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THE ROLE OF PHOSPHOLIPID IN CA^{2+}-STIMULATED ATPase ACTIVITY OF SARCOPLASMIC RETICULUM

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SUMMARY

1. The role of phospholipid in Ca^{2+}-stimulated ATPase activity of sarcoplasmic reticulum vesicles was reinvestigated using phospholipases A or C to degrade phospholipid. The byproducts of phospholipase A digestion were removed with a wash solution containing bovine serum albumin. Removal of 80–90 % of the phospholipid using phospholipase A led to loss of ATPase activity but had relatively little effect on the steady state level of phosphoenzyme. The formation of the phosphoenzyme, an intermediate in ATPase activity, was not blocked by the phospholipase A treatment, whereas the degradation of the intermediate was. Similar results were obtained with vesicles treated with phospholipase C.

2. The requirement for phospholipid in the Ca^{2+}-stimulated ATPase was clearly demonstrated. Both addition of phospholipid to the assay and rebinding of phospholipid restored ATPase activity of vesicles depleted of lipid by phospholipase A.

3. Ca^{2+} is required for phosphoenzyme formation in both normal and lipid-depleted sarcoplasmic reticulum vesicles.

INTRODUCTION

Sarcoplasmic reticulum regulates the contraction–relaxation cycle of skeletal muscle by releasing and accumulating Ca^{2+} (refs. 1–5). Uptake of Ca^{2+} against a large concentration gradient is energized by ATP through a membrane bound Ca^{2+}-stimulated ATPase. Thus the membrane of sarcoplasmic reticulum vesicles serves to maintain a barrier as well as to contain the pumping machinery, *i.e.* the Ca^{2+}-stimulated ATPase. ATPase activity involves two steps, the formation of a covalently linked phosphoenzyme intermediate and its subsequent breakdown[6–8]:

$$\text{ATP} + \text{enzyme} \xrightarrow{Ca^{2+}} P\text{-enzyme} + \text{ADP} \quad (1)$$

$$P\text{-enzyme} \longrightarrow \text{enzyme} + P_i \quad (2)$$

The overall reaction results in the translocation of two molecules of Ca^{2+} from the outside to the inside of the vesicles at the expense of one molecule of ATP[2,9].

The role of phospholipid in the function of sarcoplasmic reticulum vesicles has

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

been previously studied by degrading the lipid with phospholipases[10-13]. It was generally concluded from these studies that there is a requirement of lipid for Ca^{2+}-stimulated ATPase activity. However conflicting results were obtained about the role of phospholipid in the formation and breakdown of the phosphoenzyme intermediate. MARTONOSI[8] reported that treatment with phospholipase C did not abolish formation of the phosphoenzyme, whereas FIEHN AND HASSELBACH[13] found that delipidation by digestion with phospholipase A and subsequent washing with bovine serum albumin inactivated formation of the phosphoenzyme. The latter investigators could restore phosphoenzyme formation with fatty acids and ATPase activity with fatty acids or lysophosphatidylcholine; phospholipids were without effect. However, sarcoplasmic reticulum membranes do not contain appreciable quantities of lysophosphatides or free fatty acids[14]; most of the lipid is phospholipid. It would therefore be reasonable to expect that phospholipid is required for function.

In this study the role of lipid was reinvestigated using both phospholipase A and C. Both methods led to the same conclusion. Phospholipid is required for Ca^{2+} stimulated ATPase activity. Lipid was found to be preferentially required for the breakdown of phosphoenzyme (Eqn. 2). A preliminary report has already appeared[15].

METHODS AND MATERIALS

Preparation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were prepared as previously described[14]. The preparation included a sucrose step gradient and a wash with 0.5 M LiBr.

Removal of lipid with phospholipase A

Phospholipase A digestion was carried out using a Potter-Elvehjem homogenizer as the reaction vessel[16]. The reaction mixture contained 5 mg sarcoplasmic reticulum protein per ml, 0.3 M sucrose, 0.25 mM Ca^{2+}, 0.1 mM dithiothreitol, 1 % bovine serum albumin, 10 mM histidine and 50 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.45. After preincubation for 2 min at 32°, 5 μg phospholipase A (*Naja Naja*) was added per mg sarcoplasmic reticulum protein. A control was treated similarly but without phospholipase A. During digestion the sample was mixed by gently moving a Teflon pestle up and down several times at the beginning and then once every minute. The reaction was terminated by diluting the reaction mixture with 4 vol. of ice cold washing solution containing 0.3 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 % bovine serum albumin, 0.1 mM dithiothreitol and 10 mM histidine, pH 7.3. The mixture was centrifuged for 45 min at 40000 rev./min in a 65 Spinco rotor. This preparation is referred to as singly washed (1 bovine serum albumin wash). The washing procedure was repeated three times by resuspending the pellet in washing solution (1 mg sarcoplasmic reticulum protein per ml washing solution) and recentrifuging as before. All membrane preparations were washed finally once with 0.3 M sucrose–2.5 mM HEPES, pH 7.4, (1 mg sarcoplasmic reticulum protein per ml solution) to remove bovine serum albumin. The final pellet was then resuspended in the same buffer.

Digestion with phospholipase C

Phospholipase C digestion was carried out at 37° in a medium containing 5 mg sarcoplasmic reticulum protein per ml, 2.4 mM $CaCl_2$, 0.3 M sucrose, 1 % bovine serum

albumin and 20 mM histidine, pH 7.3. After preincubation for 2 min at 37°, 0.2 mg phospholipase C (*Clostridium perfringens*) was added per mg sarcoplasmic reticulum protein. After 30 min of digestion the mixture was diluted with 4 vol. of cold 0.3 M sucrose–2.5 mM HEPES, pH 7.4, and centrifuged for 60 min at 35000 rev./min in a 65 Spinco rotor. The pellet was washed another two times by resuspending (1 mg sarcoplasmic reticulum protein per ml) in 0.3 M sucrose–2.5 mM HEPES, pH 7.4, and centrifuging as before. The final pellet was then resuspended in the same buffer.

