Role of sarcoplasmic reticulum phospholipids in calcium ion binding activity

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Abstract The relationship between sarcoplasmic reticulum phospholipid and Ca^{2+} binding by sarcoplasmic reticulum membranes was explored. Ca^{2+} bound in the absence of ATP was defined as "ATP-independent Ca^{2+} binding," and the additional amount of Ca^{2+} bound in the presence of ATP was defined as "ATP-dependent Ca^{2+} binding." The latter was found to be very sensitive to the loss of sarcoplasmic reticulum phospholipid; the amount of Ca^{2+} bound was reduced when as little as 3% of the phospholipid was destroyed by phospholipase C. Further destruction of membrane phospholipid up to a 40% loss caused little or no further reduction of this Ca^{2+} binding. However, when the destruction of phospholipid exceeded 40%, further loss of this Ca^{2+} binding occurred, and there was an almost complete loss of this function when more than 60% of the sarcoplasmic reticulum phospholipid was destroyed.

Supplementary key words phospholipase C · "ATP-dependent Ca^{2+} binding" · "ATP-independent Ca^{2+} binding" · "ATP-dependent, oxalate-promoted Ca^{2+} transport"

The ability of SR to release and to sequester Ca^{2+} is basic to the excitation–contraction process and to the relaxation process of skeletal muscle (1). During the past 10 years, the Ca^{2+} sequestration has been intensively investigated by means of in vitro experiments utilizing SR vesicles isolated from homogenates of skeletal muscle. In most studies, measurement of Ca^{2+} transport was made in systems containing ATP, which energizes this transport, and oxalate, which promotes it. Therefore, we call the process so studied "ATP-dependent, oxalate-promoted Ca^{2+} transport," and it is well established that this process requires SR phospholipids to be functional (1).

However, SR also has the capacity to bind Ca^{2+} to the membrane structure, a function that first received careful attention in a research report published by Carvalho and Leo (2). Surprisingly, the Ca^{2+} binding function has been less extensively studied than Ca^{2+} transport; moreover, the results reported from various laboratories (3–6) have not been entirely consistent in regard to the number of classes of binding sites and the values of the apparent association constants.

Carvalho and Leo (2) also reported that at certain pCa values ATP promotes the binding of Ca^{2+} by SR membranes in exchange for other cations. As will be presented below, we have confirmed this finding and have obtained further support for the conclusion that it is due to enhanced Ca^{2+} binding to the SR membrane structure rather than to the intravesicular sequestration of free Ca^{2+}.

Phospholipid and protein are the predominant chemical substances of which SR membranes are composed (7). It is therefore likely that one or both of these substances provide the sites to which Ca^{2+} is bound. Cohen and Selinger (3) reported that hydrolysis of the SR phospholipids by treatment with phospholipase C did not influence Ca^{2+} binding (studied in the absence of ATP) by these membranes, a finding that implies that the protein provides the sites to which Ca^{2+} is bound.

Because phospholipids have been shown to be so important in "ATP-dependent, oxalate-promoted Ca^{2+} transport," we decided to further explore possible relationships between SR phospholipid and Ca^{2+} binding by SR membranes; in particular, the effect of destroying the membrane phospholipid on the ability of ATP to selectively promote Ca^{2+} binding by SR was investigated. In this study we have defined as "ATP-independent Ca^{2+} binding" that Ca^{2+} bound in the absence of ATP and as "ATP-dependent Ca^{2+} binding" the additional amount of Ca^{2+} bound in the presence of ATP.

METHODS

Preparation of fragmented SR vesicles

The following modification of the method of Ebashi and Yamanouchi (8) for the isolation of fragmented SR from

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Abbreviations: SR, sarcoplasmic reticulum; pCa, $-\log [Ca^{2+}]$; EGTA, ethylene glycol-bis(aminoethyl ether)-*N,N'*-tetraacetic acid.

