Anionic phospholipids decrease the rate of slippage on the Ca^{2+} -ATPase of sarcoplasmic reticulum

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Accumulation of Ca^{2+} by the Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum has been measured in reconstituted, sealed vesicles as a function of lipid composition. Measurements were performed in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to eliminate any effects of H^+ transport; in the presence of FCCP, addition of valinomycin had no effect on the level or rate of accumulation of Ca^{2+} showing that, in the presence of FCCP, no electrical potential built up across the membrane. Levels of accumulation were low when the phospholipid was dioleoylphosphatidylcholine (DOPC), even though DOPC supports high ATPase activity. Inclusion of 10 mol % anionic phospholipid [dioleoylphosphatidic acid (DOPA) or dioleoylphosphatidylserine (DOPS)] led to higher levels of accumulation of Ca^{2+} , 10 mol % being the optimum concentration. Cardiolipin or phosphatidylinositol 4-phosphate

were more effective than DOPA or DOPS in increasing accumulation of Ca^{2+} . Effects of anionic phospholipids were seen in the presence of an ATP-regenerating system to remove ADP, and in the presence of phosphate within the reconstituted vesicles to precipitate calcium phosphate. Rates of passive leak of Ca^{2+} from the reconstituted vesicles were slow. The Ca^{2+} -accumulation process was simulated assuming either simple passive leak of Ca^{2+} from the vesicles or assuming slippage on the ATPase, a process in which the phosphorylated intermediate of the ATPase releases bound Ca^{2+} on the cytoplasmic rather than the luminal side of the membrane. The experimental data fitted to a slippage model, with anionic phospholipids decreasing the rate of slippage.

Key words: Ca^{2+} transport, leakage, lipid–protein interactions, P-type ATPases, transport mechanism.

INTRODUCTION

The P-type ATPases couple the hydrolysis of ATP with the movement of ions across a membrane. In the case of the Ca^{2+} -ATPase of skeletal-muscle sarcoplasmic reticulum (SR), two Ca^{2+} ions and one ATP molecule bind to the ATPase so that full coupling results in the transport of 2 Ca^{2+} ions for each molecule of ATP hydrolysed. Recent experiments are consistent with a model in which two pairs of sites for Ca^{2+} exist on the Ca^{2+} -ATPase, transport of Ca^{2+} by the Ca^{2+} -ATPase corresponding to transfer of Ca^{2+} from a cytoplasmic pair of sites to a luminal pair of sites, driven by phosphorylation of the ATPase by ATP [1–3], as shown in Scheme 1. Ca^{2+} is released into the lumen of the SR from the luminal pair of sites on the phosphorylated intermediate, and dephosphorylation of E2P then allows recycling to E1 [4].

The expected ratio of two Ca^{2+} ions accumulated per ATP molecule hydrolysed is observed during the first cycle of the

ATPase, before the luminal concentration of Ca^{2+} has increased to a high level [5]; it is also observed in the presence of oxalate, which leads to precipitation of calcium oxalate in the lumen of the SR, so maintaining a low free luminal concentration of Ca^{2+} [6,7]. However, under conditions where the luminal Ca^{2+} concentration reaches millimolar concentrations, the level of Ca^{2+} accumulated becomes less than 2:1 with respect to ATP hydrolysed [8,9]. Lower levels of accumulation of Ca^{2+} than expected could arise in two ways: (i) passive leak of Ca^{2+} down its concentration gradient, either involving the Ca^{2+} -ATPase [8,10–13] or involving some other protein in the SR membrane; or (ii) slippage of the ATPase, a process in which the phosphorylated, Ca^{2+} -bound intermediate release Ca^{2+} to the cytoplasmic rather than the luminal side of the membrane (Scheme 1). The presence of a passive leak pathway has been demonstrated in experiments measuring the rate of Ca^{2+} release from Ca^{2+} -loaded SR vesicles, lacking Ca^{2+} channels (the ryanodine receptor); this leak is inhibited by micromolar concentrations of Ca^{2+} in the cytoplasm, suggesting release via the Ca^{2+} -ATPase [8]. Passive leak of Ca^{2+} has also been observed from vesicles reconstituted from phospholipids and the purified Ca^{2+} -ATPase, again suggesting that leak of Ca^{2+} could occur through the Ca^{2+} -ATPase [11–13]. Evidence also exists for a slippage pathway [8,14]. Thus uptake of Ca^{2+} by SR vesicles on addition of ATP is followed by spontaneous release of some of the accumulated Ca^{2+} , even in the continued presence of ATP and external Ca^{2+} , conditions under which simple passive leak is inhibited [9]. This spontaneous release is also observed in the presence of acetyl phosphate instead of ATP so that release does not correspond to the back-reaction [15] in which Ca^{2+} efflux is linked to the conversion of ADP into ATP [9]. Changes in the

Scheme 1

Abbreviations used: C_{12}E_8 , octa(ethylene glycol)-*n*-dodecyl ether; DOPA, dioleoylphosphatidic acid; DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SR, sarcoplasmic reticulum.