Readdition of lipid prior to assay

Unless otherwise described, reactivation of lipid-depleted membranes was carried out by combining sarcoplasmic reticulum vesicles and the lipid microdispersion in a mixture containing 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 10 mM histidine, pH 7.3, at 0°. Two different protein concentrations were used. The details are specified in the legends of Table VI and Fig. 3. The mixture was incubated for either 10 min at 32° or 4 min at 37°. Samples were then stored at 0°, unless analysis was carried out immediately.

Rebinding of lipid

Rebinding of lipid was carried out by incubating phospholipid microdispersions with sarcoplasmic reticulum vesicles under the conditions described in the legend of Table VIII.

Assays

Ca^{2+} uptake capacity (at 23°), [^{32}P]phosphoenzyme formation (at 0 or 32°) and ATPase activity (at 32°) were determined as previously described[14]. Each of these activities was measured in a medium containing 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 10 mM histidine, pH 7.3. Depending on the assay the medium contained in addition 5 mM ATP and 5 mM potassium oxalate (Ca^{2+} uptake capacity), 0.1 mM [γ-^{32}P]-ATP ([^{32}P]phosphoenzyme formation) or 2.5 mM ATP (ATPase activity). The ATPase assay was slightly modified when the reaction was carried out with samples containing lipid microdispersions. Bovine serum albumin, 50 μl of a 10 % solution, was added to 2 ml of the assay medium 5–10 sec before the reaction was stopped with 0.7 ml 15 % cold trichloroacetic acid (in place of 1.5 N $HClO_4$). This modification allowed precipitation of the lipid.

Protein was determined by the procedure of LOWRY *et al.*[17] using bovine serum albumin as a standard. Total phosphorus was measured as an estimate of lipid phosphorus[14] using a modification[18] of the method of CHEN *et al.*[19]. Free fatty acids and phospholipids were quantitated as previously described[14].

Materials

"Ultrapure" grade sucrose from Mann Research Laboratories (New York, N. Y.) was used throughout the experiments. [γ-^{32}P]ATP was prepared according to POST AND SEN[20] and was a generous gift of Dr. Robert Post (Department of Physiology, Vanderbilt University). Non-radioactive ATP was purchased from P–L Biochemicals (Milwaukee, Wisc.). Crystallized bovine serum albumin was purchased from Armour Pharmaceutical Co. (Chicago, Ill.). Sarcoplasmic reticulum lipids were prepared by extracting sarcoplasmic reticulum vesicles with chloroform–methanol (2:1, by vol.)

and back extracting[18,21] to remove non-lipid material. To prepare mixed sarcoplasmic reticulum phospholipids neutral lipids were removed by column chromatography using silicic acid (UNISIL-Clarkson Chemical, Williamsport, Penn.[22]). Mixed mitochondrial phospholipids were prepared from beef heart mitochondria[18,22]. A preparation of mixed soybean phospholipids (Asolectin) was obtained from Associated Concentrates (Woodside, New York, N. Y.). Phosphatidylcholine and lysophosphatidylcholine derived from soybean phospholipids were a generous gift of Drs. H. Genthe and H. Betzing (A. Nattermann, Cologne, Germany). The phosphatidylcholine preparation, in the form of a microdispersion, contained 8 % lysophosphatidylcholine. Lysophosphatidylcholine was dissolved in 0.1 M KCl, 1 mM EDTA and 20 mM Tris acetate, pH 8.1. Phospholipid microdispersions were prepared by solubilizing the lipid in a mixture of cholate and *n*-butanol, followed by prolonged dialysis against 20 mM Tris acetate, pH 8.1, and 1 mM EDTA at 4°[16,23]. In the case of sarcoplasmic reticulum phospholipid, a microdispersion was also prepared by sonication. A lipid film containing 3 mg lipid phosphorus was suspended by shaking[24] with 10 ml of a solution containing 0.1 M NaCl, 1 mM EDTA and 20 mM Tris acetate, pH 8.0. The suspension was clarified by sonication (Branson Sonifier, Model S110) for 10 min at 4 A at 0° under N_2 in a Rosette cell using a microprobe. The lipid microdispersion was then centrifuged for 30 min at 35000 rev./min in a 65 Spinco rotor to remove undispersed phospholipid. Oleic acid was purchased from Mann Research Lab. (New York, N. Y.). It was microdispersed by sonicating a suspension of oleic acid containing 0.9 mM oleic acid, 50 mM KCl and 10 mM histidine, pH 7.3. Phospholipase A was prepared from lyophilized *Naja Naja* venom (Miami Serpentarium Lab., Miami, Fla.). To destroy protease activity the snake venom was heated in a boiling water bath as previously described[16]. Phospholipase C (*Cl. perfringens*) was purchased from Worthington Biochemical Co. (Freehold, N. J.) and was free of detectable protease activity. It was dissolved in 1 % bovine serum albumin–10 mM histidine, pH 7.3, at a concentration of 5 mg/ml and clarified by centrifugation.

RESULTS

The degree of phospholipid breakdown was controlled by varying the time of incubation of samples with phospholipase A. The breakdown was estimated by measuring the ratio of phosphorus to protein after four washes with bovine serum albumin (Table I). Close to 90 % of the phospholipid was digested within a period of 30 min under the conditions of the experiment. Most of the fatty acids and some of the lysophosphatides were removed in the first wash[16]. Several bovine serum albumin washes were required to remove the bulk of the lysophosphatides from membranes treated with phospholipase A (Table II). The phosphorus content of control sarcoplasmic reticulum vesicles in which the phospholipase A was omitted was not decreased after four washes with bovine serum albumin.

