ACKNOWLEDGMENTS

We thank Dr. J. Luber-Narod for helpful discussions, J. Hoogasian for excellent technical assistance, and J. Allardice and C. Rassias for preparing the manuscript.

Registry No. GTP, 86-01-1; SP, 33507-63-0; [Phe^8(pBz)]SP, 130409-05-1.

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Effects of Phospholipids on the Function of $(Ca^{2+}-Mg^{2+})$-ATPase†

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Received January 23, 1990; Revised Manuscript Received August 16, 1990

ABSTRACT: The ATPase activity for the $(Ca^{2+}-Mg^{2+})$-ATPase purified from rabbit skeletal muscle sarcoplasmic reticulum is lower when reconstituted into bilayers of dimyristoleoylphosphatidylcholine [(C14:1)PC] than when it is reconstituted into dioleoylphosphatidylcholine [(C18:1)PC]. The rate of formation of phosphoenzyme on addition of ATP is slower for (C14:1)PC-ATPase than for the native ATPase or (C18:1)PC-ATPase. The reduction in rate of phosphoenzyme formation is attributed to a reduction in the rate of a conformational change on the ATPase following binding of ATP but before phosphorylation. The level of phosphoenzyme formed from P_i is also less for (C14:1)PC-ATPase than for (C18:1)PC-ATPase. At steady state at pH 6.0 in the presence of ATP Ca^{2+} is released from (C18:1)PC-ATPase into the medium, but not from (C14:1)PC-ATPase. These effects of (C14:1)PC on the ATPase are reversed by addition of androstenol to a 1:1 molar ratio with (C14:1)PC. The results are interpreted in terms of a kinetic model for the ATPase.

Membrane proteins function in an environment defined in part by the phospholipid component of the biological membrane. It is known that the phospholipid composition of all biological membranes is complex, but it is not known whether or not this complexity is required for the proper function of the membrane [see Lee (1988)]. One approach to the problem is to purify membrane proteins and reconstitute them into phospholipid bilayers of defined composition, so allowing a

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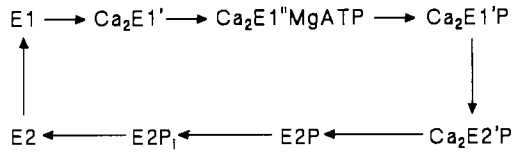
0006-2960/91/0430-342$02.50/0

Scheme I: Simplified Reaction Scheme for the ATPase



study of the effect(s) of phospholipid structure on protein function. Such studies with the (Ca^{2+}–Mg^{2+})-ATPase purified from the sarcoplasmic reticulum of skeletal muscle have shown that, for this protein, activity is sensitive to the structure of the phospholipid molecules surrounding it in the bilayer. The phospholipid supporting the 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., 1974b; Johannsson et al., 1981; Caffrey & Feigenson, 1981; East & Lee, 1982; Froud et al., 1986).

Effects of phospholipid on the activity of the ATPase can only be properly understood in terms of a comprehensive kinetic model for the ATPase. The kinetics of the ATPase may be interpreted in terms of a two-conformation model (de Meis & Vianna, 1979; Scheme I), modified in the light of later findings. It has been suggested that binding of Ca^{2+} to the ATPase is cooperative with a slow conformational change between binding of the first and second Ca^{2+} ions (Scheme II; Dupont, 1982; Champeil et al., 1983; Fernandez-Belda et al., 1984; Inesi, 1985; Froud & Lee, 1986a; Tanford et al., 1987; Inesi, 1987; Petithory & Jencks, 1988). We have suggested that a similar scheme applies to the dissociation of Ca^{2+} from the phosphorylated ATPase (Scheme III; Gould et al., 1986; Michelangeli et al., 1990b). Stahl and Jencks (1987) have shown the presence of a conformational change between the binding of MgATP to Ca_2E1' and phosphorylation which also has to be incorporated into the scheme (Scheme IV). It is also necessary to consider phosphorylation of the ATPase by CaATP, since, at high concentrations of Ca^{2+}, a significant proportion of the ATP will be present in this form. Nakamura et al. (1986), Lund and Moller (1988), and Orlowski et al. (1988) have shown that, at high concentrations of Ca^{2+}, dephosphorylation of the phosphorylated ATPase exhibits both fast and slow components, the proportion of the slow component increasing with increasing Ca^{2+} concentration attributable to binding of CaATP to the ATPase in competition with binding of MgATP. These effects can be simulated in terms of a simplified reaction scheme for phosphorylation following the binding of CaATP (Michelangeli et al., 1990b) (Scheme V).

Scheme II

$$E1 \rightarrow CaE1 \rightarrow CaE1' \rightarrow Ca_2E1'$$

Scheme III

$$Ca_2E2'P \rightarrow CaE2'P \rightarrow CaE2P \rightarrow E2P$$

Scheme IV

$$Ca_2E1' \rightarrow Ca_2E1'MgATP \rightarrow Ca_2E1''MgATP$$

Scheme V

$$Ca_2E1'CaATP \rightarrow Ca_2E1'PCa + ADP \rightarrow Ca_2E1'P + Ca^{2+}$$

Here we show that reconstitution of the ATPase into bilayers of the short chain phosphatidylcholine dimyristoleoylphosphatidylcholine ((C14:1)PC) has marked effects on the phosphorylation of the ATPase, distinct from those observed on reconstitution with the long chain phosphatidylcholine, dinervonylphosphatidylcholine ((C24:1)PC).

Materials and Methods

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

(Ca^{2+}–Mg^{2+})-ATPase was purified from the sarcoplasmic reticulum of skeletal muscle as described by East and Lee (1982). Reconstitutions of samples for steady-state measurements of ATPase activity were performed as described by East and Lee (1982). For pre-steady-state measurements requiring higher concentrations of protein, the reconstitution procedure was modified, as now described. Phospholipid (10 μmol) was mixed with buffer (400 μL; 10 mM Hepes/Tris, 15% sucrose, pH 8.0) containing $MgSO_4$ (5 mM) and potassium cholate (13 mg/mL) and sonicated to clarity in a bath sonicator (Megason). ATPase (1.25 mg) in a volume of 60 μL was then added and left for 45 min at 5 °C to equilibrate. The mixture was added to 10 mL of cold buffer (10 mM Hepes/Tris, pH 8.0) containing 2 mM dithiothreitol and spun at 200000*g* for 1 h. The pellet was rehomogenized in 200–300 μL of 10 mM Hepes/Tris, 15% sucrose, pH 7.0, to give a final protein concentration of 3–8 mg/mL. The sample was stored on ice until use. In experiments with androstenol, androstenol was added to the phospholipid at a 1:1 molar ratio of androstenol to phosphatidylcholine, prior to solubilization in the original cholate solution.

Steady-state measurements of enzyme phosphorylation by [^{32}P]P_i were carried out in 150 mM Mes/Tris, pH 6.2, containing 5 mM EGTA and the required concentrations of Mg^{2+} and P_i, at 25 °C, at a protein concentration of 0.33 mg/mL. After 20 s the reaction was quenched by addition of an equal volume of quenching solution (15% trichloroacetic acid, 0.2 M potassium phosphate). The precipitate was collected by filtration through Whatman GF/C glass fiber filters, washed with ice-cold quenching solution, and finally counted in Labscint. Controls were performed by first denaturing the ATPase with quench solution followed by addition of [^{32}P]P_i.

Steady-state measurements of enzyme phosphorylation by [γ-^{32}P]ATP were carried out in a medium containing 40 mM Hepes/Tris, 100 mM KCl, 5 mM $MgSO_4$, and 1 mM $CaCl_2$, pH 7.2, at 25 °C. The reaction was started by addition of the required concentration of ATP to the ATPase (0.1 mg/mL), followed by quenching after 10 s with ice-cold 20% trichloroacetic acid and 0.2 M phosphoric acid.