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stoichiometry of Ca^{2+} accumulation with time in reconstituted vesicles have also been interpreted in terms of slippage on the ATPase [14].

In previous studies we have reported on the effects of phospholipid structure on the rate of ATP hydrolysis by the ATPase [16,17]. These studies were performed using membrane fragments unable to accumulate Ca^{2+} so that the scalar process of ATP hydrolysis could be studied, uncomplicated by any of the vectorial processes associated with transport and leak of Ca^{2+} . Here we show that effects of phospholipid structure on accumulation of Ca^{2+} are different from those on ATP hydrolysis. To measure accumulation of Ca^{2+} , the ATPase has been reconstituted into large sealed vesicles, using a procedure based on that of Rigaud et al. [18], which gives reconstituted vesicles with very low ionic permeabilities [14,19–22].

MATERIALS AND METHODS

Octa(ethylene glycol)*n*-dodecyl ether (C_{12}E_8) and *n*-octyl- β -D-glucoside were obtained from Calbiochem and Sigma respectively, phosphatidylinositol (PtdIns) and phosphatidylinositol 4-phosphate [PtdIns(4)*P*] were from Sigma, and the other phospholipids were from Avanti Polar Lipid Inc, Alabaster, AL, U.S.A.

Light SR vesicles were prepared from rabbit skeletal muscle as described by Dalton et al. [23]; the vesicles contain predominantly Ca^{2+} -ATPase with some calsequestrin and 53 kDa glycoprotein [24], the latter two being soluble proteins that are lost during the reconstitution process. Reconstituted vesicles were prepared by a modification of the method described by Levy et al. [25]. Phospholipids (24 mg) were suspended in buffer (4.6 ml; 10 mM Pipes/100 mM K_2SO_4 , pH 7.1), containing 40 mM octyl- β -D-glucoside and sonicated in a bath sonicator (Ultrawave Model U100) for about 5 min. SR (1 mg of protein) was solubilized in 0.5 ml of buffer (10 mM Pipes/100 mM K_2SO_4 , 0.1 mM CaCl_2 , pH 7.1) containing 3 mg of C_{12}E_8 . The solubilized SR was mixed with the lipid sample to give a 1:40 ratio of protein:lipid (w/w). Detergent was removed by addition of four aliquots of washed SM_2 Bio-Beads, as described by Levy et al. [25], to give the final preparation of sealed vesicles.

Accumulation of Ca^{2+} by the reconstituted vesicles was measured at 25 °C using the indicator Arsenazo III (70 μM) to monitor the external Ca^{2+} concentration, measuring the absorption difference 720–790 nm in an Aminco-Bowman DW2000 dual wavelength spectrophotometer. The absorption change was calibrated by the incremental addition of Ca^{2+} prior to the addition of ATP to initiate uptake. The assay buffer was 10 mM Pipes, 100 mM K_2SO_4 , 5 mM MgSO_4 , pH 7.1, containing 120 μM Ca^{2+} , 70 μM Arsenazo III, 0.8 mM ATP and a protein concentration of 0.02 mg/ml. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to a concentration of 0.25 μM to make the vesicles permeable to H^+ [19]. ATPase activity was determined at 25 °C by using a coupled enzyme assay in 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, 0.53 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 maximally stimulating concentration of Ca^{2+} (free Ca^{2+} concentration $\approx 10 \mu\text{M}$).

Vesicle diameters were determined using a Coulter N4 Plus particle sizer that measures particle size by light scattering. The internal vesicle volume V_i (nm^3 per mg of lipid) was calculated from the internal vesicle radius, given by $r_i = r_o - \delta$, where r_o is

the external radius and δ is the bilayer thickness, using the equation:

$$V_i = \frac{10^{-3}}{\rho[(1 + \frac{\delta}{r_o})^3 - 1]}$$

where ρ is the density of the lipid (taken to be 1 g/ml) and the bilayer thickness δ was taken to be 4 nm. Simulations of uptake were performed using the program FACSIMILE (UKEA).