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skeletal muscle was used. 140 g of hindleg muscle was dissected from retired male breeders of Sprague-Dawley rats (purchased from Charles River Laboratories) immediately after decapitation by means of a guillotine. The muscle was further processed at 4°C as follows. Excess adipose tissue and connective tissue were trimmed off and discarded, and the muscle was minced with scissors before being suspended in 280 ml of 15 mM $NaHCO_3$. The suspension was homogenized in a Waring blender (full speed for 35 sec), and the resulting homogenate was centrifuged at 9000 *g* for 20 min. Throughout the isolation procedure the pH of the system was maintained within the range of 6.7–7.3. The supernatant was filtered through Whatman no. 1 paper, and the filtrate was centrifuged at 23,000 *g* for 1 hr. The pellet was collected and suspended in 0.6 M KCl buffered with 20 mM Tris–maleate (pH 7.0), and the suspension was allowed to stand for 1 hr before being centrifuged at 105,000 *g* for 1 hr. The pellet was collected, suspended in 50 mM KCl buffered with 20 mM Tris–maleate (pH 7.0), and either used within 24 hr or stored in the frozen state for subsequent use in Ca^{2+} binding experiments. Just prior to such use, the suspension was diluted to the approximate concentration of SR fragments desired and centrifuged at 6000 *g* for 10 min. The resultant supernate, called the "SR suspension," was utilized in the Ca^{2+} binding studies.

Procedure for measuring Ca^{2+} binding

The following procedure, a modification of the method of Carvalho and Leo (2), was used for measuring Ca^{2+} binding by SR membranes. An aliquot of SR suspension containing approximately 5 mg of protein was transferred to a centrifuge tube and centrifuged at 80,801 *g* for 35 min. The supernate was discarded and the pellet was ground in the centrifuge tube with a glass pestle until finely suspended in 5 ml of medium containing 5 μmoles of $MgCl_2$, an amount of $CaCl_2$ needed to obtain the pCa desired, 5 μmoles of EGTA, 50 μmoles of KCl, and 49.5 μmoles of imidazole buffer; the final pH of the system was 7.0. After a 10-min incubation at room temperature, the suspension was centrifuged at 80,801 *g* for 35 min at a temperature ranging from 0 to 5°C. If the effect of ATP on binding was to be determined, 0.17 ml of a 30 mM disodium ATP solution (adjusted to pH 7.0 with 2 M Tris base) was added to the suspension immediately before centrifuging along with additional $CaCl_2$ to maintain the desired pCa. This provides a concentration of ATP in the binding assay system of 1 mM.

After centrifugation the supernate was discarded, and the centrifuge tube containing the pellet was carefully rinsed with distilled, deionized water. The pellet was then suspended in 5 ml of 0.25 M sucrose by grinding with the glass pestle. This suspension was allowed to stand at room temperature for 20 min before being centrifuged at 80,801 *g* for 35 min. The supernate was discarded, and the test tube containing the pellet was rinsed with 0.25 M sucrose.

The pellet was suspended in 3.75 ml of water from which two 0.05-ml aliquots were used for protein analysis. To the remaining 3.65 ml, 0.5 ml of 50% trichloroacetic acid was added, and after 20 min the system was centrifuged at 80,000 *g* for 10 min. The supernate was removed and neutralized with Tris base, and its Ca^{2+} and Mg^{2+} content was measured by atomic absorption spectrophotometry.

Analysis of SR phospholipid and protein content

The content of phospholipid in the fragmented SR suspension was determined by the method of Sanslone et al. (7), a method that extracts more than 99.5% SR lipid. The protein content of the fragmented SR suspension was estimated as described by Yu, DeMartinis, and Masoro (9).

Use of these methods to measure the phospholipid content of six individual aliquots taken from the same membrane preparation yielded the following data: mean, 17.831 μg of lipid phosphorus/mg of protein; standard deviation, 0.472; standard error, 0.192; 95% confidence level for the mean, $\overline{X} \pm 0.494$; and 95% confidence level for a single determination, $X \pm 1.214$.