Steady-state measurements of Ca^{2+} release from the ATPase on addition of ATP were made by using Antipyralazo III as described by Champeil and Guillian (1986). Release of Ca^{2+} was followed by measuring the change in absorbance at 720–790 nm, using a Shimadzu UV3000 dual-wavelength spectrometer, at 25 °C. Samples were stirred with a "Cellspinbar" magnet (Bel-Art products) in the sample cuvette, and ATP was injected directly into the cuvette with a Hamilton syringe. ATPase (0.4 mg/mL) was added to buffer (150 mM Mes/Tris, pH 6.0, 20 mM $MgSO_4$) containing 100 μM Antipyralazo III. Aliquots of a concentrated solution of $CaCl_2$ were added to a final concentration of 50 μM to calibrate the signal. Five microliters of a concentrated solution of ATP was added to give a final ATP concentration of 40 μM, and measurements of Ca^{2+} released were corrected for the small dilution artifact observed on addition of 5 μL of buffer.

Measurements of the time dependence of Ca^{2+} release from the ATPase were performed by using a Biologic rapid filtration system, using a modification of the method described in

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

Champeil and Guillain (1986), as described in Wakabayashi et al. (1986). ATPase (50 μg) was mixed at 20 °C with 3 mL of buffer (20 mM Hepes/Tris, 100 mM KCl, 5 mM $MgSO_4$, pH 7.2, 15 μM $^{45}CaCl_2$, 100 μM [^{3}H]sucrose, and 2 mM ATP). The mixture was quickly adsorbed onto a Millipore HA filter, and, at a chosen preset time, the filter was perfused with the same buffer, but containing $^{40}CaCl_2$. The filter was then counted in a scintillation mixture. [^{3}H]Sucrose was included in the buffer to allow a determination of the wetting volume of the filter, from which the amount of Ca^{2+} trapped in the filter with water but not bound to the ATPase could be estimated (Champeil & Guillain, 1986). For experiments with the purified ATPase, the Ca^{2+} ionophore A23187 was added at 0.04 g of ionophore per gram of protein to render any sealed vesicles present in the preparation leaky to Ca^{2+}. For the reconstituted ATPase, preparations accumulated insignificant amounts of Ca^{2+} (Michelangeli et al., 1990c) so that addition of A23187 was not necessary.

The time dependence of phosphorylation of the ATPase by [γ-^{32}P]ATP at 25 °C was determined by using a Hi-Tech PQ-43 preparative quench flow system. ATPase (0.2 mg/mL) in buffer (20 mM Hepes/Tris, 100 mM KCl, 5 mM $MgSO_4$, 0.1 mM $CaCl_2$, pH 7.2) was mixed with an equal volume of [γ-^{32}P]ATP in buffer, followed by quenching with 2 mL of 1 M perchloric acid. The quenched samples were then mixed with 1 mL of 40% trichloroacetic acid and 0.2 M phosphoric acid and the precipitate was collected by filtration through Whatman GF/C glass fiber filters.

ATPase activity was measured by using a coupled enzyme assay. Samples (12 μL, equivalent to 6 μg of ATPase) were added to the medium described by East and Lee (1982), containing 40 mM Hepes/KOH, pH 7.2, 5 mM $MgSO_4$, 2.1 mM ATP, 1.01 mM EGTA, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, 7.5 IU of pyruvate kinase, and 18 IU of lactate dehydrogenase in a total volume of 2.5 mL, with $CaCl_2$ added to give the required free Ca^{2+} concentration. Free concentrations of Ca^{2+} were calculated by using the binding parameters given by Gould et al. (1986), except for those describing the binding of Ca^{2+} and Mg^{2+} to EGTA, for which the constants used by Petithory and Jencks (1988) were used, in order to facilitate comparison with their data.

For measurements of accumulation of Ca^{2+}, the ATPase was reconstituted into sealed vesicles as described by Gould et al. (1987). Phospholipid (10 mg) was dried onto the sides of glass tubes and then dispersed into buffer (600 μL, 0.4 M potassium phosphate, pH 7.4) by vortex mixing. An aliquot of 10% (w/v) potassium cholate in buffer (40 mM Hepes/KOH, pH 7.2, 100 mM KCl) was then added to give 1 mg of cholate per mg of phospholipid. The suspension was sonicated to clarity under nitrogen in a bath sonicator (Megason). An aliquot (3.8 μL) of a 10% solution of deoxycholate in buffer (40 mM Hepes/KOH, pH 8.0) was added to 0.6 mg of the ATPase (typically in 30 μL of buffer) to give a final deoxycholate to ATPase ratio of 0.6:1.0 (mg/mg). The mixture was vortexed for 5 min then spun at 10000*g* in a microfuge to remove any unsolubilized aggregates. The phospholipid and protein samples were then mixed to give a molar ratio of phospholipid:ATPase of 3000:1. The detergent was removed by two passages through Sephadex G-50, as described in Gould et al. (1987) to give sealed phospholipid vesicles containing the ATPase. In experiments with androstenol, androstenol was added to the phospholipid prior to solubilization in the original cholate solution.

Ca^{2+} accumulation by the reconstituted vesicles was followed by dual-wavelength spectrophotometry using arsenazo III to monitor the external Ca^{2+} concentration. Reconstituted vesicles were added to buffer (40 mM Hepes/Tris, pH 7.4, 100 mM KCl, 5 mM $MgSO_4$, 50 μM $CaCl_2$) at 25 °C containing arsenazo III (50 μM), to give a protein concentration of 25 μg/mL. Ca^{2+} uptake was initiated by addition of ATP to a final concentration of 0.5 mM. Uptake of Ca^{2+} was followed by measuring the change in absorbance at 675–685 nm, using a Shimadzu UV3000 dual-wavelength spectrophotometer. A calibration curve was established by addition of aliquots of known Ca^{2+} concentration to the samples prior to initiation of uptake. Uptakes were corrected for the small change in free Ca^{2+} observed on addition of ATP to buffer in the absence of protein (attributable to the binding of Ca^{2+} to ATP).

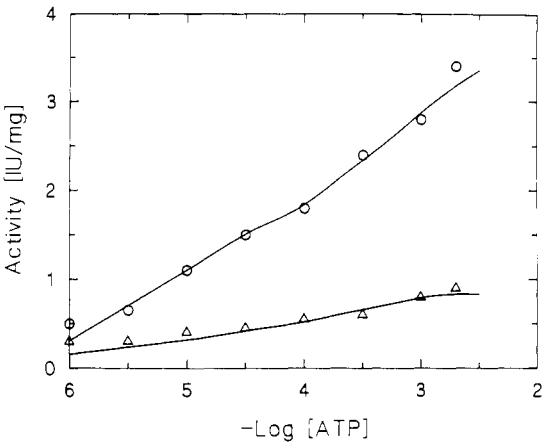


FIGURE 1: ATP concentration dependence of the activity (international units per milligram) of the native ATPase (○) and (C14:1)PC–ATPase (△) with K^+ = 16 mM, Mg^{2+} = 5.0 mM, and pCa = 4.7, pH 7.2, 25 °C. Lines represent simulations performed as described in the text, for an ATPase purity of 0.35 and an ATP stimulation factor of 8 for the native ATPase.

Concentrations of protein were estimated by using the extinction coefficient given by Hardwicke and Green (1974) or the modified Lowry procedure described by Peterson (1977). Kinetic simulations were carried out by using the FACSIMILE program (Chance et al., 1977), run on an IBM 3090 computer.