RESULTS

Mixing SR vesicles solubilized in C_{12}E_8 with dioleoyl-phosphatidylcholine (DOPC) solubilized in *n*-octyl- β -D-glucoside, followed by detergent removal with SM_2 Bio-Beads, gave a preparation of sealed vesicles able to accumulate Ca^{2+} . The level of accumulation of Ca^{2+} was identical with that observed for vesicles reconstituted by adding the solubilized SR to a preparation of pre-formed large unilamellar vesicles, using the

Figure 1 ATP-dependent accumulation of Ca^{2+} by reconstituted vesicles

(A) ATP-dependent accumulation of Ca^{2+} by reconstituted vesicles containing: (a) DOPC; (b) 90% DOPC/10% DOPA; (c) 95% DOPC/5% cardiolipin; (d) 90% DOPC/10% cardiolipin. In each case, accumulation of Ca^{2+} was initiated by addition of 0.8 mM ATP. Samples contained 0.02 mg of protein/ml at a lipid:protein ratio (w/w) of 40:1 in 10 mM Pipes, pH 7.1, 100 mM K_2SO_4 , 5 mM Mg^{2+} , 0.25 μM FCCP and an initial Ca^{2+} concentration of 120 μM . The continuous lines show experimental data and the broken lines show simulations for the slippage model with the parameters given in the text, assuming an internal volume of 174 μl /mg protein, a concentration of active, outwardly oriented ATPase of 0.03 μM and rates for the slippage step (s^{-1}) of: (a) 250; (b) 65; (c) 45; and (d) 0. (B). ATP-dependent accumulation of Ca^{2+} by reconstituted vesicles as a function of the mol% of DOPA: (a) 0; (b) 5; (c) 10; (d) 15; (e) 20.

Figure 2 Effects of phosphatidylinositols on ATP-dependent accumulation of Ca^{2+}

ATP-dependent accumulation of Ca^{2+} was determined as described in the legend to Figure 1 for reconstituted vesicles containing: (a) DOPC; (b) 90% DOPC/10% PtdIns; (c) 90% DOPC/10% PtdIns(4)P. The continuous lines show experimental data and the broken lines show simulations for the slippage model with the parameters given in the text and rates for the slippage step (s^{-1}) of: (a) 250; (b) 65; (c) 15.

procedure of Levy et al. [25] (results not shown). As reported by Levy et al. [19], we found that addition of FCCP to make the reconstituted vesicles permeable to H^+ led to increased levels of accumulation of Ca^{2+} (because the Ca^{2+} -ATPase acts as a $\text{Ca}^{2+}/\text{H}^+$ -ATPase), and that addition of valinomycin had no significant effect in the presence of FCCP, showing that no significant membrane potential built up in the presence of FCCP. All experiments reported here were therefore performed in the presence of $0.25 \mu\text{M}$ FCCP; this concentration of FCCP was found to give maximum levels of accumulation of Ca^{2+} , independent of the lipid composition.

As shown in Figure 1(A), the rate of accumulation of Ca^{2+} by the Ca^{2+} -ATPase reconstituted in DOPC decreased markedly with time, reaching a final steady-state level of accumulation of $\approx 800 \text{ nmol of } \text{Ca}^{2+}/\text{mg protein}$ after about 10 min. Incorporation of 10 mol % dioleoylphosphatidic acid (DOPA) into the vesicles increased the level of accumulation of Ca^{2+} to $\approx 1500 \text{ nmol of } \text{Ca}^{2+}/\text{mg protein}$ after 10 min (Figure 1A); the effect of 10 mol % dioleoylphosphatidylserine (DOPS) was identical with that of 10 mol % DOPA shown in Figure 1(A). The optimal concentration was 10 mol % DOPA; lower levels of accumulation were observed at 5 mol % DOPA, and 15 and 20 mol % DOPA gave slightly lower levels of accumulation than 10 mol % (Figure 1B). The effect of 10 mol % cardiolipin on accumulation of Ca^{2+} was considerably more marked than that of DOPA or DOPS (Figure 1A). Again, 10 mol % was the optimal concentration of cardiolipin with lower levels of accumulation being observed at either 5 (Figure 1A) or 20 mol % (results not shown); the effect of 5 mol % cardiolipin was comparable with that of 10 mol % DOPA (Figure 1A). The presence of 10 mol % PtdIns had an effect on accumulation of Ca^{2+} very similar to that of 10 mol % DOPA (Figure 2). However, the effect of 10 mol % PtdIns(4)P was more marked, although levels of accumulation of Ca^{2+} were not as high as those recorded in the presence of cardiolipin.