Treatment of fragmented SR with phospholipase C

The procedure of Yu, DeMartinis, and Masoro (10) was used for the treatment of SR membranes with phospholipase C with the exception that various concentrations of Ca^{2+} were added as described in the footnotes and legends of the relevant tables and figures. The amount and the nature of the phospholipase C preparations employed are also described in the relevant tables and figures.

Purification of phospholipase C

A method of purifying phospholipase C based on the procedures of Diner (11) and Macchia and Pastan (12) was developed. All procedures were carried out at 4°C. 1 g of phospholipase C, purchased from Sigma Chemical Co. and prepared by the method of MacFarlane and Knight (13), was the starting material. It was suspended in 628 ml of 10 mM Tris base and 5 mM $CaCl_2$ (pH of system, 7.6). To this, solid $(NH_4)_2SO_4$ was added until the solution was 33% saturated with this salt. The pH was adjusted to 7.6, and after 1.5 hr the system was centrifuged at 10,400 *g* for 5 min. The supernate was removed, to it sufficient solid $(NH_4)_2SO_4$ was added to reach a 50% saturation with this salt, and the pH was adjusted to 7.6. After 15 hr, the system was centrifuged at 10,400 *g* for 5 min. The precipitate was suspended in 22.5 ml of 0.01 M Tris buffer (pH 7.6) and dialyzed for 18 hr against two 4-l changes of this buffer. The dialysate was then treated with calcium phosphate gel for 45 min, and the system was centrifuged at 3900 *g* for 5 min. The precipitate was suspended in 30 ml of 25 mM sodium phosphate buffer (pH 7.5) and centrifuged at 3900 *g* for 5 min, and the pellet was collected and resuspended in the

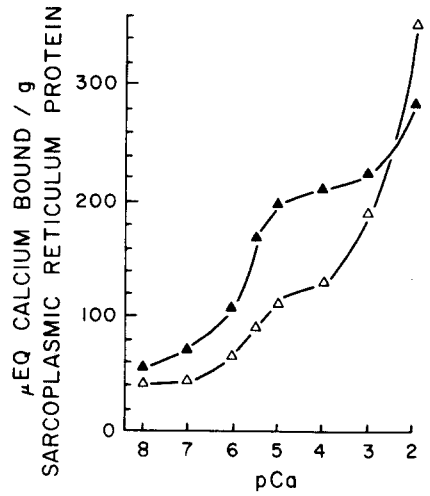

Fig. 1. Effect of pCa on calcium binding. Calcium binding measurements were carried out as described in the Methods section, except 34.25 μmoles of $MgCl_2$ was added rather than 5 μmoles. Open triangles refer to calcium binding in the absence of ATP, and closed triangles to binding in the presence of 1 mM ATP.

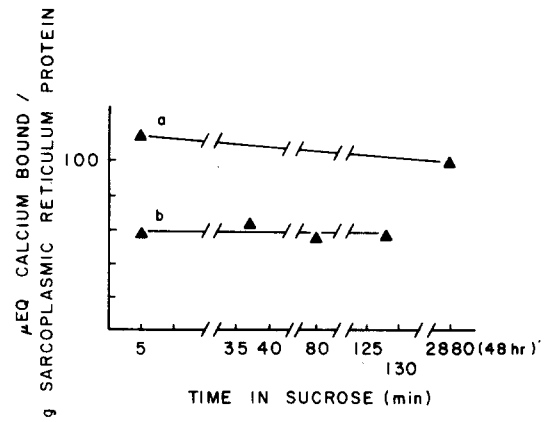

Fig. 2. Stability of the ATP-induced increment in calcium content of SR during suspension in 0.25 M sucrose. "ATP-dependent Ca^{2+} binding" at pCa 5 was carried out as described in the Methods section. SR membranes were collected by centrifugation after exposure to the ATP-containing calcium binding medium. The resulting pellet of SR was then suspended in 0.25 M sucrose as described in the Methods section and allowed to remain in suspension for the times noted on the *x* axis, after which the membranes were collected by centrifugation and analyzed for calcium as described in the Methods section. *a* and *b* refer to experiments carried out with two different preparations of SR fragments. The *y* axis refers to "ATP-dependent Ca^{2+} binding."