Table III compares the lipid composition of vesicles from which 80 % of the lipid was removed with that of the original sarcoplasmic reticulum vesicles. It was shown previously that upon digestion of mitochondria with phospholipase A from snake venom, phosphatidylethanolamine was more rapidly degraded than phosphatidylcholine (ref. 25 and S. FLEISCHER AND A. CASU, unpublished studies). In turn the acidic lipids, phosphatidylinositol and cardiolipin were broken down most

TABLE I

THE EFFECT OF PHOSPHOLIPID REMOVAL ON THE FUNCTION OF SARCOPLASMIC RETICULUM VESICLES

Sarcoplasmic reticulum vesicles were digested for varying times at 32° with phospholipase A (5 μg/mg sarcoplasmic reticulum protein) and then washed 4 times with bovine serum albumin as described in METHODS. The starting material (original, no bovine serum albumin washes) and a control (phospholipase A omitted, four bovine serum albumin washes) are included for comparison.

Sample	*Time of incubation (min)*	*Total phosphorus (μmoles P/mg protein)*	*Ca^{2+} uptake (μmoles Ca^{2+}/mg protein)*	*ATPase (μmoles P_i/min per mg protein)*	*[^{32}P]Phosphoenzyme (nmoles P/mg protein)*
Original	0	0.73	3.2	0.75	3.8
Control	30	0.775	2.2	0.81	2.4
+ Phospholipase A	1	0.445	1.1	0.68	2.7
	4	0.36	0.65	0.76	2.6
	15	0.155	0.05	0.40	2.4
	30	0.09	0	0.10	1.7
	60	0.05	—	0.03	1.3

TABLE II

THE EFFECT OF WASHING WITH BOVINE SERUM ALBUMIN ON THE FUNCTION OF SARCOPLASMIC RETICULUM VESICLES DIGESTED WITH PHOSPHOLIPASE A

Sarcoplasmic reticulum vesicles were digested with phospholipase A for 15 min and then repeatedly washed with bovine serum albumin. The starting material (original) and a control (phospholipase A was omitted) are included for comparison. Standard deviation is given when values were determined for at least five preparations.

Sample	*Number of bovine serum albumin washes*	*Total phosphorus (μmoles P/mg protein)*	*Ca^{2+} uptake (μmoles Ca^{2+}/mg protein)*	*ATPase (μmoles P_i/min per mg protein)*	*[^{32}P]Phosphoenzyme (nmoles P/mg protein)*
Original	0	0.765 ± 0.070	3.6 ± 0.9	0.90 ± 0.10	3.9 ± 0.4
Control	1	0.76	—	0.9	2.8
	4	0.780 ± 0.045	2.4	0.90 ± 0.20	2.4 ± 0.6
+ Phospholipase A	0	—	0	3.5	—
	1	0.41	0	0.9	—
	3	0.19	—	0.35	1.9
	4	0.140 ± 0.015	0	0.15 ± 0.07	1.9 ± 0.4

slowly. This observation holds also for sarcoplasmic reticulum vesicles. Small amounts of free fatty acid, lysophosphatidylcholine and lysophosphatidylethanolamine were not washed out even after four bovine serum albumin washes (Table III).

Sarcoplasmic reticulum vesicles appeared in the electron microscope as closed membranous vesicles. When 80 % of the phospholipid was removed by treatment with phospholipase A and washing with bovine serum albumin, the vesicles appeared smaller and some of the membranes were no longer closed vesicles. Typical electron micrographs of control and lipid-depleted sarcoplasmic reticulum vesicles are shown in Fig. 1. The trilaminar appearance of the membrane was distinctly widened by

TABLE III

PHOSPHOLIPID AND FREE FATTY ACID CONTENT OF SARCOPLASMIC RETICULUM VESICLES TREATED WITH PHOSPHOLIPASE A AND WASHED WITH BOVINE SERUM ALBUMIN

Sarcoplasmic reticulum vesicles were digested with phospholipase A for 15 min and washed 4 times with bovine serum albumin. The starting material (Original) is included for comparison and was not washed with bovine serum albumin.

	Lipid content (nmoles lipid/mg protein)	
	Original sarcoplasmic reticulum	*Lipid-depleted sarcoplasmic reticulum*
Free fatty acid	22	11
Total phospholipid	760	126
Phosphatidylcholine	552	64
Phosphatidylethanolamine	103	2
Phosphatidylinositol	66	13
Phosphatidylserine	14	2
Sphingomyelin	7.5	6
Lysophosphatidylcholine	—	21
Lysophosphatidylethanolamine	—	12
Other phospholipids	17.5	6

lipase treatment and the membrane assumed an asymmetric appearance. The outer rim of the vesicles was more intensely stained than the inner rim. A decrease in the size of sarcoplasmic reticulum vesicles was also observed, when the phospholipids were degraded with phospholipase C[26].

Removal of 80 % of the phospholipid was accompanied by the selective release of protein from the membrane (Fig. 2). Polyacrylamide gel electrophoresis on soaked, acid gels[14] showed that one protein band (mol. wt. approx. 50000) was completely missing while a second one (mol. wt. approx. 60000) was reduced in intensity. Both proteins were retained in a control and in preparations from which only about 50 % of the phospholipid was removed. Treatment of mitochondrial membranes with phospholipase A also resulted in the selective release of protein[27].

