Separate effects of long-chain phosphatidylcholines on dephosphorylation of the Ca^{2+} -ATPase and on Ca^{2+} binding

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The steady-state activity of the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum (SR) is low when reconstituted into bilayers of the long-chain phosphatidylcholines dierucyl phosphatidylcholine [di(C22:1)PC] or dinervonyl phosphatidylcholine [di(C24:1)PC]. In di(C24:1)PC the ATPase binds a single Ca^{2+} ion, whereas in di(C22:1)PC it binds two, as in the native SR [Starling, East and Lee (1993) *Biochemistry* **32**, 1593–1600]. In di(C22:1)PC, rates of phosphorylation of the ATPase by ATP and the rate of ATP-induced Ca^{2+} dissociation are slightly lower than in the native ATPase. However, a much more marked decrease is observed in di(C22:1)PC in the rate of

dephosphorylation of the phosphorylated ATPase, which explains the low steady-state ATPase activity. The level of phosphorylation of the ATPase by P_i was little affected by reconstitution in di(C22:1)PC, suggesting that the rate of phosphorylation by P_i is also decreased. The very similar effects of di(C22:1)PC and di(C24:1)PC (Starling, East and Lee (1995) *Biochem. J.* **310**, 875–879) on phosphorylation and dephosphorylation suggest that changes in these steps and the change in Ca^{2+} binding stoichiometry observed in di(C24:1)PC represent independent changes on the ATPase.

INTRODUCTION

Chemical labelling and antibody studies have suggested that the Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum (SR) contains ten transmembrane α -helices [1–4], consistent with the low-resolution structure for the ATPase derived from cryo-electron microscopic images [5]. One face of most of the helices is relatively highly conserved throughout the endoplasmic reticulum/SR family of Ca^{2+} -ATPases, whereas the amino acid residues on the other face are highly variable, although always hydrophobic [6]. The helices can be arranged as a bundle with the non-conserved, hydrophobic residues making up most of the outer surface of the bundle. It is presumed that the bulk phospholipids of the membrane interact with this surface [6].

A membrane protein has a distinct hydrophobic thickness that is determined by the length and orientations of its transmembrane α -helices. The lipid bilayer will also have an equilibrium thickness that is determined by the length of the lipid fatty acyl chains and by physical parameters of the system such as temperature. In a biological membrane, the hydrophobic thicknesses of the membrane proteins and of the lipid bilayer have to match because of the high energetic cost of exposing hydrophobic regions of the protein or the bilayer to water. One possible solution when the thicknesses do not match is for the lipid fatty acyl chains to distort around the protein (thinning or thickening) with relatively little change in the membrane protein [7]. A consequence of the required distortion of the fatty acyl chains is that the energy of interaction between a protein and a lipid with too short or too long fatty acyl chains will be less favourable than that for a lipid whose fatty acyl chain length exactly matches the requirement of the protein [7]. However, the Ca^{2+} -ATPase binding constants for phosphatidylcholines have been shown to vary little with fatty acyl chain length [8]. This suggests that rather than the fatty acyl

chains distorting around a relatively rigid membrane protein, the ATPase is able to undergo a rather facile distortion to match the thickness of the bilayer. This distortion would most probably be a tilting of α -helices.

Site-directed mutagenesis and chemical labelling have suggested that the two Ca^{2+} -binding sites on the Ca^{2+} -ATPase are located in the transmembrane region, involving putative transmembrane helices M4, M5, M6 and M8 [9,10]. It has been proposed that the two Ca^{2+} -binding sites are located in a channel-like structure [11] bounded by these four helices [12]. Binding of Ca^{2+} to the ATPase is co-operative, and it has been suggested that, after binding of the first Ca^{2+} ion, a reorientation of the helices leads to formation of the second, high-affinity Ca^{2+} -binding site [12]. For the Ca^{2+} -ATPase reconstituted in bilayers of phosphatidylcholines with chain lengths of C_{12} , C_{14} or C_{24} , only a single Ca^{2+} ion binds to the ATPase, suggesting that distortion of the ATPase in thin or thick bilayers prevents the conformational changes on the ATPase normally involved in Ca^{2+} binding [13,14].