RESULTS

ATPase Activity. As shown in Figure 1 the dependence of ATPase activity on the concentration of ATP measured at maximally stimulating concentrations of Ca^{2+} is complex, with ATPase activity increasing with increasing concentrations of ATP in both the micro- and millimolar ranges. On reconstitution with (C14:1)PC, ATPase activity is reduced at all concentrations of ATP; the data provide no evidence for any marked reduction in the affinity of the ATPase for ATP. The effect of Ca^{2+} on the activity of the ATPase is also complex (Figure 2A), with low concentrations of Ca^{2+} activating the ATPase, attributable to binding to the Ca^{2+} binding sites in E1, and high concentrations of Ca^{2+} inhibiting the ATPase, attributable both to binding of Ca^{2+} to the phosphorylated ATPase (E2P) with subsequent decrease in the rate of phosphoenzyme decay and to the formation of CaATP, which is hydrolyzed more slowly by the ATPase than MgATP (Shigekawa et al., 1983; Yamada et al., 1986; Lund & Moller, 1988; Orlowski et al., 1988; Michelangeli et al., 1990b). For the native ATPase or for the ATPase reconstituted by dilution into buffer, as here, membrane fragments are formed that are unable to accumulate Ca^{2+}, so that possible effects on Ca^{2+}-leak pathways do not need to be considered. Effects of Ca^{2+} on the activity of the native ATPase and (C18:1)PC–ATPase are very similar (Figure 2A). The activity of

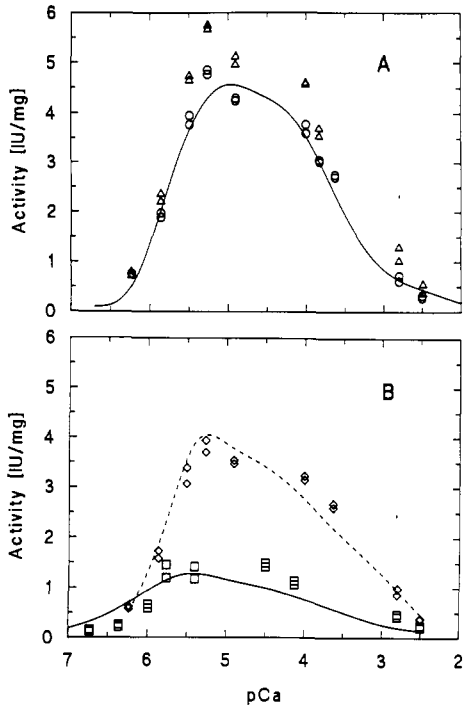


FIGURE 2: Dependence of ATPase activity on pCa measured at pH 7.2, Mg^{2+} = 5 mM, K^+ = 12 mM, ATP = 2.1 mM, and 25 °C. (A) native ATPase (○); (C18:1)PC–ATPase (△). (B) (C14:1)PC–ATPase (□); (C14:1)PC–ATPase + androstenol 1:1 (◇). The solid lines in (A) and (B) represent simulations of ATPase activity for native ATPase and (C14:1)PC–ATPase, respectively, performed as described in the text, for an ATPase purity of 0.5 and an ATP stimulation factor of 5 for the native ATPase.

(C14:1)PC–ATPase is affected by Ca^{2+} concentration over the same ranges that affect the activity of the native ATPase despite the lower activity for this system (Figure 2B). Addition of androstenol to (C14:1)PC–ATPase to a 1:1 molar ratio of androstenol to (C14:1)PC increases activity to values similar to those observed for (C18:1)PC–ATPase, again with a very similar Ca^{2+} dependence (Figure 2B). The data provide no evidence for any marked change in Ca^{2+} affinity being important for the low activity of (C14:1)PC–ATPase.

Plots of ATPase activity against Ca^{2+} concentration for the native ATPase are known to show positive cooperativity (de Meis, 1981). We have reported that least-squares fits to the Hill equation give values for the Hill coefficient n between 1.5 and 1.9, the value varying between preparations of the ATPase (Rooney & Lee, 1983). Figure 3 compares the Ca^{2+} dependence of ATPase activity for native and reconstituted ATPase, measured at 37 °C, at which temperature the higher rates allow more accurate rate determinations. Fits to the Hill equation give n values of 1.41 ± 0.07, 1.43 ± 0.08, and 1.14 ± 0.05 for native ATPase, (C18:1)PC–ATPase, and (C14:1)PC–ATPase, respectively.

In any analysis of ATPase activity, an important parameter is the proportion of the ATPase in the preparation in active form. This has been equated with the maximum possible level of phosphorylation observed with [γ^{32}-P]ATP under conditions (high Ca^{2+}) where the rate of dephosphorylation is low (Table I). For the purified ATPase, it has been shown that the maximal level of phosphorylation varies considerably between preparations, even for preparations that appear pure (>95%) on the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis; for our preparations of ATPase, levels of phosphorylation between 2.3 and 5 nmol/mg of protein are observed, corresponding to preparations containing a fraction of active ATPase between 0.26 and 0.58, respectively (Michelangeli et al., 1990b). It is shown in Michelangeli et al. (1990c) that the maximal level of phoshphorylation for any particular preparation of the ATPase is unchanged on reconstitution. Maximal levels of phosphorylation for the preparation of ATPase used to collect the data shown in Figure 2 observed after a 10-s incubation with high concentrations of ATP correspond to a purity of 0.5 (Table I). At lower concentrations of ATP, significant hydrolysis of ATP occurs during the incubation period, giving lower levels of phosphorylation when measured at 10 s. Maximal levels of phosphorylation are unaffected by reconstitution with (C14:1)PC or (C24:1)PC (Table I). For the preparation of ATPase used to collect the data shown in Figure 1, the maximal level of phosphorylation corresponded to a purity of 0.35 (data not shown).

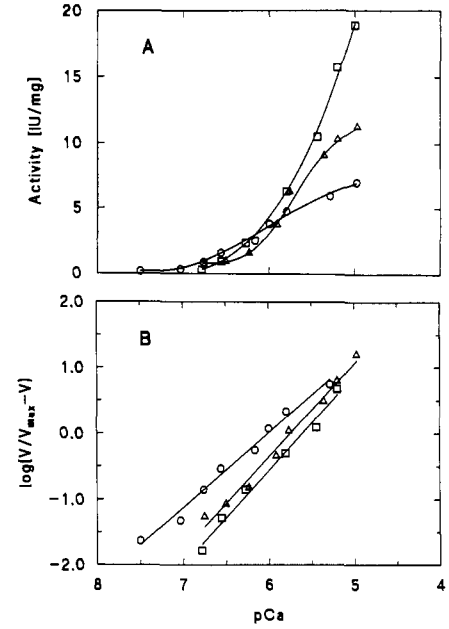


FIGURE 3: (A) Dependence of ATPase activity on pCa measured at pH 7.2, Mg^{2+} = 5 mM, K^+ = 12 mM, ATP = 2.1 mM, and 37 °C: native ATPase (△); (C18:1)PC–ATPase (□); (C14:1)PC–ATPase (○). (B) Hill plot of the data in (A), calculated by assuming maximal ATPase activities of 12, 19, and 7 IU/mg of protein for native ATPase, (C18:1)PC–ATPase, and (C14:1)PC–ATPase, respectively.

Table I: Phosphorylation of the ATPase with [γ-^{32}P]ATP[a]

ATP concn (μM)	level of phosphorylation (nmol/mg)					
	native		(C14:1)PC		(C14:1)PC + androstenol (1:1) exptl	(C24:1)-PC exptl
	exptl	calcd	exptl	calcd		
100	4.4	4.4	4.4	4.1	4.4	4.4
50	4.0	4.4	4.8	4.1		4.4
20	3.5	4.2	3.5	4.0	3.7	4.3
5	2.3	3.9	3.3	3.6	2.0	3.7
2.5	1.1	1.3	2.7	2.8	1.1	3.3
1	0.5	0.3	1.7	1.0	0.5	2.3

[a] ATPase (0.1 mg/mL) was incubated with the given concentration of [γ-^{32}P]ATP for 10 s in the medium given under Materials and Methods containing 1 mM Ca^{2+} at pH 7.2, 25 °C, before quenching the reaction. The calculated steady-state levels of phosphorylation were calculated as described in the text, for an ATPase purity of 0.5 and an ATP stimulation factor of 8.

