Effects of Phosphatidylcholine Fatty Acyl Chain Length on Calcium Binding and Other Functions of the (Ca^{2+}–Mg^{2+})-ATPase†

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ABSTRACT: The ATPase activity of the (Ca^{2+}–Mg^{2+})-ATPase purified from skeletal muscle sarcoplasmic reticulum and reconstituted into phosphatidylcholine bilayers of defined composition depends on the fatty acyl chain length of the surrounding phospholipid. The stoichiometry of Ca^{2+} binding to the ATPase is also sensitive to fatty acyl chain length, changing from the normal two Ca^{2+} ions bound per ATPase molecule to one Ca^{2+} ion bound for the ATPase reconstituted with phosphatidylcholines of chain lengths C12, C14, or C24. For the ATPase reconstituted with mixtures of phosphatidylcholines where one phosphatidylcholine supports a Ca^{2+} binding stoichiometry of two and the other a stoichiometry of one, a highly cooperative change in binding stoichiometry with change in phospholipid composition is observed, suggesting that the effects of phospholipids follow from binding to a large number of sites at the lipid–protein interface of the ATPase. For the ATPase reconstituted with either 1-myristoyl-2-oleoylphosphatidylcholine or 1-oleoyl-2-myristoylphosphatidylcholine, the stoichiometry of Ca^{2+} binding is the normal two per ATPase molecule. Effects of short-chain phosphatidylcholines on Ca^{2+} binding stoichiometry and on ATPase activity can be reversed by addition of androstenol, oleic acid, methyl oleate, or oleyl alcohol but these molecules have no effect on the ATPase reconstituted with dinervonylphosphatidylcholine (C24:1). For the ATPase reconstituted with phosphatidylcholines with chain lengths between C16 and C22, release of the two bound Ca^{2+} ions is sequential, with release of the second Ca^{2+} being inhibited by high concentrations of Ca^{2+} in the bathing medium. For the ATPase reconstituted with phosphatidylcholines of chain lengths C14 or C24, release of the single bound Ca^{2+} is only slightly inhibited by the presence of Ca^{2+} in the medium. For the ATPase reconstituted with phosphatidylcholines of chain lengths between C16 and C24, removal of bound Ca^{2+} results in a decrease in tryptophan fluorescence intensity, whereas for the ATPase reconstituted with phosphatidylcholines of chain lengths C12 or C14, removal of bound Ca^{2+} results in an increase in tryptophan fluorescence intensity. In mixtures of phosphatidylcholines, changes in the tryptophan response mirror changes in Ca^{2+} binding stoichiometry.

The (Ca^{2+}–Mg^{2+})-ATPase of skeletal muscle sarcoplasmic reticulum (SR)[1] translocates two Ca^{2+} ions per molecule of ATP hydrolyzed (Hasselbach & Makinose, 1961). An essential step in the mechanism of the ATPase is the binding of the two Ca^{2+} ions to sites of high affinity on the cytoplasmic side of the membrane, a step which alters the conformation of the ATPase from one in which it can be phosphorylated by P_i to one in which it can be phosphorylated by MgATP (de Meis, 1981). Site-directed mutagenesis has indicated that the Ca^{2+} binding sites are located in membrane-spanning regions of the ATPase (Clarke et al., 1989), a result consistent with experiments in which the ATPase has been modified with fluorescent carbodiimides (Pick & Racker, 1979; Chadwick & Thomas, 1983; Munkonge et al., 1989; Sumbilla et al., 1991; Mata et al., 1993).

The relationship between the two Ca^{2+} binding sites on the ATPase is still unclear. Binding of Ca^{2+} exhibits positive cooperativity. This could follow from sequential binding to two sites with differing intrinsic affinities for Ca^{2+} or from a conformational change on the ATPase either before the first Ca^{2+} ion can bind (as in the E2–E1 model, where E2 and E1 represent conformations with low and high affinities for Ca^{2+} respectively) or following the binding of the first Ca^{2+} ion with the creation of a second Ca^{2+} binding site (as in the reaction sequence $E1 \rightarrow E1Ca \rightarrow E1'Ca \rightarrow E1'Ca_2$). The observation of biphasic kinetics for the dissociation of Ca^{2+} from the ATPase in the presence of external Ca^{2+} is consistent with a structure in which the first Ca^{2+} that binds to the ATPase binds deeply in a crevice or channel with the second Ca^{2+} ion binding more peripherally and preventing access of the first Ca^{2+} to the external medium (Dupont, 1982; Nakamura, 1986; Moutin & Dupont, 1991; Orlowski & Champeil, 1991).

Whatever the exact relationship between the two Ca^{2+} binding sites, for the ATPase in the SR membrane it is necessary for two Ca^{2+} ions to bind before the ATPase can be phosphorylated by ATP (de Meis, 1981; Petithory & Jencks, 1988; Coan & DiCarlo, 1990). We have found, however, that the stoichiometry of Ca^{2+} binding and the dependence of phosphorylation on Ca^{2+} binding varies with the structure of the phospholipids surrounding the ATPase in the membrane. The phospholipid optimal for ATPase activity is dioleoylphosphatidylcholine (di(C18:1)PC), and phospholipids with longer or shorter fatty acyl chains or different head groups support lower activity (Caffrey & Feigenson, 1981; East & Lee, 1982; Froud et al., 1986a; Lee, 1988, 1991). For the ATPase reconstituted with di(C18:1)PC, the stoichiometry of Ca^{2+}

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[1] Abbreviations: di(C12:0)PC, dilauroylphosphatidylcholine; di(C14:1)PC, dimyristoleoylphosphatidylcholine; di(C16:1)PC, dipalmitoleoylphosphatidylcholine; di(C18:1)PC, dioleoylphosphatidylcholine; di(C20:1)PC, dieicosenoylphosphatidylcholine; di(C22:1)PC, dierucoylphosphatidylcholine; di(C24:1)PC, dinervonylphosphatidylcholine; (C14:0,C18:1)PC, 1-myristoyl-2-oleoylphosphatidylcholine; (C18:1,-C14:0)PC, 1-oleoyl-2-myristoylphosphatidylcholine; SR, sarcoplasmic reticulum.

