with mixtures of (C18:1,CHS)PC and di(Br_2C18:0)PC results in higher fluorescence intensities than in di(Br_2C18:0)PC alone (Figure 3A), indicating that (C18:1,CHS)PC can bind at the lipid–protein interface, displacing di(Br_2C18:0)PC. Correspondingly, as shown in Figure 3B, (Br_2C18:0,Br_2CHS)PC can quench the fluorescence of the ATPase reconstituted with di(C18:1)PC. The data fits to eq 2, with a value for K of 0.6.

Figure 7 shows fluorescence quenching in mixtures of di(C18:1)PC and (C18:1,CHS)PC brominated in either the fatty acyl chain or the cholesterol ring. As shown, the data fits to Eq 2 again with a K value of 0.6, but assuming a smaller maximal quenching of 40%.

DISCUSSION

We have suggested two classes of binding site on the Ca^{2+}-ATPase for hydrophobic molecules such as sterols, fatty acids, and alcohols, one at the lipid–protein interface (annular sites) and one at other sites on the ATPase (nonannular sites), possibly between transmembrane α-helices (Simmonds et al., 1982, 1984; Lee et al., 1983, 1991; Jones & Lee, 1985; Froud et al., 1986a; Rooney et al., 1987; Michelangeli et al., 1990a).

This model developed largely from studies of the interactions of sterols with the ATPase (Simmonds et al., 1982). Strength of binding at the lipid–protein interface was determined from measurements of the ability of a sterol to displace the bromine-containing phospholipid di(Br_2C18:0)PC from the surface of the ATPase; the fraction of sites at the lipid–protein interface occupied by di(Br_2C18:0)PC was determined from the extent of quenching of the tryptophan fluorescence of the ATPase by the bromine atoms. It was found that cholesterol bound only very weakly at the lipid–protein interface (Simmonds et al., 1982), whereas cholesteryl hemisuccinate bound more strongly, with a binding constant one-half that of a phospholipid (Simmonds et al., 1984). Although cholesterol bound only very weakly to annular sites, 5,6-dibromocholestan-3β-ol was found to be a very efficient quencher of the tryptophan fluorescence of the ATPase, suggesting that it must be able to bind elsewhere on the ATPase (Simmonds et al., 1982).

The existence of binding sites for cholesterol on the ATPase other than at the lipid–protein interface was also suggested by activity studies. Addition of cholesterol up to a 1:1 molar ratio with phospholipid had no effect on the activity of the ATPase reconstituted with di(C18:1)PC (Simmonds et al., 1982), but when the ATPase was first depleted of phospholipids and then reconstituted with cholesterol, a very low activity was observed (Warren et al., 1975). These studies together suggest that, in the presence of phospholipid, cholesterol is largely excluded from the lipid–protein interface, but that, in the absence of phospholipid, cholesterol can bind at the interface, this resulting in an ATPase with very low activity. In contrast, addition of cholesterol to the ATPase reconstituted with the short-chain phospholipid di(C14:1)PC resulted in a marked increase in activity (Simmonds et al., 1982; Starling et al., 1993), this increase paralleling a change in the stoichiometry of Ca^{2+} binding from one Ca^{2+} ion bound per ATPase molecule in di(C14:1)PC to two Ca^{2+} ions bound at high molar ratios of cholesterol (Starling et al., 1993).

The effects of cholesterol on the function of the ATPase reconstituted in di(C14:1)PC could, in principle, follow indirectly from changes in some property of the lipid phase (e.g., fluidity or thickness) or could follow directly from binding to nonannular sites on the ATPase. To distinguish between these possibilities, we have synthesized a series of phosphatidylcholines containing one acyl chain and one cholesterol moiety (Figure 1). Figure 2 shows that incorporation of the cholesterol-containing phosphatidylcholines into di(C18:1)-PC increases the fluorescence polarization of diphenylhexatriene, the effect being slightly more marked than for cholesterol or cholesteryl hemisuccinate when the concentration of cholesterol-containing phosphatidylcholine is expressed as the molar ratio of sterol to fatty acyl chain.