Simulations of ATPase activity for the native ATPase in terms of Schemes I–V need to take into account the level of active ATPase in the preparation, as described above. It has also been observed that preparations of ATPase that have identical ATPase activities when measured at low (μM) concentrations of ATP can have different ATPase activities when measured at high (mM) concentrations of ATP. This

Table II: Kinetic Parameters Describing Phosphorylation of Native ATPase and (C18:1)PC–ATPase[a]

reaction	equilib const	forward rate const
$Ca_2E1'MgATP \rightleftharpoons Ca_2E1''MgATP$	0.5	700 s^{-1}
$Ca_2E1''MgATP \rightleftharpoons Ca_2E1'PMgADP$	1	5000 s^{-1}
$Ca_2E1'PMgADP \rightleftharpoons Ca_2E1'P + MgADP$	2.7×10^{-9}	0.4 s^{-1}
$Ca_2E1'PMgADP \rightleftharpoons Ca_2E1'PMg + ADP$	3.5×10^{-5} [b]	1300 s^{-1}
$Ca_2E1'P + Mg^{2+} \rightleftharpoons Ca_2E1'PMg$	6×10^6	[c]
$Ca_2E1'P \rightleftharpoons Ca_2E2'P$	0.03	[d]
$Ca_2E1'PCaATP$[e] $\rightleftharpoons Ca_2E1'PCa + ADP$	7×10^{-4}	700 s^{-1}
$Ca_2E1'P + Ca^{2+} \rightleftharpoons Ca_2E1'PCa$	21	6.2 M^{-1} s^{-1}

[a] Other rate constants given by Michelangeli et al. (1989a–c), Stefanova et al. (1987), and Froud and Lee (1986). [b] Value at pH 7.2; pH dependence of the equilibrium constant given by the pH dependence of binding of Mg^{2+} to ADP in free solution. [c] Set fast. [d] Set fast, equal for Mg^{2+} and MgATP bound and unbound forms, and independent of pH. [e] Binding constants and rates of binding for CaATP equal to 5× those for MgATP.

has been attributed to different levels of stimulation of the slow step CaE2'P → CaE2P by ATP (Gould et al., 1986; Michelangeli et al., 1990b); this is taken into account by treating the "ATP-stimulation factor" as a variable in the simulations. Figure 1 shows a simulation of ATPase activity as a function of ATP for the native ATPase, with the parameters given in Table II, other constants being given by Gould et al. (1986), Stefanova et al. (1987), and Michelangeli et al. (1990b) with the experimentally determined proportion of active ATPase and assuming an ATP-stimulation factor of 8. Figure 2 shows simulations of ATPase activity for the native ATPase and (C18:1)PC–ATPase as a function of Ca^{2+} concentration, for a different preparation of the ATPase of higher purity, with an ATP-stimulation factor of 5.

Phosphorylation of the ATPase. To help locate the slow step(s) for (C14:1)PC–ATPase responsible for the low ATPase activity, we measured the time course of phosphorylation of the ATPase by [γ^{32}-P]ATP. As shown in Figure 4A, the rate of phosphorylation of the native ATPase increases with increasing ATP concentration from 2 to 25 μM, and the level of phosphorylation increases slightly when the concentration of Ca^{2+} is increased from 100 μM to 1 mM. Simulations using the parameters in Table II match the experimental data well (Figure 4A). As shown in Figure 4B the rate of phosphorylation of (C14:1)PC–ATPase is considerably slower than that of the native ATPase; the level of phosphorylation is again increased when the concentration of Ca^{2+} is increased from 100 μM to 1 mM. Figure 5 shows that the rate of phosphorylation of (C18:1)PC–ATPase by 5 μM ATP is very similar to that observed for native ATPase and that addition of androstenol to (C14:1)PC–ATPase to a 1:1 molar ratio of androstenol to (C14:1)PC increases the rate of phosphorylation to that seen with (C18:1)PC–ATPase. For (C24:1)PC–ATPase, the steady-state level of phosphorylation of the ATPase appears to be somewhat less than for the other systems, possibly attributable to some denaturation of the ATPase during the reconstitution process, but the rate of phosphorylation is within a factor of 2 of that seen for (C18:1)PC (Figure 5) and does not exhibit the marked reduction observed for (C14:1)PC–ATPase (Figure 4B).

The ATPase can also be phosphorylated by P_i in the presence of Mg^{2+} and absence of external Ca^{2+} (de Meis, 1981; Froud & Lee, 1986b). Figure 6 shows equilibrium levels of phosphorylation of the native ATPase as a function of the concentration of P_i in the presence of 10 mM Mg^{2+} at pH 6.2. Very low levels of phosphorylation are observed for (C14:1)PC–ATPase, although addition of androstenol to a 1:1 molar ratio with (C14:1)PC increases levels of phosphorylation

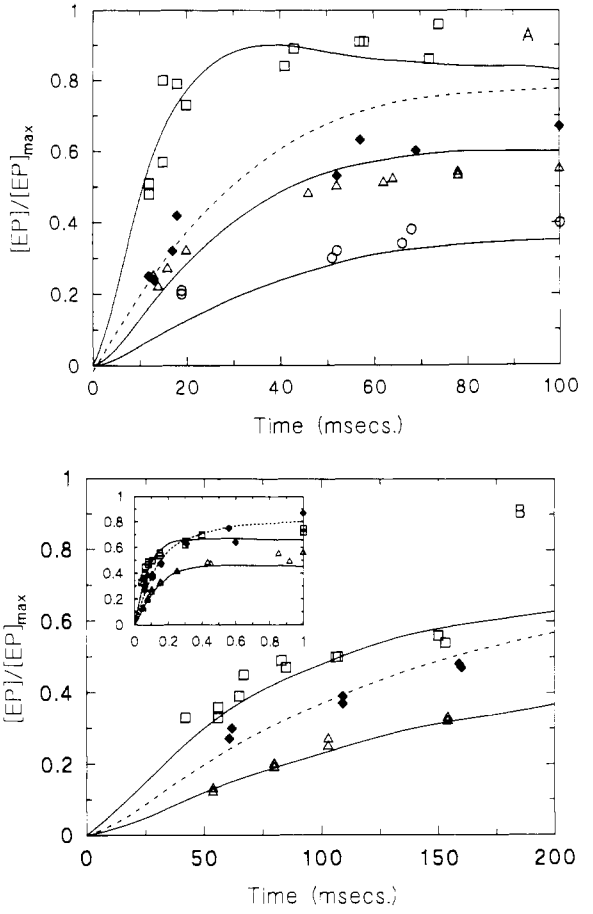


FIGURE 4: Rate of phosphorylation of (A) native ATPase or (B) (C14:1)PC–ATPase by [γ^{32}-P]ATP at pH 7.2, Mg^{2+} = 5 mM, K^+ = 100 mM, and 25 °C at Ca^{2+} concentrations of 0.1 mM (○, △, □) or 1 mM (◆) and ATP concentrations of 2 μM (○), 5 μM (△, ◆), and 25 μM (□). The final protein concentration was 0.1 mg/mL and EP_{max} was put equal to 2.8 nmol/mg of protein (corresponding to an ATPase purity of 0.33). The curves are simulations performed with the parameters in Table II as described in the text, with an ATP stimulation factor of 8 for the native ATPase. The insert in (B) shows the rate of phosphorylation ($[EP]/[EP]_{max}$) on a 1-s time scale.