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binding is two Ca^{2+} ions bound per ATPase molecule, as for the ATPase in the native membrane, but on reconstitution with di(C14:1)PC, the stoichiometry changes to one Ca^{2+} ion bound per ATPase molecule (Michelangeli et al., 1990b). Despite the changed stoichiometry, the ATPase reconstituted with di(C14:1)PC can still be phosphorylated by ATP in the presence of Ca^{2+}, albeit at a slower rate, and will still hydrolyze ATP, although again at a slower rate than normal (Michelangeli et al., 1991).

Here we investigate the effects of a wider range of phospholipids on the stoichiometry of Ca^{2+} binding to the ATPase and study effects of mixtures of phospholipids.

MATERIALS AND METHODS

Phospholipids were obtained from Avanti Polar Lipids, androstenol was from Steraloids, and oleyl alcohol, methyl oleate, and oleic acid were from Aldrich. The (Ca^{2+}–Mg^{2+})-ATPase was purified from skeletal muscle sarcoplasmic reticulum as described in East and Lee (1982). Reconstitutions were performed largely as described in Michelangeli et al. (1990b). Typically, phospholipid (10 μmol) was mixed with buffer (400 μL; 10 mM Hepes/Tris and 15% sucrose, pH 8.0) containing $MgSO_4$ (5 mM) and potassium cholate (12 mg/mL) and sonicated to clarity in a bath sonicator (Megason). ATPase (1.25 mg) in a volume of 20–30 μL was then added and, for phospholipids of chain lengths C14–C18, 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 phospholipids with chain lengths C20–C24, samples were equilibrated for 1 h at room temperature, and for di(C12:0)PC samples were equilibrated for 45 min on ice. For studies of the effects of mixtures of two phospholipids, sonicated dispersions of the two phospholipids were prepared in cholate and then mixed in the appropriate proportions before addition of the ATPase and reconstitution as described above. For experiments with androstenol, oleyl alcohol, methyl oleate, and oleic acid, these were added to the phospholipid prior to dispersion in the original cholate solution.

Binding of $^{45}Ca^{2+}$ to the ATPase was measured using the double labeling method described elsewhere (Michelangeli et al., 1990b). The ATPase was added to buffer (2 mL; 20 mM Hepes/Tris, pH 7.2, 100 mM KCl, and 5 mM $MgSO_4$) containing 100 μM $^{45}Ca^{2+}$ and 500 μM [^{3}H]sucrose to give a final protein concentration of 200 μg/mL. Samples (0.5 mL) were filtered through a Millipore HAWP filter (0.45 μm), dried overnight in air, and counted in OptiPhase HiSafe 3. The amount of [^{3}H]sucrose trapped on the filter was used to calculate the wetting volume of the filter, and the amount of $^{45}Ca^{2+}$ calculated to be in this volume was subtracted from the total $^{45}Ca^{2+}$ on the filter to give that bound to the ATPase. A correction was also applied for $^{45}Ca^{2+}$ nonspecifically bound to the filter, typically amounting to 1 nmol of Ca^{2+} bound/mg of protein.

The time dependence of Ca^{2+} release from the ATPase was determined using a Biologic rapid filtration system, at room temperature (typically 20 °C). A suspension of the ATPase in buffer, 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, 100 mM KCl, and 5 mM $MgSO_4$) containing either EGTA (2 mM) or $^{40}Ca^{2+}$ (1 mM). The filter was then counted, and the $^{45}Ca^{2+}$ bound to the ATPase was calculated as described above.

If dissociation of Ca^{2+} from the ATPase can be described as the sequential process given by

Scheme I

$$E1Ca_2 \xrightarrow{k_1} E1Ca \xrightarrow{k_2} E1$$

then the rate of loss of Ca^{2+} is described by the equation

$$[Ca^{2+}]_B{}^0 = \frac{[Ca^{2+}]_B{}^t}{2(k_2 - k_1)}((2k_2 - k_1)\exp^{-k_1 t} - k_1 \exp^{-k_2 t}) \quad (1)$$

where $[Ca^{2+}]_B{}^0$ and $[Ca^{2+}]_B{}^t$ are the concentrations of bound Ca^{2+} at times zero and t, respectively. When the rate constant k_1 is ca. 15-fold or more greater than k_2, it can be shown that the rate of loss of bound Ca^{2+} is represented by the sum of two exponentials of equal amplitude.

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, 1 mM $CaCl_2$. The reaction was started by addition of 100 μM [γ-^{32}P]ATP and, after incubation at 25 °C 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 fiber filters. The filter was washed three times with 15 mL of cold 25% trichloroacetic acid in 0.13 M potassium phosphate and 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 $MgSO_4$, 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. ATPase activities were measured using a coupled enzyme assay. The 10-μL aliquots of the reconstitution mixture (equivalent to 30 μg of the ATPase) were diluted 250-fold into a medium containing 40 mM Hepes/KOH (pH 7.2), 100 mM KCl, 5 mM $MgSO_4$, 2.1 mM ATP, 1.01 mM EGTA, 0.41 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) in a total volume of 2.5 mL at 25 °C, with $CaCl_2$ added to give a maximally stimulating concentration of Ca^{2+} (free Ca^{2+} concentration typically 10 μM). 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).