The main cytoplasmic region of the ATPase, containing the ATP binding domain and the phosphorylation domain (in which is located Asp-351, the residue on the ATPase phosphorylated by ATP), is located between helices M4 and M5 [15]. The phosphorylation and nucleotide-binding domains on the ATPase are located some distance from the lipid bilayer surface [16]. Transport of Ca^{2+} by the ATPase therefore involves long-range interactions between the phosphorylation domain and the transmembrane α -helices. The observed changes in the rates of phosphorylation and dephosphorylation on reconstitution of the ATPase into bilayers of short- and long-chain phospholipids must also involve long-range interactions between the phosphorylation domain and the transmembrane region of the ATPase [17,18]. The question addressed here is whether the

Abbreviations used: di(C18:1)PC, dioleoyl phosphatidylcholine; di(C22:1)PC, dierucyl phosphatidylcholine; di(C24:1)PC, dinervonyl phosphatidylcholine; E1, E2, different conformations of the ATPase enzyme; EP, phosphorylated ATPase; SR, sarcoplasmic reticulum.

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changes at the Ca^{2+} -binding sites and in the phosphorylation domain are linked or independent changes. We studied the ATPase reconstituted in dioleoyl phosphatidylcholine [di(C22:1)PC] because in this lipid the stoichiometry of Ca^{2+} binding is the normal two Ca^{2+} ions per ATPase molecule but the ATPase activity is low, indicating changes in the rate of at least one of the steps important for the steady-state activity [14].

MATERIALS AND METHODS

Dioleoyl phosphatidylcholine [di(C18:1)PC], di(C22:1)PC and dinervonyl phosphatidylcholine [di(C24:1)PC] were obtained from Avanti Polar Lipids. The Ca^{2+} -ATPase was purified from skeletal muscle SR as described previously [8] and concentrations of ATPase were estimated by using a specific absorption coefficient of $1.2 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ for a solution in 1% (w/v) SDS [19]. Reconstitutions were performed as described previously [14]. Phospholipid ($10 \mu\text{mol}$) in buffer [$400 \mu\text{l}$; 10 mM Hepes/Tris, pH 8.0, containing 15% (w/v) sucrose, 5 mM MgSO_4 , 5 mM ATP and 12 mg/ml potassium cholate] was sonicated to clarity in a bath sonicator. ATPase (1.25 mg) in a volume of 20–30 μl was then added and left for 15 min at room temperature and 45 min at 5 °C to equilibrate. For measurements of steady-state activities or rates of Ca^{2+} dissociation, the samples were diluted into buffer and stored on ice until use. For measurements of phosphorylation or dephosphorylation, for which higher concentrations of ATPase were required, samples were added to precooled Oakridge tubes containing ice-cold buffer (10 mM Hepes/Tris, pH 8.0) and centrifuged at $200000 g$ for 1 h at 4 °C; pellets were rehomogenized and suspended in buffer [10 mM Hepes/Tris, 15% (w/v) sucrose] to a concentration of 3–8 mg/ml, and stored at –80 °C until use.

Measurements of ATPase activities were performed at 25 °C by using a coupled enzyme assay in which aliquots of the reconstitution mixture were diluted into a medium containing 40 mM Hepes/KOH, pH 7.2, 100 mM KCl, 5 mM MgSO_4 , 2.1 mM ATP, 1.1 mM EGTA, 0.41 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 units) and lactate dehydrogenase (18 units) in a total volume of 2.5 ml. The reaction was initiated by the addition of an aliquot of a 25 mM CaCl_2 solution to a cuvette containing the ATPase and the other reagents to give a free Ca^{2+} concentration of $10 \mu\text{M}$.

The time dependence of phosphorylation-induced Ca^{2+} release from the ATPase was determined with a Biologic rapid filtration system. A suspension of the ATPase in buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl and 5 mM MgSO_4) containing $100 \mu\text{M}$ $^{45}\text{Ca}^{2+}$ and $500 \mu\text{M}$ [^3H]sucrose, corresponding to 50 μg of ATPase, was loaded on to a Millipore HAWP filter (0.45 μm) and then rapidly perfused with the same buffer containing $100 \mu\text{M}$ $^{40}\text{CaCl}_2$ and 2 mM ATP. The filters were dried overnight in air and counted in OptiPhase 'HiSafe' 3. The amount of [^3H]sucrose trapped on the filter was used to calculate the wetting volume of the filter, and the amount of $^{45}\text{Ca}^{2+}$ calculated to be in this volume was subtracted from the total $^{45}\text{Ca}^{2+}$ on the filter to give that bound to the ATPase.