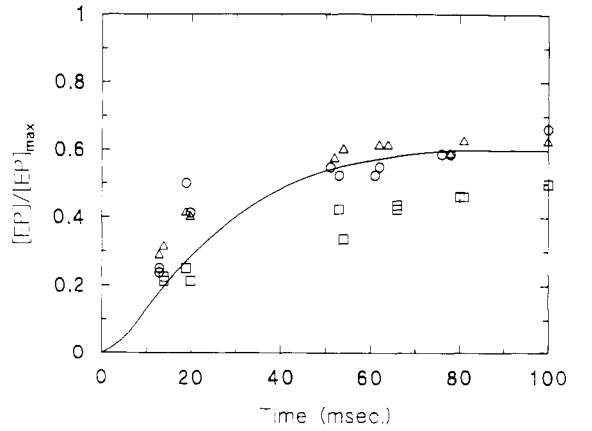


FIGURE 5: Rate of phosphorylation of the ATPase by 5 μM [γ^{32}-P]ATP at pH 7.2, Mg^{2+} = 5 mM, K^+ = 100 mM, and 25 °C at a Ca^{2+} concentration of 0.1 mM: (C18:1)PC–ATPase (○); (C14:1)-PC–ATPase + androstenol 1:1 (△); (C24:1)PC–ATPase (□). The final protein concentration was 0.1 mg/mL and $[EP]_{max}$ was put equal to 2.2 nmol/mg of protein (corresponding to an ATPase purity of 0.26). The curve is the simulation shown in Figure 3A for the native ATPase at 5 μM ATP and Ca^{2+} = 100 μM.

to close to those observed for the native ATPase. Levels of phosphorylation of (C24:1)PC–ATPase by P_i were unmeasurably low (data not shown). Phosphorylation of the ATPase

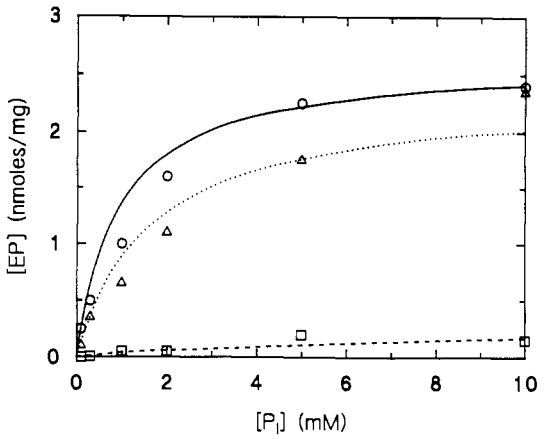
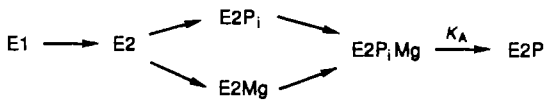


FIGURE 6: Equilibrium level of phosphorylation of the ATPase (nmol/mg of protein) by P_i at pH 6.2, Mg^{2+} = 10 mM, and 25 °C in the absence of K^+ and Ca^{2+}: (C14:1)PC–ATPase (□); (C14:1)-PC–ATPase + androstenol 1:1 (△); (C18:1)PC–ATPase (○). The solid line represents a simulation with the parameters given by Froud and Lee (1986b) and the broken and dotted lines represent simulations with the parameters given by Froud and Lee (1986b) except for the equilibrium constant for phosphorylation, which has been reduced by a factor of 3 0 [broken line, (C14:1)PC] and 2 [dotted line, (C14:1)PC + androstenol], and for the modified value for the equilibrium E1/E2 for (C14:1)PC–ATPase (see text). An ATPase purity of 0.6 was assumed for the simulations.

by P_i can be described by the following scheme (de Meis, 1981):



with effective equilibrium constants for each of the steps calculated as described by Froud and Lee (1986b). At pH 6.2 in the absence of K^+, the effective equilibrium constant for phosphorylation [K_A in Froud and Lee (1986)] is 14.3, and, as shown in Figure 6, this gives a good fit to the data obtained here for the native ATPase. For (C14:1)PC–ATPase, the effective increase in the equilibrium constant E1/E2 (Froud et al., 1986; Michelangeli et al., 1990c) would produce only a very small decrease in the level of phosphorylation expected under the conditions of Figure 6. However, the data for (C14:1)PC–ATPase is consistent with a 30-fold decrease in the equilibrium constant K_A (Figure 6). The effect of addition of androstenol to (C14:1)PC–ATPase can be simulated by assuming an increase in K_A to half that for the native ATPase (Figure 6).

Dissociation of Ca^{2+} from ATPase. Information about the steady-state balance between E1 and E2 (phosphorylated and nonphosphorylated) forms of the ATPase in the presence of ATP can be obtained from measurements of Ca^{2+} release from the ATPase, since the conversion of Ca_2E1' to E2P (Scheme I) will lead to an increase in the concentration of Ca^{2+} in the medium. Figure 7 shows experiments using Antipyralazo III to measure the external concentration of Ca^{2+}. On addition of ATP to (C18:1)PC–ATPase incubated in the presence of 50 μM Ca^{2+}, a distinct release of Ca^{2+} into the medium is observed, which reverses after hydrolysis of the ATP. The observed differences in the signal before addition of ATP and after its complete hydrolysis correspond to the effect of dilution. The Ca^{2+} release shown in Figure 7 corresponds to 5.5 nmol of Ca^{2+} released per milligram of protein (Table III). For (C14:1)PC–ATPase, no release of Ca^{2+} is observed after addition of ATP, the change in external Ca^{2+} concentration corresponding simply to the dilution that occurs on addition of the solution of ATP (Figure 7). For (C14:1)PC–ATPase

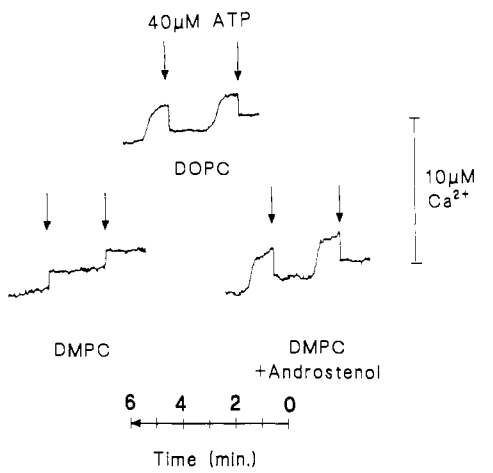


FIGURE 7: ATP-induced changes in the Ca^{2+} concentration in the medium following Ca^{2+} release from the ATPase, measured by using Antipyralazo III. ATPase (0.3 mg/mL) was suspended in Mes/Tris buffer (pH 6.0) containing 20 mM Mg^{2+}, 0.1 mM Antipyralazo III, and 50 μM Ca^{2+}. At times denoted by the arrows, 40 μM ATP was added in a volume of 5 μL. An upward deflection denotes an increase in the concentration of free Ca^{2+} in the medium. Following complete hydrolysis of the added ATP, the external Ca^{2+} concentration returns to a new equilibrium level, and the change in this equilibrium level equals the effect of dilution caused by addition of 5 μL of buffer.

Table III: Steady-State Release of Ca^{2+} from ATPase in the Presence of ATP[a]

system	Ca^{2+} released (nmol/mg of protein) exptl	calcd[b]
native	5.5	5.7
(C18:1)PC	5.9	5.7
(C14:1)PC	0	0
(C24:1)PC	0.4	
(C18:1)PC + androstenol (1:1)	5.9	
(C14:1)PC + androstenol (1:1)	5.6	

[a] ATPase was incubated at pH 6 in 20 mM Mg^{2+} and 50 μM Ca^{2+}, and the Ca^{2+} released on addition of 40 μM ATP was measured as described in the caption of Figure 7. [b] Calculated by using the parameters given in Tables II, III, and V, assuming an ATPase purity of 0.5 and an ATP stimulation factor of 8.

plus androstenol, however, Ca^{2+} release is comparable to that for (C18:1)PC–ATPase (Figure 7, Table III).

