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Biochimica et Biophysica Acta 1196 (1994) 1–13

BBA Biochimica et Biophysica Acta

The relationship between phospholipid content and Ca^{2+}-ATPase activity in the sarcoplasmic reticulum

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Received 6 July 1993; revised 11 July 1994

Abstract

The relationship between the phospholipid composition of sarcoplasmic reticulum and the activity of the Ca^{2+}, Mg^{2+}-stimulated ATPase was analyzed by digestion of membrane phospholipids with phospholipase C and A_2 enzymes of diverse specificity and by detergent extraction. Phospholipase C of *Clostridium perfringens* and *Clostridium welchii*, that hydrolyze preferentially phosphatidylcholine (PC), inhibited the Ca^{2+}-ATPase activity parallel with the depletion of phosphatidylcholine from the membrane. Phospholipase C of *Bacillus cereus* hydrolyzed in addition to PC, phosphatidylethanolamine (PE) and phosphatidylserine (PS), causing complete inhibition of Ca^{2+}-stimulated ATPase activity. Digestion of sarcoplasmic reticulum with the phospholipase A_2 of snake or bee venom produced similar effects. The phosphatidylinositol (PI)-specific phospholipases of *B. cereus* and *Bacillus thuringiensis* caused less than 10% inhibition of the Ca^{2+}-ATPase, accompanied by the hydrolysis of more than 70% of the phosphatidylinositol content of the membrane, without significant change in PC, PE and PS content. The inhibition of ATPase activity by the C type phospholipases was nearly completely reversed by octaethyleneglycol dodecyl ether ($C_{12}E_8$). These experiments suggest that the full phospholipid content of native sarcoplasmic reticulum ($\simeq$ 100 mol phospholipid per mol Ca^{2+}-ATPase), is required for ATPase activity and there is no indication that PE, PS, and PI play a specific role in ATP hydrolysis. Extraction of sarcoplasmic reticulum phospholipids by detergents such as deoxycholate, cholate and $C_{12}E_8$ also caused proportional inhibition of ATPase activity with the decrease in phospholipid content; the parallel extraction of PC, PE and PI left the phospholipid composition largely unchanged during delipidation. These observations do not support the requirement for a 'lipid annulus' of $\simeq$ 30 phospholipid molecules/Ca^{2+}-ATPase as proposed by Hesketh et al. ((1976) Biochemistry 15, 4145–4151) or the specific interaction of phosphatidylethanolamine with the ATPase molecule proposed by Bick et al. ((1991) Arch. Biochem. Biophys. 286, 346–352).

Keywords: Sarcoplasmic reticulum; ATPase, Ca^{2+}-; Phospholipid

1. Introduction

The Ca^{2+} transport ATPase of sarcoplasmic reticulum [1,2] is inhibited by digestion of membrane phospholipids with phospholipase C of *C. welchii* [3]. With the exception of a brief initial period, the inactivation of the Ca^{2+}-ATPase and the hydrolysis of phospholipids followed parallel courses (Fig. 1) and the maximal inhibition of ATPase activity was 75–90%. As the 'cephalin' (phosphatidylethanolamine and phosphatidylserine) content of the membrane was not affected by the *C. welchii* phospholipase C, Kielley and Meyerhof concluded [3] that choline-containing phospholipids (primarily phosphatidylcholine) are essential for the Ca^{2+}-ATPase activity. The products of phospholipase C action – diglycerides and phosphorylcholine – did not contribute to the inhibition [3], as they are both released from the membrane [4,5].

The loss of phosphatidylcholine and ATPase activity was confirmed in several laboratories using both phospholipase C [4,6–8] and phospholipase A [9–11].

The inhibition of ATPase activity by lipid depletion was attributed to the inhibition of the hydrolysis of phosphoenzyme intermediate (E ~ P) [7,8,10,12–17]. The formation of E ~ P was inhibited only after the removal of > 90% of membrane phospholipids [17,18]. The inhibited ATPase

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; $C_{12}E_8$, octaethylene glycol dodecyl ether; E ~ P, phosphorylated intermediate of Ca^{2+}-ATPase; SR, sarcoplasmic reticulum.

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SSDI 0005-2736(94)00198-7

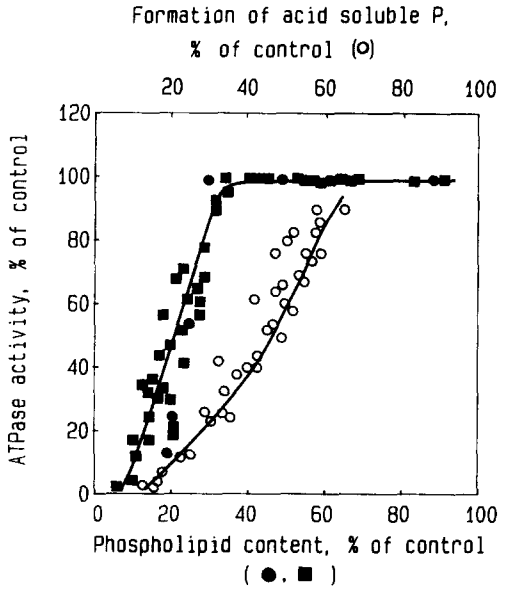

Fig. 1. Comparison of published data on the relationship between Ca^{2+}-stimulated ATPase activity and phospholipid content in sarcoplasmic reticulum. The relationship between residual phospholipid content and ATPase activity after extraction of sarcoplasmic reticulum phospholipids with cholate was reproduced by permission from Fig. 1 of Hesketh et al. [29] (●) and from Fig. 2 of Hidalgo et al. [17] (■). These data provided support for a lipid annulus of 30–35 phospholipid molecules, that is essential for ATPase activity. The relationship between the hydrolysis of phosphatidylcholine by *C. welchii* phospholipase C and the ATPase activity (○) is shown for comparison from Fig. 1 of the classical paper of Kielley and Meyerhof [3]. For details, see original publications. Clarification of the difference between these two sets of observations is the purpose of our report.

activity of the phospholipase treated preparations was fully reactivated by micellar dispersions of a variety of phospholipids, lysophospholipids, fatty acids and detergents, indicating that the phospholipid requirement of the Ca^{2+}-ATPase is non-specific [4,6–9,11,15,19–24].

A qualitatively different relationship between phospholipid content and ATPase activity was observed when the sarcoplasmic reticulum phospholipids were depleted by extraction with detergents, followed by sucrose gradient centrifugation [17,25–30]. Under these conditions nearly two-thirds of the phospholipid content of the membrane could be removed without apparent inhibition of ATPase activity, thus reducing the phospholipid content to about 30 moles of phospholipid per mol of Ca^{2+}-ATPase. Complete, irreversible loss of ATPase activity occurred when the phospholipid content was further decreased from 30 to $\simeq$ 10–20 mol/mol of Ca^{2+}-ATPase (Fig. 1). These observations, together with physical data, led to the proposition that a 'lipid annulus' of $\simeq$ 30 phospholipid molecules around the Ca^{2+}-ATPase is required for maximal ATPase activity; the non-annular, bilayer lipids constituting two-thirds of the lipid complement of sarcoplasmic reticulum, were assumed to have no direct influence on the Ca^{2+}-ATPase.

In disagreement with these observations, Moore et al. [31] reported a parallel decrease of phospholipid content and ATPase activity using cholate extraction according to Meissner et al. [32] to deplete membrane phospholipids. The large difference between the minimum lipid requirement of ATPase activity derived from phospholipase experiments and from the detergent extraction studies of Hesketh et al. [29], and Hidalgo et al. [17] also remained unexplained.

The physical evidence adduced in support of the lipid annulus underwent major reevaluation in the intervening years [33–39], raising doubts about the proposed stoichiometry, dynamics and composition of annular lipids.

The main topic of this report is a reinvestigation of the relationship between phospholipid content and ATPase activity in sarcoplasmic reticulum preparations depleted of phospholipids by digestion with phospholipase C and A_2 enzymes of different specificity, or by extraction with deoxycholate, cholate and $C_{12}E_8$.

A related unresolved problem is the possible role of minor phospholipid species in ATPase activity and Ca^{2+} transport. The dominant phospholipid component of rabbit sarcoplasmic reticulum is phosphatidylcholine (65.9 mol%), followed by phosphatidylethanolamine (17.4 mol%), phosphatidylinositol (10.7 mol%), sphingomyelin (4.7 mol%), phosphatidylserine (0.8 mol%), and cardiolipin (0.4 mol%) [40]. The phospholipid composition of purified Ca^{2+}-ATPase [41] is essentially identical to that of the sarcoplasmic reticulum [40].