To test whether the decrease in the rate of accumulation of Ca^{2+} with time was due to depletion of ATP or build up of ADP

Figure 3 Accumulation of Ca^{2+} and hydrolysis of ATP in the presence of an ATP-regenerating system

(A) ATP-dependent accumulation of Ca^{2+} by reconstituted vesicles containing (a,b) 90% DOPC/10% DOPA or (c,d) 100% DOPC was measured in the absence (b,d) or presence (a,c) of an ATP-regenerating system consisting of pyruvate kinase (200 units) and phosphoenolpyruvate (0.4 mM). Sample conditions are as described in the legend to Figure 1. The broken lines show simulations performed as described in the text with slippage rates (s^{-1}) of (a,b) 65; (c,d) 250. (B) Hydrolysis of ATP by reconstituted vesicles containing 90% DOPC/10% DOPA, measured at pH 7.2 in 40 mM Hepes, 100 mM KCl, 2.1 mM ATP and 5 mM Mg^{2+} using a coupled enzyme assay. The protein concentration was $1.2 \mu\text{g}/\text{ml}$.

Table 1 Steady-state ATPase activities for reconstituted vesicles

ATPase activities were measured using the experimental conditions described in the Materials and methods section.

Lipid	ATPase activity (units/mg protein)	
	$-\text{C}_{12}\text{E}_8$	$+\text{C}_{12}\text{E}_8^*$
SR vesicles†	4.3 ± 0.4	4.4 ± 0.4
DOPC	2.5 ± 0.2	4.9 ± 0.4
90% DOPC/10% DOPA	2.6 ± 0.2	5.2 ± 0.5
90% DOPC/10% DOPS	2.2 ± 0.2	5.5 ± 0.5
90% DOPC/10% cardiolipin	2.0 ± 0.2	3.6 ± 0.3

* At a concentration of $0.8 \text{ mg}/\text{ml}$; † in the presence of A23187 at a concentration of $5 \mu\text{g}/\text{ml}$.

as a result of hydrolysis of ATP by the Ca^{2+} -ATPase, the effect of an ATP-regenerating system consisting of pyruvate kinase and phosphoenolpyruvate was studied (Figure 3). This led to some-

Figure 4 The effect of luminal phosphate on accumulation of Ca^{2+}

(A) ATP-dependent accumulation of Ca^{2+} by reconstituted vesicles containing (a,b) 90% DOPC/10% DOPA or (c,d) 100% DOPC was measured in the absence (b,d) or presence (a,c) of a luminal phosphate concentration of 50 mM. Other conditions were as described in the legend to Figure 1.

what higher levels of accumulation of Ca^{2+} at longer times, the effect being larger for the ATPase in 90% DOPC/10% DOPA than in DOPC alone (Figure 3A). However, even in the presence of the regenerating system, the rate of accumulation of Ca^{2+} still decreased with time (Figure 3A). In contrast, the rate of hydrolysis of ATP by the ATPase reconstituted in 90% DOPC/10% DOPA, measured in the presence of an ATP-regenerating system, did not change with time, remaining constant until all the ATP and phosphoenolpyruvate had been consumed (Figure 3B). Thus the stoichiometry of net accumulation of Ca^{2+} to ATP hydrolysed decreases with time. A similar observation has been made for native SR vesicles in the absence of any Ca^{2+} -precipitating agent in the lumen of the vesicles [14].

The rate of hydrolysis of ATP in the reconstituted system was about one-half of that in the SR vesicles used for the reconstitution, suggesting that the distribution of ATPase molecules across the membrane was close to random (Table 1). This was confirmed by addition of low concentrations (0.8 mg/ml) of the detergent C_{12}E_8 to the reconstituted vesicles to make them leaky to ATP. C_{12}E_8 at 0.8 mg/ml had no significant effect on the activity of the purified Ca^{2+} -ATPase [26], and also had no significant effect on the ATPase activity of SR vesicles measured in the presence of the Ca^{2+} ionophore A23187 (Table 1). Addition of C_{12}E_8 to the reconstituted vesicles led to about a doubling of ATPase activity, showing that in the reconstituted vesicles the distribution of ATPase molecules across the membrane is close to random, with about 50% having a 'right-side-out' orientation. The rate of hydrolysis of ATP was not significantly changed by the presence of 10 mol% anionic phospholipid (Table 1). This is consistent with measurements of ATPase activity for the ATPase reconstituted into membrane fragments, where ATPase activity was unaffected by the presence of 10 mol% DOPA or DOPS and decreases at higher mole fractions of anionic phospholipid [23].

