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Effects of Phospholipids on Binding of Calcium to (Ca^{2+}–Mg^{2+})-ATPase†

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ABSTRACT: The (Ca^{2+}–Mg^{2+})-ATPase purified from skeletal muscle sarcoplasmic reticulum binds two Ca^{2+} ions per ATPase molecule. On reconstitution into bilayers of dioleoylphosphatidylcholine ((C18:1)PC) or dinervonylphosphatidylcholine ((C24:1)PC) the stoichiometry of binding remains unchanged, but when the ATPase is reconstituted into bilayers of dimyristoleoylphosphatidylcholine ((C14:1)PC) the stoichiometry changes to one Ca^{2+} ion per ATPase molecule. Nevertheless, the level of phosphorylation is the same for the ATPase reconstituted with (C18:1)PC or (C14:1)PC. The effect of (C14:1)PC on the stoichiometry of Ca^{2+} binding is prevented by androstenol at a 1:1 molar ratio with the phospholipid.

The activity of the (Ca^{2+}–Mg^{2+})-ATPase purified from the sarcoplasmic reticulum of skeletal muscle is known to be sensitive to the structure of the phospholipid molecules surrounding it in phospholipid bilayers. The phospholipid supporting highest ATPase activity is dioleoylphosphatidylcholine ((C18:1)PC),[1] and phospholipids with either shorter or longer fatty acyl chains or different headgroups support lower activities (Warren et al., 1974; Johannsson et al., 1981; Caffrey & Feigenson, 1981; East & Lee, 1982; Froud et al., 1986a). The low ATPase activity supported by phosphatidylcholines with short fatty acyl chains can be increased very considerably by addition of a wide variety of hydrophobic molecules, including sterols, fatty acids, and alcohols, alkanes, and pyrethroids (Johannsson et al., 1981; Simmonds et al., 1982, 1984; Jones & Lee, 1985; Jones et al., 1985, 1986; Froud et al., 1986b; Michelangeli et al., 1989). The low ATPase activities supported by phosphatidylcholines with long fatty acyl chains, however, are unaltered by addition of such hydrophobic molecules (Froud et al., 1986b). This argues that the changes to the ATPase that follow from reconstitution with a short-chain phosphatidylcholine are very different to those that follow from reconstitution with a long-chain phosphatidylcholine. We have been attempting to define the steps in the reaction sequence for the ATPases that are sensitive to phospholipid structure (Froud et al., 1986b; Michelangeli et al., manuscript in preparation). Here we focus on the steps in which Ca^{2+} binds to the ATPase on the cytoplasmic side of the membrane.

The binding of two Ca^{2+} ions to the native ATPase is a critical step since only in the Ca^{2+}-bound form can the ATPase by phosphorylated by MgATP; in the Ca^{2+}-free conformation the ATPase can instead be phosphorylated by P_i (de Meis & Vianna, 1979). Two Ca^{2+} ions bind per ATPase molecule in a cooperative manner, involving at least one slow conformational change. Rates of binding and dissociation of $^{45}Ca^{2+}$ and of changes in the tryptophan fluorescence of the ATPase that

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[1] Abbreviations: (C14:1)PC, dimyristoleoylphosphatidylcholine; (C18:1)PC, dioleoylphosphatidylcholine; (C24:1)PC, dinervonylphosphatidylcholine; SR, sarcoplasmic reticulum.

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follow from Ca^{2+} binding have been tentatively interpreted in terms of Scheme I (Dupont, 1982; Champeil et al., 1983; Fernandez-Belda et al., 1984; Inesi, 1985; Froud & Lee, 1986; Tanford et al., 1987; Inesi, 1987; Petithory & Jencks, 1988). In this model a site of moderate affinity is immediately available for binding and a second high affinity site becomes available only after a slow conformational change CaE1–CaE1′ triggered by binding of the first Ca^{2+} to E1. Here we show that on reconstitution of the ATPase with the short-chain phospholipid dimyristoleoylphosphatidylcholine ((C14:1)PC) there is a major change in the ATPase, resulting in binding of a single Ca^{2+} ion but with no loss of the ability of the ATPase to be phosphorylated by ATP.

Scheme I

$$E1 \rightleftharpoons CaE1 \rightleftharpoons CaE1' \rightleftharpoons Ca_2E1'$$

Materials and Methods

(C14:1)PC, (C18:1)PC, and dinervonylphosphatidylcholine ((C24:1)PC) were obtained from Avanti Polar Lipids. Hepes (Ultrol) was from Calbiochem and AnalaR water from BDH.