As nearly complete exchange of sarcoplasmic reticulum phospholipids with dioleoylphosphatidylcholine [25–27] or with $C_{12}E_8$ [42] did not inhibit the enzymatic activity, none of the major phospholipid classes appear specifically required for ATP hydrolysis. Indeed FTIR spectroscopy provided no indication for preferential association of Ca^{2+}-ATPase with phosphatidylethanolamine [43,44]. The suggested stimulation of ATP-dependent Ca^{2+} transport by phosphatidylethanolamine [18,45–48] may have been due to the opening of leakage pathways for compensating ion fluxes and does not imply a direct role of phosphatidylethanolamine in the coupling of ATP hydrolysis to Ca^{2+} translocation [49].

In contrast to these observations, Bick et al. [50] reported a preferential retention of plasmalogenic phosphatidylethanolamine and phosphatidylserine by the Ca^{2+}-ATPase during solubilization with $C_{12}E_8$, and suggested that 6–8 phospholipid molecules "are tightly bound to the protein and are not easily extracted by extreme detergent treatment and that these phospholipids are required for enzyme turnover and calcium translocation".

Using phospholipases with differing specificities for phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine [51–55], we selectively varied the PC, PE and PS content of sarcoplasmic reticulum and analyzed the effect on ATPase activity. All phospholipid classes appear accessible to phospholipases in the native membrane and

the data do not support a specific requirement for phosphatidylethanolamine in the turnover of the Ca^{2+}-ATPase. Neither could we confirm the preferential retention of PE by the sarcoplasmic reticulum during extraction with $C_{12}E_8$, deoxycholate or cholate.

The third topic of our investigation centered on the role of phosphatidylinositides in Ca^{2+}-dependent ATP hydrolysis. Varsanyi et al. [56,57] observed that phosphorylation of $\simeq 1$ mol phosphatidylinositol per $100\,000 \times g$ protein in isolated Ca^{2+}-ATPase preparations was accompanied by 2-fold stimulation of the Ca^{2+}-ATPase activity.

We analyzed the relationship between the PI content of sarcoplasmic reticulum and the Ca^{2+}-stimulated ATPase activity using phosphatidylinositol specific phospholipases isolated from *B. cereus* and *B. thuringiensis* that specifically degrade phosphatidylinositol [58–60]. Only a slight inhibition of ATPase activity ($< 10\%$) accompanied the hydrolysis of PI, in accord with the relatively small contribution of PI to the lipid content of sarcoplasmic reticulum.

The principal conclusion of the work is that the whole phospholipid complement of sarcoplasmic reticulum ($\simeq$ 100 mol per mol of ATPase) is required for optimal ATPase activity and progressive removal of phospholipids causes a proportional impairment of the enzyme. The inhibition of ATPase activity caused by the depletion of PC, PI and PE content of sarcoplasmic reticulum roughly corresponds to the relative amounts of the various phospholipids in the membrane. As all these effects can be reversed by $C_{12}E_8$, there is no indication of a specific requirement for particular phospholipid classes in ATPase activity.

2. Experimental procedures

2.1. Materials

The following four preparations of phospholipase C were obtained from Sigma, St. Louis, MO: phospholipase C from *C. perfringens*, type XIV; phospholipase C from *C. welchii*; phospholipase C from *B. cereus*, type XI, and phosphatidylinositol specific phospholipase C from *B. cereus*. Phospholipase A_2 from *Crotalus dur. terr.* and from bee venom were also supplied by Sigma. The phosphatidylinositol-specific phospholipase from *B. thuringiensis* was kindly donated by Dr. B.N. Singh of the Department of Microbiology, SUNY Health Science Center at Syracuse, New York.

The detergents were supplied by the following companies: $C_{12}E_8$, Behring Diagnostics (Calbiochem), La Jolla, CA; sodium deoxycholate, Fisher Scientific, Fairlawn, NJ; and sodium cholate by Sigma, St. Louis, MO. Lactate dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle), ATP, ADP, NADH, phospho*enol*pyruvate, dithiothreitol, fatty acid free bovine serum albumin, 3-(*N*-morpholino)propanesulfonic acid were also products of Sigma. A23187 was purchased from Calbiochem. Phospholipids were supplied by the following companies: phosphatidylserine (bovine brain), Sigma; phosphatidylethanolamine (bacterial), phosphatidylinositol (plant), and sphingomyelin (bovine brain), Applied Science Laboratories, State College, PA; phosphatidylcholine (egg yolk), Makor Chemical, Jerusalem, Israel; asolectin (95% purified soy phosphatides), Associated Concentrates, Woodside, NY.

Thin-layer chromatography silica gel G-60 plates were purchased from Fisher and Merck, Darmstadt, Germany.

2.2. Methods

Isolation of sarcoplasmic reticulum

Sarcoplasmic reticulum vesicles were isolated from rabbit muscles essentially as described by Nakamura et al. [15]. The animals were fasted for 1 day prior to the isolation in order to deplete the glycogen content. The preparations were frozen in liquid nitrogen and stored before use in a medium of 0.3 M sucrose, 10 mM Tris-maleate buffer, pH 7.0, at a protein concentration of 20–30 mg/ml in polyethylene containers at -70°C. Immediately before use the microsomes were thawed, diluted 10-fold with a solution of 0.1 M KCl, 10 mM imidazole, pH 7.0, and 5 mM $MgCl_2$, and sedimented by centrifugation in a Type 35 fixed-angle Spinco rotor at 25 000 rpm ($72\,900 \times g$) for 40 min. The sedimented microsomes were resuspended in the appropriate test medium to final concentrations of 2–20 mg/ml.

Protein was determined by the Lowry method [61], biuret [62], or by the bicinchoninic acid method [63].

ATPase assay

ATPase activities were measured either by a coupled enzyme system of pyruvate kinase and lactate dehydrogenase [64], or by the analysis of inorganic phosphate liberation from ATP using the Fiske-SubbaRow [65] technique.

(a) In the coupled enzyme assay the microsomes were incubated at 25°C in 1 ml of medium I containing 0.1 M KCl, 0.02 M Tris-HCl, pH 7.5, 0.9 mM $CaCl_2$ (or as stated in the legends to the figures), 5 mM $MgCl_2$, 0.42 mM phospho*enol*pyruvate, 0.2 mM NADH, 7.5 IU of pyruvate kinase and 18 IU of lactate dehydrogenase ± 2 μM A23187; the microsomal protein concentration was 1–20 μg/ml. After brief preincubation 1 ml of medium II was added to start the reaction. Medium II contained 0.1 M KCl, 0.02 M Tris-HCl, pH 7.5, 1 mM EGTA, 5 mM $MgCl_2$, 10 mM ATP, and phosphoenolpyruvate, NADH, pyruvate kinase, and lactate dehydrogenase at the same concentrations as in medium I. Further additions are indicated in the legends. The absorbance was measured at 340 nm at 25°C using a Perkin-Elmer Lambda 3-B dual wavelength spectrophotometer. The recorded absorbance was linear up to at least 5–10 min of reaction time. For

measurement of the Ca^{2+}-insensitive (basal) ATP hydrolysis, Ca^{2+} was omitted from the medium. The Ca^{2+}-insensitive ATP hydrolysis rate was usually less than 5% of the rate of ATP hydrolysis in Ca^{2+}-containing medium.

(b) The rate of inorganic phosphate liberation from ATP was determined in a few cases by incubation of microsomes (0.05–0.1 mg of protein/ml) in an assay medium of 0.1 M KCl, 10 mM imidazole, pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, 0.5 mM EGTA, 0.45 mM $CaCl_2 \pm 1$ μM A23187 at 25°C for 1–5 min. The reaction was stopped with trichloroacetic acid, and the inorganic phosphate was determined according to Fiske and SubbaRow [65]. In the assay of basal (Ca^{2+}-insensitive) ATPase activity, the Ca^{2+} was omitted from the medium.

Assay of phospholipid content

For determination of phospholipids, microsomes were extracted with chloroform-methanol according to the method of Folch et al. [66] and the phospholipids were separated by thin-layer chromatography on Silica Gel G using chloroform-methanol-concentrated NH_4OH (65:25:4, v/v) as solvent. For two-dimensional separation the solvent systems were: chloroform-methanol-concentrated NH_4OH (65:35:5, v/v) and chloroform-acetone-methanol-acetic acid-H_2O (50:20:10:10:5, v/v). Normally the phospholipids were detected by exposure to iodine vapors. For the identification of aminophospholipids (phosphatidylethanolamine and phosphatidylserine), ninhydrin was used [67]; sphingomyelin and cerebrosides were detected by the benzidine reaction [68,69].

Total phospholipid phosphorus was determined from the Folch extracts after perchloric acid digestion according to Dittmer and Wells [70] by a modification of the Bartlett method [71]. The same procedures were used for the determination of individual phospholipids after thin-layer chromatography.

Electron microscopy

The specimens were stained with 1% uranyl acetate, pH 4.3 and viewed in a Siemens Elmiskop I electron microscope at an accelerating voltage of 60 kV.