RESULTS

The (Ca^{2+}–Mg^{2+})-ATPase can be reconstituted into phospholipid bilayers by mixing the purified ATPase with excess phospholipid in cholate solution followed by a 250-fold dilution to decrease the concentration of cholate below its critical micelle concentration. The procedure results in the formation of membrane fragments which are unable to accumulate Ca^{2+} and which thus show full (uncoupled) ATPase activity (Warren et al., 1974a,b). Figure 1 shows ATPase activities measured at 25 °C for the (Ca^{2+}–Mg^{2+})-ATPase reconstituted with a series of phosphatidylcholines containing mono-unsaturated fatty acyl chains of lengths between C14 and C24 and with di(C12:0)PC. Phase transition temperatures for all these phospholipids are below 25 °C (Lee, 1983) so that all will be in the liquid crystalline phase under the conditions of these experiments. The profile of activities, with low activities measured for the ATPase in di(C12:0)PC, di(C14:1)PC, and di(C24:1)PC, is very similar to that reported previously at 37

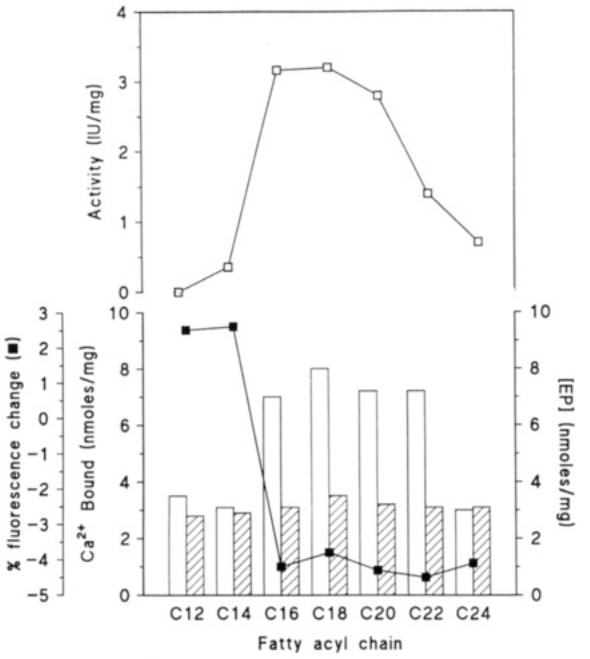

FIGURE 1: Effects of phosphatidylcholine fatty acyl chain length on the (Ca^{2+}–Mg^{2+})-ATPase. The ATPase was reconstituted with phosphatidylcholines of the given length, all being in the liquid crystalline phase. Symbols: □, ATPase activities measured at 25 °C; ■, % change in tryptophan fluorescence intensity on addition of EGTA to the ATPase initially in the presence of Ca^{2+}; hatched bars, maximal level of phosphorylation of the ATPase (nanomoles per milligram of protein) by ATP in the presence of 1 mM Ca^{2+}; open bars, Ca^{2+} bound (nanomoles per milligram of protein).

Table I: Effects of Mixed-Chain Phosphatidylcholines on the (Ca^{2+}–Mg^{2+})-ATPase

phospholipid	[EP][a] (nmol/mg of protein)	Ca^{2+} bound (nmol/mg of protein)
SR[b]	3.5	6.9
egg yolk phosphatidylcholine	3.2	5.7
(C18:1,C14:0)PC	3.3	6.1
(C14:0,C18:1)PC	3.2	6.6

[a] Maximal level of phosphorylation observed with 100 μM [γ-^{32}P]ATP in the presence of 1 mM Ca^{2+}, as described in the text. [b] Unreconstituted, purified ATPase.

°C (Caffrey & Feigenson, 1981; Froud et al., 1986a,b; Lee, 1991). Maximal levels of phosphorylation of the reconstituted ATPase were 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. As described elsewhere (Michelangeli et al., 1990a,b), and as observed by others [see, for example, Stahl and Jencks (1984) and Orlowski and Champeil (1991)], maximal levels of phosphorylation vary between preparations of ATPase. The maximal level of phosphorylation observed for the preparation used to obtain the data shown in Figure 1 was 3.5 nmol of [EP]/mg of protein (Table I), corresponding to 40% of that expected for a pure protein of molecular weight 115 000. As shown in Figure 1, the maximal level of phosphorylation of the ATPase is unaltered by reconstitution. In contrast, the stoichiometry of Ca^{2+} binding changes on reconstitution with di(C12:0)PC, di(C14:1)PC, or di(C24:1)PC from the normal two Ca^{2+} ions bound per molecule of phosphorylatable ATPase (Table I) to one Ca^{2+} ion bound. In previous experiments, we had observed the change in stoichiometry on reconstitution with di(C14:1)PC, but although we had observed reduced binding of Ca^{2+} on reconstitution with di(C24:1)PC compared