The time course of release of Ca^{2+} from the ATPase was determined by using a rapid-filtration technique. For the native ATPase, release of Ca^{2+} from the ATPase following addition of ATP gives the rate of the $Ca_2E1P \rightarrow E2P$ step (Champeil & Guillain, 1986). For (C14:1)PC–ATPase, however, this technique will not work, since no Ca^{2+} is released on addition of ATP (Figure 7, Table III). We therefore adopted an alternative approach (Wakabayashi et al., 1986) in which the ATPase incubated with $^{45}Ca^{2+}$ and ATP was adsorbed onto filters and then perfused for fixed times with $^{40}Ca^{2+}$ and ATP. As shown in Figure 8, rapid release of $^{45}Ca^{2+}$ from (C14:1)PC–ATPase is observed under these conditions. The initial level of $^{45}Ca^{2+}$ bound to the ATPase has a stoichiometry of close to 1:1 with respect to active ATPase, in agreement with our previous observations (Michelangeli et al., 1990c). In the presence of androstenol at a 1:1 molar ratio with (C14:1)PC, initial Ca^{2+} binding is close to 2:1 with respect to active ATPase and the rate of release of $^{45}Ca^{2+}$ is somewhat slower (Figure 8).

Accumulation of Ca^{2+}. If the ATPase is reconstituted at a high molar ratio of phospholipid to protein by the column method, then sealed vesicles are formed that can accumulate Ca^{2+} from the external medium on addition of ATP. In the

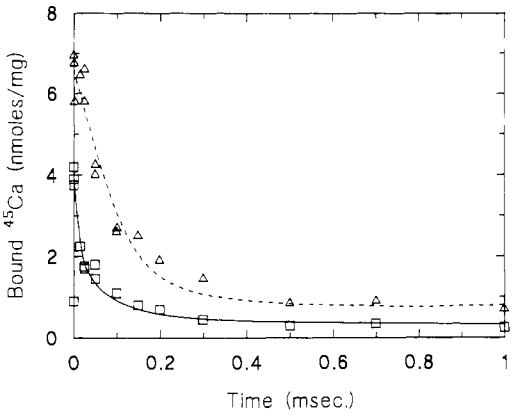


FIGURE 8: Rate of dissociation of Ca^{2+} measured by rapid filtration for phosphorylated (C14:1)PC–ATPase (□) and (C14:1)PC–ATPase + androstenol 1:1 (△), measured in a medium containing 15 μM Ca^{2+}, 5 mM Mg^{2+}, 2.0 mM ATP, and 100 mM KCl, pH 7.2, 20 °C. ATPase, 50 μg, incubated in the above medium with $^{45}Ca^{2+}$ was adsorbed on a filter and then perfused for the given times with the same medium but containing $^{40}Ca^{2+}$. The level of $^{45}Ca^{2+}$ bound to the ATPase (nmol/mg of protein) is plotted against perfusion time. For (C14:1)PC–ATPase, the solid line represents a simulation with the parameters in Table I, assuming an ATPase purity of 0.5.

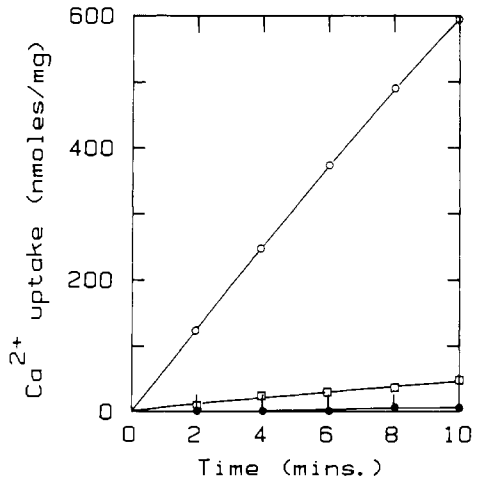


FIGURE 9: Accumulation of Ca^{2+} (nmol/mg of protein) by vesicles reconstituted with ATPase and (C18:1)PC (□), (C14:1)PC (●); and (C14:1)PC + androstenol (○). Vesicles were reconstituted in the presence of 0.4 M potassium phosphate (pH 7.4) at a molar ratio of lipid to ATPase of 3000:1. For vesicles containing androstenol, the molar ratio of androstenol to phospholipid was 1:1. Calcium accumulation was measured at 25 °C in a medium containing 40 mM Hepes/Tris, 100 mM KCl, 5 mM $MgSO_4$, 50 μM $CaCl_2$, and 50 μM arsenazo III, pH 7.4. Accumulation of Ca^{2+} was initiated by addition of 0.5 mM ATP.

absence of a Ca^{2+}–precipitating agent, levels of accumulated Ca^{2+} are very small, but these become higher if the vesicles are reconstituted in the presence of 0.4 M phosphate (Navarro et al., 1984; Gould et al., 1987). As shown in Figure 9, Ca^{2+} uptake can be measured at 25 °C for the ATPase reconstituted into vesicles of (C18:1)PC, whereas for the ATPase reconstituted into vesicles of (C14:1)PC uptake is unmeasurable. Reconstitution with a 1:1 molar ratio of androstenol and (C14:1)PC, however, results in a marked increase in the level of accumulation.

Discussion

The ATPase activity of the (Ca^{2+}–Mg^{2+})-ATPase is dependent on the structure of the phospholipids surrounding it in the membrane; the activity of the ATPase reconstituted into bilayers of a phospholipid such as (C14:1)PC with short fatty acyl chains or (C24:1)PC with long fatty acyl chains is less than that in (C18:1)PC, the phospholipid of optimal chain length (Johannsson et al., 1981; Caffrey & Feigenson, 1981; East & Lee, 1982). Despite the marked effects of phospholipid structure on the activity of the ATPase, the relative binding constants for phospholipids to the ATPase are almost independent of chain length (Caffrey & Feigenson, 1981; East & Lee, 1982; Froud et al., 1986), arguing against structural changes involving large changes in free energy on reconstitution of the ATPase into bilayers of (C14:1)PC or (C24:1)PC. Further, it is clear that effects of short- and long-chain phospholipids on the structure of the ATPase must be different, since the effect of (C14:1)PC on ATPase activity can be reversed by addition of hydrophobic molecules such as androstenol, whereas addition of such molecules has little effect on the activity of the ATPase reconstituted with (C24:1)PC (Simmonds et al., 1982, 1984; Michelangeli et al., 1989).

Table IV: Kinetic Parameters Changed for the (C14:1)PC–ATPase System

reaction	equilib const	forward rate const
$E1 \rightleftharpoons E1'$	9[a]	*b*
$E1' + Ca^{2+} \rightleftharpoons CaE1''$[c]	5.7×10^7	1.8×10^9 M^{-1} s^{-1}
$E1'H + Ca^{2+} \rightleftharpoons CaE1''H$[c]	2.3×10^6	7.4×10^7 M^{-1} s^{-1}
$E1'H_2 + Ca^{2+} \rightleftharpoons CaE1''H_2$[c]	1.2×10^6	3.8×10^7 M^{-1} s^{-1}
$CaE1'MgATP \rightleftharpoons CaE1''MgATP$	0.02	28 s^{-1}
$CaE1'P \rightleftharpoons CaE2'P$	0.002	*b*
$E2P' + Ca^{2+} \rightleftharpoons E2'PCa$	*d*	*d*
$E2'P \rightleftharpoons E2 + P_i$	*e*	*e*

[a] From Michelangeli et al. (1989d). [b] Set fast. [c] Parameters same as for binding of the second Ca^{2+} to native ATPase (Michelangeli et a., 1989d). [d] Parameters equal to those for dissociation of the first Ca^{2+} from $E2PCa_2$ for native ATPase (Michelangeli et al., 1989c). [e] Equilibrium constant for phosphorylation reduced by a factor of 30 from that for the native ATPase; other parameters as given by Froud and Lee (1986b).

The reconstituted ATPase used in the experiments reported here was prepared by exchange of the phospholipids around the ATPase with excess endogenous phospholipid in cholate, followed by dilution into assay buffer (East & Lee, 1982). It is important to establish complete mixing and substitution of phospholipids in this process. Such an analysis has been reported previously (Warren et al., 1974a,b). Complete substitution is also confirmed by the data reported here. Thus, given our observation that (C14:1)PC–ATPase can only bind a single Ca^{2+} ion compared to the two ions bound by native ATPase (Michelangeli et al., 1990c), it might have been thought that (C14:1)PC–ATPase would be unable to hydrolyze ATP, so that the observation that on reconstitution of the ATPase with (C14:1)PC an ATPase activity was observed ca. 25% of that for the native ATPase (Figure 1) would imply that 25% of the ATPase molecules in the sample were not reconstituted with (C14:1)PC. However, it is also observed that on reconstitution of the ATPase with (C14:1)PC, the level of phosphorylation of the ATPase by P_i decreases by a factor of ca. 20 (Figure 6), inconsistent with any such interpretation and implying that the proportion of any unreconstituted ATPase in the preparation cannot be greater than ca. 5%.