3. Results

3.1. The effect of various phospholipase C enzymes on the total phospholipid content of sarcoplasmic reticulum

Phospholipase C enzymes of three distinct specificities were used. The phospholipase C of *C. perfringens* and *C. welchii* rapidly hydrolyze phosphatidylcholine, somewhat slower sphingomyelin, but do not attack phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol [54,55]. The phospholipid content of sarcoplasmic reticulum was rapidly reduced by these enzymes to $\simeq 0.2$ μmol per mg protein and further incubation even at high enzyme concentration (3 IU/ml) caused little change in phospholipid content (Fig. 2).

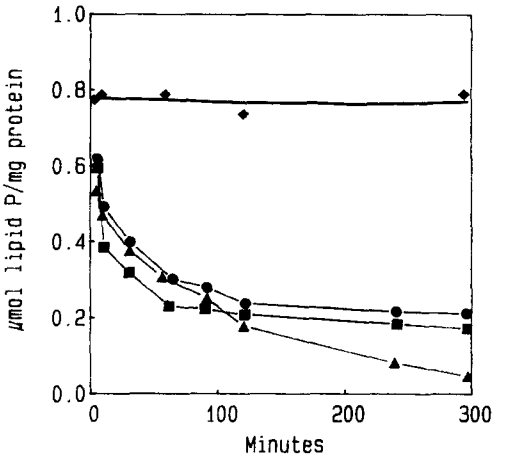

Fig. 2. Hydrolysis of sarcoplasmic reticulum phospholipids by various phospholipases. Sarcoplasmic reticulum vesicles were digested with phospholipase C from *C. perfringens* (●), *C. welchii* (■), and *B. cereus* (▲) in a medium of 0.1 M KCl, 10 mM imidazole, pH 7.3 and 0.1 mM $CaCl_2$, at 25°C for times indicated on the abscissa. The concentration of sarcoplasmic reticulum proteins was 4 mg/ml and of phospholipases 1 IU/ml. Control samples were incubated under the same conditions, without phospholipase C (♦). Aliquots taken at intervals were extracted according to Folch et al. [66], the phospholipids were dissolved in chloroform/methanol (2:1, v/v) and analyzed for phospholipid phosphorus according to Dittmer and Wells [70].

The phospholipase C of *B. cereus* has a broader specificity and in addition to PC and SM, it hydrolyzes PE and PS, at a slightly slower rate [52,54]. As a result, the phospholipid content of sarcoplasmic reticulum was reduced by *B. cereus* phospholipase C to levels below 0.05 μmol/mg protein (Fig. 2).

The phosphatidylinositol-specific phospholipase C enzymes of *B. thuringiensis* and *B. cereus* cleave relatively selectively phosphatidylinositol [58–60]. The PI-specific phospholipases caused only $\simeq$ 8–10% change in the total phospholipid content of sarcoplasmic reticulum (not shown), that corresponds to the PI content of 8–10 mol%.

3.2. Changes in the ATPase activity and phospholipid composition of sarcoplasmic reticulum during treatment with Cl. perfringens and Cl. welchii phospholipase C

During digestion with phospholipase C either from *C. perfringens* (Fig. 3A) or from *C. welchii* (Fig. 3B) the ATPase activity of sarcoplasmic reticulum decreases without a significant lag-phase, parallel with the decrease in phosphatidylcholine content. A residual Ca^{2+}-sensitive ATPase activity is retained even after prolonged digestion, consistent with the retention of some undigested phospholipids.

The phosphatidylethanolamine and phosphatidylinositol content of sarcoplasmic reticulum was not affected significantly by either enzymes and these phospholipids may

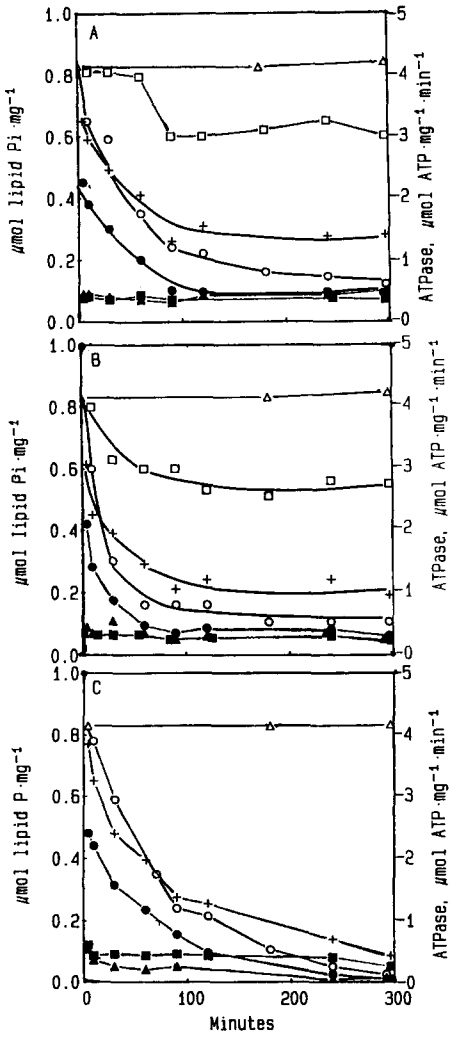

Fig. 3. Phospholipid composition and ATPase activity of sarcoplasmic reticulum after digestion with *C. perfringens* (A) *C. welchii* (B). and *B. cereus* (C) phospholipase C. Sarcoplasmic reticulum vesicles were digested with the various phospholipases as described in Fig. 2, and the extracted phospholipids were separated by two-dimensional thin layer chromatography. The phospholipid spots scraped from thin layer plates were subjected to perchloric acid digestion [70], and the lipid phosphorus was determined by Bartlett's method [71]. Total lipid phosphorus was analyzed from the phospholipid extracts by the same method without prior separation by thin layer chromatography. ATPase activity was measured in the presence of 0.5 mM Ca^{2+}, 0.5 mM EGTA and 2 μM A23187, with or without $C_{12}E_8$ (4 mg/mg protein) as described under Methods. Symbols: △, ATPase activity of control microsomes incubated without phospholipase C, assayed without $C_{12}E_8$; ○, ATPase activity of phospholipase C-treated microsomes assayed without $C_{12}E_8$; □, ATPase activity of phospholipase C-treated microsomes assayed with 4 mg $C_{12}E_8$. Phospholipid composition: ●, phosphatidylcholine; ▲, phosphatidylethanolamine; ■, phosphatidylinositol; +, total phospholipid P.

account for the residual ATP hydrolysis. Sphingomyelin and phosphatidylserine are minor components and their role in ATPase activity is uncertain.

The ATPase activity of the lipid-depleted membranes was nearly completely restored by 4 mg $C_{12}E_8$ per mg protein in the assay system, even after several days of incubation at 2–4°C. The extent of restoration was sensitively affected by the Ca^{2+} and $C_{12}E_8$ concentration of the medium (see below). Complete restoration of ATPase activity could be obtained in most cases only by optimizing the conditions of each experiment.

3.3. The ATPase activity and phospholipid composition of sarcoplasmic reticulum during digestion with B. cereus phospholipase C

The *B. cereus* phospholipase C hydrolyzed both phosphatidylcholine and phosphatidylethanolamine, decreasing the Ca^{2+}-sensitive ATPase activity to below 10% of the control level (Fig. 3C). This is consistent with the suggestion that the residual ATPase activity after treatment with *C. welchii* or *C. perfringens* phospholipase C was indeed supported by phosphatidylethanolamine. The ATPase activity of the lipid-depleted membranes was largely restored by 4 mg $C_{12}E_8$/mg protein, indicating that neither phosphatidylcholine nor phosphatidylethanolamine were specifically required for ATP hydrolysis.

The phosphatidylinositol content of the sarcoplasmic reticulum remained largely unaffected by *B. cereus* phospholipase C and may account for the small residual ATPase activity after 200 min of digestion.