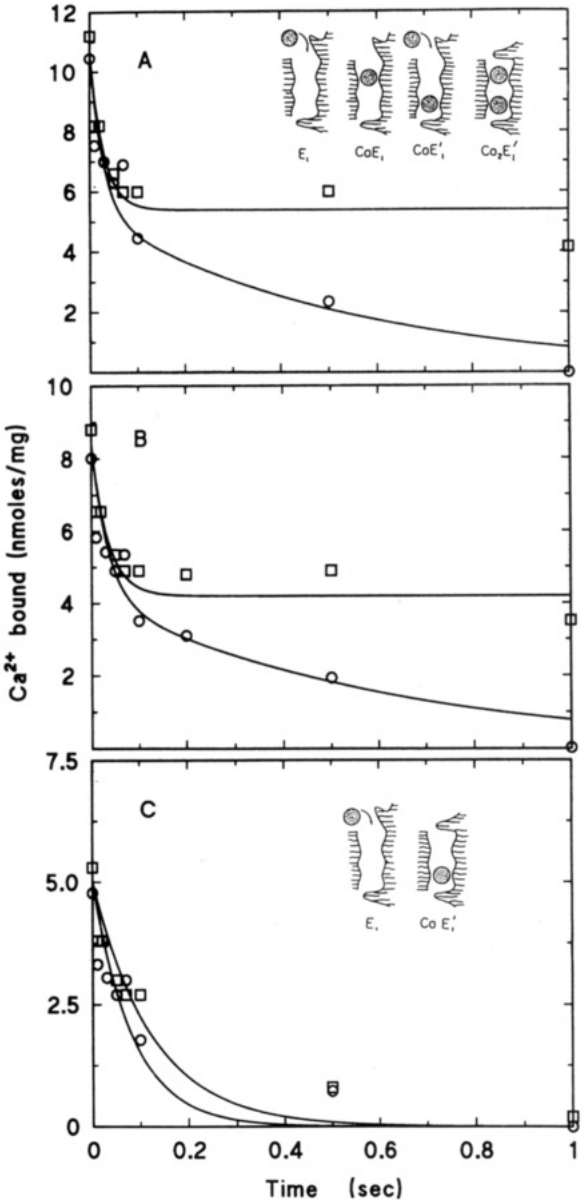

FIGURE 2: Rapid filtration measurement of the rate of Ca^{2+} dissociation from the ATPase reconstituted with (A) di(C20:1)PC, (B) di(C22:1)PC, and (C) di(C24:1)PC. The ATPase was incubated with $^{45}Ca^{2+}$ in a medium containing 100 μM $^{45}Ca^{2+}$, 5 mM Mg^{2+}, and 100 mM KCl, pH 7.2, 20 °C, and then adsorbed on a filter and perfused with buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl, 5 mM Mg^{2+}) containing either 2 mM EGTA (○) or 1 mM $^{40}Ca^{2+}$ (□). The level of $^{45}Ca^{2+}$ bound to the ATPase (nanomoles per milligram of protein) is plotted against the perfusion time (s). Curves represent single- or double-exponential decays, calculated with the parameters given in Table II. The inserts show a possible model for dissociation of Ca^{2+} for the ATPase reconstituted with (A) di(C20:1)PC or (C) di(C24:1)PC.

to di(C18:1)PC, the level of binding appeared to be greater than on reconstitution with di(C14:1)PC. We therefore checked the value of binding given in Figure 1 by measuring the level of Ca^{2+} binding as a function of time of reconstitution with di(C24:1)PC in parallel to measurements of ATPase activity. The ATPase was incubated with di(C24:1)PC in cholate and samples were taken at various times for assay of ATPase activities. It was found that ATPase activity decreased from an original value of 4.9 IU/mg to a steady level of 0.6 IU/mg after 1 h; activities were found to be stable for up to 2 h of incubation with di(C24:1)PC. Measurements of Ca^{2+} binding were found to be constant at 3.2 nmol/mg for incubations between 1 and 2 h (data not shown).

Table II: Dissociation of $^{45}Ca^{2+}$ from the (Ca^{2+}–Mg^{2+})-ATPase[a]

	rate constants (s^{-1})		
	1 mM $^{40}Ca^{2+}$	2 mM EGTA[b]	
lipid	k_1	k_1	k_2
SR	24.0	24.0	1.8
di(C16:1)PC	35.4	35.4	1.5
di(C20:1)PC	35.3	35.3	1.9
di(C22:1)PC	28.7	28.7	1.7
di(C24:1)PC	8.0	12.0	-
di(C14:1)PC + di(C24:1)PC[c]	35.3	35.3	1.9

[a] $^{45}Ca^{2+}$ dissociation measured in 20 mM Hepes/Tris, pH 7.2, 100 mM KCl, 5 mM Mg^{2+} containing either 2 mM EGTA or 1 mM $^{40}Ca^{2+}$. [b] Fitted to the sum of two exponentials of equal amplitude, with the rate k_1 fixed at the value obtained on washing with $^{40}Ca^{2+}$. [c] Molar ratio of di(C14:1)PC:di(C24:1)PC of 4:6.

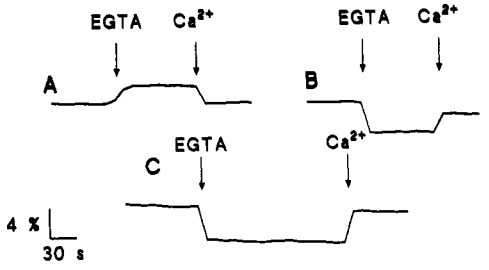

FIGURE 3: Effects of EGTA and Ca^{2+} on the tryptophan fluorescence intensity of the ATPase reconstituted with (A) di(C12:0)PC, (B) di(C16:1)PC, or (C) di(C24:1)PC. The ATPase was incubated in 2.5 mL of buffer (20 mM Hepes/KOH, pH 7.2, 5 mM Mg^{2+}, 100 mM KCl) containing 100 μM Ca^{2+}, and then EGTA (12.5 μL) and Ca^{2+} (6 μL) were added to give final concentrations of 1.2 and 2.4 mM, respectively.

The changed stoichiometry of Ca^{2+} binding following reconstitution with di(C24:1)PC was also confirmed by measuring the time course of release of Ca^{2+} from the reconstituted ATPase. For the ATPase reconstituted with di(C20:1)PC or di(C22:1)PC, it is observed that whereas on washing with EGTA essentially all the bound $^{45}Ca^{2+}$ is lost from the ATPase, on washing with 1 mM $^{40}Ca^{2+}$ only half the $^{45}Ca^{2+}$ is lost (Figure 2); an identical result was obtained for the ATPase reconstituted with di(C16:1)PC (data not shown). When the ATPase was reconstituted with di(C24:1)PC, the results were distinctly different, with loss of $^{45}Ca^{2+}$ being essentially complete on washing with either EGTA or $^{40}Ca^{2+}$ (Figure 2).

We found that, because of the excess lipid present in the system, a maximum of about 100 μg of reconstituted ATPase could be adsorbed on the filters, compared to about 300 μg for the native ATPase. As a consequence, levels of bound $^{45}Ca^{2+}$ that could be achieved for the reconstituted system were less than for native ATPase and the Ca^{2+} dissociation curves were correspondingly less well defined. Nevertheless, data for the dissociation of $^{45}Ca^{2+}$ in the presence of $^{40}Ca^{2+}$ fitted well to a single exponential (Figure 2, Table II). For the ATPase reconstituted with di(C24:1)PC, dissociation of $^{45}Ca^{2+}$ in the presence of EGTA also fitted well to a single exponential (Figure 2, Table II), but for the ATPase reconstituted with phosphatidylcholines with chain lengths between C16 and C22 dissociation of $^{45}Ca^{2+}$ in the presence of EGTA could not be fitted to a single exponential. Although in these cases the precision of the data was insufficient to obtain unambiguous fits to a double exponential, satisfactory fits could be obtained to a double exponential if the two components were fixed to be of equal intensity (see eq 1) with one rate constant fixed at the value obtained in the presence of $^{40}Ca^{2+}$ (Figure 2, Table II).