Steady-state measurements of phosphorylation by [^{32}P]P_i were performed 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 and a protein concentration of 0.2 mg/ml [18]. The time dependence of phosphorylation of the ATPase by [γ - ^{32}P]ATP at 25 °C was determined with a Biologic QFM-5 system. ATPase (0.2 mg/ml) was incubated in 20 mM Hepes/Tris, pH 7.2, containing 5 mM MgSO_4 , 100 mM KCl and $100 \mu\text{M}$ CaCl_2 . This was mixed with an equal volume of the same buffer containing $100 \mu\text{M}$ [γ - ^{32}P]ATP, followed by quenching with 25% (w/v)

trichloroacetic acid/0.13 M phosphoric acid. The precipitated protein was filtered, washed and counted. The time dependence of dephosphorylation of the ATPase phosphorylated with [^{32}P]P_i was determined by first incubating the ATPase (4 mg/ml) in 12.5 mM Mes/Tris, pH 6.0, containing 10 mM EGTA, 1 mM [^{32}P]P_i, 20 mM MgSO_4 and 14% (v/v) DMSO. One volume of this suspension was mixed in the Biologic QFM-5 system with 16 vol. of 100 mM Mes/Tris, pH 7.5, containing 100 mM KCl, 4 mM MgSO_4 and 5.3 mM ATP, followed by quenching as described above.

RESULTS AND DISCUSSION

The ATPase activity of the Ca^{2+} -ATPase reconstituted into bilayers of di(C22:1)PC and measured at pH 7.2, 25 °C, saturating concentrations of Ca^{2+} ($10 \mu\text{M}$) and 2.1 mM ATP is 1.3 units/mg of protein, compared with values of 3.2 units/mg of protein and 0.7 unit/mg of protein in di(C18:1)PC and di(C24:1)PC respectively. Although ATPase activities are low in both di(C22:1)PC and di(C24:1)PC, a major difference is that whereas the ATPase molecule binds one Ca^{2+} ion in di(C24:1)PC, in di(C22:1)PC it binds two, the normal value [14]. This is illustrated in Figure 1. Both the native ATPase and the ATPase reconstituted in di(C22:1)PC show a specific binding of approx. 7 nmol of Ca^{2+} per mg of protein, when the total binding of approx. 9 nmol of $^{45}\text{Ca}^{2+}$ per mg of protein is corrected for non-specific Ca^{2+} binding of approx. 2 nmol of Ca^{2+} per mg of protein (represented by that amount of Ca^{2+} not released after 1 s of washing with a solution containing $100 \mu\text{M}$ $^{40}\text{Ca}^{2+}$). The maximal level of phosphorylation of the ATPase was approx. 3 nmol of phosphorylated enzyme (EP) per mg of protein (Table 1), giving a stoichiometry of Ca^{2+} binding to the ATPase of approx. 2:1.

The mechanism of inhibition of the ATPase is interpreted in terms of the E1–E2 scheme shown in Scheme 1; in the scheme the E1PC₂ and E2PC₂ intermediates are bracketed because recent experiments have cast doubt on the existence of two distinct forms of the phosphorylated ATPase [20]. It is proposed that, in

Figure 1 ATP-induced release of $^{45}\text{Ca}^{2+}$ from the ATPase

The native ATPase (○) or the ATPase reconstituted in di(C22:1)PC (□) was equilibrated in buffer (20 mM Hepes/Tris, pH 7.2, 100 mM KCl, 20 mM Mg^{2+}) containing $100 \mu\text{M}$ $^{45}\text{Ca}^{2+}$ and 0.5 mM [^3H]sucrose and then adsorbed on a Millipore filter. The loaded filter was perfused for the given times with the same buffer containing $100 \mu\text{M}$ unlabelled Ca^{2+} and 2 mM ATP, and the level of $^{45}\text{Ca}^{2+}$ bound to the ATPase was determined. The solid line shows a single exponential fit to the data for the ATPase in di(C22:1)PC.

Table 1 Effect of di(C22:1)PC on phosphorylation of the ATPase by P_i

The ATPase was incubated in 150 mM Mes/Tris, pH 6.2, in the presence of 5.0 mM EGTA and the given concentrations of Mg^{2+} and P_i , and the level of phosphorylation was determined.

	Level of phosphorylation (nmol of EP per mg of protein)	
	Native ATPase	ATPase in di(C22:1)PC
$[\text{P}_i] = 1 \text{ mM}$:		
1 mM Mg^{2+}	0.7	0.8
3 mM Mg^{2+}	1.1	1.3
5 mM Mg^{2+}	1.3	1.5
10 mM Mg^{2+}	1.3	1.5
$[\text{Mg}^{2+}] = 10 \text{ mM}$:		
1 mM P_i	1.3	1.4
2 mM P_i	2.0	1.6
3 mM P_i	2.2	2.0
5 mM P_i	2.6	2.4
10 mM P_i	3.0	2.9

the E1 conformation, the ATPase possesses two outward-facing Ca^{2+} -binding sites of high affinity. After binding of Ca^{2+} and ATP the enzyme is phosphorylated and undergoes a conformational change to E2PCa_2 , from which Ca^{2+} is lost to the lumen of the SR. Dephosphorylation of E2P allows recycling to E1.