As shown in Figure 3, cholesterol-containing phospholipids are able to bind at the lipid–protein interface of the ATPase. Thus the extent of fluorescence quenching observed in mixtures of di(Br_2C18:0)PC and (C18:1,CHS)PC decreases with increasing mole fraction of (C18:1,CHS)PC, and (Br_2C18:0,Br_2CHS)PC is able to quench the fluorescence of the ATPase in di(C18:1)PC. The fluorescence quenching curves fit to eq 2 with a binding constant for the cholesterol-containing phosphatidylcholine relative to di(C18:1)PC of 0.6. Thus the annular binding constant for (C18:1,CHS)PC is about one-half that for di(C18:1)PC and is very similar to that for cholesteryl hemisuccinate (Simmonds et al., 1984). Phospholipids are normally miscible in the liquid crystalline phase (Lee, 1977), but we have not determined whether or not this is also true for the cholesterol-containing phospholipids. However, the fluorescence quenching results shown in Figure 3 indicate that the distribution of phospholipids around the ATPase is very similar to the average phospholipid composition of the membrane. In a membrane where it has been established that phospholipids are present in both liquid crystalline and gel phases, the ATPase has been shown to partition preferentially into the regions of liquid crystalline lipid (East & Lee, 1982). Very similar quenching profiles were obtained with the lipids (Br_2C18:0,CHS)PC and (C18:1,Br_2CHS) brominated on the fatty acyl chain and on cholesterol, respectively (Figure 7), suggesting little selectivity in which "face" of the cholesterol-containing phospholipid binds to the ATPase.

In its effects on the function of the Ca^{2+}-ATPase, (C14:1,CHS)PC resembles di(C14:1)PC and not cholesterol. Thus the ATPase activity of the Ca^{2+}-ATPase in (C14:1,CHS)PC is comparable to that in di(C14:1)PC (Table 1), and addition of (C14:1,CHS)PC to the ATPase in di(C14:1)PC has no significant effect on activity (Figure 5), whereas addition of cholesterol to the ATPase in di(C14:1)PC results in a large increase in activity (Starling et al., 1993). The stoichiometry of Ca^{2+} binding to the ATPase in (C14:1,CHS)PC is one Ca^{2+} ion bound per ATPase molecule, as it is in di(C14:1)PC (Figure 5), and whereas addition of cholesterol to the ATPase in di(C14:1)PC increases the stoichiometry of Ca^{2+} binding to two Ca^{2+} ions bound per ATPase molecule (Starling et al., 1993), addition of (C14:1,CHS)PC has no effect on the stoichiometry of Ca^{2+} binding (Figure 5). However, addition of cholesterol to the ATPase in (C14:1,CHS)PC has the same effect as addition of cholesterol to the ATPase in di(C14:1)PC: the stoichiometry of Ca^{2+} binding increases to two Ca^{2+} ions bound per ATPase molecule, and the ATPase activity increases (Figure 6). (C14:1,CHS)PC also behaves in an analogous manner to di(C14:1)PC in mixtures with (C18:1,CHS)PC. As shown in Figure 4, the stoichiometry of Ca^{2+} binding is two Ca^{2+} ions bound per ATPase molecule in (C18:1,CHS)PC. This stoichiometry is unchanged in mixtures with di(C14:1)PC or (C14:1,CHS)PC down to a mole fraction of (C18:1,CHS)PC of 0.5, beyond which the stoichiometry changes to one Ca^{2+} ion bound per ATPase molecule.

The parallel between di(C14:1)PC and (C14:1,CHS)PC is also seen in their effects on the response of the tryptophan fluorescence of the ATPase to Ca^{2+}. Removal of Ca^{2+} from the ATPase in the native membrane by addition of EGTA results in a decrease in fluorescence intensity, whereas for the ATPase reconstituted with di(C14:1)PC removal of Ca^{2+} in the presence of Mg^{2+} results in an increase in fluorescence intensity (Michelangeli et al., 1990b; Starling et al., 1993). Addition of sterols to the ATPase reconstituted in di(C14:1)PC causes a reversal of this effect, with a decrease in fluorescence intensity on removal of Ca^{2+} being observed at molar ratios of sterol above ca. 0.5, at which concentration the stoichiometry of Ca^{2+} binding changes from one Ca^{2+} ion bound per ATPase molecule to the normal two Ca^{2+} ions bound per ATPase molecule (Starling et al., 1993). As shown in Figure 5, addition of EGTA to the Ca^{2+}-bound ATPase results in an increase in fluorescence intensity in (C14:1,CHS)-PC, with the magnitude and sign of the change being unaltered in mixtures of (C14:1,CHS)PC and di(C14:1)PC. However, as shown in Figure 6, addition of cholesterol to the ATPase in (C14:1,CHS)PC reverses the sign of the fluorescence change at molar ratios of cholesterol above 0.4, this again being the point at which the stoichiometry of Ca^{2+} binding changes.