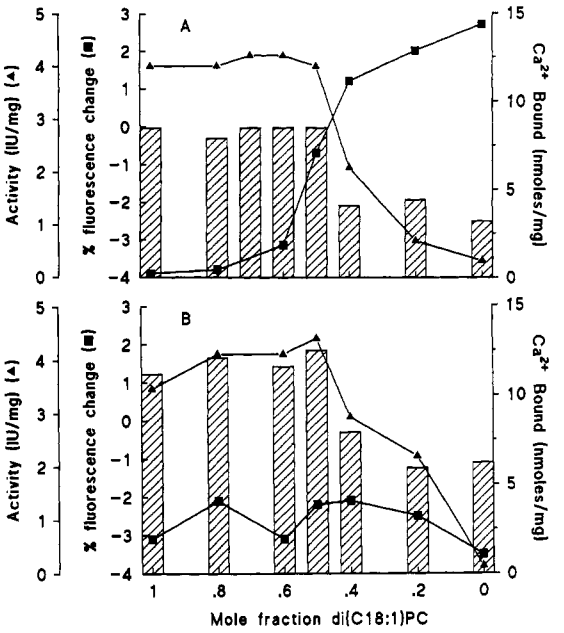

FIGURE 4: Effects on the (Ca^{2+}–Mg^{2+})-ATPase of mixtures of di-(C18:1)PC with either (A) di(C14:1)PC or (B) di(C24:1)PC at the given molar ratios. Symbols: ▲, ATPase activities measured at 25 °C; ■, % change in tryptophan fluorescence intensity on addition of EGTA to the ATPase initially in the presence of Ca^{2+}; hatched bars, Ca^{2+} bound (nanomoles per milligram of protein).

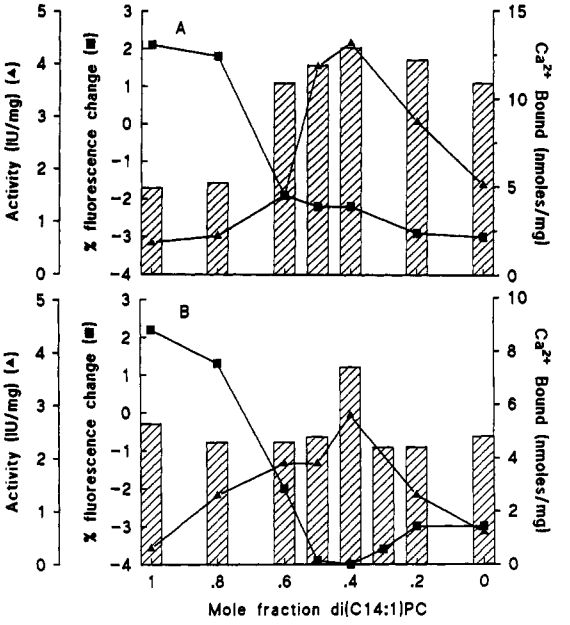

FIGURE 5: Effects on the (Ca^{2+}–Mg^{2+})-ATPase of mixtures of di-(C14:1)PC with either (A) di(C22:1)PC or (B) di(C24:1)PC at the given molar ratios. Symbols: ▲, ATPase activities measured at 25 °C; ■, % change in tryptophan fluorescence intensity on addition of EGTA to the ATPase initially in the presence of Ca^{2+}; hatched bars, Ca^{2+} bound (nanomoles per milligram of protein).

Binding of Ca^{2+} to the ATPase can also be followed from changes in the tryptophan fluorescence of the ATPase (Dupont et al., 1988). Although removal of Ca^{2+} from the native ATPase by addition of EGTA results in a decrease in fluorescence intensity, removal of Ca^{2+} from the ATPase reconstituted with di(C12:0)PC results in an increase in intensity as shown in Figure 3. Removal of Ca^{2+} from the ATPase reconstituted with di(C14:1)PC also results in an increase in fluorescence intensity (Michelangeli et al., 1990b) (Figure 1). For the ATPase reconstituted with phosphatidylcholines of chain lengths between C16 and C24 inclusive,

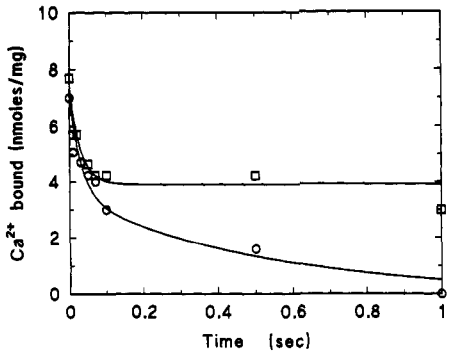

FIGURE 6: Rapid filtration measurement of the rate of Ca^{2+} dissociation from the ATPase reconstituted with a 4:6 molar ratio of di(C14:1)PC to di(C24:1)PC. The ATPase was incubated with $^{45}Ca^{2+}$ and then perfused with buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl, 5 mM Mg^{2+}) containing either 2 mM EGTA (○) or 1 mM $^{40}Ca^{2+}$ (□) as described in the legend to Figure 2. Curves represent single- and double-exponential decays, with the parameters given in Table II.