Mixing the ATPase incubated in the presence of Ca^{2+} with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ leads to the rapid formation of phosphoenzyme. For the native ATPase the rate of phosphorylation observed with $50 \mu\text{M}$ ATP fits to a single exponential with a rate of $74.7 \pm 9.8 \text{ s}^{-1}$ and an amplitude of $2.6 \pm 0.1 \text{ nmol}$ of EP per mg of protein (Figure 2). The rate of phosphorylation of the native ATPase is very similar to that reported previously for the ATPase in di(C18:1)PC ($78.7 \pm 10.0 \text{ s}^{-1}$) [18]. For the ATPase in di(C22:1)PC, the rate is slightly lower ($58.7 \pm 4.7 \text{ s}^{-1}$) but the amplitude is the same ($2.7 \pm 0.1 \text{ nmol}$ of EP per mg of protein). For the ATPase reconstituted in di(C24:1)PC the rate of phosphorylation is $60.7 \pm 3.0 \text{ s}^{-1}$ [18]. A slight decrease in the rate of phosphorylation is therefore observed in long-chain phosphatidylcholines irrespective of the stoichiometry of Ca^{2+} binding; the decrease in the rate of this step is, however, insufficient to explain the 2.5-fold decrease in the steady-state rate of ATP hydrolysis.

The rate of the Ca^{2+} transport step ($\text{E1PCa}_2 \rightarrow \text{E2P}$ in terms of Scheme 1) was determined by incubating the ATPase with $^{45}\text{Ca}^{2+}$, adsorbing it on a Millipore filter, and then perfusing the filter for determined periods of time with a solution containing $^{40}\text{Ca}^{2+}$ and ATP. As long as the rate of phosphorylation of the ATPase is faster than the rate of dissociation of $^{45}\text{Ca}^{2+}$ from the ATPase, the rate of loss of $^{45}\text{Ca}^{2+}$ from the ATPase in this

experiment is equal to the rate of the Ca^{2+} transport step. The loss of $^{45}\text{Ca}^{2+}$ from the ATPase fits to a single exponential process, with rates of 19.4 ± 4.3 and $15.7 \pm 3.9 \text{ s}^{-1}$ for the native ATPase and for the ATPase in di(C22:1)PC, respectively (Figure 1). For the ATPase in di(C24:1)PC, the rate of dissociation for the single bound Ca^{2+} ion was found to be $16.3 \pm 3.6 \text{ s}^{-1}$. It is clear that any effects on the rate of this step are very small, although again in both long-chain lipids there does seem to be a small decrease in rate.

The rate of dephosphorylation of the phosphorylated ATPase was determined by first phosphorylating the ATPase with $[\gamma\text{-}^{32}\text{P}]\text{P}_i$ at pH 6.0 in the absence of Ca^{2+} and presence of DMSO followed by mixing with an excess of a pH 7.5 buffer containing KCl and ATP to induce dephosphorylation. Rates of dephosphorylation of 12.9 ± 2.2 and $4.7 \pm 1.1 \text{ s}^{-1}$ were found for the native ATPase and for the ATPase in di(C22:1)PC respectively (Figure 3). These rates can be compared with the rate of dephosphorylation of $12.5 \pm 1.1 \text{ s}^{-1}$ found for the ATPase in di(C18:1)PC [18]. The 64% decrease in the rate of dephosphorylation observed in di(C22:1)PC explains the 60% decrease in steady-state ATPase activity.

The back reaction, the phosphorylation of the ATPase by P_i , can be studied if the ATPase is incubated with P_i and Mg^{2+} at acid pH in the absence of Ca^{2+} . Levels of phosphorylation of the ATPase in di(C22:1)PC as a function of P_i and Mg^{2+} concentration are very similar to those observed for the native ATPase (Table 1). Thus the affinities of the ATPase for P_i and Mg^{2+} and the equilibrium constant for the phosphorylation step ($\text{E2MgP}_i \rightleftharpoons \text{E2P}$) must be unaffected by reconstitution in di(C22:1)PC. Because the rate of dephosphorylation has decreased by a factor of approx. 3 on reconstitution in di(C22:1)PC, the rate of phosphorylation must have decreased by the same amount.