The function of sarcoplasmic reticulum vesicles was drastically effected by the breakdown of phospholipid. Treatment of sarcoplasmic reticulum vesicles with phospholipase A, without washing stimulated ATPase activity. This was because fatty acids and lysophosphatides were formed during phospholipase A digestion. Both stimulate ATPase activity and cause loss of Ca^{2+} uptake[11,13]. The incubation medium contained some bovine serum albumin but it was not enough to tie up these by-products (Table II). The first bovine serum albumin wash removed most of the fatty acids and some of the lysophosphatides as already found for mitochondrial membranes treated with phospholipase A[16]. The ATPase rate was reduced to approximately the value in normal vesicles when half or more of the lysophosphatides remained. Removal of the residual lysophosphatides (*i.e.* removal of 80 % of the phospholipid) reduced the ATPase activity to low levels (about 15–20 %) (Table II). In extensively treated vesicles Ca^{2+} uptake capacity was not restored by washing. At intermediate levels of lipid breakdown and removal, the Ca^{2+} uptake capacity was more rapidly reduced than ATPase activity (Table I).

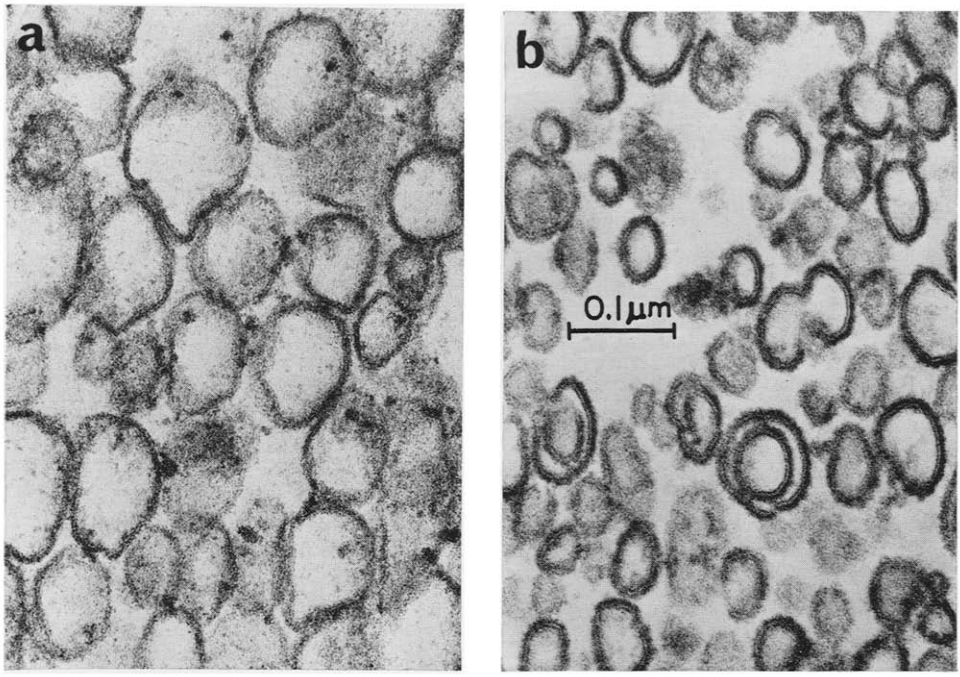

Fig. 1. Electron micrographs of native and lipid-depleted sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum preparations described in Table I were used: (a) Control; (b) + phospholipase A for 15 min and washed 4 times with bovine serum albumin. Samples were fixed in 1% OsO_4, embedded and sectioned as previously described[14]. Magnification 140000 ×.

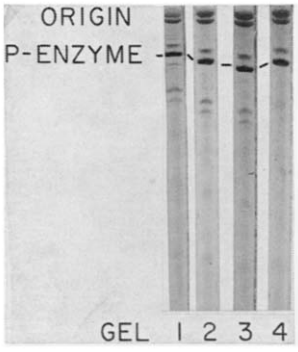

Fig. 2. Separation of sarcoplasmic reticulum proteins of native and lipid-depleted membranes by gel electrophoresis. Soaked, acid gels containing 7% polyacrylamide were prepared and run as previously described[14]. They were stained with 1% Amido Schwartz in 7% acetic acid. Sarcoplasmic reticulum preparations, 20 μg sarcoplasmic reticulum protein per gel, described in Table I were used. Gel 1, original; Gel 2, control; Gel 3, + phospholipase A for 4 min; Gel 4, + phospholipase A for 15 min.

Under optimal conditions most of the ATPase is present in the phosphorylated form, *i.e.* the formation of the [^{32}P]phosphoenzyme is more rapid than its breakdown[14, 28]. Characterization of lipid-depleted membranes showed that the steady state level of the [^{32}P]phosphoenzyme was reduced by 40–60 % by the phospholipase

A extraction procedure. However, at levels of phospholipid removal which led to complete loss of Ca^{2+} uptake capacity and to a severe reduction in ATPase activity, [^{32}P]phosphoenzyme concentration was only slightly lower than in control vesicles (Tables I and II). It was previously shown that the optimal steady state concentration of the [^{32}P]phosphoenzyme is reached within 2–3 sec at 0 or 32° for native sarcoplasmic reticulum vesicles[7,14,28]. Removal of phospholipid did not reduce this time for obtaining maximal levels of [^{32}P]phosphoenzyme.

[^{32}P]Phosphoenzyme levels of native and lipid-depleted vesicles were similarly effected by Ca^{2+} and Mg^{2+} (Table IV). Approx. 0.1 and 0.2 nmole ^{32}P/mg protein were bound to native and lipid-depleted vesicles respectively when the free Ca^{2+} concentration was drastically lowered by addition of 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) and the incubation was carried out for 6 or 60 sec at 0°. Approximately half of the level of [^{32}P]phosphoenzyme was formed when Mg^{2+} was omitted from the incubation medium containing 0.1 mM $CaCl_2$. At 20 mM Ca^{2+} the normal level of [^{32}P]phosphoenzyme was obtained, even when no Mg^{2+} was added.