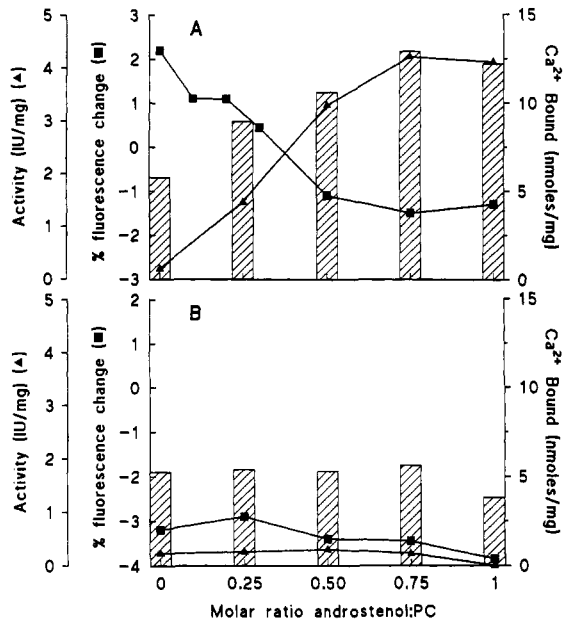

FIGURE 7: Effects on the (Ca^{2+}–Mg^{2+})-ATPase of mixtures of androstenol and (A) di(C14:1)PC or (B) di(C24:1)PC. Symbols: ▲, ATPase activities measured at 25 °C; ■, % change in tryptophan fluorescence intensity on addition of EGTA to the ATPase initially in the presence of Ca^{2+}; hatched bars, Ca^{2+} bound (nanomoles per milligram of protein).

removal of Ca^{2+} results in a decrease in fluorescence intensity, reversed by subsequent addition of Ca^{2+} as shown in Figure 3.

Effects of mixtures of di(C18:1)PC with either di(C14:1)PC or di(C24:1)PC are shown in Figure 4. ATPase activities are fairly constant in these mixtures for di(C18:1)PC contents between 100% and ca. 50%, with activities then gradually decreasing to levels characteristic of di(C14:1)PC or di(C24:1)PC, respectively. In contrast, the stoichiometry of Ca^{2+} binding changes sharply from two Ca^{2+} ions bound per ATPase molecule for mixtures containing 50% or more di(C18:1)PC to one Ca^{2+} ion bound per ATPase molecule for mixtures containing 40% or less di(C18:1)PC (Figure 4). As shown in Table I, the stoichiometry of Ca^{2+} binding to the ATPase reconstituted with the mixed chain phospholipids (C18:1,C14:0)PC and (C14:0,C18:1)PC is two Ca^{2+} ions bound per ATPase molecule, as for a 1:1 mixture of di(C18:1)PC and di(C14:1)PC (Figure 4A). For mixtures of

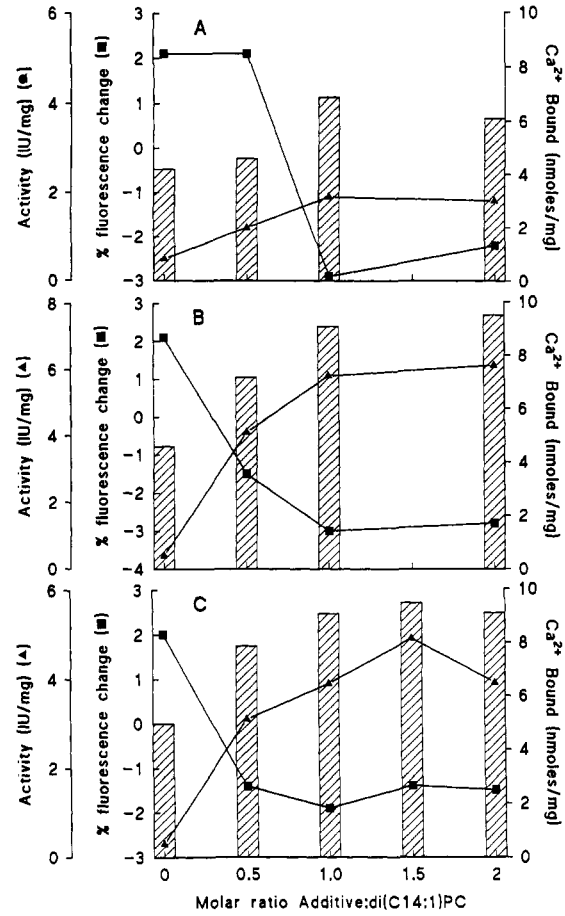

FIGURE 8: Effects on the (Ca^{2+}–Mg^{2+})-ATPase of mixtures of di(C14:1)PC with (A) oleic acid, (B) methyl oleate, and (C) oleyl alcohol at the given molar ratios. Symbols: ▲, ATPase activities measured at 25 °C; ■, % change in tryptophan fluorescence intensity on addition of EGTA to the ATPase initially in the presence of Ca^{2+}; hatched bars, Ca^{2+} bound (nanomoles per milligram of protein).

di(C18:1)PC and di(C14:1)PC, the response of trytophan fluorescence to removal of Ca^{2+} also changes sign in parallel to the changes in Ca^{2+} binding stoichiometry (Figure 4A); in mixtures of di(C18:1)PC with di(C24:1)PC, changes in tryptophan fluorescence on removal of Ca^{2+} are essentially independent of the phospholipid composition (Figure 4B).

Effects on the ATPase of mixtures of di(C14:1)PC with the longer chain phospholipids di(C22:1)PC and di(C24:1)-PC are shown in Figure 5. Both the stoichiometry of Ca^{2+} binding and the fluorescence response to the removal of Ca^{2+} are normal in mixtures of di(C24:1)PC and di(C22:1)PC containing 40% or more di(C22:1)PC, but in mixtures containing only 20% di(C22:1)PC, the stoichiometry of Ca^{2+} binding and the fluorescence responses are as observed for the ATPase reconstituted with di(C14:1)PC alone. Effects of these lipid mixtures on ATPase activities are more complex; addition of increasing amounts of di(C14:1)PC to di(C22:1)PC results in an increase in ATPase activity to a maximum value observed in mixtures containing 60% di(C22:1)PC, but addition of further di(C14:1)PC results in decreasing activities (Figure 5A). For the ATPase reconstituted with mixtures of di(C14:1)PC and di(C24:1)PC, the stoichiometry of Ca^{2+} binding is one Ca^{2+} bound per ATPase molecule for all mixtures except that containing 40% di(C14:1)PC, where the stoichiometry of Ca^{2+} binding changes to close to two Ca^{2+} ions bound per ATPase molecule. To confirm this change in binding stoichiometry, we studied the time courses of release of $^{45}Ca^{2+}$ from the ATPase reconstituted with a 4:6 molar

ratio of di(C14:1)PC and di(C24:1)PC (Figure 6). As shown, washing with 1 mM $^{40}Ca^{2+}$ results in the release of half the bound $^{45}Ca^{2+}$ whereas washing with EGTA results in release of essentially all the bound $^{45}Ca^{2+}$, a result characteristic of the ATPase with two bound Ca^{2+} ions per ATPase molecule (see Figure 2).