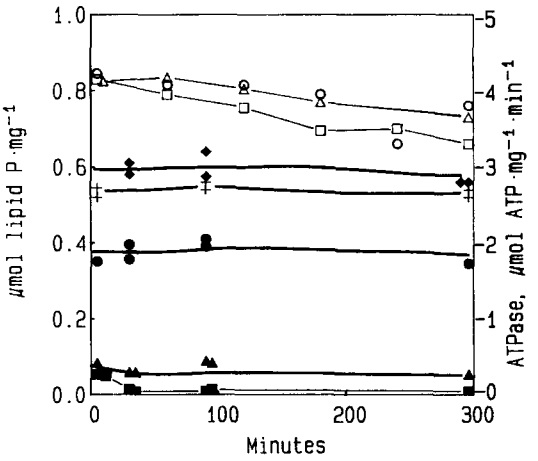

Fig. 4. The ATPase activity and phospholipid composition of sarcoplasmic reticulum during digestion with the phosphatidylinositol-specific phospholipase C of *B. cereus* and *B. thuringiensis*. The treatment of sarcoplasmic reticulum vesicles with PI-specific phospholipases was carried out in a medium of 0.1 M KCl, 10 mM imidazole, pH 7.4, at 25°C for times indicated on the abscissa. The protein concentration of sarcoplasmic reticulum was 4.0 mg/ml and of phospholipases 0.5 IU/ml. ATPase and phospholipid assays were performed as described in Figs. 2–4. Symbols: △, control ATPase; ○, ATPase activity of sarcoplasmic reticulum digested with PI-specific phospholipase C from *B. cereus*; □, ATPase activity of sarcoplasmic reticulum digested with PI-specific phospholipase C from *B. thuringiensis*. ATPase assays were performed with 0.5 mM Ca^{2+}, 0.5 mM EGTA and 2 μM A23187, without $C_{12}E_8$, otherwise as described in Methods. Phospholipids were analyzed after separation by thin layer chromatography; ●, phosphatidylcholine; ▲, phosphatidylethanolamine; ■, phosphatidylinositol; +, total lipid P, control; ◆, total lipid P after phospholipase treatment. The phospholipid data obtained by the two phospholipases were similar and are identified by the same symbols.

The products of phospholipase C action – diglycerides and phosphorylcholine – are both released from the membrane [5] and are not responsible for the inhibition of ATPase activity [3]. This conclusion is further supported by the observation that partial extraction of diglycerides from phospholipase C-treated freeze-dried preparations by dry ether according to Drabikowski et al. [72], did not restore the ATPase activity but subsequent addition of $C_{12}E_8$ produced essentially complete reactivation (not shown).

3.4. The ATPase activity and phospholipid composition of sarcoplasmic reticulum during digestion with phosphatidylinositol-specific phospholipase C (PI-PLC) from B. thuringiensis and B. cereus

The phosphatidylinositol-specific phospholipases from these two different sources had little ($< 10\%$) effect on ATPase activity; a slight decrease in hydrolysis rate was also observed in some control samples that were incubated without phospholipase C (Fig. 4). The phosphatidylinositol content of sarcoplasmic reticulum was sharply reduced already after 30 min digestion, but the PC and PE content of the membranes remained unaffected.

These observations argue against a specific role of phosphatidylinositol in ATPase activity, but the suggested involvement of $\simeq 1$ mol PI/mol ATPase in the regulation of ATP hydrolysis [56,57] is difficult to exclude.

3.5. The experimental conditions affecting the restoration of ATPase activity by $C_{12}E_8$ after phospholipid depletion with phospholipase C of B. cereus

The somewhat variable restoration of ATPase activity in phospholipase C-treated membranes by $C_{12}E_8$ prompted us to explore the experimental conditions that may differentially affect ATPase activity in native and reconstituted membranes.

The optimal Ca^{2+} concentration for ATPase activity increased by $\simeq 0.5$ pCa units upon addition of 4.46 mg $C_{12}E_8$/mg protein to native sarcoplasmic reticulum vesicles (Fig. 5). Therefore the ATPase assay of phospholipase C-treated preparations reconstituted with $C_{12}E_8$ must be performed at a relatively high ($\simeq 100$ μM) free Ca^{2+} concentration to make them comparable with the control enzyme. ATPase assays performed at the optimal Ca^{2+} concentration of the native vesicles (5–10 μM) are likely to yield 40–50% lower ATPase activity in the presence of $C_{12}E_8$.

The dependence of ATPase activity on $C_{12}E_8$ concentration is also complex. When assayed at free Ca^{2+} concentration of $9 \cdot 10^{-6}$ M the optimal activation of ATP hydrolysis by the vesicles treated with *B. cereus* phospholipase C was at a $C_{12}E_8$ concentration of $\simeq 30$ μg/ml (1.5 mg $C_{12}E_8$/mg protein); this peak of activation was followed by a sharp decline to a lower steady level of ATP

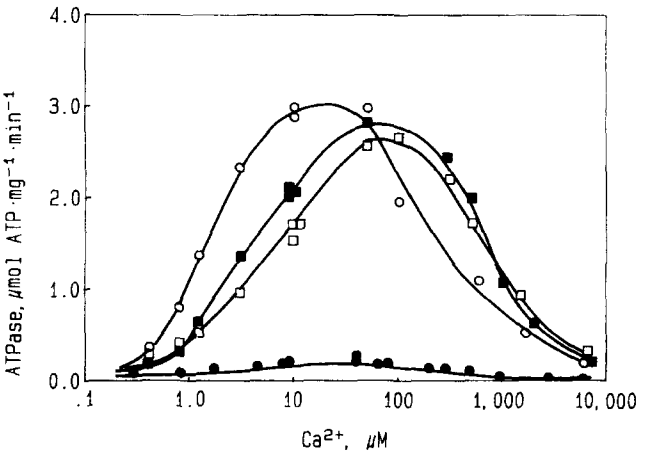

Fig. 5. Effect of $C_{12}E_8$ on the [Ca^{2+}] dependence of ATPase activity in control sarcoplasmic reticulum and in sarcoplasmic reticulum digested with phospholipase C of *B. cereus*. Sarcoplasmic reticulum vesicles were digested with phospholipase C of *B. cereus* at 25°C for 5 h in a medium of 0.1 M KCl, 10 mM imidazole, pH 7.3, SR protein concentration: 3.54 mg/ml; phospholipase C concentration, 1.3 IU/ml (■,●). Control microsomes were incubated under similar conditions without phospholipase C (□,○). ATPase assay was performed as described under Methods in the presence of 2 μM A23187, either without $C_{12}E_8$ (○,●) or with 4.46 mg $C_{12}E_8$/mg protein (□,■). The SR protein concentration during ATPase assay was 8.9 μg/ml. The free Ca^{2+} concentration of the assay system was established by varying the total Ca^{2+} concentration between 0.45 and 1.5 mM at a fixed EGTA concentration of 0.5 mM or by varying the EGTA concentration between 0.5 mM and 1.5 mM at a fixed total Ca^{2+} concentration of 0.45 mM.

hydrolysis at $C_{12}E_8$ concentrations of 0.1–1.0 mg/ml (Fig. 6A). The ATPase activity of native sarcoplasmic reticulum showed the opposite behavior; it was inhibited at 30 μg/ml $C_{12}E_8$ concentration, and returned to higher steady level at 0.1–1.0 mg/ml $C_{12}E_8$ (Fig. 6A).

A different pattern was seen when the ATPase activities were measured at 0.55 mM Ca^{2+} concentration. The specific activities were generally lower due to inhibition by Ca^{2+}, but the control and delipidated membranes reached similar ATPase activities above 20 μg/ml $C_{12}E_8$ concentration (Fig. 6B). These observations suggest that the ATPase of delipidated membranes undergoes rapid inactivation at low Ca^{2+} and high $C_{12}E_8$ concentration to a form that is no longer activated by $C_{12}E_8$, and this effect is prevented by 0.55 mM Ca^{2+}.

Ca^{2+}-dependent changes in the sensitivity of delipidated Ca^{2+}-ATPase to $C_{12}E_8$ may also explain that in our experiments maximal reactivation of Ca^{2+}-ATPase was usually obtained at $C_{12}E_8$ concentration of 2–4 mg/mg ATPase, while in the experiments of Dean and Tanford [42], 50 mg $C_{12}E_8$/mg protein was required for maximal reactivation.

3.6. The effect of phospholipase A_2 on the ATPase activity and phospholipid composition of sarcoplasmic reticulum

Phospholipase A_2 cleaves phosphatidylcholine into lysophosphatidylcholine and fatty acids. Phosphatidyl-

ethanolamine and phosphatidylserine are also attacked, but sphingomyelin and phosphatidylinositol are not hydrolyzed. As fatty acids and lysophosphatidylcholine are known to activate the hydrolysis of ATP by lipid depleted sarcoplasmic reticulum [4,6], fatty acid free serum albumin was included in the reaction mixture to neutralize the effects of reaction products.

Treatment of sarcoplasmic reticulum vesicles with bee venom phospholipase A_2 or with *Crotalus durissus terrificus* venom phospholipase A_2 in the presence of serum albumin, produced rapid inhibition of ATPase activity (Fig. 7A), accompanied by parallel loss of phosphatidylcholine and a corresponding increase in lysophosphatidylcholine content (Fig. 7B). There was also a decrease in phosphatidylethanolamine, while the phosphatidylinositol content decreased only slightly (Fig. 7B). The bee venom and snake venom phospholipases produced similar effects. The ATPase activity of phospholipase A_2-treated sarcoplasmic reticulum was only moderately activated by 2.5 mg $C_{12}E_8$ per mg protein (Fig. 7A); it is not known whether $C_{12}E_8$ binds to serum albumin.