phosphate buffer. This step was repeated five times. The pellet then was mixed for 50 min with 20 ml of 1 M $(NH_4)_2SO_4$ (pH 7.5) and centrifuged at 3900 *g* for 5 min, and the supernate was collected. The pellet was treated in a similar fashion two more times, and the three supernates were pooled and dialyzed for 18 hr against two 4-l volumes of a solution containing 10 mM Tris–HCl (pH 7.6) and 5 mM $CaCl_2$. This dialysate was concentrated and layered on a Sephadex G-100 bead column. The eluate was collected in 2-ml fractions, which were analyzed for protein and phospholipase C activity. Those containing the phospholipase C activity were pooled. Phospholipase C activity was assayed by the method of Ottolenghi (14). The eluate was also assayed for neuraminidase activity by the method of Macchia and Pastan (12) and for proteolytic activity by the method of Cohen and Hirsch (15).

This purification procedure yielded a phospholipase C preparation with a specific activity 5.4-fold greater than the commercial preparation used as the starting material. The specific activity of neuraminidase was reduced to 20% of that of the commercial preparation, an activity that represents a negligible quantity in terms of the amount of enzyme preparation used to hydrolyze the membrane phospholipids. The purified preparation contained no detectable proteinase activity.

RESULTS

The aim of the initial experiment was the establishment of those conditions under which maximal "ATP-dependent Ca^{2+} binding" by fragmented SR occurs. To this end, an investigation of the effect of pCa on Ca^{2+} binding by SR was carried out. In these experiments the desired pCa was obtained by choosing appropriate concentrations of $CaCl_2$ and EGTA; all other solutes were added in amounts noted in the Methods section, with the exception that 19 μmoles of $MgCl_2$ was added. As shown in **Fig. 1,** the maximal "ATP-dependent Ca^{2+} binding" (i.e., the increment in Ca^{2+} binding caused by the addition of ATP) occurs at pCa 5; therefore, this pCa value was used in all further experiments. At pCa 5, the addition of ATP was found to cause approximately as much Mg^{2+} to be lost from SR membrane binding sites as it caused Ca^{2+} to be bound (not shown in Fig. 1). Experiments were conducted for the purpose of determining the amount of $MgCl_2$ that results in maximal "ATP-dependent Ca^{2+} binding" at pCa 5. Over a range of added $MgCl_2$ of from 0 to 34.25 μmoles, the "maximal ATP-dependent Ca^{2+} binding" was found to occur with the addition of 5 μmoles of $MgCl_2$. Thus, this amount of $MgCl_2$ was used in all subsequent experiments.

To determine whether our modification of the method of assaying Ca^{2+} binding by SR developed by Carvalho and Leo (2) measures binding alone or binding plus intravesicular sequestration of free Ca^{2+}, the following experiments were done. The SR vesicles were incubated at pCa 5 with and without ATP; they were isolated by centrifugation and then suspended in 0.25 M sucrose. As is evident from **Fig. 2,** the ATP-induced increase in Ca^{2+} content of the SR fragments suspended in this sucrose medium for 5 min is about the same as in those suspended in this way for 37, 80, 130, or 2880 min. Since Duggan and Martonosi (16) found that intravesicularly sequestered Ca^{2+} is rapidly released (time course ranging from seconds to minutes), these data provide strong evidence that the Ca^{2+} remaining with the membranes after suspension for 5 min in 0.25 M sucrose probably represents a bound