Effects of androstenol, oleic acid, methyl oleate, and oleyl alcohol on the ATPase reconstituted with di(C14:1)PC and di(C24:1)PC are shown in Figures 7 and 8. Whereas addition of these molecules to the ATPase reconstituted with di(C14:1)PC increases activity and restores the stoichiometry of Ca^{2+} binding to the normal two Ca^{2+} ions bound per ATPase molecule (Figures 7,8), addition of androstenol to the ATPase reconstituted with di(C24:1)PC has no effect (Figure 7); similarly, addition of oleic acid, methyl oleate, or oleyl alcohol to the ATPase reconstituted with di(C24:1)PC has no effect on ATPase activity, on Ca^{2+} binding, or on the fluorescence response to the removal of Ca^{2+} (data not shown).

DISCUSSION

Membrane proteins are unique in that part of their environment is composed of phospholipid molecules. Any mismatch between the thickness of the hydrophobic region of the ATPase and of the phospholipid bilayer is unlikely to result in significant exposure of these regions of the protein to water because the Gibbs free energy of exposure of hydrophobic residues to water is high (Tanford, 1973). Thus, changes in conformation of either the protein or the phospholipid can be expected to minimize the mismatch. Since binding constants for phosphatidylcholines to the ATPase have been shown to be independent of chain length (Caffrey & Feigenson, 1981; East & Lee, 1982; Froud et al., 1986c), major distortion of the phospholipids seems unlikely. Rather, if different conformational states of the ATPase have different hydrophobic thicknesses, then differences in solvation energies could lead to changes in the relative energies of the conformational states, the state with the narrower hydrophobic region being favored by the phospholipid with the shorter chain. Since phospholipid binding is independent of fatty acyl chain length, free energy differences between conformations would have to be small compared to kT per annular lipid, but with about 30 phospholipid molecules binding to the ATPase (East et al., 1985), a cooperative transition between conformations would seem possible. Although measurements using fluorescence energy transfer have suggested that aggregation of the ATPase is more extensive in di(C14:1)PC than in di(C18:1)PC (Munkonge et al., 1988), this does not appear to involve significant changes in the interactions between hydrophobic regions of the ATPase, since fluorescence quenching experiments suggest equal binding of di(C14:1)PC and di(C18:1)PC to the ATPase (Caffrey & Feigenson, 1981; East & Lee, 1982; Froud et al., 1986a,b,c; Michelangeli et al., 1990c).

As shown in Figure 1, although ATPase activities measured at 25 °C are dependent on the fatty acyl chain length of the surrounding phosphatidylcholines in reconstituted systems, maximal levels of phosphorylation of the ATPase by [γ-^{32}P]-ATP are identical for the native ATPase and for all the reconstituted ATPases (Figure 1, Table I). However, the stoichiometry of Ca^{2+} binding changes from two Ca^{2+} ions bound per active ATPase molecule for the native ATPase and for the ATPase reconstituted with phospholipids of chain lengths C16–C22 to one Ca^{2+} ion bound per active ATPase molecule for the ATPase reconstituted with di(C12:0)PC, di(C14:1)PC, or di(C24:1)PC. The change in stoichiometry of Ca^{2+} binding is also demonstrated in measurements of the kinetics of dissociation of $^{45}Ca^{2+}$ from the ATPase. For the native ATPase, or for the ATPase reconstituted with phospholipids with chain lengths between C16 and C22, release of the two bound Ca^{2+} ions is sequential, with the second Ca^{2+} only being released after release of the first, as demonstrated by the very slow rate of release of the second Ca^{2+} ion in the presence of high (1 mM) concentrations of Ca^{2+} in the bathing medium (Figure 2). In contrast, for the ATPase reconstituted with di(C24:1)PC, a high concentration of Ca^{2+} in the bathing medium has only a slight inhibitory effect on the release of Ca^{2+} (Figure 2C), as reported previously for the ATPase reconstituted with di(C14:1)PC (Michelangeli et al., 1990b). In terms of a gated channel model for the ATPase, two Ca^{2+} ions can bind in the channel in the native ATPase and phosphorylation of the ATPase with closing of the outer channel gate can only occur with two Ca^{2+} ions in the channel (see insert Figure 2A). In contrast, for the ATPase reconstituted with di(C12:0)PC, di(C14:1)PC, or di(C24:1)PC, only a single Ca^{2+} ion can bind in the channel, and phosphorylation of the ATPase occurs with just one Ca^{2+} ion bound (insert to Figure 2C).

