Uncoupling of Ca\(^{2+}\) Transport in Sarcoplasmic Reticulum as a Result of Labeling Lipid Amino Groups and Inhibition of Ca\(^{2+}\)-ATPase Activity by Modification of Lysine Residues of the Ca\(^{2+}\)-ATPase Polypeptide*

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Limited labeling of amino groups with fluorescamine in fragmented sarcoplasmic reticulum vesicles inhibits Ca\(^{2+}\)-ATPase activity and Ca\(^{2+}\) transport. Under the labeling conditions used, 80% of the label reacts with phosphatidylethanolamine and 20% with the Ca\(^{2+}\)-ATPase polypeptide. This degree of labeling does not result in vesicular disruption or in loss of vesicular proteins and does not increase the membrane permeability to Ca\(^{2+}\). Fluorescamine labeling of a purified Ca\(^{2+}\)-ATPase devoid of aminophospholipids also inhibits Ca\(^{2+}\)-ATPase activity, suggesting that labeling of lysine residues of the enzyme polypeptide is responsible for the inhibition of Ca\(^{2+}\)-ATPase activity in sarcoplasmic reticulum. Fluorescamine labeling interferes with phosphoenzyme formation and decomposition in both the native vesicles and the purified enzyme; addition of ATP during labeling, and with less effectiveness ADP or AMP, protects both partial reaction steps. Addition of a nonhydrolyzable ATP analog protects phosphoenzyme formation but not decomposition. The inhibition of Ca\(^{2+}\) transport but not of Ca\(^{2+}\)-ATPase occurs in sarcoplasmic reticulum vesicles labeled in the presence of ATP, indicating that the transport reaction is uncoupled from the Ca\(^{2+}\)-ATPase reaction. The inhibition of Ca\(^{2+}\) transport but not of Ca\(^{2+}\)-ATPase activity is also found in sarcoplasmic reticulum vesicles in which only phosphatidylethanolamine has reacted with fluorescamine. Furthermore, the extent of labeling of phosphatidylethanolamine is correlated with the inhibition of Ca\(^{2+}\) transport rates. The inhibition of Ca\(^{2+}\) transport is a reflection of the inhibition of Ca\(^{2+}\) translocation and is not due to an increase in Ca\(^{2+}\) efflux. We propose that labeling of phosphatidylethanolamine perturbs the lipid environment around the enzyme, producing a specific defect in the Ca\(^{2+}\) translocation reaction.

Fragmented sarcoplasmic reticulum vesicles transport Ca\(^{2+}\) coupled to the hydrolysis of ATP. The elementary steps of the Ca\(^{2+}\)-ATPase reaction have been studied in detail (for recent reviews, see Refs. 1 and 2). It is generally agreed that the reaction is initiated by binding of Ca\(^{2+}\) to the high affinity sites of the enzyme, followed by enzyme phosphorylation. This results in Ca\(^{2+}\) translocation presumably triggered by a conformational change in the enzyme molecule. After Ca\(^{2+}\) is translocated, the enzyme undergoes a decrease in affinity for Ca\(^{2+}\), allowing Ca\(^{2+}\) release to the vesicular interior. The dephosphorylation of the enzyme resulting in P\(_i\) liberation completes the reaction cycle.

The isolated SR vesicles have a relatively simple protein and lipid composition (3). This, together with the considerable knowledge of the mechanism of the Ca\(^{2+}\)-ATPase reaction, makes the SR system particularly suitable for studying the relationship between structure and function. With this aim in mind, a complex of fluorescamine with cycloheptaamyllose was used in previous studies as a nonpermeant covariant label to analyze the disposition of proteins and aminophospholipids in SR. It was found that the Ca\(^{2+}\)-ATPase was significantly labeled with CFC, indicating that a considerable fraction of the Ca\(^{2+}\)-ATPase polypeptide is exposed to the outside of the SR vesicles; calsequestrin did not react with CFC, indicating that it is buried in the vesicular interior (4). In addition, the labeling pattern of PE, the major aminophospholipid present in purified SR, indicates that PE has a highly asymmetric disposition, with 70–80% of it located in the external side of the membrane bilayer (4). The same asymmetric disposition of PE has been observed after labeling SR with other amino-labeling reagents (5, 6).