The importance of leak pathways in determining the level of accumulation of Ca^{2+} was shown by trapping phosphate in the lumen of the reconstituted vesicles to complex the Ca^{2+} ions transported into the lumen. As shown in Figure 4, the presence of 50 mM phosphate led to higher levels of accumulation of Ca^{2+} for vesicles containing 10 mol% DOPA, with relatively little

Figure 5 Passive release of Ca^{2+} from vesicles loaded with Ca^{2+}

ATP-dependent accumulation of Ca^{2+} by reconstituted vesicles containing (a) 90% DOPC/10% DOPA or (b) 100% DOPC was measured over an approx. 2 min period under the conditions described in Figure legend 1, in a buffer containing 12.5 mM glucose. At the time shown by the arrowheads, 56 units of hexokinase were added to stop accumulation of Ca^{2+} .

effect on vesicles of DOPC alone. This result suggests that the presence of anionic phospholipids leads to a higher luminal concentration of free Ca^{2+} , so that the solubility limit for formation of $\text{Ca}_3(\text{PO}_4)_2$ is exceeded.

The rate of simple passive leak of Ca^{2+} from the reconstituted vesicles was determined by allowing vesicles to accumulate Ca^{2+} in the presence of both ATP and glucose, and then adding hexokinase to remove unreacted ATP (Figure 5). For vesicles containing only DOPC, a small, rapid burst of release of Ca^{2+} was seen after removal of ATP, followed by a very slow release phase; for vesicles containing 10 mol% DOPA, the small, rapid burst phase was not seen. We conclude that the rate of passive leak of Ca^{2+} is slow under the conditions used in the accumulation experiments.

The sizes of the vesicle preparations, measured using light scattering, varied somewhat between preparations, but typically had external diameters of 120 ± 30 nm; no significant effect of anionic phospholipids on the size of the vesicles was observed. Rigaud et al. [18] reported that reconstitution of bacteriorhodopsin gave vesicles of diameters between 100 and 200 nm at low and high detergent concentrations respectively, and Levy et al. [19] reported that reconstitution of the Ca^{2+} -ATPase at low detergent concentrations gave vesicles of 100 nm diameter.

DISCUSSION

The lipid compositions of all biological membranes are complex and it is unclear to what extent this complexity is required for the proper functioning of the membrane and the proteins embedded in it. In previous studies we have shown that a relatively wide range of phospholipid structure will support a high rate of ATP hydrolysis by the Ca^{2+} -ATPase; the phospholipid acyl chain length has to be between C_{16} and C_{20} to give the optimal bilayer thickness, the phospholipids have to be in the liquid-crystalline, bilayer phase, and the optimal headgroup is zwitterionic, either phosphatidylcholine or phosphatidylethanolamine [16,17]. The only exception to this simple pattern is that the presence of small amounts of $\text{PtdIns}(4)\text{P}$ leads to increased ATPase activity [27,28].

Figure 6 Simulations of Ca^{2+} accumulation

Simulations were performed using the parameters in Table 2, unless otherwise stated, with a concentration of active, outwardly oriented ATPase of $0.045 \mu\text{M}$, a luminal volume of $174 \mu\text{l/mg}$ protein, and initial Ca^{2+} and ATP concentrations of $120 \mu\text{M}$ and 0.8 mM respectively. **(A)** shows the effect of simple passive leak, with rates (s^{-1}) for the leak step ($\text{Ca}^{2+}_{\text{i}} \rightarrow \text{Ca}^{2+}_{\text{o}}$) of (a) 0; (b) 5×10^{-6} ; (c) 1×10^{-5} ; (d) 3×10^{-5} . **(B)** shows the effect of slippage, with rates (s^{-1}) for the slippage step ($\text{E2PCa}_2 \rightarrow \text{E2} + \text{Ca}^{2+}_{\text{o}} + \text{Ca}^{2+}_{\text{i}}$) of (a) 0; (b) 50; (c) 150; and (d) 500. **(C)** Effect of the affinity of E2P for Ca^{2+} on accumulation of Ca^{2+} in the presence of slippage. The rate of slippage was fixed at 500 s^{-1} with a Ca^{2+} affinity of (a) 33 mM; (b) 10 mM; (c) 3.3 mM; and (d) 1.1 mM. **(D)** Effect of ATPase concentration on accumulation of Ca^{2+} in the presence of slippage. The rate of slippage was fixed at 50 s^{-1} (a–c) or 500 s^{-1} (d–f) with concentrations of active, outwardly oriented ATPase molecules of (a,d) $0.18 \mu\text{M}$; (b,e) $0.09 \mu\text{M}$; and (c,f) $0.045 \mu\text{M}$. In curves a, d, and e, Ca^{2+} accumulation ceases when the added ATP has been consumed.