(Ca^{2+}–Mg^{2+})-ATPase was purified from sarcoplasmic reticulum of rabbit skeletal muscle as described by East and Lee (1982). The final preparation contained 30 phospholipid molecules per ATPase molecule and was >95% pure based on Coomassie Blue staining of sodium dodecyl sulfate–polyacrylamide gels. Reconstitutions were performed largely as described by East and Lee (1982). Phospholipid (1 μmol) was mixed with buffer (40 μL; 50 mM potassium phosphate, 1 M KCl, and 0.25 M sucrose, pH 8) containing $MgSO_4$ (5 mM) and potassium cholate (12 mg/mL) and sonicated to clarity in a bath sonicator (Megason). ATPase (0.125 mg) in a volume of 3–10 μL was then added and left for 1 h at 5 °C to equilibrate before being diluted with buffer (200 μL) and stored on ice until use. In experiments with androstenol, androstenol was added to phospholipid prior to solubilization in the original cholate solution.

Fluorescence measurements were made in buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl, 5 mM $MgSO_4$, and 100 μM Ca^{2+}) by using a Perkin-Elmer LS 3B fluorimeter. Tryptophan fluorescence was excited at 295 nm and observed at 330 nm, for ATPase concentrations of 1.0 μM and 0.2 μM for the native and reconstituted ATPase, respectively. The Ca^{2+} dependence of fluorescence was observed by addition of EGTA to the ATPase in the Ca^{2+}-containing buffer to give the required free Ca^{2+} concentration. Free concentrations of Ca^{2+} were calculated by using the binding constants for Ca^{2+}, Mg^{2+}, and H^+ to EGTA given by Godt (1974), as used by Petithory and Jencks (1988), corresponding to effective dissociation constants at pH 7.2 of 2.9×10^{-7} and 1.4×10^{-2} for Ca^{2+} and Mg^{2+}, respectively.

Binding of $^{45}Ca^{2+}$ to the ATPase was measured by using the filter method as described by Champeil and Guillain (1986) with double labeling as also detailed by Yamaguchi and Watanabe (1989). The ATPase was added to buffer (1 mL; 20 mM Hepes/Tris, pH 7.2, 100 mM KCl, and 5 mM $MgSO_4$) containing 50 μM $^{45}CaCl_2$ and 500 μM [^{3}H]sucrose and EGTA to give the required free concentration of Ca^{2+}, at 20 °C, to give a final protein concentration of 50 μg/mL. The suspension was filtered through a Millipore HAWP filter (0.45 μm) and the filter counted in Labscint. The maximum amount of protein that could be adsorbed to the filters was determined by filtration of ATPase, followed by detection of any ATPase in the filtrate by tryptophan fluorescence. It was found that whereas up to 200 μg of the native ATPase or SR could be adsorbed to the filter, for the reconstituted ATPase the maximum was 50 μg, due to the excess lipid. The amount of [^{3}H]sucrose trapped on the filter was used to calculate the wetting volume for the filter, and the amount of $^{45}Ca^{2+}$ calculated to be present 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 $^{45}Ca^{2+}$ binding observed for pure lipid samples prepared as above but in the absence of ATPase, which was equivalent to 1 nmol of Ca^{2+} bound/mg of protein.

Measurements of the time dependence of Ca^{2+} release from the ATPase were performed by using a Biologic rapid filtration system, at 20 °C. Reconstituted ATPase to which 2% A23187 (w/w protein) had been added was suspended to 15 μg of protein/mL in buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl, 5 mM Mg^{2+}, 15 μM $^{45}Ca^{2+}$, and 0.5 mM [^{3}H]glucose) containing A23187 (0.3 μg/mL). Three milliliters of the suspension, corresponding to 45 μg of the ATPase, was then loaded onto a Millipore HA filter and slowly perfused with 0.5 mL of the above buffer to remove cholate and eliminate any contaminating Ca^{2+} contributed by the reconstitution medium. The filter was then rapidly perfused with buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl, and 5 mM Mg^{2+}) containing either EGTA (2 mM) or $^{40}Ca^{2+}$ (1 mM). The filter was then counted, and corrections were made for the amount of Ca^{2+} trapped in the filter with water as described above.