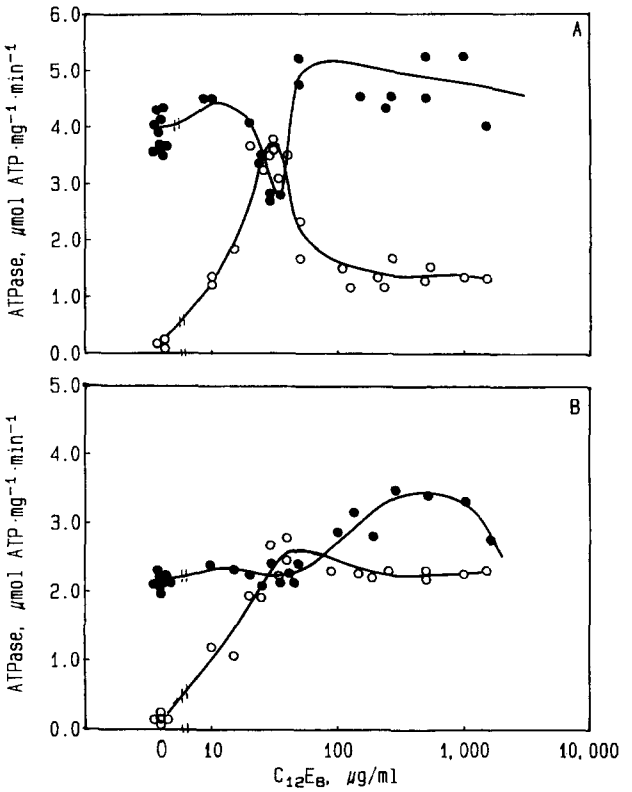

Fig. 6. Dependence of ATPase activity on $C_{12}E_8$ concentration at a free Ca^{2+} concentration of 9 μM (A) and 550 μM (B). Sarcoplasmic reticulum vesicles were digested with *B. cereus* phospholipase C in a medium of 0.1 M KCl, 10 mM imidazole, pH 7.4, and 0.1 mM $CaCl_2$ for 2 hrs at 25°C (○). Protein concentration of sarcoplasmic reticulum was 4 mg/ml and that of phospholipase C, 2 IU/ml. Control sarcoplasmic reticulum was incubated under identical conditions without phospholipase C (●). ATPase assays were performed as described under Methods in the presence of 2 μM A23187 and $C_{12}E_8$ at concentrations indicated on the abscissa. The concentration of EGTA was 0.5 mM and the total Ca^{2+} concentration was adjusted to yield free Ca^{2+} concentrations of 9 μM (A) and 550 μM (B). The higher Ca^{2+} concentration provided protection against irreversible loss of ATPase activity in the phospholipase C treated preparation at $C_{12}E_8$ concentrations higher than 100 μg/ml.

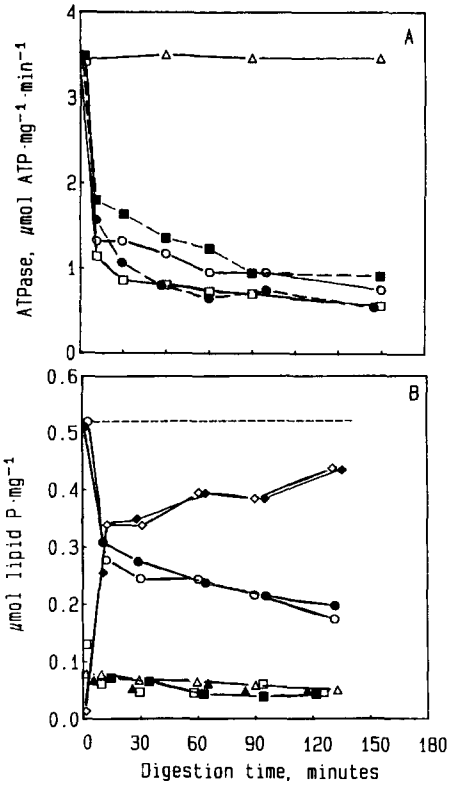

Fig. 7. The effect of phospholipase A_2 from bee venom and from *Crotalus dur. terr.* venom on the ATPase activity (A) and phospholipid composition (B) of sarcoplasmic reticulum. (A) Sarcoplasmic reticulum vesicles (4 mg protein/ml) were digested witb 2.5 IU/ml bee venom phospholipase A_2 (□,■) or *Crotalus* phospholipase A_2 (○,●) in a medium of 0.1 M KCl, 10 mM Tris-maleate, pH 7.0, 5 mM $CaCl_2$ and 4% fatty acid free serum albumin at 25°C for times indicated on the abscissa. Aliquots were taken for assay of ATPase activity in the presence of 2 μM A23187, with 2.5 mg $C_{12}E_8$/mg protein (■,●) or without $C_{12}E_8$ (□,○). Control samples were incubated under the same conditions without phospholipase A_2 and assayed for ATPase activity with 2 μM A23187, but without $C_{12}E_8$ (△). (B) Sarcoplasmic reticulum vesicles were incubated with bee venom phospholipase A_2 (○,□,△,◇), or snake venom phospholipase A_2 (●,■,▲,◆), as described under A. Samples taken at intervals were extracted according to Folch et al. [66], the phospholipids were separated by one-dimensional thin layer chromatography, and the phospholipid composition was determined according to Dittmer and Wells [70]. Symbols, ○,●, phosphatidylcholine; □,■, phosphatidylethanolamine; △,▲, phosphatidylinositol; ◇,◆, lysophosphatidylcholine. The phospholipid content of control sarcoplasmic reticulum incubated without phospholipase remained unchanged during the experiment. The level of control phosphatidylcholine-P is indicated by a broken line.

From each series of experiments additional samples were collected after 60 and 130 min digestion with phospholipase A_2, diluted 20-fold with 0.1 M KCl, 10 mM imidazole, pH 7.3 and centrifuged at 80000 × g for 1 h; these samples provided data on the phospholipid composition of the sedimented membranous elements. Phospholipase A_2-treated samples extracted with chloroform-

methanol without prior centrifugation provided data on the total phospholipid content. Sarcoplasmic reticulum vesicles not exposed to phospholipase A_2 served as control.

In control samples there was only a trace amount of lysophosphatidylcholine, even after 2 h of incubation, and all major phospholipids were fully recovered after centrifugation in the sediment fraction (Table 1). By contrast, after treatment with either phospholipase A_2 the recovery of phospholipid phosphorus following centrifugation was significantly reduced (Table 1). The decrease in lysophosphatidylcholine content was particularly striking, indicating that much of the lysophosphatidylcholine formed from PC by phospholipase A_2 was released from the membrane. Similarly, there was a nearly complete loss of fatty acids from the sedimented membranes due to binding to serum albumin. Therefore, the progressive loss of ATPase activity during the treatment of sarcoplasmic reticulum with phospholipase A_2 can be attributed to the proportional loss of phosphatidylcholine and phosphatidylethanolamine from the membrane.

3.7. The effect of detergents on phospholipid content and ATPase activity

Extraction of sarcoplasmic reticulum phospholipids with deoxycholate

Sarcoplasmic reticulum was incubated for 30 min in ice with deoxycholate (0.1–1.0 mg/mg protein) in a medium of 0.1 M KCl, 50 mM Tris-Cl, pH 8.0, 5 mM ATP, and 5 mM DTT. After sucrose gradient centrifugation the top layer and the ATPase-containing layer were analyzed for ATPase activity and lipid composition (Fig. 8). The ATPase activity of the control samples measured in the absence of A23187 was relatively low ($\simeq 1.3$ μmol mg^{-1} min^{-1}) due to inhibition by the accumulated Ca^{2+} and

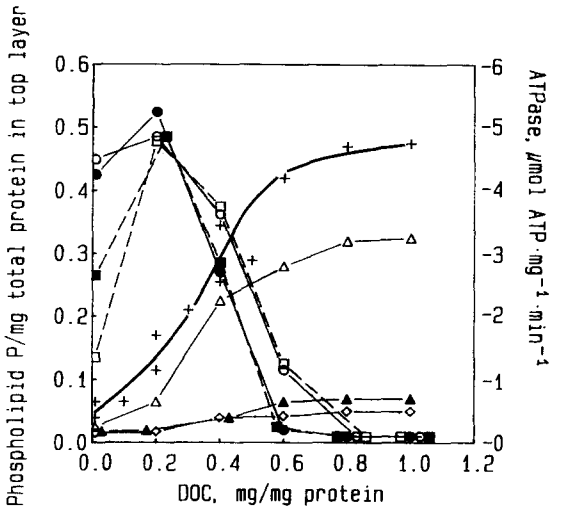

Fig. 8. Extraction of sarcoplasmic reticulum phospholipids with deoxycholate. Sarcoplasmic reticulum was treated with deoxycholate (0.1–1.0 mg/mg protein) in a medium of 1.0 M KCl, 50 mM Tris-HCl, pH 8.0, 5 mM ATP and 5 mM DTT for 30 min at 0°C. The protein concentration was 4 mg/ml. Samples were taken for ATPase activity measurements immediately after the end of incubation. The remaining samples were applied for sucrose gradient (25–50 percent sucrose in a medium of 1 M KCl, 50 mM Tris-HCl, pH 8.0, 5 mM DTT) and centrifuged in SW 40 Ti rotor at 35000 rpm (= 200000× *g*) overnight. The top layers and the ATPase bands were collected and analyzed for protein concentration, ATPase activity and phospholipid composition. Symbols: ○,□, ATPase activity before centrifugation; ●,■, ATPase activity after centrifugation. The ATPase activities were measured with 2 μM A23187 in the assay medium (○,●) or without A23187 (□,■). The phospholipids released by deoxycholate are indicated by the phospholipid composition of the top layer calculated on the basis of the total protein applied to the sucrose gradient. △, phosphatidylcholine; ▲, phosphatidylethanolamine; ◇, phosphatidylinositol; + total phospholipid P. The phospholipid content of the top layer ($\simeq 0.5$ μmol P mg^{-1}) is $\simeq 30\%$ less than the total phospholipid content of control sarcoplasmic reticulum. This difference is largely accounted for by the presence of free phospholipids swept into the heavier protein containing fractions.