The effect of $\text{PtdIns}(4)\text{P}$ is specific, i.e. the presence of phosphatidylinositol, phosphatidylserine or phosphatidic acid at low mole fractions has no effect on activity, and at higher mole fractions leads to inhibition [23]. The native SR membrane contains about 2 mol % phosphatidylserine and 8 mol % of PtdIns and $\text{PtdIns}(4)\text{P}$, but no $\text{PtdIns}(4,5)\text{P}_2$ [29,30]. The absence of $\text{PtdIns}(4,5)\text{P}_2$ means that SR lacks the capacity for $\text{Ins}(1,4,5)\text{P}_3$ generation [30].

Here we explore the possibility that the role of anionic phospholipids in the SR membrane is to maximize accumulation of Ca^{2+} , rather than to maximize the rate of hydrolysis of ATP. Using the procedure developed by Rigaud et al. [18], the Ca^{2+} -ATPase can be reconstituted into sealed vesicles showing a very low ionic permeability. The large size of the vesicles and the large luminal volume per ATPase molecule mean that luminal Ca^{2+} concentrations rise very slowly during Ca^{2+} transport, so that the time course of accumulation can be easily followed.

Accumulation of Ca^{2+} by the reconstituted vesicles was measured in the presence of FCCP to eliminate any effects of H^+ gradients or membrane potentials [19]. As shown in Figure 1, levels of accumulation of Ca^{2+} by the Ca^{2+} -ATPase in reconstituted vesicles are higher in the presence of anionic phospholipids than in DOPC alone, the effect of cardiolipin being greater than that of DOPS or DOPA. The effect of anionic phospholipid reaches a maximum at about 10 mol %, higher concentrations leading to slightly lower levels of accumulation. The effect of PtdIns on accumulation of Ca^{2+} is very similar to that of DOPS or DOPA, but the effect of $\text{PtdIns}(4)\text{P}$ is more marked. The observation that the largest effects with the anionic

phospholipids are seen with cardiolipin and $\text{PtdIns}(4)\text{P}$ suggests that the negative charge borne by the phospholipid is important. Nevertheless, it is not the total negative charge on the membrane that is important since 20 mol % DOPA does not have the same effect as 10 mol % cardiolipin (Figure 1).

One possible mechanism for the effect of anionic phospholipids could be via formation of an anionic phospholipid- Ca^{2+} complex in the lumen of the vesicles; by reducing the free luminal concentration of Ca^{2+} , the level of the phosphorylated intermediate, E2PCa_2 , would be reduced, leading to reduced inhibition of the ATPase (Scheme 1). Effective concentrations of anionic phospholipids in the luminal volume are high. The vesicle diameter measured using light scattering was typically $120 \pm 30 \text{ nm}$. A vesicle diameter of 120 nm gives a calculated internal volume of $174 \mu\text{l/mg}$ protein, in agreement with the value of $175 \mu\text{l/mg}$ of protein estimated by Levy et al. [19]. If the membrane contains 10 mol % anionic phospholipid, distributed randomly across the membrane, the concentration of anionic lipid on the inner surface, expressed in terms of the luminal volume, is 15 mM. However, if this were to complex significant amounts of Ca^{2+} , the level of accumulation of Ca^{2+} would be expected to increase linearly with increasing concentration of anionic phospholipid, whereas the effect of anionic phospholipid is seen to saturate at 10 mol % (Figure 1B). Furthermore, the presence of 10 mol % oleic acid, which partitions strongly into the membrane [31], would also be expected to lead to increased levels of accumulation of Ca^{2+} , and this was not observed (results not shown). Finally, if increased levels of accumulation of Ca^{2+} in the presence of anionic phospholipid were to follow from a

Table 2 Rate constants used in the simulations of Ca^{2+} accumulation shown in Figure 4

Step	Rate constant	
	Forward	Reverse
$\text{E2} \rightarrow \text{E1}$	13 s^{-1}	47 s^{-1}
$\text{E1} + \text{Ca}_0 \rightarrow \text{E1Ca}$	$1.0 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$	100 s^{-1}
$\text{E1Ca} + \text{Ca}_0 \rightarrow \text{E1Ca}_2$	$1.0 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$	100 s^{-1}
$\text{E1Ca}_2 + \text{ATP} \rightarrow \text{E1Ca}_2\text{ATP}$	$1.0 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$	$1.0 \times 10^3 \text{ s}^{-1}$
$\text{E1Ca}_2\text{ATP} \rightarrow \text{E2PCa}_2$	100 s^{-1}	0
$\text{E2PCa}_2 \rightarrow \text{E2PCa} + \text{Ca}_i$	60 s^{-1}	$1.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$
$\text{E2PCa} \rightarrow \text{E2P} + \text{Ca}_i$	30 s^{-1}	$9.0 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$
$\text{E2P} \rightarrow \text{E2}$	100 s^{-1}	0