TABLE IV

THE EFFECT OF Ca^{2+} AND Mg^{2+} ON [^{32}P]PHOSPHOENZYME FORMATION OF NATIVE AND LIPID-DEPLETED SARCOPLASMIC RETICULUM VESICLES

Lipid-depleted membranes (0.14 μmole P/mg protein) were obtained by phospholipase A digestion for 15 min, followed by 4 washes with bovine serum albumin (*cf.* Table II). [^{32}P]Phosphoenzyme formation was measured at 0° in a medium containing 1 mg sarcoplasmic reticulum protein per ml, 0.1 M KCl, 0.1 mM [γ-^{32}P]ATP, 10 mM histidine, pH 7.3, and the Ca^{2+}, Mg^{2+} and EGTA concentrations indicated.

Components added			*[^{32}P]Phosphoenzyme (nmoles P/mg protein)*	
Mg^{2+} (mM)	*Ca^{2+} (mM)*	*EGTA (mM)*	*Original sarcoplasmic reticulum*	*Lipid-depleted sarcoplasmic reticulum*
5	0.1	0	3.85	1.7
5	0.1	1.0	0.1	0.20
5	0	1.0	0.05	0.15
30	0	5.0	0.05	—
30	0.1	0	4.1	1.5
0	0.1	0	1.9	1.1
0	20.0	0	4.25	1.8

Removal of phospholipid dramatically increased the stability of the ^{32}[P]phosphoenzyme intermediate (Table V). EGTA was added to ^{32}P-labelled membranes to chelate Ca^{2+} and thereby to block further [^{32}P]phosphoenzyme formation. In native sarcoplasmic reticulum vesicles, the [^{32}P]phosphoenzyme level approached zero within 20 sec of the addition of EGTA, whereas in lipid-depleted vesicles the [^{32}P]-phosphoenzyme level was practically unchanged. Hence, it appeared that breakdown of [^{32}P]phosphoenzyme was prevented by removal of lipid. On the other hand the addition of ADP to both preparations caused a rapid loss of ^{32}P label. Thus it would appear that the back reaction, *i.e.* the formation of ATP from [^{32}P]phosphoenzyme and ADP[29] as well as the forward reaction were not effected by removal of 80 % of lipid.

TABLE V

THE EFFECT OF EGTA AND ADP ON THE STABILITY OF [^{32}P]PHOSPHOENZYME OF NATIVE AND LIPID-DEPLETED SARCOPLASMIC RETICULUM VESICLES

Lipid-depleted membranes were obtained by phospholipase A digestion for 15 min, followed by four washes with bovine serum albumin (*cf.* Table II), or by phospholipase C digestion for 30 min (*cf.* Table VIII). The membranes (1 mg sarcoplasmic reticulum protein per ml) were incubated for 6 sec at 0° in a medium containing 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM [γ-^{32}P]ATP and 10 mM histidine, pH 7.3. EGTA and ADP were then added and the reaction was stopped with trichloroacetic acid at the indicated time. The steady-state concentration of [^{32}P]phosphoenzyme shown in the first row was maintained for at least 45 sec at 0°.

Component	*Added (mM)*	*Time (sec)*	[^{32}P]*Phosphoenzyme (nmoles P/mg protein)*		
			Original sarcoplasmic reticulum	*Lipid-depleted sarcoplasmic reticulum*	
				Phospholipase A	*Phospholipase C*
—	—	—	4.3	1.8	3.5
EGTA	2.0	3	1.25	1.6	3.2
	2.0	20	0.1	1.55	2.4
ADP	1.0	3	0.6	0.2	0.9
	1.0	20	0.5	0.15	0.6

TABLE VI

ATPase ACTIVITY AND [^{32}P]PHOSPHOENZYME FORMATION OF LIPID-DEPLETED AND CONTROL VESICLES UPON ADDITION OF LIPID

Lipid-depleted sarcoplasmic reticulum vesicles (0.10–0.15 μmole P/mg protein) were prepared by phospholipase A digestion for 15 min, followed by four washes with bovine serum albumin (*cf.* Table II). Control vesicles (0.76 μmole P/mg protein) were obtained by treating sarcoplasmic reticulum vesicles in the same manner but omitting the phospholipase A from the incubation mixture. Addition of lipid was carried out for 4 min at 37° in a medium containing 1 mg sarcoplasmic reticulum protein per ml, 0.1 M KCl, 5 mM Mg^{2+}, 0.1 mM Ca^{2+}, 10 mM histidine, pH 7.3, and lipid. Aliquots were used for measuring ATPase activity at 32° and [^{32}P]phosphoenzyme formation at 0° and 32°.

Lipid		*Lipid added (μmoles/mg protein)*	*ATPase (μmoles P_i/min per mg protein)*	[^{32}P]*Phosphoenzyme (nmoles P/mg protein)*	
				at 0°	*at 32°*
Control vesicles					
—		—	0.9	2.3	2.1
Mitochondrial phospholipid		0.8	1.1	2.3	1.9
Lysophosphatidylcholine		1.0	2.7	2.1	2.1
Oleate		0.4	1.1	2.1	2.2
Lipid-depleted vesicles					
—		—	0.05–0.25	1.8	1.8
Sarcoplasmic reticulum phospholipid		2.0	1.3	1.9	2.2
Mitochondrial phospholipid	(1)	1.3	0.8	1.8	—
	(2)	3.5	1.1	1.9	1.6
Soybean phospholipid		2.7	1.2	1.6	1.5
Lysophosphatidylcholine	(1)	1.0	1.2	1.7	—
	(2)	2.4	1.7	1.7	1.5
Oleate		0.4	0.9	2.2	1.95

Lipid-depleted vesicles from which 80 % of the phospholipid was removed, contained a residual ATPase activity of approx. 0.15 μmole P_i/min per mg protein or about 20 % of the original activity (Table II). The addition of phospholipid microdispersions to the lipid-depleted sarcoplasmic reticulum vesicles in the assay medium reactivated ATPase activity 5-fold or more (Fig. 3 and Table VI). Phospholipids from sarcoplasmic reticulum vesicles, mitochondria and soybean were all effective.