Steady-state measurements of enzyme phosphorylation by ATP were carried out in a medium containing 20 mM Hepes/Tris, pH 7.2, 5 mM $MgSO_4$, 100 mM KCl, 1 mM $CaCl_2$, and 0.1 mg/mL ATPase, in a total volume of 1 mL. The reaction was started by addition of 100 μM [γ-^{32}P]ATP and, after incubation at 25 °C for 10 s, was quenched with ice-cold 25% trichloroacetic acid and 0.2 M potassium phosphate. The quenched protein was allowed to precipitate by incubating on ice for 15 min. The precipitate was collected by filtration through Whatman GF/C glass fiber filters, washed three times with 15 mL of cold 12% trichloroacetic acid and 0.2 M potassium phosphate, and finally counted in 4 mL of Labscint. We also measured phosphorylation of ATPase samples previously adsorbed onto Millipore HAWP filters; as for the $^{45}Ca^{2+}$ binding experiments, 1 mL of buffer containing the ATPase at 50 μg/mL was first deposited on the filter, followed by perfusion of 1 mL of the [^{32}P]ATP-containing medium described above. After 10 s, 1 mL 40% trichloroacetic acid containing 5 mM ATP was passed through the filter to quench the reaction. The filter was washed with 10 mL of cold 12% trichloroacetic acid, 5 mM ATP followed by 20 mL of cold 12% trichloroacetic acid, and 0.2 M potassium phosphate and finally counted in 4 mL of Labscint.

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). Simulations were carried out by using the FACSIMILE program (Chance et al., 1977) run on an IBM 3090 computer.

Results

A recurring problem in establishing the stoichiometry of Ca^{2+} binding to the ATPase is the possible presence of a variable and relatively large proportion of inactive ATPase in preparations of the ATPase that appear pure on the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Gafni & Boyer, 1984). Measured binding stoichiometries are therefore usually compared to a maximal level of phosphorylation of the ATPase, determined under conditions where the rate of dephosphorylation is low. Thus Table I first shows maximal phosphorylation levels for the ATPase reconstituted with a variety of phospholipids. Within experimental error,

Table I: Maximal Levels of Phosphorylation and Ca^{2+} Binding for the Reconstituted ATPase[a]

system	[EP] (nmol/mg of protein)[b]	Ca^{2+} bound (nmol/mg of protein)[b]
native ATPase	3.0 ± 0.3	9.5 ± 1.1
(C18:1)PC–ATPase	2.7 ± 0.2	9.6 ± 1.4
(C14:1)PC–ATPase	3.2 ± 0.2	4.5 ± 1.5
(C24:1)PC–ATPase	3.2 ± 0.2	7.7 ± 0.3
(C18:1)PC–ATPase + androstenol[c]		7.8 ± 1.1
(C14:1)PC–ATPase + androstenol[c]	2.8 ± 0.3	11.4 ± 1.0
native ATPase[d]	3.9 ± 0.4	

[a] ATPase adsorbed onto Millipore HAWP filters. For phosphorylation, the medium contained 20 mM Hepes/Tris, pH 7.2, 5 mM $MgSO_4$, 100 mM KCl, 1 mM $CaCl_2$, and 100 μM [γ-^{32}P]ATP. The same medium was used to determine Ca^{2+} binding, except for the absence of ATP and a Ca^{2+} concentration of 50 μM. [b] Mean of three determinations. [c] Molar ratio of phospholipid:androstenol, 1:1. [d] For ATPase not adsorbed onto a filter.

the levels of maximal phosphorylation measured for the ATPase reconstituted into (C14:1)PC, (C18:1)PC, or (C24:1)PC are equal to that observed with the native ATPase (Table I). For these experiments, the maximal level of phosphorylation of the native ATPase was 3.9 nmol/mg when the ATPase was first phosphorylated and then collected by filtration. This level of phosphorylation corresponds to 43% active protein, based on a molecular weight of 115000 for the ATPase, and is typical for our preparations of ATPase [see Michelangeli et al. (1990b)]. When the native ATPase was first adsorbed onto Millipore filters and then phosphorylated, there was a slight decrease in the measured maximal level of phosphorylation.