These studies show therefore that cholesterol anchored to the glycerol backbone of a phosphatidylcholine molecule is

unable to produce the same changes in function for the ATPase in di(C14:1)PC as observed with free cholesterol. However, cholesterol has the same effect when added to the ATPase reconstituted with either (C14:1,CHS)PC or di(C14:1)PC. Since cholesterol and the cholesterol-containing phospholipids all increase the fluorescence polarization of diphenylhexatriene incorporated into bilayers of di(C18:1)PC, changes in fatty acyl chain order as monitored by fluorescence polarization cannot be involved in the changes in the function of the ATPase seen on addition of cholesterol. Effects of cholesterol on bilayer thickness are also unlikely to be involved, since we would expect very similar bilayer thicknesses in bilayers of (C14:1,CHS)PC and of mixtures of di(C14:1)PC plus cholesterol. We conclude that cholesterol can bind to the ATPase at sites not accessible to phospholipids, and that the observed effects of cholesterol on the function of the ATPase follow from binding to these (nonannular) sites and not from changes in the phospholipid bilayer component of the membrane.

The nature of the nonannular binding site(s) for cholesterol on the ATPase remains to be determined. It is likely that the sites are highly hydrophobic and probably involve hydrogen bonding to the cholesterol -OH moiety. Cholesterol has been suggested to bind to nonannular sites on the nicotinic acetylcholine receptor (Jones & McNamee, 1988). Since it has been shown that local anesthetics compete for binding with spin-labeled analogues of cholesterol, it is possible that both bind to the same site on the receptor, probably involving clusters of Ser residues in the M2 transmembrane helix (Taylor et al., 1991). In the 10-helix model for the ATPase (MacLennan et al., 1985; Mata et al., 1992) single Ser residues occur in helices M1, M3, and M10, but helix M8 contains a pair of Ser residues at positions 902 and 915, and helix M9 contains three, at positions 936, 940, and 942. Helices M5 and M7 contain Tyr residues, in helix M5 in the sequence Tyr^{763}-Leu-Ile-Ser-Ser, and in helix M7 at positions 837 and 843. A number of potential binding sites therefore exist in the transmembrane region of the ATPase.

REFERENCES

Caffrey, M., & Feigenson, G. W. (1981) *Biochemistry 20*, 1949–1961.

Dupont, Y., Guillain, F., & Lacapere, J. J. (1988) *Methods Enzymol. 157*, 206–219.

East, J. M., & Lee, A. G. (1982) *Biochemistry 21*, 4144–4151.

East, J. M., Melville, D., & Lee, A. G. (1985) *Biochemistry 24*, 2615–2623.

Froud, R. J., East, J. M., Jones, O. T., & Lee, A. G. (1986a) *Biochemistry 25*, 7544–7552.

Froud, R. J., East, J. M., Rooney, E. K., & Lee, A. G. (1986b) *Biochemistry 25*, 7535–7544.

Johannsson, A., Smith, G. A., & Metcalfe, J. C. (1981) *Biochim. Biophys. Acta 641*, 416–421.

Jones, O. T., & Lee, A. G. (1985) *Biochemistry 24*, 2195–2202.

Jones, O. T., & McNamee, M. G. (1988) *Biochemistry 27*, 2364–2374.

Lee, A. G. (1977) *Biochim. Biophys. Acta 472*, 237–281.

Lee, A. G., East, J. M., Jones, O. T., McWhirter, J., Rooney, E. K., & Simmonds, A. C. (1983) *Biochim. Biophys. Acta 732*, 441–454.

Lee, A. G., East, J. M., & Balgavy, P. (1991) *Pestic. Sci. 32*, 317–327.

MacLennan, D. H., Brandl, C. J., Korczak, B., & Green, N. M. (1985) *Nature 316*, 696–700.

Mata, A. M., Matthews, I., Tunwell, R. E. A., Sharma, R. P., Lee, A. G., & East, J. M. (1992) *Biochem. J. 286*, 567–580.

Michelangeli, F., East, J. M., & Lee, A. G. (1990a) *Biochim. Biophys. Acta 1025*, 99–108.

Michelangeli, F., Orlowski, S., Champeil, P., Grimes, E. A., East, J. M., & Lee, A. G. (1990b) *Biochemistry 29*, 8307–8312.

Rooney, E. K., Gore, M. G., & Lee, A. G. (1987) *Biochemistry 26*, 3688–3697.

Simmonds, A. C., East, J. M., Jones, O. T., Rooney, E. K., McWhirter, J., & Lee, A. G. (1982) *Biochim. Biophys. Acta 693*, 398–406.

Simmonds, A. C., Rooney, E. K., & Lee, A. G. (1984) *Biochemistry 23*, 1432–1441.

Starling, A. P., East, J. M., & Lee, A. G. (1993) *Biochemistry 32*, 1593–1600.

Taylor, P., Abramson, S. N., Johnson, D. A., Valenzuela, C. F., & Herz, J. (1991) *Ann. N. Y. Acad. Sci. 625*, 568–587.

Warren, G. B., Houslay, M. D., Metcalfe, J. C., & Birdsall, N. J. M. (1975) *Nature 255*, 684–687.