Table 1
The phospholipid composition of sarcoplasmic reticulum after treatment with bee venom or snake venom phospholipase A_2

Treatment	Incubation time (min)	Centrifugation	Phospholipid content (nmol mg^{-1})				
			PC	lyso PC	PE	PI	total lipid P
Control	0	–	513.3	–	123.5	73.8	769.5
Control	120	–	521.9	–	127.8	78.8	784.8
Control	120	+	501.4	–	130.0	81.4	763.2
Bee venom PL A_2	60	–	241.9	395.2	60.2	59.0	772.4
Bee venom PL A_2	60	+	170.9	123.3	41.9	52.8	401.2
Bee venom PL A_2	130	–	171.4	419.0	40.0	43.5	691.0
Bee venom PL A_2	130	+	127.6	93.3	42.3	43.0	317.6
Crotalus dur. terr. PL A_2	60	–	240.0	399.0	49.5	62.6	766.1
Crotalus dur. terr. PL A_2	60	+	186.1	141.1	55.0	59.0	452.6
Crotalus dur. terr. PL A_2	130	–	191.4	425.2	48.0	49.2	730.4
Crotalus dur. terr. PL A_2	130	+	141.9	112.3	43.3	49.2	355.9

Sarcoplasmic reticulum vesicles were digested with phospholipase A_2 as described in the legend to Fig. 10. After 60 and 130 minutes of digestion samples were processed for phospholipid analysis as described in Methods. Parallel aliquots were diluted 20-fold with 0.1 M KCl, 10 mM imidazole, pH 7.4, and centrifuged at 80000 × *g* for 1 h. The sedimented membranes were extracted with chloroform/methanol according to Folch et al. [66] and processed for phospholipid analysis. Membranes not exposed to phospholipase A_2 served as control. The traces of lysophosphatidylcholine in the control samples were not determined quantitatively.

was activated about 2-fold at an initial deoxycholate concentration of 0.2 mg/mg protein. In the presence of A23187 the control ATPase activity was 4–5 μmol mg^{-1} min^{-1} and the activation by deoxycholate was less pronounced. These changes in ATPase activity occurred without major loss of sarcoplasmic reticulum phospholipids.

At higher concentration of deoxycholate (0.4–0.8 mg/mg protein), the ATPase activity was progressively inhibited, with proportional release of phospholipids into the top layer of the gradient. The parallel changes in PC, PE and PI content do not support preferential interaction of either phospholipid classes with the Ca^{2+}-ATPase.

Half-maximal inhibition of ATPase activity coincides with the loss of $\simeq$ half of the phospholipid complement of the membrane. This is in essential agreement with the relationship of phospholipid content to ATPase activity in the phospholipase C-treated vesicles (Figs. 2 and 3). The inhibition of DOC-treated Ca^{2+}-ATPase could not be reversed by the addition of $C_{12}E_8$. This irreversible inhibition cannot be attributed to the loss of phospholipids per se, since lipid-depleted preparations obtained by phospholipase C treatment could be fully reactivated with $C_{12}E_8$ even after several days of incubation in ice.

The activation of Ca^{2+}-ATPase at low deoxycholate concentration and the parallel loss of ATPase activity and phospholipid content between 0.2 and 0.8 mg deoxycholate per mg protein (Fig. 8) are in disagreement with the data of Hesketh et al. [29] and of Hidalgo et al. [17], who showed no change in ATPase activity until the phospholipid content of the membrane was reduced to 30–35% of the control value (Fig. 1).

The data of Hesketh et al. [29] and of Hidalgo et al. [17] do not show the well known activation of Ca^{2+}-ATPase by deoxycholate that is clearly seen in Fig. 8. Although Hidalgo et al. [17] commented in the text that there was activation of ATP hydrolysis at 0.1 mg DOC/mg protein, this activation was not reflected in Fig. 2 of their report. By disregarding this activation, Hesketh et al. [29] and Hidalgo et al. [17] may have chosen an arbitrary low value for maximal ATPase activity, thus shifting the apparent inhibition to greater than 70% lipid depletion.

Extraction of sarcoplasmic reticulum phospholipids with cholate

Treatment of sarcoplasmic reticulum with cholate under the conditions of Moore et al. [31] followed by sucrose gradient centrifugation causes little or no change in the phospholipid content of the membrane up to $\simeq$ 0.6 mg cholate per mg protein, while the Ca^{2+}-stimulated ATPase is activated (Fig. 9A,B). At higher cholate concentration there is a parallel decrease in ATPase activity and phospholipid content (Fig. 9A,B). Half-maximal decrease in phospholipid content is accompanied by half-maximal inhibition of ATPase activity when tested in the presence of A23187 (Fig. 10). Similar changes in ATPase activity were observed before and after sucrose gradient centrifuga-

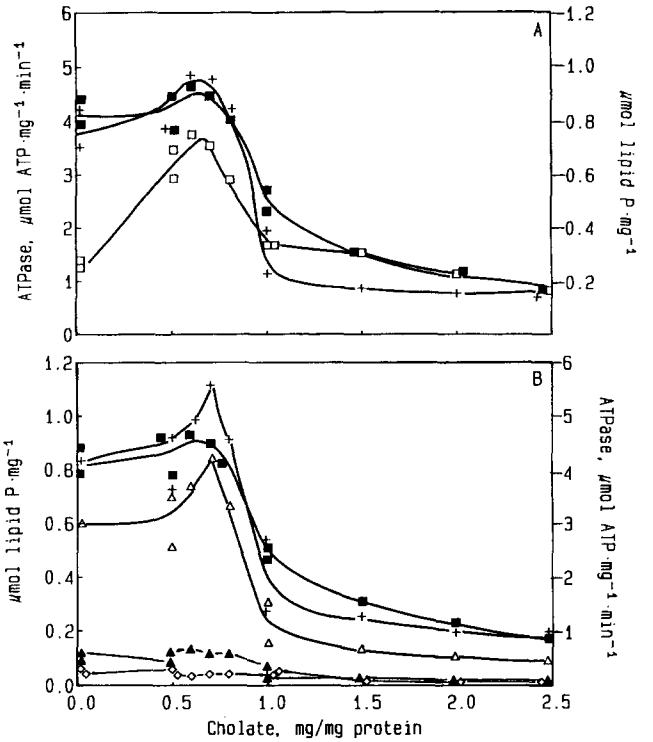

Fig. 9. Extraction of sarcoplasmic reticulum phospholipids with cholate. Sarcoplasmic reticulum vesicles (4 mg protein/ml) were extracted with cholate (0.5–2.5 mg/mg protein) in a medium of 0.3 M sucrose, 0.5 M KCl, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 0.1 mM $CaCl_2$ and 10 mM Tris, pH 8.0 for 10 min at 0°C. The samples were loaded on a gradient of 25–50% sucrose in 0.5 M KCl, 10 mM Tris-HCl, pH 8.0, 5 mM DTT and 1 mM $CaCl_2$ and centrifuged at 35000 rpm in SW 40 Ti rotor ($\simeq 200000 \times g$) overnight. The top layers and the layers containing the Ca^{2+}-ATPase were analyzed for protein content, ATPase activity (A), and phospholipid composition (B), as described under Methods. Symbols: (A) ATPase activity of the ATPase fraction of the sucrose gradient assayed with 2 μM A23187 (■) or without A23187 (□). Total phospholipid-P content (+). (B) Phospholipid composition: △, phosphatidylcholine; ▲, phosphatidylethanolamine; ◇, phosphatidylinositol; +, total phospholipid-P content. ATPase activity assayed with 2 μM A23187 (■).