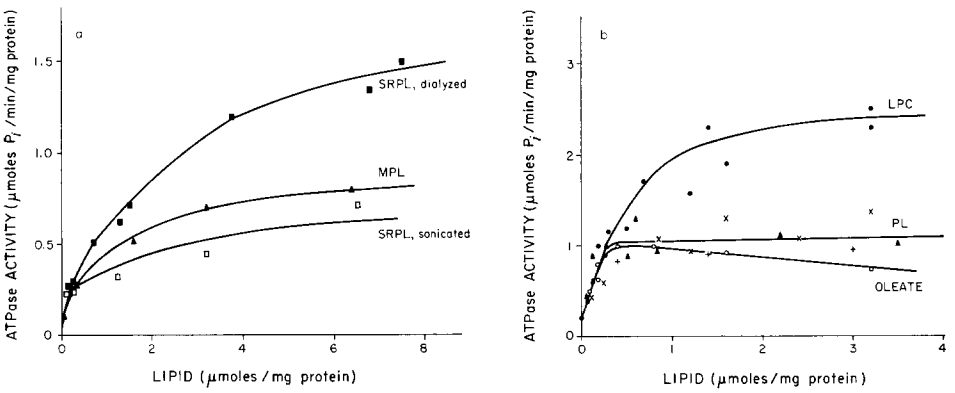

Fig. 3. Restoration of ATPase activity of lipid-depleted membranes by addition of lipid. Sarcoplasmic reticulum vesicles were digested with phospholipase A for 24 min (a) or 15 min (b) and washed 4 times with bovine serum albumin (*cf.* Table II). The lipid-depleted vesicles were preincubated for either 10 min at 32° or 4 min at 37° in a medium containing 30 μg sarcoplasmic reticulum protein per ml, 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM histidine, pH 7.3, and the lipid microdispersion. ATPase activity was subsequently determined at 32°. □—□, sarcoplasmic reticulum phospholipid (SRPL), sonicated; ■—■, sarcoplasmic reticulum phospholipid, dialyzed; ▲—▲, mitochondrial phospholipid (MPL); ●—●, lysophosphatidylcholine (LPC); ×—×, soybean phospholipids; +—+, phosphatidylcholine; ○—○, oleate. PL, phospholipid. (a) Lipid-depleted vesicles with 0.10 μmole P/mg protein. (b) Lipid-depleted vesicles with 0.15 μmole P/mg protein.

On the other hand the level of [^{32}P]phosphoenzyme was not changed by the readdition of lipid (Table VI). The restoration of ATPase activity as a function of phospholipid concentration is shown in Figs. 3a and b. Saturating values of ATPase activity were approached at high phospholipid to protein ratios. Some variation in the efficiency of reactivation by lipid was observed which was mainly due to the extent of lipid depletion of sarcoplasmic reticulum vesicles. The reactivation tended to be more efficient when less lipid was removed (*cf.* Figs. 3a and 3b). In addition we found greater efficiency of lipid reactivation when lipid and protein were mixed together in high concentrations as in Table VI. Sonicated sarcoplasmic reticulum phospholipids were not quite as effective in restoring ATPase activity as those prepared by the dialysis technique. At low concentrations oleate, lysophosphatidylcholine and phospholipids were all equally effective in restoring ATPase activity (Fig. 3b). At high concentrations oleate had an inhibitory effect[11,13], whereas lysophosphatidylcholine activated ATPase activity approximately to twice the level obtained by phospholipid. Lysophosphatidylcholine at high concentrations markedly clarified the sarcoplasmic reticulum suspension probably due to its detergent properties. This could be best observed by increasing the protein concentration to 1.0 mg/ml (Table VI). The sample was largely clarified at 1–2 μmoles lysophosphatidylcholine per ml. It would thus appear that the enhanced ATPase activity was related, at least in part, to this clarifying effect.

ATPase activity and [^{32}P]phosphoenzyme formation in native and lipid-depleted membranes were dependent on Ca^{2+} (Table IV). The same was also true for membranes reactivated by the addition of lipid. We found that the addition of 1 mM EGTA in place of 0.1 mM Ca^{2+} to the assay medium containing lipid-depleted sarcoplasmic reticulum vesicles and phospholipid gave an ATPase activity of approx. 0.10 μmole P_i/min per mg protein and a [^{32}P]phosphoenzyme concentration of approx. 0.10 nmole P per mg protein.

Rebinding of sarcoplasmic reticulum and mitochondrial phospholipid by lipid-depleted vesicles was accomplished by incubating the membranes with prespun phospholipid microdispersions. Sarcoplasmic reticulum vesicles were then centrifuged through a layer of 25% sucrose to separate the unbound phospholipid from the vesicles. The pellet was analyzed for phospholipid and ATPase activity (Table VII). The amount of lipid rebound increased with larger amounts of added lipid and was not appreciably affected by small amounts of Mg^{2+} in the incubation medium. The effec-

TABLE VII

REBINDING OF PHOSPHOLIPID TO LIPID-DEPLETED MEMBRANES

Lipid-depleted membranes were obtained by phospholipase A digestion for 24 min, followed by four washes with bovine serum albumin. A control was treated similarly but without phospholipase A. Lipid rebinding was carried out for 12 min at 32° in a medium containing 1 mg sarcoplasmic reticulum protein per ml, 0.1 M KCl, 15 mM Tris–acetate *plus* 15 mM histidine, pH 7.4, and EDTA, Mg^{2+} and lipid concentrations as indicated. The suspension was then placed on top of a solution of 25% sucrose and 5 mM HEPES, pH 7.4, and centrifuged for 45 min at 40000 rev./min in a SW 50L rotor. The pellet was resuspended in 0.3 M sucrose–2.5 mM HEPES, pH 7.4. 50–80% of the protein of lipid-depleted vesicles and 30–60% of that of the control were recovered in the pellet. Sarcoplasmic reticulum and mitochondrial phospholipid microdispersions prepared by the dialysis technique[16] were used.