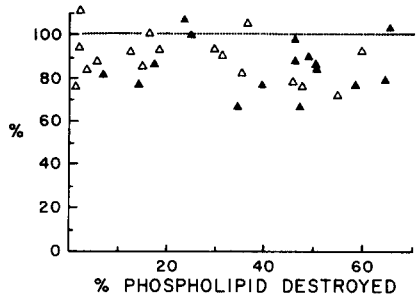

Fig. 3. Effects of destruction of SR phospholipid with phospholipase C on "ATP-independent Ca^{2+} binding." The line refers to amount of Ca^{2+} bound at pCa 5 by SR not treated with phospholipase C and expressed on *y* axis as 100%. Closed triangles refer to amount of Ca^{2+} bound after treatment with commercial phospholipase C, and open triangles refer to amount of Ca^{2+} bound after treatment with purified phospholipase C, both expressed as percentages of that obtained with the same SR preparation not treated with phospholipase C. A range of 0.13–0.39 units (0.027–0.080 mg/mg of SR protein) of commercial phospholipase C (*C. welchii;* type 1, Sigma Chemical Co.), a range of 0.067–0.134 units (0.005–0.010 mg/mg of SR protein) of purified phospholipase C, and a range of 0–1 mM $[Ca^{2+}]$ were used to obtain the broad range of phospholipid destruction noted on the *x* axis.

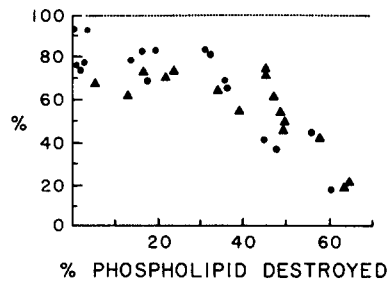

Fig. 4. Effects of destruction of SR phospholipid with phospholipase C on "ATP-dependent Ca^{2+} binding." The line refers to amount of Ca^{2+} bound at pCa 5 by SR not treated with phospholipase C and expressed on *y* axis as 100%. Triangles refer to amount of Ca^{2+} bound after treatment with commercial phospholipase C, and circles refer to amount of Ca^{2+} bound after treatment with purified phospholipase C, both expressed as percentages of that obtained with the same SR preparation not treated with phospholipase C. A range of 0.13–0.39 units (0.027–0.080 mg/mg of SR protein) of commercial phospholipase C (*C. welchii;* type 1, Sigma Chemical Co.), a range of 0.067–0.134 units (0.005–0.010 mg/mg of SR protein) of purified phospholipase C, and a range of 0–1 mM $[Ca^{2+}]$ were used to obtain the broad range of phospholipid destruction noted on the *x* axis.

form rather than free Ca^{2+} stored intravesicularly against a concentration gradient. In light of these results, in all subsequent experiments the SR membranes were suspended in sucrose for 20 min before assaying for bound Ca^{2+}. (The 20-min suspension in 0.25 M sucrose was also utilized in the pCa and $[Mg^{2+}]$ studies described above.)

The effect of treating the SR vesicles with phospholipase C on "ATP-independent Ca^{2+} binding" is shown in **Fig. 3.** The destruction of phospholipid by the commercial or the purified phospholipase C preparation usually, but not invariably, causes a small decrease in "ATP-independent Ca^{2+} binding"; there was no relationship between the extent of phospholipid destruction and the extent of reduction of Ca^{2+} binding. These findings are similar to those reported by Cohen and Selinger (3).

In contrast, "ATP-dependent Ca^{2+} binding" by SR is consistently decreased by treatment with the commercial or the purified phospholipase C preparation (**Fig. 4**). The extent to which "ATP-dependent Ca^{2+} binding" is decreased by loss of SR phospholipid did not change between 3 and 40% phospholipid destruction. Statistical analysis of the binding data in this range of phospholipid loss revealed "ATP-dependent Ca^{2+} binding" to be significantly reduced by both the commercial preparation ($P < 0.005$) and the purified preparation ($P < 0.001$). At greater than 40% destruction of SR phospholipid there is a further marked fall in "ATP-dependent Ca^{2+} binding," with an almost total loss with 60% or more destruction of phospholipid.