In previous publications we have established a consistent kinetic model for the ATPase (Gould et al., 1986; Stefanova et al., 1987; Michelangeli et al., 1990b). We show here that it is possible to simulate the kinetic properties of the ATPase reconstituted with (C14:1)PC in terms of discrete changes in that model resulting from the change in phospholipid structure. All simulations reported here for (C18:1)PC–ATPase have used the parameters reported by Gould et al. (1986), by Stefanova et al. (1987), by Michelangeli et al. (1990b), and in Table II, and all simulations for (C14:1)PC–ATPase have used these same parameters except for those modified as listed in Table IV.

In Michelangeli et al. (1990c) we showed that the binding stoichiometry of (C14:1)PC–ATPase was one Ca^{2+} per ATPase molecule rather than the normal two Ca^{2+} per ATPase molecule observed for the native ATPase or for the ATPase reconstituted with (C18:1)PC or (C24:1)PC. We also showed that addition of androstenol to (C14:1)PC–ATPase returned the binding of Ca^{2+} to the normal 2:1 stoichiometry. The affinity of (C14:1)PC–ATPase for Ca^{2+} was comparable to that for the second Ca^{2+} binding to CaE1′ in the normal ATPase (Scheme II), and we showed that this was consistent with the formation of a conformation E1′ for (C14:1)PC–ATPase whose affinity for Ca^{2+} was the same as that of CaE1′ in the native ATPase, according to Scheme VI (Michelangeli et al., 1990c). The Ca^{2+} binding data were consistent with a value of K_2 of 9 for (C14:1)PC–ATPase, K_1 being the same for native ATPase and for (C14:1)PC–ATPase (Michelangeli et al., 1990c). Figure 2 shows the dependence of ATPase activity on the concentration of Ca^{2+} for (C14:1)PC–ATPase, (C14:1)PC–ATPase plus androstenol, and (C18:1)PC–ATPase. For activity data at 37 °C, Hill plots of ATPase activity in the pCa range 7.5–5 give cooperativity parameters n of 1.14 ± 0.05, 1.41 ± 0.07, and 1.43 ± 0.008 for (C14:1)PC–ATPase, native ATPase, and (C18:1)PC–ATPase, respectively (Figure 3). The cooperativity parameters for the latter two systems are comparable to values we have reported previously (Rooney & Lee, 1983) and the cooperativity parameter close to 1 for the former system is consistent with the suggested binding of a single Ca^{2+} ion to (C14:1)PC–ATPase (Michelangeli et al., 1990c). Concentrations of Ca^{2+} giving half-maximal rates are calculated to be 1.0, 1.7, and 2.5 μM, respectively, consistent with the slightly higher affinity for Ca^{2+} reported for (C14:1)PC–ATPase (Michelangeli et al., 1990c).

Scheme VI

$$E2 \xrightarrow{K_1} E1 \xrightarrow{K_2} E1' \rightarrow CaE1''$$

It is not possible to change the stoichiometry of Ca^{2+} binding to the ATPase without considerable changes elsewhere in the reaction cycle, because the product of the equilibrium constants around the ATPase cycle must be constant (being equal to the equilibrium constant for the hydrolysis of ATP). Thus for the native ATPase at pH 7.2, the product of the binding constants of Ca^{2+} to E1 is 4×10^{11} (Michelangeli et al., 1990b,c) and the ratio of the binding constants of E1 to Ca^{2+} to the binding constants for the phosphorylated form E2P to Ca^{2+} is 6×10^7 (Michelangeli et al., 1990b,c). For (C14:1)PC–ATPase, the binding constant for Ca^{2+} is 9.2×10^5 at pH 7.2. (Michelangeli et al., 1990c). With an equilibrium constant K_2 of 9, if the only other equilibrium constant to change for (C14:1)PC–ATPase is that for binding of Ca^{2+} to the phosphorylated intermediate, then that binding constant would have to be 0.1 M, a seemingly unrealistic value for binding of Ca^{2+} to what is presumably a negatively charged site. It seems more likely that other equilibria in the reaction cycle are also modified.

Comparison of the rate of phosphorylation of the native ATPase by ATP (Figure 4A) with that for (C14:1)PC–ATPase (Figure 4B) shows that the rate of formation of phosphoenzyme from Ca^{2+}-bound ATPase is much slower for (C14:1)PC–ATPase than for native ATPase. The increase in the rate of phosphorylation with increasing concentration of ATP is comparable for native ATPase and (C14:1)PC–ATPase, showing that the low rate of phosphorylation for (C14:1)PC–ATPase cannot be attributed to a reduced affinity for ATP for (C14:1)PC–ATPase; the dependence of ATPase activity on ATP concentration (Figure 1) is also inconsistent with a marked reduction in ATP affinity for (C14:1)PC–ATPase. If it is assumed that phosphorylation of (C14:1)PC–ATPase by ATP follows a pathway comparable to that for the native ATPase, despite the changed stoichiometry of binding of Ca^{2+}, then phosphorylation of (C14:1)PC–ATPase can be written by analogy with Scheme IV as

$$CaE1'' \rightarrow CaE1'MgATP \rightarrow CaE1''MgATP \rightarrow CaE1'PMgADP$$

A slow rate of phosphorylation could follow from either a slowing of the conformational change CaE1′MgATP → CaE1″MgATP or a slowing of phosphorylation. Simulations show that, with a slow rate of phosphorylation, the effect of Ca^{2+} concentration on the level of phosphoenzyme is more marked than is observed experimentally, whereas, for a slowing of the conformational change, the change in level of phosphoenzyme with increasing Ca^{2+} concentration matches that observed experimentally (Figure 4B). The experimental data are consistent with the rate of the conformational change being 25-fold slower for (C14:1)PC–ATPase than for native ATPase (Table IV). As shown in Figure 5, this effect of (C14:1)PC on the rate of phosphoenzyme formation is reversed by addition of androstenol at a 1:1 molar ratio with lipid. The data in Figure 5 also shows that (C24:1)PC has little effect on the rate of phosphoenzyme formation.

Measurements of the equilibrium levels of phosphorylation of the ATPase by P_i also show marked effects of (C14:1)PC on phosphorylation of the ATPase. The experimental data are consistent with a 30-fold decrease in the equilibrium constant for phosphorylation of the ATPase on reconstitution with (C14:1)PC, the effect again being largely reversed by addition of androstenol (Figure 6). Reconstitution with (C24:1)PC has even more marked effects on phosphorylation by P_i than reconstitution with (C14:1)PC, levels of phosphoenzyme being unmeasurably low (data not shown), probably implying an even more marked change in the equilibrium constant for phosphorylation.

Slowing the CaE1′MgATP → CaE1″MgATP conformational change would be expected to lead to a build up of CaE1′MgATP for (C14:1)PC–ATPase at steady state in the presence of Ca^{2+} and ATP. This can be detected by measurement of Ca^{2+} release from the ATPase incubated with Ca^{2+} on addition of ATP (Figure 7). As shown in Figure 7, addition of ATP to (C18:1)PC–ATPase incubated with Ca^{2+} leads to a release of 5.9 nmol of Ca^{2+}/mg of protein (Table III), attributable to the formation of E2P from Ca_2E1'. For an ATPase preparation containing 50% active protein, a steady-state release of 5.7 nmol Ca^{2+}/mg of protein is calculated from the parameters given in Tables II and by Michelangeli et al. (1990b,c) (Table III). For (C14:1)PC–ATPase, no Ca^{2+} release is observed on addition of ATP to the ATPase incubated in Ca^{2+}, compatible with a marked buildup of CaE1′MgATP under these conditions at pH 6 (Table III). Again, the effect of (C14:1)PC is reversed by addition of androstenol, whereas addition of androstenol to (C18:1)PC–ATPase has no effect on Ca^{2+} release (Table III).