reduction in the level of free luminal Ca^{2+} , the presence of luminal phosphate would have a smaller effect in the presence of anionic phospholipid than in its absence, and the opposite effect is seen (Figure 4). We therefore conclude that complexing of luminal Ca^{2+} by anionic phospholipids is not the explanation for the observed effects of anionic phospholipids on the accumulation of Ca^{2+} .

An alternative explanation for the effects of anionic phospholipids is that they decrease the rate of a leak pathway for Ca^{2+} across the membrane of the reconstituted vesicles. Two types of leak pathway can be distinguished. The first is simple passive leak of Ca^{2+} down its concentration gradient, mediated either by the Ca^{2+} -ATPase or by some other protein in the membrane. The other is slippage on the ATPase, a process in which the phosphorylated intermediate of the ATPase releases Ca^{2+} on the cytoplasmic side of the membrane rather than on the luminal side (Scheme 1). Experiments in which ATP is removed from the system by addition of hexokinase in the presence of glucose show that the rate of passive leak is very slow (Figure 5), suggesting that slippage must be the important factor.

Simulation of a simplified form of the reaction scheme shows that passive leak and slippage result in very different time-dependencies of accumulation of Ca^{2+} (Figure 6). Rate constants for the uptake pathway in Scheme 1 can be assigned on the basis of experimental data (Table 2). The rate constants for the $\text{E2} \rightarrow \text{E1}$ change were calculated from the parameters described in Henderson et al. [32]. The affinity for ATP [33], the rate of phosphorylation [23] and the rate of dephosphorylation [34] of the phosphorylated intermediate E2P have been set at the experimentally determined values. Ca^{2+} binding has been simplified to a two-step binding process, since this was found to give the same result as the full binding model described by Henderson et al. [32]. The rate of the dissociation of the first Ca^{2+} ion from the phosphorylated intermediate E2PCa_2 has been set at twice that of the second Ca^{2+} ion to give a single exponential decay for the Ca^{2+} -dissociation process, with an overall rate constant of 30 s^{-1} , as observed experimentally [35]; the affinity of the two luminal Ca^{2+} -binding sites on E2P for Ca^{2+} has been estimated to be 2–3.5 mM [36], and has been set here at 3.3 mM. The internal volume of the vesicles has been set at $174 \mu\text{l}/\text{mg}$ protein. The only variables in the simulation are then the rates of passive leak and slippage.

In the presence of passive leak, the level of accumulation of Ca^{2+} increases almost linearly with time until the rate of transport of Ca^{2+} into the vesicles equals the rate of leak out, at which time the maximal level of accumulation is achieved; this maximal level decreases with increasing leak rate (Figure 6A). In contrast, in

Figure 7 Simulation of ATP hydrolysis for the Ca^{2+} -ATPase with slippage

The simulations were performed using the conditions described in Figure 3B and show the time-dependence of hydrolysis of ATP in the presence of a regenerating system with a concentration of active, outwardly oriented ATPase of $0.003 \mu\text{M}$ (equivalent to $1.2 \mu\text{g}$ of protein/ml, with 50% of the protein being outwardly oriented and 60% of the total protein being active, as in the experiment shown in Figure 3B). Slippage rates (s^{-1}) are as follows: (a) 500; (b) 65.

the presence of slippage, accumulation of Ca^{2+} increases non-linearly with time and effects of slippage are seen at very short times; the level of accumulation of Ca^{2+} decreases with increasing rate of slippage (Figure 6B). These very different patterns of effect mean that passive leak and slippage can easily be distinguished; the experimental data shown in Figures 1 and 2 are typical of slippage. The exact shapes of the accumulation curves in the slippage model depend on a number of parameters in the simulation. Increasing the affinity of E2P for Ca^{2+} leads to decreased levels of accumulation of Ca^{2+} , but with the time-dependence of accumulation remaining non-linear (Figure 6C). Decreasing the vesicle volume has the same effect as increasing the affinity of E2P for Ca^{2+} . Increasing the concentration of active, outwardly oriented Ca^{2+} -ATPase molecules leads to faster accumulation of Ca^{2+} , but the faster depletion of the added ATP results in accumulation curves easily distinguishable from those resulting from decreasing the rate of slippage (Figure 6D).