Labeling SR with CFC results in marked inhibition of both Ca\(^{2+}\)-ATPase and Ca\(^{2+}\) transport, although the labeled vesicles retain their structural integrity (4). The inhibition of the Ca\(^{2+}\)-ATPase reaction caused by CFC labeling is due to inhibition of the phosphorylation step and can be prevented by the presence of ATP during labeling (7). In contrast, the presence of ATP during labeling does not prevent the inhibition of Ca\(^{2+}\) transport, which is due presumably to a specific defect in the translocation reaction (7). Since in previous experiments even limited labeling with CFC resulted in labeling of both protein and lipid amino groups, it was not possible to ascertain unambiguously whether the labeling of lysine residues in the Ca\(^{2+}\)-ATPase polypeptide or the modification of the polar head group of PE produces the inhibition of Ca\(^{2+}\) transport.

In the present study, fluorescamine was chosen instead of CFC to label SR amino groups since labeling with fluorescamine allows more precise control of the extent of labeling and, as will be shown below, produces inhibitory effects similar to those of CFC. However, labeling with fluorescamine has to be restricted to a few amino groups; addition of 10 or more mol of fluorescamine/10\(^5\) g of SR protein results in loss of

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calsequestrin (4). In experiments using a purified Ca\textsuperscript{2+}-ATPase devoid of amino phospholipids, it was found that the inhibition of phosphoenzyme formation, and of the Ca\textsuperscript{2+}-ATPase reaction, observed after labeling with fluorescamine is due to modification of a limited number of lysine residues of the Ca\textsuperscript{2+}-ATPase polypeptide chain. Furthermore, when the SR vesicles were incubated with chloroquine under conditions in which the label reacts only with the polar head group of PE results in inhibition of Ca\textsuperscript{2+} transport but not of Ca\textsuperscript{2+}-ATPase activity. We propose that labeling of PE perturbs the interactions between the enzyme and its surrounding phospholipids resulting in the observed defect in the Ca\textsuperscript{2+} translocation reaction, which is presumably more sensitive to perturbations of lipid-substrate interactions than the phosphorylation step and the subsequent release of inorganic phosphate.

**EXPERIMENTAL PROCEDURES**

**Preparation of SR, Purified Ca\textsuperscript{2+}-ATPase, and Phospholipid-replaced Ca\textsuperscript{2+}-ATPase—**Fragmented SR was prepared from rabbit skeletal white (fast) muscle as described in detail previously (8), except that muscle was homogenized in 0.1 M KCl, 20 mM Tris-maleate, pH 7.0, instead of in 0.3 M sucrose, 20 mM Tris-maleate, pH 7.0. Also, care was taken to eliminate the very light density material that contains significant amounts of transverse tubule vesicles (9), by sedimenting the muscle microsomes at 0000–7500 × g instead of 150,000 × g. The SR vesicles were resuspended in 0.3 M sucrose, 20 mM Heps, pH 7.2, and stored frozen at −20 °C. For the preparation of a phospholipid-replaced ATPase (devoid of PE), the Ca\textsuperscript{2+}-ATPase enzyme, purified from SR by extraction with deoxycholate, was used as starting material. To purify the Ca\textsuperscript{2+}-ATPase, the procedure of Warren et al. (10) was modified in that the solubilization with deoxycholate was carried out with a total of 600 mg of SR, and the resulting suspension was layered on top of discontinuous sucrose gradients (15) containing DOPC (DOPC-ATPase) was obtained by centrifugation in a Beckman SW 27 rotor at 100,000 × g. After collecting the Ca\textsuperscript{2+}-ATPase from the interface of the two sucrose solutions, it was diluted 10-fold in 0.3 M sucrose, 20 mM Tris-maleate, pH 7.0, and was collected by centrifugation at 4 °C for 60 min at 100,000 × g. The resulting pellets, resuspended in 1.0 M KCl, 0.3 M sucrose, 5 mM dithiothreitol, 50 mM potassium phosphate buffer, pH 8.0, at a protein concentration of 10 mg/ml, were incubated with slow stirring at 0 °C for 2 h with a sonicated mixture of deoxycholate and DOPC. A weight ratio of protein/deoxycholate/DOPC of 1:1:2 was used. The purified enzyme complex (DOPC-ATPase) was then dialyzed against the lipid solution at different times after starting the Ca\textsuperscript{2+} uptake reaction as described previously (11).