Table I also shows the level of $^{45}Ca^{2+}$ bound to the ATPase at 50 μM Ca^{2+}, pH 7.2. For all systems except (C14:1)PC–ATPase, the amount of Ca^{2+} bound to the ATPase was similar, slightly greater than twice the maximal level of phosphorylation; the level of Ca^{2+} binding observed for (C14:1)PC–ATPase is, however, significantly reduced, to about half that observed for the other systems. Similar results were obtained at pH 6 (data not shown). Levels of Ca^{2+} binding for the native ATPase greater than the expected level of two Ca^{2+} per phosphorylatible ATPase molecule have been observed by others under similar conditions (Inesi et al., 1980) and can be attributed either to binding to additional weak binding sites on the ATPase or to binding to Ca^{2+} sites on ATPase molecules that are not phosphorylatible by MgATP [see also Barrabin et al. (1984) and Gafni and Boyer (1984)]. The observed reduction in Ca^{2+} binding potentially could occur through a reduction in the affinity of the ATPase for Ca^{2+}. Figure 1 shows Ca^{2+} binding to the native ATPase and to (C18:1)PC–ATPase and (C14:1)PC–ATPase as a function of free Ca^{2+} concentration at pH 7.2. Despite some scatter in the experimental values, it is clear that the low level of Ca^{2+} binding to (C14:1)PC–ATPase is not simply attributable to a low affinity for Ca^{2+}. The same conclusion, that the apparent affinity of (C14:1)PC–ATPase for Ca^{2+} is comparable to that of (C18:1)PC–ATPase, can be drawn from measurements of ATPase activity as a function of Ca^{2+} concentration (F. Michelangeli, J. M. East, and A. G. Lee, unpublished observations).

Froud and Lee (1986) presented a set of binding parameters to describe binding of Ca^{2+} to the ATPase as a function of pH in terms of Scheme I, largely based on the binding data of Hill and Inesi (1982). The absolute values of the binding constants for Ca^{2+} depend on the assumed value of the Ca^{2+}–EGTA binding constant, since this determines the free Ca^{2+} concentrations calculated to be present in the binding experiments; in this paper Ca^{2+} binding constants have been

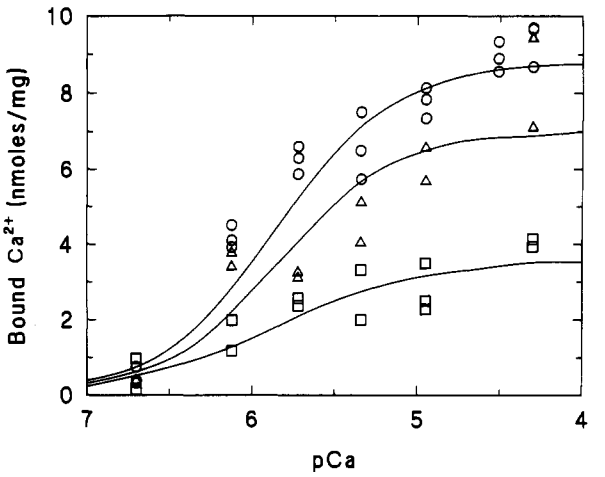

FIGURE 1: Binding of Ca^{2+} (nmol/mg of protein) to native ATPase (○), (C18:1)PC–ATPase (△), and (C14:1)PC–ATPase (□) as a function of pCa at pH 7.2, Mg^{2+} = 5 mM, 20 °C. The curves are simulations calculated as described in the text, assuming maximal levels of Ca^{2+} binding of 8.8, 7, and 3.5 nmol/mg of protein for native ATPase, (C18:1)PC–ATPase, and (C14:1)PC–ATPase, respectively. The relatively large scatter in data points is due to the limited amount of the reconstituted ATPase that can be adsorbed to the filter (see Methods).

Table II: Parameters Used To Describe Binding of Ca^{2+} to the ATPase[a]

reaction	symbol[b]	Figure 1 K_a^c	Figure 1 $K_a^{eff\,d}$	Figure 3 K_a^c	Figure 3 $K_a^{eff\,d}$
E1 + Ca^{2+} ⇌ CaE1	K_{C1}	7.25×10^5	10.5×10^5	1.45×10^5	2.1×10^5
CaE1 ⇌ CaE1′	K_{C2}	1.8	1.8	1.8	1.8
CaE1′ + Ca^{2+} ⇌ Ca_2E1′	K_{C3}	5.7×10^7	9.2×10^5	5.7×10^7	9.2×10^5