Lipid	*[Mg^{2+}–EDTA] (mM)*	*Total phosphorus*		*Rebound P/added P*	*ATPase activity (μmoles P_i/min per mg protein)*
		μmoles P added per mg protein	*μmoles P in pellet per mg protein*		
Control vesicles					
—	—	—	0.81	—	1.0
SR	1.7–0.7	1.93	1.23	0.22	1.8
MPL	1.7–0.7	1.87	1.12	0.17	3.6
Lipid-depleted vesicles					
—	—	—	0.10	—	0.03
SR	0.9–0.8	0.26	0.14	0.15	0.10
	0.9–0.8	0.65	0.21	0.17	0.10
	0.9–0.8	2.60	0.69	0.23	0.41
	0.9–0.8	8.05	1.4	0.16	0.64
	1.7–0.7	0.71	0.31	0.30	0.45
	1.7–0.7	2.1	0.72	0.29	0.90
	3.4–0.3	2.6	0.73	0.24	0.45
MPL	1.7–0.7	0.49	0.30	0.41	0.64
	1.7–0.7	0.68	0.33	0.34	0.43
	1.7–0.7	1.47	0.45	0.24	0.57
	1.7–0.7	1.97	0.55	0.23	0.55
	1.7–0.7	3.3	0.65	0.17	0.52

Abbreviations: SR, sarcoplasmic reticulum; MPL, mitochondrial phospholipid.

tive Mg^{2+} concentration, *i.e.* the difference in concentration between Mg^{2+} and EDTA was varied from 0.1–3 mM. At a high lipid to protein ratio the lipid rebound exceeded that of the amount in native vesicles. Control vesicles also bound appreciable amounts of lipid when the incubation was carried out at high lipid and protein concentrations as in Table VII.

Rebinding of lipid resulted in a reactivation of ATPase activity in lipid-depleted membranes and a stimulation of ATPase activity in control vesicles. Similar binding of lipid and stimulation of ATPase activity was also observed when sarcoplasmic reticulum phospholipid microdispersions prepared by sonication instead of by the dialysis technique were added to control vesicles. Sonicated sarcoplasmic reticulum phospholipids were used to show that residual amounts of cholate present in the dialyzed samples were not alone responsible for the stimulation of the ATPase activity in the experiments described in Fig. 3 and Tables VI and VII. It should also be noted that reactivation and rebinding were obtained with phospholipid microdispersions which contained less than 0.02 mole % of free fatty acid and 1–3 mole % of lysophosphatides. In agreement with this the addition of bovine serum albumin (0.25 %) to the assay mixture of lipid-rebound sarcoplasmic reticulum vesicles did not decrease appreciably ATPase activity.

TABLE VIII

PROPERTIES OF SARCOPLASMIC RETICULUM VESICLES TREATED WITH PHOSPHOLIPASE C

Sarcoplasmic reticulum vesicles were digested with phospholipase C for 30 min at 37° and washed as described in METHODS. The starting material (original) and a control (phospholipase C omitted) are included for comparison.

Sample	*Total phosphorus (μmoles P/mg protein)*	*ATPase (μmoles P_i/min per mg protein)*	*[^{32}P]Phosphoenzyme (nmoles P/mg protein)*
Original	0.81	0.76	4.0
Control	0.78	0.87	3.5
Phospholipase C treated	0.25	0.27	3.5

Digestion of 70 % of the phospholipid by phospholipase C decreased ATPase activity severalfold (Table VIII). [^{32}P]Phosphoenzyme formation was little affected by this treatment. In this regard phospholipase C digestion was somewhat gentler than that of the phospholipase A procedure. A comparison of membranes treated with phospholipase A or C showed that both behaved similarly with respect to the presence of EGTA and ADP (Table V). Hence both treatments allow qualitatively the same conclusion, that is the removal of phospholipid blocked the breakdown of the [^{32}P]-phosphoenzyme. Less than 0.2 nmole ^{32}P per mg protein were bound to the membrane in the presence of 2 mM EGTA. This dependence of Ca^{2+} for [^{32}P]phosphoenzyme formation was similar for native sarcoplasmic reticulum vesicles as well as for those treated with phospholipases A or C.

DISCUSSION

It is now well accepted that a number of enzymes associated with membranes require phospholipid for function[16,30]. Phospholipase C was used by KIELLEY AND

MEYERHOF[10] in studying Ca^{2+}-stimulated ATPase activity of a "lipoprotein" fraction of muscle. MARTONOSI *et al.*[11] extended these studies. They found that up to 60 % of the lipid was degraded with loss of 80–90 % of ATPase activity and Ca^{2+} uptake capacity. Addition of phospholipid to the assay mixture restored both functions. Rebinding of phospholipid was not studied. The problem with the use of phospholipase C is that diglyceride is not removed from the membrane so that it is not clear that reactivation of activity by lipid is referable only to rebinding by lipid which is required for function, rather than to a reversal of diglyceride inhibition.