Because the purified phospholipase C preparation is not a totally pure enzyme preparation (see Methods section), the question arose as to whether the effects noted above were actually due to phospholipid destruction or to some other action of the enzyme preparation used. Several ancillary studies, presented below, suggest that phospholipid destruction is the factor responsible for this loss of "ATP-dependent Ca^{2+} binding."

A known contaminant that the purification procedures did not totally remove from the commercial phospholipase C preparation is neuraminidase. Therefore, the effect of neuraminidase on Ca^{2+} binding by SR was studied. From the results in **Table 1,** it seems evident that levels of neuraminidase much higher than could be present in the phospholipase C preparation do not affect "ATP-dependent Ca^{2+} binding" or cause sialic acid to be released from the SR preparation.

Phospholipase C is a heat-stable enzyme. As shown in **Table 2,** the amount of "ATP-dependent Ca^{2+} binding" is the same in SR membranes in which 30–50% of the phospholipid is destroyed by heat-treated commercial phospholipase C as in membranes in which the same

TABLE 1. Effects of neuraminidase on "ATP-dependent calcium binding"

Time Incubated with Neuraminidase[a]	Not Treated with Neuraminidase	Treated with Neuraminidase
min	*μeq Ca^{2+} bound/g SR protein*	
30	80	77
80	76	78

[a] Neuraminidase (Sigma type V; sp act, 100 nmoles of sialic acid released/mg of protein/min, assayed by the method of Macchia and Pastan [12]) was added at a concentration of 0.067 mg/mg of SR protein. This represents about one order of magnitude of neuraminidase activity above that present in the commercial phospholipase C used in the experiments presented in Figs. 3 and 4 and Table 2, and more than two orders of magnitude of neuraminidase activity above that present in the purified phospholipase C used in the experiments presented in Figs. 3 and 4 and Table 2. The "ATP-dependent calcium binding" was measured at pCa 5. Under conditions used for phospholipase C treatment, neuraminidase did not release sialic acid from the SR.

amount of SR phospholipid was destroyed by either the commercial phospholipase C or by the purified phospholipase C, which were not heat-treated. These results are consistent with phospholipase C being the material in the enzyme preparation responsible for inhibiting "ATP-dependent Ca^{2+} binding."

It seemed possible that the action of phospholipase C on Ca^{2+} binding by SR membranes could be the result of the solubilization of SR protein; i.e., the loss of a particular protein could be the reason for the changes in Ca^{2+} binding induced by this enzymatic treatment. Experiments designed to test this revealed that treatment with sufficient phospholipase C to markedly inhibit "ATP-dependent Ca^{2+} binding" did not induce solubilization of SR membrane proteins. The methods used would have detected a 0.5% solubilization of SR protein by phospholipase C if it had occurred.

Moreover, as stated in the Methods section, the purified phospholipase C preparation is free from protease activity. Therefore, this enzyme preparation is not likely to have caused proteolysis of SR protein.

To examine the possibility that the phospholipase C preparation affects Ca^{2+} binding because of the presence of some toxic contaminant, the following experiment was done. Phospholipase C was added to the SR vesicles at a temperature of 0°C, and the mixture was immediately diluted 10-fold with ice-cold medium. The system was quickly centrifuged, and the SR vesicles were collected and tested for Ca^{2+} binding activity. This treatment did not affect Ca^{2+} binding. Therefore, purified phospholipase C does not contain toxic agents that alter Ca^{2+} binding by rapidly combining with the SR membranes. This conclusion is further strengthened by the fact that *Clostridium welchii* antitoxin, which is known to inhibit the activity of this phospholipase C, when added to the SR preparation along with phospholipase C prevented both the destruction of SR phospholipids and the inhibition of "ATP-dependent Ca^{2+} binding."