[a] Proton binding constants and E2/E1 equilibrium constant as given by Froud and Lee (1986). [b] As defined by Froud and Lee (1986). [c] Absolute binding constant for the nonprotonated forms (Froud & Lee, 1986). [d] Effective binding constant at pH 7.2, 5 mM Mg^{2+}.

slightly modified to agree with the Ca^{2+}–EGTA binding constants given by Godt (1974) (Table II). In addition, the apparent variability in Ca^{2+} binding between preparations of the ATPase observed by Rooney and Lee (1983) was taken into account by assuming, in these experiments, a slightly different value of the constant K_{C1} for the binding of the first Ca^{2+} to the ATPase. As shown in Figure 1, Ca^{2+} binding data for the native ATPase and for (C18:1)PC–ATPase can be simulated by using the parameters given in Table II. In contrast, the binding data for (C14:1)PC–ATPase fits to binding to a single site with a binding constant of 7.7×10^5.

Ca^{2+} binding can also be studied through changes in tryptophan fluorescence of the ATPase [see references in Dupont et al. (1988)]. Addition of Ca^{2+} to either the native ATPase or (C18:1)PC–ATPase results in an increase in the intensity of tryptophan fluorescence (Figure 2A), whose pCa dependence is shown in Figure 3. In marked contrast, addition of Ca^{2+} to (C14:1)PC–ATPase results in a small decrease in fluorescence intensity (Figures 2B and 3), with a slightly higher apparent Ca^{2+} affinity for (C14:1)PC–ATPase than for (C18:1)PC–ATPase (50% effects at pCa values of 5.9 and 5.5 for (C14:1)PC–ATPase and (C18:1)PC–ATPase, respectively). It has been argued that the change in tryptophan fluorescence follows from the CaE1–CaE1′ conformational change, with the E2, E1, and CaE1 forms having relatively

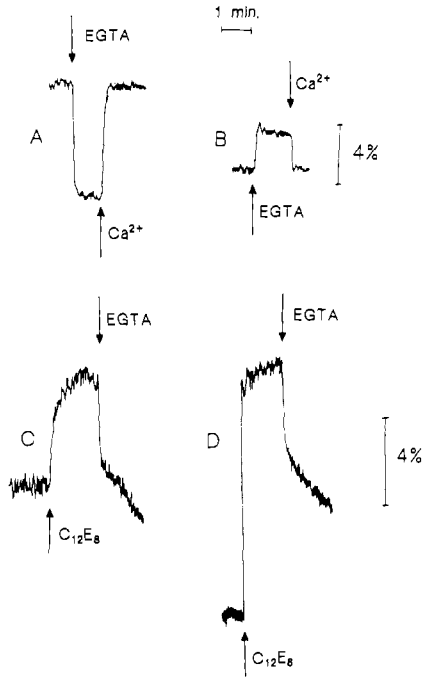

FIGURE 2: Effects of Ca^{2+} and EGTA on the tryptophan fluorescence intensity of (C18:1)PC–ATPase (A and C) and (C14:1)PC–ATPase (B and D). (A and B) Effects of addition of EGTA followed by Ca^{2+} at final concentrations of 1.2 and 2.4 mM, respectively, on the ATPase initially incubated with 100 μM Ca^{2+}, at 20 °C, pH 7.2. (C and D) Effects of addition of $C_{12}E_8$ followed by EGTA at final concentrations of 2 mg/mL and 2.0 mM, respectively, on the ATPase initially incubated with 100 μM Ca^{2+}.

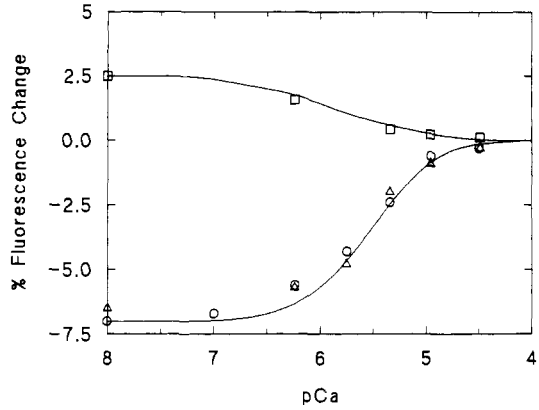

FIGURE 3: Percent change in tryptophan fluorescence intensity as a function of pCa for native ATPase (○), (C18:1)PC–ATPase (△), and (C14:1)PC–ATPase (□) at pH 7.2, Mg^{2+} = 5 mM, 20 °C. The curves are simulations calculated as described in the text.