It has been suggested that formation of the phosphorylated ATPase results in increased hydrophobicity for the nucleotide-

Figure 2 Rate of phosphorylation of the ATPase with ATP

The ATPase (0.2 mg/ml protein) was incubated in buffer (20 mM Hepes/Tris, pH 7.2, 5 mM Mg^{2+} , 100 mM KCl, 100 μM Ca^{2+}) and mixed in a 1:1 ratio with the same buffer containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to give a final concentration of $50 \mu\text{M}$ ATP. After the given times the mixture was quenched with 25% (w/v) trichloroacetic acid and the level of phosphorylated ATPase (nmol of EP per mg of protein) determined: $\circ$, native ATPase; $\square$, ATPase in di(C22:1)PC. The solid and broken lines represent best fits to single-exponential processes for the native ATPase and for the ATPase in di(C22:1)PC respectively.

Scheme 1

Mechanism of inhibition of the ATPase interpreted in terms of the E1-E2 scheme (see text for details).

Figure 3 The rate of dephosphorylation of the phosphorylated ATPase

The enzyme syringe held ATPase (4 mg/ml protein) in 12.5 mM Mes/Tris, pH 6.0, containing 10 mM EGTA, 1 mM [32 P]P_i, 20 mM Mg²⁺ and 14% (v/v) DMSO. The second syringe held 100 mM Mes/Tris, pH 7.5, containing 100 mM KCl, 4 mM Mg²⁺ and 5.3 mM ATP. The contents of the enzyme syringe were mixed in a 1:16 volume ratio with the dephosphorylation mixture and the reaction was quenched at the given times with 25% (w/v) trichloroacetic acid: ○, native ATPase; □, ATPase in di(C22:1)PC. The solid lines show best fits to single-exponential processes, with the rates given in the text.

binding site on the ATPase, perhaps as a result of the closing of a cleft between the phosphorylation and nucleotide-binding domains of the ATPase [21]. It has also been suggested that the initial, rate-controlling, step in phosphorylation of the ATPase by ATP is a relocation of the nucleotide-binding and phosphorylation domains to bring the terminal phosphate of ATP close to Asp-351, the residue in the phosphorylation domain to be phosphorylated [22]. The observed decrease in the rate of phosphorylation of the ATPase by ATP in di(C22:1)PC, taken together with the observed decrease in the rate of dephosphorylation and the calculated decrease in the rate of phosphorylation by P_i, suggests a decrease in the forward and backward rate constants for the conformation change for the ATPase in di(C22:1)PC.

Conclusions

Reconstitution of the Ca²⁺-ATPase in di(C14:1)PC results in a large decrease in the rates of both phosphorylation and dephosphorylation, whereas although reconstitution in di(C24:1)PC also results in a large decrease in the rate of dephosphorylation the decrease in the rate of phosphorylation is only small [18]. In both di(C14:1)PC and di(C24:1)PC, the stoichiometry of Ca²⁺ binding is altered with only a single Ca²⁺ ion binding per ATPase molecule [18]. It is therefore possible that the observed changes in the rates of phosphorylation and dephosphorylation are linked directly to the changes in the Ca²⁺-

binding region of the ATPase responsible for the change in Ca²⁺ binding stoichiometry. The results reported here show that this is not so. The Ca²⁺-ATPase in di(C22:1)PC binds two Ca²⁺ ions per ATPase molecule, but the changes in the rates of phosphorylation and dephosphorylation are very similar in di(C22:1)PC and di(C24:1)PC. Because the phosphorylation domain of the ATPase is located some distance from the lipid bilayer surface [16], changes in phosphorylation and dephosphorylation require long-range interactions in the ATPase; changes in the lipid bilayer, sensed by the trans-membrane α -helices of the ATPase, are transmitted to the phosphorylation domain. The phosphorylation domain seems to be more sensitive to changes in the lipid bilayer than are the Ca²⁺-binding sites because changes in the former occur in di(C22:1)PC whereas for the latter changes are only observed in di(C24:1)PC. The phospholipids in the SR membrane contain approx. 10–15 mol % C₂₀ fatty acyl chains, but less than 3% of C₂₂ chains [23]. It has been reported that fatty acid-metabolizing enzymes in animal tissues show considerable discrimination against erucic acid [24]. It is therefore likely that C₂₀ fatty acyl chains are the longest compatible with full activity for many other membrane proteins.

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