The effect that phosphorylcholine, the major water-soluble product of phospholipase C action on SR membranes, has on Ca^{2+} binding was also examined. Phosphorylcholine at concentrations that might be expected from the action of phospholipase C on SR membranes did not inhibit "ATP-dependent Ca^{2+} binding" (**Fig. 5**) or "ATP-independent Ca^{2+} binding" (data now shown).

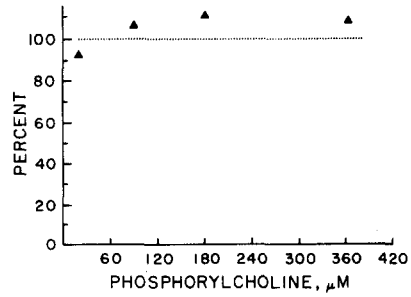

Fig. 5. Effect of treatment of SR membranes with phosphorylcholine on "ATP-dependent Ca^{2+} binding." A phosphorylcholine concentration of 480 μM would be the approximate concentration of phosphorylcholine in the system if 100% of the SR membrane phosphatidylcholine were hydrolyzed by phospholipase C. The line refers to amount of Ca^{2+} bound at pCa 5 by SR to which phosphorylcholine was not added and expressed on the *y* axis as 100%. Triangles refer to amount of Ca^{2+} bound when concentration of phosphorylcholine indicated on the *x* axis was added.

The other product of phospholipase C action is diglyceride, which probably remains associated with the SR membrane. It is difficult to know what effect, if any, the accumulation of this substance had in the experiments just presented. It is theoretically possible that a lipase present in either (*a*) the phospholipase C preparation or (*b*) the SR membrane converts some of this diglyceride to FFA, which in turn competes with the SR for the available Ca^{2+}. However, this is not a likely mechanism of inhibition of "ATP-dependent Ca^{2+} binding" by phospholipase C because (*a*) no relationship was found between the amount of a phospholipase C preparation used and the extent of inhibition of Ca^{2+} binding when SR phospholipid destruction ranged between 3 and 40% and (*b*) although phospholipase C has been found to monotonously inhibit SR ATPase activity (10, 17), FFA stimulates this activity at low concentrations and inhibits it at high concentrations (17).

TABLE 2. Influence of heating phospholipase C on its effect on "ATP-dependent calcium binding"

Enzyme Preparation Used to Treat SR Membranes	Ca^{2+} Bound[a]
	μeq/g SR protein
Commercial phospholipase C	59 ± 3.3 (n = 8)
Heated commercial phospholipase C[b]	58 ± 0.9 (n = 3)
Purified phospholipase C	62 ± 8.1 (n = 6)

[a] For each enzyme preparation the data presented are those in which between 30 and 50% of the SR phospholipid is destroyed by the phospholipase C. The "ATP-dependent calcium binding" was measured at pCa 5. Values are means ± SEM.

[b] Heated for 8 min at 97°C in 10 mM phosphate buffer (pH 7.5) prior to use.

DISCUSSION

Although much work has been done on the relationship between SR phospholipid and "ATP-dependent, oxalate promoted Ca^{2+} transport" by isolated SR vesicles (10, 17), little has been done on the role of these phospholipids on Ca^{2+} binding by SR. Cohen and Selinger (3) did report that phospholipase C treatment did not influence "ATP-independent Ca^{2+} binding," which is in contrast to our claim of a small but statistically significant loss of this SR activity. However, a review of their paper reveals their data and ours to be in agreement even if our conclusions differ somewhat.