low tryptophan fluorescence intensities and the CaE1′ and Ca_2E1′ forms having higher fluorescence intensities (Fernandez-Belda et al., 1984; Michelangeli et al., 1990a). Figure 3 shows that the experimental data for the native ATPase and for (C18:1)PC–ATPase can be simulated well with these assumptions and the binding parameters given in Table II, assuming a maximal increase in fluorescence intensity of 7% for the native ATPase and for (C18:1)PC–ATPase. The data for (C14:1)PC–ATPase can be fitted by assuming binding to a single site with a binding constant of 7.7×10^5 with a 2.5% decrease in fluorescence intensity on binding Ca^{2+} (Figure 3).

It is important to establish that the effects seen on reconstitution with (C14:1)PC are fully reversible. Previously this has been done through kinetic measurements, demonstrating that exchange of the phospholipids on (C14:1)PC–ATPase for (C18:1)PC leads to full recovery of ATPase activity (Warren et al., 1974). It is also possible to use the fluorescence response

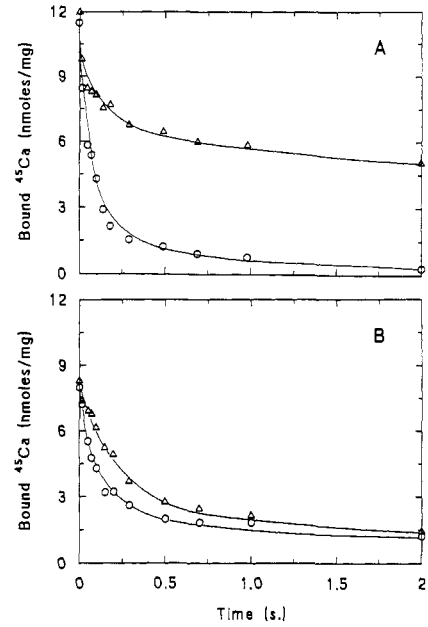

FIGURE 4: Rapid filtration measurements of the rate of calcium dissociation from (A) (C18:1)PC–ATPase and (B) (C14:1)PC–ATPase. The ATPase was incubated with $^{45}Ca^{2+}$ in a medium containing 15 μM $^{45}Ca^{2+}$, 5 mM Mg^{2+}, and 100 mM KCl, pH 7.2, 20 °C and 0.3 μg/mL A23187 was adsorbed on a filter and then perfused for the given times 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 (nmol/mg of protein) is plotted against perfusion time (s).

to Ca^{2+} and EGTA to demonstrate reversibility, as illustrated in Figure 2. In previous studies we have shown that high concentrations (2 mg/mL) of the detergent $C_{12}E_8$ can displace essentially all the phospholipids from around the ATPase (de Foresta et al., 1989). Addition of $C_{12}E_8$ to (C18:1)PC–ATPase incubated with a high (100 μM) concentration of Ca^{2+} results in a small increase in fluorescence intensity, and subsequent addition of EGTA results in a rapid decrease in intensity, comparable to that seen for (C18:1)PC–ATPase in the absence of $C_{12}E_8$ (Figure 2C), followed by a slower change attributable to irreversible denaturation in the presence of both EGTA and $C_{12}E_8$, as reported previously by Andersen et al. (1982). Addition of $C_{12}E_8$ to (C14:1)PC–ATPase incubated with Ca^{2+} results in a larger increase in fluorescence intensity (Figure 2D), consistent with the (C14:1)PC–ATPase being in a lower fluorescent state in the presence of Ca^{2+} than (C18:1)PC–ATPase. However, the decrease in fluorescence intensity observed on addition of EGTA is identical with that seen for (C18:1)PC–ATPase solubilized in the presence of $C_{12}E_8$ (Figure 2), indicating that comparable conformational states of the ATPase are obtained on displacing phospholipid from the ATPase when starting from either (C18:1)PC–ATPase or (C14:1)PC–ATPase; effects of (C14:1)PC on the ATPase are therefore reversible.