FIEHN AND HASSELBACH[13] also used phospholipase C. Ether was used to extract diglyceride to preclude its complicating role. Free fatty acid and lysophosphatidylcholine could restore ATPase activity but phospholipids were without effect. To improve upon the limitation of the phospholipase C method, these workers also used phospholipase A to degrade the phospholipids and washed with bovine serum albumin to remove breakdown products. Again ATPase activity and Ca^{2+} uptake capacity were lost by the phospholipase A treatment. Fatty acids and lysophosphatidylcholine could restore ATPase but phospholipids could not. The reactivation of Ca^{2+} stimulated ATPase is not necessarily a restoration of function. Fatty acids are known to have profound effects on some enzymes both stimulating as well as inhibiting, *e.g.* the stimulation of ATPase activity by fatty acids in mitochondria is nonfunctional, in that it is no longer oligomycin sensitive[16].

The phospholipase A method represents the gentlest treatment for removal of phospholipid which includes removal of the byproducts of digestion[16]. Most of our studies were therefore carried out with vesicles prepared by this method. Phospholipids were degraded with phospholipase A and the breakdown products were then removed by repeated washing with bovine serum albumin. ATPase activity was lost in extensively extracted vesicles but could be restored to about the level of intact native vesicles by the addition of phospholipid. To demonstrate a lipid requirement, restoration of enzyme activity should be correlated with rebinding of lipid[16,22]. We could show for the first time that rebinding of phospholipid to sarcoplasmic reticulum vesicles depleted of lipid resulted in reactivation of ATPase activity. The reactivation was due to phospholipid and not to contamination by free fatty acid or lysophosphatides since negligible amounts of the latter were present in our phospholipid microdispersions. Some of the phospholipid was also bound to normal sarcoplasmic reticulum vesicles (Table VII) indicating that the rebinding of lipid to lipid-depleted vesicles may not fully result in the original arrangement of the membrane. ATPase activity in these lipid-rich vesicles was increased several-fold. A similar effect was observed when fatty acids or lysophosphatides were added to sarcoplasmic reticulum vesicles. Generally the stimulation of ATPase activity is accompanied by a decrease of Ca^{2+} uptake capacity and may therefore represent a type of control phenomenon[11,13]. The pump may be stimulated to compensate for the leak, or ATPase activity and Ca^{2+} transport may be uncoupled. This raises the question of whether we obtained optimal reactivation of ATPase activity in the lipid-depleted membranes by the addition and rebinding of phospholipid. This question is not readily answered at this stage. A broader approach is required which addresses itself to reconstitution of the compartment as well as the overall transport process. In this regard, our failure to restore transport in extensively treated sarcoplasmic reticulum vesicles (80–90 % of the lipid extracted) may in part be related to the loss of specific proteins (Fig. 2).

Removal of specific sarcoplasmic reticulum proteins was reported by DUGGAN AND MARTONOSI[31] to result in loss of Ca^{2+} uptake capacity.

The transport of Ca^{2+} involves the formation and dissociation of a phosphoenzyme intermediate (*cf.* Eqns. 1 and 2). Removal of 80 % or more of the phospholipid preferentially affected the second reaction, *i.e.* the breakdown of [^{32}P]phosphoenzyme. MARTONOSI[8] found that phospholipase C treatment increased the [^{32}P]phosphoenzyme concentration and came to the conclusion that phosphoenzyme formation did not require lipid whereas ATPase activity did. These conclusions would agree with our studies. However, his interpretation is severely weakened in that: (1) maximal levels of [^{32}P]phosphoenzyme formation were not obtained; and (2) diglycerides formed during treatment with phospholipase were present and might cause further complications. The second reservation does not seem to be serious in so far as [^{32}P]phosphoenzyme formation is concerned since vesicles treated with the phospholipases A or C gave qualitatively similar results (Table V). FIEHN AND HASSELBACH[13] reported a 6-fold decrease of the steady state concentration of [^{32}P]phosphoenzyme on removal of lipid by the phospholipase A procedure and a restoration of [^{32}P]phosphoenzyme formation to half of the starting concentration upon addition of oleate but not of lysophosphatidylcholine or phospholipid. We cannot explain these results, especially since the extent of lipid removal was not defined.

In our studies addition of lipid to lipid-depleted vesicles did not result in any appreciable change in [^{32}P]phosphoenzyme concentration, whereas ATPase activity was enhanced 5-fold or more. It follows that lipid is clearly necessary for the breakdown of the [^{32}P]phosphoenzyme.

The level of the [^{32}P]phosphoenzyme was greatly enhanced by Ca^{2+} in native as well as in lipid-depleted membranes. This is in contrast to the finding of MARTONOSI[8] who reported that in phospholipase C treated membranes [^{32}P]phosphoenzyme formation was not dependent on Ca^{2+}. Further we found that the addition of ADP to previously formed [^{32}P]phosphoenzyme caused a rapid loss of ^{32}P-label in the intermediate in both normal and lipid-depleted sarcoplasmic reticulum vesicles, suggesting that ATP formation from [^{32}P]phosphoenzyme and ADP was not blocked by removal of phospholipid.

Our working hypothesis involves a reformulation of Eqns. 1 and 2 as follows:

$$ATP + 2\,(Ca^{2+})^{outside} + \text{enzyme} \rightleftharpoons P\text{-enzyme–}Ca_2 + ADP \qquad (3)$$

$$P\text{-enzyme–}Ca_2 \xrightleftharpoons[\text{(Translocation?)}]{\text{Lipid}} \text{enzyme} + P_i + 2(Ca^{2+})^{inside} \qquad (4)$$

ATP energizes the formation of a P-enzyme–Ca_2 complex with Ca^{2+} on the outside of the vesicles. Ca^{2+} is then translocated to the inside of the vesicle where it is released and the P-enzyme linkage is hydrolyzed. It is this step which is strongly dependent on lipid. We cannot say that the formation of the [^{32}P]phosphoenzyme was completely independent of lipid since a residual amount of phospholipid (10–20 %) was not extracted from the membranes in our experiments.

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