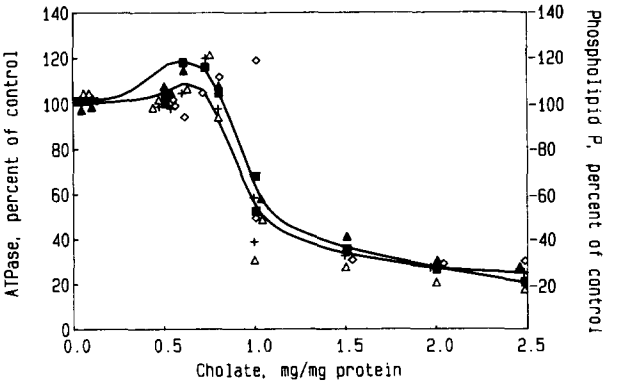

Fig. 10. The relationship between ATPase activity and phospholipid composition at various cholate concentrations. The data shown in Fig. 9A and B were recalculated as percent of control values measured in the absence of cholate to demonstrate the strictly parallel relationship of ATPase activity to phospholipid content, and the non-selective extraction of all major phospholipid species. Symbols as in Fig. 9.

tion (not shown). These observations confirm the earlier data of Moore et al. [31], but disagree with the constant ATPase activity reported by Hesketh et al. [29] at lipid contents between 100 and 40 mol/mol Ca^{2+}-ATPase.

The parallel decrease in PC and PE content with increasing cholate concentration (Figs. 9B and 10) is consistent with the FTIR data of Jaworsky and Mendelsohn [43,44] that indicated no specific interaction of Ca^{2+}-ATPase with PC or PE. The PI content of sarcoplasmic reticulum was reduced significantly only at cholate concentrations higher than 1 mg per mg protein (Figs. 9B and 10); this would indicate some resistance of PI to extraction with cholate, but the significance of this observation remains to be explored.

Extraction of sarcoplasmic reticulum phospholipids with $C_{12}E_8$

Two experimental conditions were used to analyze the effect of $C_{12}E_8$ on the phospholipid content and ATPase activity of sarcoplasmic reticulum.

Condition A was similar to that used by Bick et al. [50]. The extraction was performed in a calcium and potassium free medium containing 8.8 mM Tes, pH 7.4, 1.1 mM Tris and 0.034 M sucrose for 30 min at 4°C, and aliquots were taken for ATPase activity measurements. The samples were applied for sucrose gradient centrifugation (0.3–1.8 M sucrose) and the phospholipid composition was analyzed in the top layer of the gradient. As shown in Fig. 11, the loss of phospholipids was accompanied by parallel

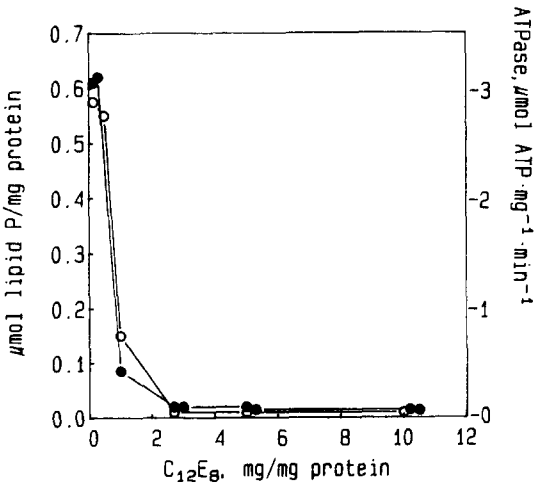

Fig. 11. Extraction of sarcoplasmic reticulum phospholipid with $C_{12}E_8$ in a low ionic strength, low Ca^{2+} medium. Sarcoplasmic reticulum vesicles (4 mg protein/ml) suspended in 10 mM Tes buffer, pH 7.4 were treated with $C_{12}E_8$ (0.5–10 mg/mg protein) at 4°C for 30 min. The samples were loaded onto sucrose gradient (0.3 M–1.8 M sucrose in 0.1 M KCl, 10 mM imidazole, pH 7.4) and centrifuged in SW 40 Ti rotor for 15 h at 35000 rpm ($\simeq 200000 \times g$) at 4°C. The top layer and the ATPase containing bands were collected and analyzed for protein concentration, ATPase activity and phospholipid content. Symbols: ○, the total phospholipid P content of the ATPase bands; ●, ATPase activity of the ATPase band assayed as described under Methods in the presence of 2 μM A23187.

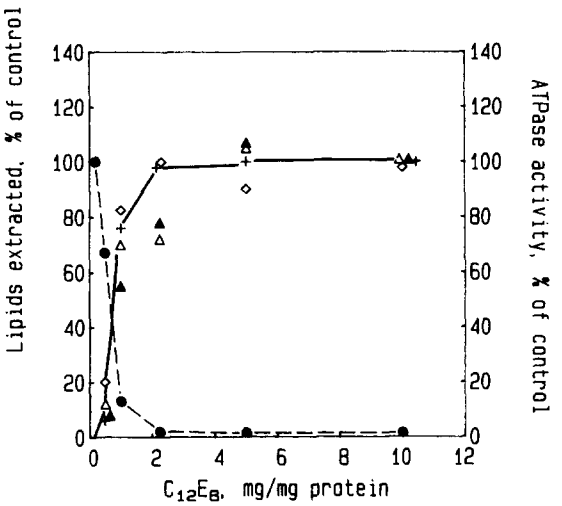

Fig. 12. Relationship between ATPase activity and phospholipid composition during treatment of sarcoplasmic reticulum with $C_{12}E_8$ in a low-ionic strength, low-Ca^{2+} medium. For experimental conditions, see legend to Fig. 11. The phospholipid composition of the top layer obtained by sucrose gradient centrifugation was analyzed. Symbols: △, phosphatidylcholine; ▲, phosphatidylethanolamine; ◇, phosphatidylinositol; +, total phospholipid-P; ●, ATPase activity of the ATPase band measured in the presence of 2 μM A23187.

inhibition of Ca^{2+}-ATPase. The parallel displacement of PC, PE, and PI from the sarcoplasmic reticulum by $C_{12}E_8$, measured by their appearance in the top layer of the

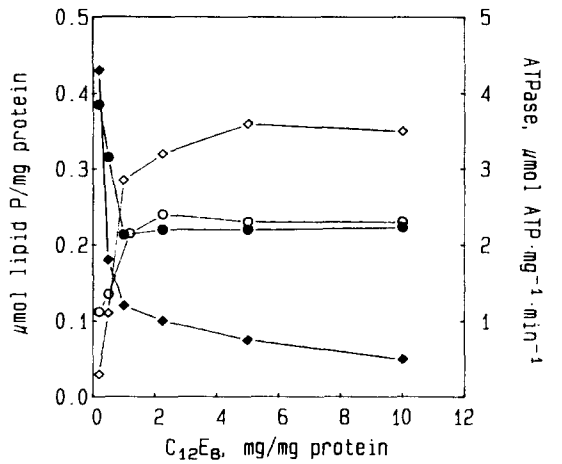

Fig. 13. Relationship between ATPase activity and phospholipid content during extraction of sarcoplasmic reticulum lipids with $C_{12}E_8$ in a high K^+, high Ca^{2+} medium. Sarcoplasmic reticulum vesicles (4 mg protein/ml) were treated with $C_{12}E_8$ (0.5–10 mg/mg protein) in a medium of 1 M KCl, 50 mM Tris-HCl, pH 8.0, 5 mM DTT and 5 mM $CaCl_2$ at 4°C for 30 min; aliquots were taken for ATPase activity and the samples were loaded onto a sucrose gradient (25%–50% sucrose in 1 M KCl, 50 mM Tris-HCl, pH 8.0, 5 mM DTT and 5 mM $CaCl_2$) and centrifuged overnight at 4°C in an SW 40 Ti rotor at 35000 rpm ($\simeq 200000 \times g$). The layers of the sucrose gradient were collected and analyzed for protein and phospholipid composition, as described under Methods. Symbols: ATPase activity assayed without A23187 (○) or in the presence of 2 μM A23187 (●). Total phospholipid content of the top layer (◇) and of the ATPase band (◆) expressed on the basis of the total protein applied to the sucrose gradient.

sucrose gradient (Fig. 12) indicates no preferential retention of either of these phospholipids by the Ca^{2+}-ATPase, in contrast to the data of Bick et al. [50]. At $C_{12}E_8$ concentrations higher than 2.5 mg/mg protein, the inhibition of ATPase activity and the release of phospholipids was essentially complete.