The time course of release of Ca^{2+} from the reconstituted ATPase was determined by using a rapid-filtration technique. For (C18:1)PC–ATPase, 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, at a rate twice that seen for the EGTA wash (Figure 4A). Very similar results are obtained for the native ATPase, in agreement with previously published reports (Dupont, 1984; Inesi, 1987). The results obtained with (C14:1)PC–ATPase are, however, distinctly different (Figure 4B). In this case, loss of $^{45}Ca^{2+}$ is essentially complete on washing with either EGTA or 1 mM $^{40}Ca^{2+}$, with the rate of

loss of $^{45}Ca^{2+}$ being slightly greater for washing with EGTA.

Discussion

The phospholipid composition of all biological membranes is complex, but it is not known whether this complexity is essential for the proper functioning of the cell. In reconstituted membrane systems it is possible to study, in detail, the effects of different phospholipids on the activity of a membrane protein, to establish the requirements of a particular protein for optimal activity, and to probe the molecular basis for this requirement. For (Ca^{2+}–Mg^{2+})-ATPase, such studies have shown that the optimum chain length for activity is C_{18} and that phospholipids with fatty acyl chains shorter than C_{16} or longer than C_{20} support low ATPase activities (Warren et al., 1974; Johannsson et al., 1981; Caffrey & Feigenson, 1981; East & Lee, 1982; Froud et al., 1986a). Previous studies have suggested that short- and long-chain phospholipids may have distinctly different effects on the ATPase, since the activity of the ATPase reconstituted with short-chain phospholipids can be increased by the addition of a wide variety of hydrophobic molecules, including androstenol, whereas such molecules have little effect on the activity of the ATPase reconstituted with long-chain phospholipids (Johannsson et al., 1981; Simmonds et al., 1982, 1984; Jones & Lee, 1985; Jones et al., 1985, 1986; Froud et al., 1986b; Michelangeli et al., 1989). Here we confirm this suggestion and demonstrate that reconstitution in the short-chain phospholipid (C14:1)PC alters a very fundamental feature of the ATPase, the stoichiometry of binding of Ca^{2+} to the ATPase, whereas in the long-chain phospholipid (C24:1)PC this stoichiometry is unaltered.

The data in Table I and Figure 1 show that binding of $^{45}Ca^{2+}$ to (C14:1)PC–ATPase is about half that to the native ATPase or to (C18:1)PC–ATPase, with a stoichiometry of binding close to 1:1 for (C14:1)PC–ATPase and 2:1 for the other systems. The decrease in binding for (C14:1)PC–ATPase cannot be attributed to denaturation of the ATPase on reconstitution with (C14:1)PC, since effects of (C14:1)PC–ATPase on the activity of the ATPase are reversible (Warren et al., 1974), as are effects on tryptophan fluorescence intensities (Fig. 2), and, in addition, maximal levels of phosphorylation of the ATPase by [γ-^{32}P]ATP are comparable for the native ATPase and for the ATPase reconstituted with either (C14:1)PC or (C18:1)PC (Table I). Binding of Ca^{2+} to native ATPase or to (C18:1)PC–ATPase (Figure 1) fits within experimental error to a cooperative binding scheme (Scheme I), with the binding parameters given in Table II taken from Froud and Lee (1986) but modified for the change in assumed value for the Ca^{2+}–EGTA binding constant (see Results). In contrast, binding of Ca^{2+} to (C14:1)PC–ATPase is consistent with binding to a single site with a slightly higher affinity of 7.7×10^5 (Figures 1 and 3). This effect of (C14:1)PC on Ca^{2+} binding is not observed if the ATPase is reconstituted with a 1:1 molar ratio of (C14:1)PC:androstenol, whereas androstenol has no effect on Ca^{2+} binding for the ATPase reconstituted with (C18:1)PC (Table I). The pCa dependence of tryptophan fluorescence shown in Figure 3 is consistent with the $^{45}Ca^{2+}$ binding data and the observation of a decrease in fluorescence intensity on addition of Ca^{2+} to (C14:1)PC–ATPase as opposed to the increase observed for the native ATPase and for (C18:1)PC–ATPase also confirms a major change in the nature of the Ca^{2+} binding process. Again, the tryptophan fluorescence response for the ATPase reconstituted with a 1:1 molar ratio of (C14:1)PC:androstenol is identical with that for the native ATPase (data not shown).