For the native ATPase, dissociation of Ca^{2+} from $Ca_2E2'P$ and the conformation change CaE2′P → CaE2P are slow and partially rate-controlling (Michelangeli et al., 1990b). We therefore measured the rate of dissociation of Ca^{2+} from the phosphorylated intermediate of (C14:1)PC–ATPase to see whether this step had been slowed compared to the corresponding step for the native ATPase (the dissociation of the first Ca^{2+} from $Ca_2E2'P$). (C14:1)PC–ATPase was incubated with $^{45}Ca^{2+}$ and ATP and allowed to reach steady state before being adsorbed onto a filter and perfused with $^{40}Ca^{2+}$ and ATP (Figure 8). Under the conditions of this experiment, simu-

lations suggest that (C14:1)PC–ATPase will be present largely as a mixture of CaE1′MgATP, CaE1′P, and CaE1′PMgATP. The rate of loss of $^{45}Ca^{2+}$ will then reflect both the rate of dissociation to the outside of Ca^{2+} from CaE1′MgATP (which is predominant under these conditions) and the rate of dissociation of Ca^{2+} from CaE2P′ and CaE2P′MgATP formed following the CaE1′P → CaE2′P and CaE1′PMgATP → CaE2′PMgATP transitions. As shown in Figure 8 the experimental data can be simulated well by assuming that the rates of these steps are unchanged from those of the corresponding steps for the native ATPase (Table IV). Also as shown in Figure 8, addition of androstenol to (C14:1)PC–ATPase increases the stoichiometry of Ca^{2+} binding to two Ca^{2+} per ATPase molecule, as reported elsewhere (Michelangeli et al., 1990c), and results in a reduced rate of Ca^{2+} release, to a rate comparable to that observed for the native ATPase (Champeil & Guillain, 1986).

Slowing the conformational change CaE1′MgATP → CaE1″MgATP is not sufficient to explain the experimentally observed slow rate of ATP hydrolysis for (C14:1)PC–ATPase (Figure 1). However, the experimentally determined ATPase activities can be simulated if it is assumed that the rate of the back-reaction CaE2′P → CaE1′P is increased 15-fold, corresponding to a 15-fold decrease in the equilibrium constant CaE2′P/CaE1′P (Figure 1).

We also studied Ca^{2+} uptake for the ATPase reconstituted into sealed vesicles containing a high internal concentration of phosphate as precipitating agent (Navarro et al., 1984; Gould et al., 1987). As shown in Figure 9, appreciable levels of Ca^{2+} accumulation are observed under these conditions for the ATPase reconstituted into vesicles of (C18:1)PC but not for the ATPase reconstituted into vesicles of (C14:1)PC. Reconstitution with a 1:1 molar ratio of androstenol to (C14:1)PC results in very much greater levels of uptake, comparable to those observed previously on reconstitution with phosphatidylethanolamines (Navarro et al., 1984; Gould et al., 1987). Net accumulation of Ca^{2+} in these experiments is a balance between uptake and leak, the leak rate being relatively high and dependent on phospholipid structure (Gould et al., 1987). The immeasurable levels of Ca^{2+} accumulation for the ATPase reconstituted with (C14:1)PC could reflect a low rate of Ca^{2+} influx combined with a high rate of efflux.

All the kinetic data we have reported here can be interpreted in terms of the change in binding stoichiometry for (C14:1)-PC–ATPase to one Ca^{2+} per ATPase molecule reported in Michelangeli et al. (1990c), with ATP hydrolysis proceeding by the normal pathway (Scheme I) but with a reduced rate for the conformational change CaE1′MgATP → CaE1″MgATP, a reduction in the equilibrium constant CaE2′P/CaE1′P, and a reduction in the equilibrium constant for phosphorylation (Table IV). To be thermodynamically consistent it is, however, necessary to introduce at least one more conformational change for (C14:1)PC–ATPase. For the native ATPase, the set of equilibrium constants used in the simulations reported here (Table II; Gould et al., 1986; Stefanova et al., 1987; Michelangeli et al., 1990b) give a free energy change $\Delta G°$ of −6.76 kcal/mol for the reaction $MgATP^{2-} \rightleftharpoons MgADP^{-} + H_2PO_4^{-}$. This can be compared to the value of −6.95 kcal/mol calculated from the experimental data of Rosing and Slater (1972) and the Mg^{2+}-binding constants given by Fabiato and Fabiato (1979). For (C14:1)PC–ATPase, the product of the equilibrium constants (Table IV) around the cycle gives a value for $\Delta G°$ of −1 kcal/mol, and the product of equilibrium constants needs to be increased by a factor of 8.4×10^3 to match that for the native ATPase. This can most easily be accommodated by introducing a new conformational change for (C14:1)PC–ATPase following loss of Ca^{2+} from the phosphorylated ATPase and before dephosphorylation, with no change in the actual equilibrium constant for phosphoenzyme formation from P_i:

$$CaE2'P \rightarrow E2'P \rightarrow E2P \rightarrow E2P_i \rightarrow E2$$

with the value of the equilibrium constant E2P/E2′P being 2.6×10^5. If the rate of this conformation change were fast, then simulations show that it would affect none of the simulations presented above for (C14:1)PC–ATPase. The new conformational change could be analogous to that following binding of MgATP to Ca_2E1'. Stahl and Jencks (1987) have suggested that this conformational change could correspond to a relocation of the nucleotide binding and phosphorylation domains on the ATPase, bringing the γ-phosphate of ATP close to the aspartate to be phosphorylated on the ATPase. The new conformational change could then be the reverse of this process for the phosphorylated form E2P, corresponding to a separation of the two domains before dephosphorylation. In terms of such a model, for the native ATPase formation of E2′P would be favored following phosphorylation with P_i, leading to a higher level of phosphorylation for the native ATPase than for (C14:1)PC–ATPase, for which E2P would be favored. For the native ATPase, the effective equilibrium constant for phosphorylation would be increased by a factor of $(1.0 + K)$ by introduction of the E2P → E2′P change, where K = E2′P/E2P. The reduction in the effective equilibrium constant for phosphorylation of the ATPase by P_i by a factor of 30 following reconstitution with (C14:1)PC (Table IV) would be consistent with a value for K of 29 for the native ATPase and ca. 10^{-5} $[1.0/(2.6 \times 10^5)]$ for (C14:1)PC–ATPase with no change in the equilibrium constant for the step $E2P_iMg$ → E2PMg. Dupont and Pougeois (1983) have suggested that on formation of the phosphorylated intermediate from native ATPase and P_i, the hydrophobicity of the nucleotide-binding site increases significantly, consistent with the proposal made here of a domain movement bringing the phosphorylation and nucleotide-binding domains together.

In a study of the inhibition of the ATPase by nonylphenol, we found that nonylphenol strongly inhibited the E2 → E1 transition and that this became the major slow step responsible for the low ATPase activity in the presence of nonylphenol (Michelangeli et al., 1990a). The observations presented here show a very different pattern of inhibition on reconstitution with (C14:1)PC and suggest considerable conformational flexibility for the ATPase.

Acknowledgments

We thank Stephane Orlowski and Phillipe Champeil for their help with the experiment reported in Figure 8 and for much stimulating discussion.

Registry No. ATPase, 9000-83-3; ATP, 56-65-5; (C14:1)PC, 18194-24-6; (C18:1)PC, 4235-95-4; (C24:1)PC, 51779-96-5; Ca, 7440-70-2; androstenol, 12041-97-3; phosphate, 14265-44-2.

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