Dissociation of Ca^{2+} from the ATPase can be followed directly using $^{45}Ca^{2+}$ or by measuring changes in tryptophan fluorescence of the ATPase. Under the conditions used for the Ca^{2+} dissociation experiments (5 mM Mg^{2+}, 100 mM KCl, pH 7.2), the change in tryptophan fluorescence intensity observed on removal of Ca^{2+} from the unreconstituted ATPase by mixing with EGTA has been shown to be double exponential (Moutin & Dupont, 1991). Although we have found that the time course of dissociation of $^{45}Ca^{2+}$ from the unreconstituted ATPase on washing with EGTA does not fit to a single exponential, the noise level of the experiments does not allow an unambiguous fit to a double-exponential process. However, within experimental error, fluorescence decay curves and $^{45}Ca^{2+}$ dissociation curves are identical suggesting both that the fluorescence changes directly monitor occupancy of the Ca^{2+} binding sites on the ATPase and that the release of $^{45}Ca^{2+}$ under these conditions is bi-exponential (A. P. Starling, I. Henderson, J. M. East and A. G. Lee, unpublished data). For sequential dissociation of Ca^{2+} (Scheme I), the rate of dissociation of the first Ca^{2+} observed on washing with $^{40}Ca^{2+}$ gives the rate constant k_1, whereas dissociation in the presence of EGTA should fit to the sum of two exponentials with rate constants k_1 and k_2 of equal amplitude if $k_1 \gg k_2$ (eq 1). As shown in Figure 2 and Table II, the data can be fitted in this way. Values for the rate constants are very similar for the ATPase in the native SR membrane and in phosphatidylcholines with fatty acyl chain lengths between C16 and C22. The $^{45}Ca^{2+}$ dissociation data for the ATPase reconstituted with di(C24:1)PC fits to a single exponential for washing with either EGTA or $^{40}Ca^{2+}$, with a somewhat slower rate in the latter case. A similar observation was made previously for the ATPase reconstituted with di(C14:1)PC with rate constants of 8.8 and 3.7 s^{-1} for washing with EGTA and $^{40}Ca^{2+}$ respectively (Michelangeli et al., 1990b). It has been observed that lanthanide ions bind to the ATPase at sites other than at the Ca^{2+} binding sites and decrease the rate of dissociation of Ca^{2+} (Ogurusu et al., 1991; Henao et al., 1992). It is possible that, at high concentrations, Ca^{2+} can bind to these same sites and decrease the rate of dissociation of Ca^{2+}.

Since binding affinities for phosphatidylcholines at the lipid–protein interface of the ATPase are independent of fatty acyl chain length (Caffrey & Feigenson, 1981; East & Lee, 1982), the composition of the lipid bilayer around the ATPase will be the same as the bulk composition. As shown in Figure 4, the stoichiometry of Ca^{2+} binding to the ATPase changes in

a highly cooperative way with phospholipid composition in mixtures of di(C18:1)PC and either di(C14:1)PC or di(C24:1)PC. The high cooperativity suggests a conformation change dependent on phospholipid binding at a large number of sites at the lipid–protein interface, rather than one dependent on phospholipid binding to just one site (or a small number of sites) on the ATPase. Changes in ATPase activity show a more complex dependence on composition showing that changes must occur in more than just Ca^{2+} binding when the phospholipids surrounding the ATPase are changed; it has, for example, been shown that rate constants for phosphorylation and dephosphorylation also change on reconstitution with di(C14:1)PC (Michelangeli et al., 1991).

For the ATPase reconstituted with mixed chain phosphatidylcholines containing both C14:0 and C18:1 chains, the binding stoichiometry is the normal 2:1, independent of the position of the two chains in the phosphatidylcholine (Table I), despite the fact that the chain at the 1-position extends further into the bilayer than that at the 2-position, because of a bend in the fatty acyl chain at the 2-position (Pearson & Pascher, 1979).

For the ATPase reconstituted with mixtures of di(C14:1)PC and di(C24:1)PC, a change in Ca^{2+} binding stoichiometry to 2:1 is observed at 40% di(C14:1)PC (Figures 5B and 6). This suggests a relationship between binding stoichiometry and a critical membrane thickness, as does the observation that in mixtures of di(C14:1)PC with di(C22:1)PC, the Ca^{2+} binding stoichiometry stays at 2:1 at higher proportions of di(C14:1)PC than in mixtures with di(C18:1)PC (Figures 4A and 5A). However, the relationship between binding stoichiometry and "average chain length" cannot be a simple one. Studies of the ATPase in single phospholipids (Figure 1) show that chain lengths between C16 and C22 are compatible with a binding stoichiometry of 2:1, but "average chain lengths" in this range obtained with mixtures of di(C14:1)PC and di(C24:1)PC show a binding stoichiometry of 1:1 (Figure 5B).

As shown in Figures 7 and 8, and as reported previously (Simmonds et al., 1982; Froud et al., 1986c; Michelangeli et al., 1990c), addition of androstenol, oleic acid, oleyl alcohol, or methyl oleate all increase the activity of the ATPase reconstituted with di(C14:1)PC. We now show that they also return the stoichiometry of Ca^{2+} binding to the normal two Ca^{2+} ions bound per ATPase molecule (Figures 7 and 8). Similarly, addition of androstenol to the ATPase reconstituted with di(C12:0)PC also increases ATPase activity and changes the stoichiometry of Ca^{2+} binding to 2:1 (data not shown). In contrast, addition of androstenol to the ATPase reconstituted with di(C24:1)PC has no effect on either activity or the stoichiometry of Ca^{2+} binding (Figure 7B); addition of oleic acid, methyl oleate, or oleyl alcohol also has no effect (data not shown). Reversal of the effects of di(C14:1)PC by these additives could follow from an increase in the thickness of the phospholipid bilayer or from conformational changes resulting from direct binding to the ATPase since fluorescence quenching data suggest that such binding does occur (Froud et al., 1986b; Michelangeli et al., 1990c). The observation of more gradual changes in Ca^{2+} binding stoichiometry than observed for the ATPase reconstituted with mixtures of phospholipids (Figures 7 and 8) suggest that effects follow from binding to a restricted number of sites on the ATPase, rather than to a change in the phospholipid bilayer, which, as described above, would be expected to be highly cooperative.

Removal of bound Ca^{2+} from the native ATPase results in a ca. 4% decrease in the intensity of the fluorescence of tryptophan residues, reversed by addition of Ca^{2+} (Moutin & Dupont, 1991). Similar changes are observed for the ATPase reconstituted with phosphatidylcholines with chain lengths between C16 and C24 (Figure 3), but for the ATPase reconstituted with di(C12:0)PC or di(C14:1)PC, removal of bound Ca^{2+} results in an increase in fluorescence intensity (Figures 1 and 3). Since many of the Trp residues on the ATPase are located close to the membrane–water interface (Froud et al., 1986b), changes in the thickness of the membrane could result in different degrees of exposure of the Trp residues to water or the phospholipid polar head groups, and so to different fluorescence responses to the same conformational change on the ATPase.

In terms of the gated pore model for the ATPase (see Figure 2), it would appear that phosphatidylcholines with chain lengths between C16 and C22 are required to prevent the outer gate closing with only one Ca^{2+} ion bound in the channel.

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