Despite the effect of (C14:1)PC on Ca^{2+} binding, (C14:1)PC–ATPase is phosphorylated by ATP in the presence of Ca^{2+} to give levels of phosphorylation comparable to those observed for the native ATPase (Table I). Further, although the rate of phosphorylation of (C14:1)PC–ATPase is less than that of the native ATPase (F. Michelangeli, P. Champeil, J. M. East, and A. G. Lee, unpublished observations), the rate of hydrolysis of ATP by (C14:1)PC–ATPase is only about a factor of 4 less than that of (C18:1)PC–ATPase or native ATPase (Froud et al., 1986b), implying that (C14:1)PC–ATPase with one bound Ca^{2+} adopts a conformation comparable to that of the native ATPase with two bound Ca^{2+} so that phosphorylation and dephosphorylation can occur.

The fluorescence experiment in Figure 3 suggests a slightly higher apparent affinity for (C14:1)PC–ATPase than for (C18:1)PC–ATPase (50% effects seen at pCa values of 5.9 and 5.5, respectively). This is consistent with previous studies of the effects of phospholipids on the E1/E2 equilibrium for the ATPase. Addition of Ca^{2+} to the native ATPase labeled with fluorescein isothiocyanate results in quenching of fluorescence (Pick, 1982; Froud et al., 1986b), attributable to the change to the Ca_2E1' form. For the labeled ATPase reconstituted with (C14:1)PC, the fluorescence quenching observed on addition of Ca^{2+} is considerably less than that observed for the native ATPase (1.4% compared to 5.6% at pH 7; Froud et al., 1986b), suggesting that even in the absence of Ca^{2+}, (C14:1)PC–ATPase is largely in a low-fluorescence form similar to Ca_2E1' for the native ATPase. In terms of the E1/E2 conformational scheme for the ATPase (de Meis & Vianna, 1979), it has been estimated that the fraction of the ATPase in the E1 form at pH 7 in the absence of Ca^{2+} changes from 0.3 for (C18:1)PC–ATPase to 0.8 for (C14:1)PC–ATPase (Froud & Lee, 1986); this 2.7-fold increase in the fraction of the ATPase in the E1 form would result in an increase in Ca^{2+} affinity for the ATPase corresponding to a shift in pCa value giving 50% occupation of the Ca^{2+} binding sites of 0.43 (log 2.7), comparable to the observed shift of 0.4.

Differences in the Ca^{2+} binding properties of (C18:1)PC–ATPase and (C14:1)PC–ATPase can also be demonstrated in measurements of the kinetics of release of Ca^{2+} from the ATPase. For (C18:1)PC–ATPase, 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 4A). These results are very similar to those observed for the native ATPase (Dupont, 1984; Inesi, 1987) and interpreted in terms of sequential binding of the two Ca^{2+} ions in a channellike structure (Inesi, 1987). For (C14:1)PC–ATPase, a high concentration of Ca^{2+} in the bathing medium has only a very slight inhibitory effect on release of Ca^{2+} (Figure 4B). In terms of a channellike model, this would be consistent with binding of Ca^{2+} to the innermost Ca^{2+} binding site in the channel with an affinity equal to that for binding to the same site in (C18:1)PC–ATPase but with very weak binding of Ca^{2+} to the second, outermost Ca^{2+} binding site. The relative lack of effect of a high bathing Ca^{2+} concentration on release of $^{45}Ca^{2+}$ from (C14:1)PC–ATPase compared to its large effect for (C18:1)PC–ATPase also helps confirm that effects seen for (C18:1)PC–ATPase follow from interactions between two Ca^{2+} ions bound within one ATPase molecule, rather than from any possible interactions between two ATPase molecules.

The effects of androstenol are worth further comment. Fluorescence quenching experiments with brominated phospholipids and a brominated derivative of androstenol suggest

that androstenol is excluded from the lipid–protein interface (annular sites) of the ATPase but that androstenol can bind to other (nonannular) sites, either at protein–protein interfaces between dimers or higher aggregates of the ATPase or within the ATPase molecule (F. Michelangeli, J. M. East, and A. G. Lee, unpublished observations). The reversal of the effects of (C14:1)PC by androstenol suggest a marked conformational flexibility for the ATPase with marked sensitivity to binding at the nonannular sites.

Registry No. ATPase, 9000-83-3; (C14:1)PC, 56750-90-4; (C18:1)PC, 4235-95-4; (C24:1)PC, 51779-96-5; Ca, 7440-70-2; androstenol, 12041-97-3.

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