Under condition B the extraction with $C_{12}E_8$ was done in a medium of 1 M KCl, 50 mM Tris-Cl, pH 8.0, 5 mM DTT and 5 mM $CaCl_2$, for 30 min at 4°C; after assay of ATPase activity the samples were applied for sucrose gradient centrifugation. Under these conditions much of the ATPase activity was retained, even at a $C_{12}E_8$ concentration of 10 mg/mg protein, that caused the release of 80–90% of the phospholipid complement of sarcoplasmic reticulum (Fig. 13). It remains to be determined whether the observed decrease in the specific activity of Ca^{2+}-ATPase at $C_{12}E_8$ concentrations of 0.5–2.5 mg/mg protein could be prevented by further changes in experimental conditions.

The release of PC and PE into the top layer of the sucrose gradient again showed similar dependence on $C_{12}E_8$ concentration (Fig. 14). These observations do not support the claim of Bick et al. [50] of a preferential association of PE with the Ca^{2+}-ATPase in the presence of $C_{12}E_8$.

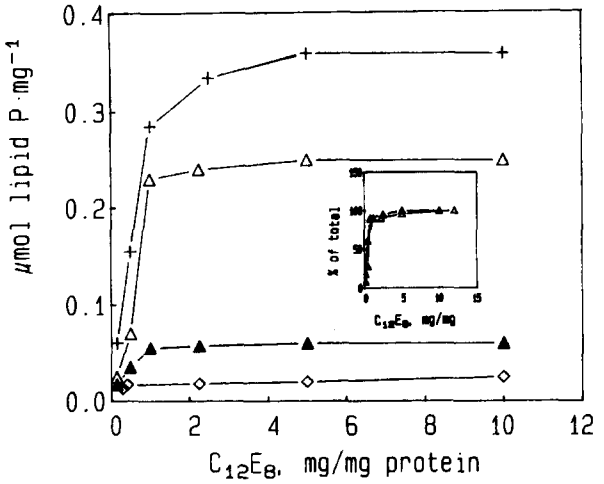

Fig. 14. The phospholipid composition of sarcoplasmic reticulum treated with $C_{12}E_8$ in a high-K^+, high-Ca^{2+} medium. The experiment was performed as described in legend to Fig. 13. The phospholipid composition of the top layer of sucrose gradient was analyzed by thin layer chromatography, as described under Methods. Symbols: +, total lipid-P; △, phosphatidylcholine; ▲, phosphatidylethanolamine; ◇, phosphatidylinositol. The inset shows the phosphatidylcholine (△) and phosphatidylethanol-amine (▲) content of the top layer expressed as percent of the values obtained at 10 mg $C_{12}E_8$/mg protein.

3.8. Electron microscopy of delipidated sarcoplasmic reticulum vesicles and the Ca^{2+}-induced crystallization of Ca^{2+}-ATPase in the presence of $C_{12}E_8$

The sarcoplasmic reticulum membranes retain their vesicular structure after extensive digestion with *B. cereus* phospholipase C, that reduced the phospholipid content below 10 percent of control level. The average diameter of the sarcoplasmic reticulum vesicles was reduced after phospholipase treatment to 730–800 Å, compared with ≃ 1000–1180 Å for the average diameter of vesicles in native sarcoplasmic reticulum. This represents a 36–62% decrease in the estimated surface area, assuming spherical shape. Assuming that the average density of ATPase molecules in the native sarcoplasmic reticulum was 30 000/μm^2 surface area [73] the estimated density after phospholipase C treatment rises to 46 000–60 000/μm^2, forcing the ATPase molecules into close contact with each other. This degree of crowding is likely to hinder protein dynamics and may be the principal cause of the inhibition of ATPase activity in lipid-depleted membranes.

Addition of 0.5 mg $C_{12}E_8$ per mg protein caused partial solubilization of the lipid-depleted vesicles, with the formation of fibrous lamellar structures, that developed into multilamellar crystalline arrays upon increasing the $C_{12}E_8$ concentration to 2 mg/mg protein. These ordered arrays are identical in appearance to the multilamellar Ca^{2+}-ATPase crystals that form in native sarcoplasmic reticulum, or in purified Ca^{2+}-ATPase preparations after solubilization with $C_{12}E_8$ in the presence of 10–30 mM Ca^{2+} [74–77].

4. Discussion

The observations presented in this report demonstrate a simple relationship between the phospholipid content of sarcoplasmic reticulum and the Ca^{2+}-stimulated ATPase activity.

Depletion of the phospholipid content of sarcoplasmic reticulum by phospholipase C enzymes with preferential activity toward phosphatidylcholine – such as the phospholipase C from *C. welchii* and *C. perfringens* – reversibly inhibit the ATPase activity in proportion to the hydrolysis of phosphatidylcholine. The phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol content remains unchanged and may be responsible for the residual ATPase activity. The functional significance of sphingomyelin is uncertain because it may be associated with contaminating surface membrane and T-tubule elements.

Digestion of sarcoplasmic reticulum with the broad specificity phospholipase C from *B. cereus* causes nearly complete loss of ATPase activity with parallel and essentially complete degradation of PC, PE and PS. Even under these conditions the ATPase activity was fully restored by $C_{12}E_8$.

By contrast, the phosphatidylinositol-specific phospholipase C of *B. cereus* and *B. thuringiensis* selectively hydrolyzed PI with only slight (< 10%) inhibition of

ATPase activity and left the PC, PE and PS content unaltered.

The simplest interpretation of these observations is:

(a) The optimal ATPase activity requires the full complement of sarcoplasmic reticulum phospholipids.
(b) There is no specific requirement for either of the main phospholipid classes, as their contribution to ATPase activity is proportional to their content in the membrane.
(c) The hydrolysis of PC, PE, and PI by their respective phospholipases does not indicate major differences in their accessibility.

The density of Ca^{2+}-ATPase molecules in rabbit sarcoplasmic reticulum membrane may be as high as 31 000–34 000 molecules/μm^2 surface area [73,78]; therefore the ATPase molecules are likely to be in frequent contact in the native membrane and may even form loose aggregates.

Decreasing the membrane surface area by hydrolysis or extraction of phospholipids may inhibit the ATPase activity by forcing the ATPase molecules into tighter aggregates and decreasing the conformational flexibility that is required for ATP hydrolysis and Ca^{2+} transport. There is indeed a decrease in the average diameter of sarcoplasmic reticulum vesicles during treatment with phospholipases [4,5], indicating a decrease in the surface area, and the inhibition of ATPase activity in lipid-depleted membranes parallels the decrease in the mobility of the protein measured by saturation transfer electron paramagnetic resonance spectroscopy [79]. Such a mechanism was proposed earlier by Nakamura and Martonosi [80] to explain the inhibition of ATPase activity in reconstituted ATPase-dipalmitoylphosphatidylcholine vesicles, where phase separation promotes the formation of ATPase aggregates [81].

A more complex relationship between phospholipid content and ATPase activity was observed after delipidation of sarcoplasmic reticulum with detergents such as deoxycholate, cholate, and $C_{12}E_8$.

In each case there was a large activation of ATP hydrolysis at low concentrations of the detergents with little or no change in phospholipid content. This activation is presumably related to the incorporation of detergents into the membrane with increase in the area and fluidity of the bilayer and a secondary increase in protein dynamics.

Increasing the detergent concentration above the level that produced maximal activation caused abrupt displacement of membrane phospholipids with proportional inhibition of ATPase activity. The close correlation between the phospholipid content and the ATPase activity (Figs. 8–11) is consistent with the observations made using phospholipases (Fig. 3 and 4) and agrees with earlier data of Moore et al. [31], but it is quite different from the data of Hesketh et al. [29] and Hidalgo et al. [17]. Instead of the constant ATPase rate between 100% and 35% lipid content shown in the earlier studies [17,29], we find a steady decline of ATP hydrolysis parallel with the decrease in phospholipid content, indicating that in the sarcoplasmic reticulum of fast-twitch muscle the full complement of sarcoplasmic lipids is required for maximal ATPase activity.

Similarly, there is no clear evidence that phosphatidylethanolamine and phosphatidylserine would specifically interact with the Ca^{2+}-ATPase and would be required for catalytic activity, as suggested by Bick et al. [50]. The proposed requirement for phosphatidylinositol [56,57] is yet to be confirmed. These observations do not rule out the possibility that PE, PS, and PI may fulfill some specific role in Ca^{2+} transport, but leave the burden of proof of such a role for future studies. The complex phospholipid composition of sarcoplasmic reticulum, may be essential for other metabolic functions.

The work contained in this report was done on sarcoplasmic reticulum isolated from fast-twitch muscle. In sarcoplasmic reticulum of slow-twitch and cardiac muscles the Ca^{2+}-ATPase density is lower and the relationship between phospholipid content and ATPase activity may be different. The evaluation of this relationship is likely to be complicated by the greater heterogeneity of protein composition and the lesser purity of slow-twitch skeletal and cardiac sarcoplasmic reticulum preparations.

Acknowledgements

This study was supported by research grants from the National Science Foundation (DMB88-23077) and from the Muscular Dystrophy Association.

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