The effect of SR phospholipid destruction on "ATP-dependent Ca^{2+} binding" has not been studied by other workers. Carvalho (18) did some related work on the effect of phospholipases on the release of Ca^{2+} from SR membranes that had been bound to them by "ATP-dependent Ca^{2+} binding." He reported that this bound Ca^{2+} could be released by treating these membranes with either phospholipase A or C; unfortunately, in these studies the extent to which the SR membrane phospholipid had been destroyed by the enzymatic treatment was not measured.

In the present study the effect of SR phospholipid destruction on "ATP-dependent Ca^{2+} binding" was directly measured, and the results indicate that phospholipids play a role in "ATP-dependent Ca^{2+} binding" by SR membranes. Indeed, they suggest that this binding activity is exquisitely sensitive to the loss of membrane phospholipid, with the amount of Ca^{2+} bound being significantly reduced when as little as 3% of the membrane phospholipid is destroyed. Moreover, almost complete loss of "ATP-dependent Ca^{2+} binding" occurs when more than 60% of the membrane phospholipid is destroyed. The mechanism by which phospholipid is involved in this binding has not been defined, but the fact that a 3% destruction of SR phospholipid causes as much loss as a 40% destruction provides an important base for such consideration.

A simple and possible explanation is that the SR phospholipids most accessible to attack by phospholipase C are the sites of "ATP-dependent Ca^{2+} binding." A loss of 3% of the membrane phospholipid is sufficient to account for the observed loss in "ATP-dependent Ca^{2+} binding" by SR membranes. Although acidic phospholipids are the prime binders of Ca^{2+}, Seimiya and Ohki (19) have recently reported conditions under which phosphatidylcholine binds Ca^{2+}. It should be noted that phospholipase C of *C. welchii* primarily, although not exclusively, attacks phosphatidylcholine in SR membranes (17) and that phosphatidylcholine makes up about 70% of rat skeletal muscle SR phospholipid, with phosphatidylethanolamine and phosphatidylinositol making up most of the rest (7). In this explanation, it must be postulated that a specific morphological compartment of phosphatidylcholine or one of the other phospholipid subclasses present is the site of "ATP-dependent Ca^{2+} binding." Further experimental explorations by methods such as analyses of hydrolysis products will not define this site morphologically. Nor since phosphatidylcholine is so abundant and so susceptible to attack by phospholipase C would such analyses be likely to provide unequivocal information in regard to the phospholipid subclass involved.

An alternate and equally plausible explanation is that these phospholipid molecules function as modulators of Ca^{2+} binding by SR protein binding sites and confer ATP sensitivity to the system. In support of this view is the abundance of literature that establishes that SR protein contains Ca^{2+} binding sites (5, 20, 21).

It has been suggested that "ATP-dependent Ca^{2+} binding" and "ATP-dependent, oxalate promoted Ca^{2+} transport" are identical processes. However, the data presented in the present report and other unpublished data obtained in our laboratory strongly indicate that these processes differ markedly, in certain characteristics at least. Although each is disrupted by phospholipase C destruction of SR phospholipid, it requires a loss of more than 60% of the SR phospholipid before the Ca^{2+} transport and its associated ATPase are significantly affected (10, 17), while "ATP-dependent Ca^{2+} binding" is significantly inhibited by the destruction of less than 10% of the membrane phospholipid. Indeed, no other SR process thus far studied is as sensitive to phospholipase C destruction of membrane phospholipid as is the "ATP-dependent Ca^{2+} binding" function. Further evidence that the "ATP-dependent, oxalate promoted Ca^{2+} transport" differs from the "ATP-dependent Ca^{2+} binding" comes from our unpublished research on the photoinactivation of SR membrane functions; kinetic analysis of these photoinactivation data by the method of Ray and Koshland (22) reveals "ATP-dependent, oxalate promoted Ca^{2+} transport" to be much more sensitive to photoinactivation than "ATP-dependent Ca^{2+} binding," the latter in this case being measured by the method of Ohnishi and Ebashi (23).

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