The simulations also show that passive leak and slippage predict different effects on the rate of hydrolysis of ATP as a function of time. As expected, simple passive leak predicts a highly non-linear rate of ATP hydrolysis at low levels of leak, ATPase activity being low at long times due to inhibition of hydrolysis of the phosphorylated intermediate E2PCa_2 at high luminal concentrations of Ca^{2+} (Scheme 1). However, in the slippage model, the existence of a hydrolysis pathway for E2PCa_2 allows hydrolysis of ATP even at high luminal concentrations of Ca^{2+} . Thus, in contrast to the curved plots for Ca^{2+} accumulation, calculated rates of hydrolysis are linear, as shown in Figure 7; rates of 5.6 and $4.4 \text{ units}/\text{mg}$ outwardly oriented active protein are calculated at slippage rates of 500 and 50 s^{-1} respectively with the parameters given in Table 2 (Figure 7). The slippage model is therefore consistent with the observed decrease with time of the ratio of net Ca^{2+} accumulation to ATP hydrolysed. With about half of the ATPase molecules present being active, as defined by the maximum level of phosphorylation by ATP [37,38], ATPase activities are comparable with the experimental

Scheme 2

Table 3 Rates of slippage ($\text{E2PCa}_2 \rightarrow \text{E2P} + 2 \text{Ca}^{2+}$) for the ATPase in reconstituted lipid vesicles

Lipid	Slippage rate (s^{-1})
DOPC	250
DOPC/10% DOPA	65
DOPC/10% DOPS	65
DOPC/10% PtdIns	65
DOPC/10% PtdIns(4) <i>P</i>	15
DOPC/5% cardiolipin	45
DOPC/10% cardiolipin	0

values given in Table 1. Thus the slippage model is able to reproduce the essential features of the experimental data shown in Figures 1 and 2.

To account for the effect of the ATP-regenerating system (Figure 3), it is necessary to include the effects of ADP in the simulation. This can be achieved using a fuller description of the phosphorylation process, as in Scheme 2, which includes a slow conformational change between binding ATP and the phosphorylation step [39]. Simulations were performed with $k_{1f} = 2.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{1b} = 20 \text{ s}^{-1}$, $k_{2f} = 220 \text{ s}^{-1}$, $k_{2b} = 100 \text{ s}^{-1}$, $k_{4f} = 1300 \text{ s}^{-1}$ and $k_{4b} = 1.7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$; rates for the other steps are assumed to be fast, described by a dissociation constant K_{ADP} of $10 \mu\text{M}$ and an equilibrium constant K_3 of 1 [33,39–41].

Figure 1 compares the results of simulations of the slippage model with the experimental data in the absence of an ATP-regenerating system. In the simulations, an internal volume of $174 \mu\text{l}/\text{mg}$ protein was assumed with a random orientation of the ATPase across the membrane, in agreement with experimental data (Table 1). The proportion of active ATPase in the SR preparations can be determined by measuring the maximal level of phosphorylation observed with ATP in the presence of high concentrations of Ca^{2+} ; maximal levels of phosphorylation have been shown to vary between preparations, but are typically about $3.2 \text{ nmol}/\text{mg}$ protein [42], corresponding to 35% of the ATPase being active. With these parameters, the simulations match the experimental data with slippage rates of 250 s^{-1} , 65 s^{-1} and 0 in the absence of anionic phospholipid and in the presence of 10 mol% DOPA and cardiolipin respectively, and with a slippage rate of 45 s^{-1} in the presence of 5 mol% cardiolipin (Table 3). Rates of slippage in the presence of PtdIns are the same as in the presence of DOPS or DOPA, with the rate of slippage in the presence of PtdIns(4)*P* being lower (Table 3). The effects of the regenerating system for the ATPase in DOPC and 90% DOPC/10% DOPA can also be reproduced using the values for slippage rate given in Table 3, as shown in Figure 3.

We conclude that slippage on the ATPase is high in the absence of anionic phospholipids. The observation that the

optimal concentration of anionic phospholipid (10 mol%) equals the mole fraction of anionic phospholipid in the native SR membrane [29,30] suggests that reducing slippage might be important for the physiological function of the Ca^{2+} -ATPase. The observation that PtdIns(4)*P* is more effective in reducing slippage than is PtdIns suggests a possible mechanism for control of the Ca^{2+} accumulation by the ATPase; PtdIns-4-kinase has been detected in SR, as has a Ca^{2+} -dependent phosphomonoesterase capable of catalysing the breakdown of PtdIns(4)*P* [43,44].

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