Since DOPC-ATPase contains 0.77 μmol of phosphorus/mg of protein (77 mol/10 g), this indicates that the upper limit for the amount of PE still associated with the enzyme is 0.4 mol/10 g.

**Measurement of Fluorescence Incorporation into SR Lipids—**A fraction of the lipid extracts containing 0.1–0.25 μmol of phosphorus was applied to Silica Gel G thin layer plates and the phospholipids were separated by running the plate in one dimension with chloroform/methanol/water (56:25:4). This solvent system allows complete separation of PE (RF = 0.34) from fluorescamine-labeled PE (RF = 0.23) and from the rest of the SR lipids (RF = 0.70). The amount of phosphorus present in the PE spot, the labeled PE spot, and in the combined spots for the other phospholipids were determined by perchloric acid hydrolysis as previously described (11). The only fluorescent spot containing measurable amounts of phospholipid was fluorescamine-labeled PE.

**Enzymatic Determinations—**Ca\textsuperscript{2+}-ATPase activities were measured colorimetrically as described previously (11) or by means of a radiometer pH stat. All measurements were carried out at 22 °C. The reaction solution contained 0.1 M KCl, 5 mM MgCl\textsubscript{2}, 0.1 mM Ca\textsuperscript{2+}, 4 mM ATP, 20 mM Tris-maleate, pH 7.0, and 0.05 mg of protein/ml. The buffer was omitted when the reaction was carried out in the pH stat using a pH of 7.0.

**Steady state levels of phosphoenzyme as well as P\textsubscript{i} liberation rates at 0 °C were measured using \[\gamma\textsuperscript{32P}\]ATP as described previously (11).** The reaction mixture contained 0.1 M KCl, 5 mM MgCl\textsubscript{2}, 0.1 mM Ca\textsuperscript{2+}, 10 μmol of \[\gamma\textsuperscript{32P}\]ATP, 20 mM Tris-maleate, pH 7.0, and 0.1 mg of protein/ml. The reaction was stopped by adding 7% trichloroacetic acid. To measure phosphoenzyme, 0.5-ml fractions were filtered through HA Millipore filters, previously washed with 1 ml of 0.1 M ATP. After extensive washing of the acid-denatured protein retained in the filter as described previously (11), the filters were dried and counted in a liquid scintillation counter. The amount of P\textsubscript{i} liberated was determined by extraction of the phosphomolybdate complex into an organic phase. A 0.7-ml fraction of the acid-quenched sample was centrifuged to remove precipitated protein, and 0.1 ml of 37% trichloroacetic acid, containing 0.3 mg/ml of phosphomolybdate, 10 mM KOAc, 10 mM NaOH, and 1 ml of isobutyric acid (35:65, v/v) were added to 0.4 ml of the supernatant. The mixture was stirred for 45 s and centrifuged for 5 min to separate the phases, and 0.5 ml of the organic phase was counted in a liquid scintillation counter. The amount of liberated P\textsubscript{i} was calculated by using a calibration curve obtained by carrying out the same extraction procedure as above with standard phosphate solutions containing \[\gamma\textsuperscript{32P}\]ATP. Initial rates of phosphoenzyme formation were measured at 4 °C by means of a three-syringe Durrum multimixer Model 131 as described in detail elsewhere (14). A solution containing 0.1 M KCl, 10 mM MgCl\textsubscript{2}, 0.1 mM Ca\textsuperscript{2+}, 20 mM Tris-maleate, pH 7.0, and 2 mg of protein/ml was mixed with an equal volume of a solution containing 0.1 M KCl, 20 mM Tris-maleate, pH 7.0, and 0.025 μmol of \[\gamma\textsuperscript{32P}\]ATP. The reaction was quenched at different times by addition of 10% trichloroacetic acid. The amount of phosphoenzyme formed was measured as described above.

**Calcium Uptake in the Presence of Oxalate—**Calcium uptake was measured at 22 °C in a solution containing 0.1 M KCl, 5 mM K-oxalate, 5 mM MgCl\textsubscript{2}, 0.1 mM Ca\textsuperscript{2+}, 20 mM Tris-maleate, pH 7.0, and 0.025 mg of protein/ml. The reaction was started by addition of 5 mM ATP and was stopped at different times by filtering 1.0 ml of the reaction through HA Millipore filters. After filtration, the filter strips were washed with 2 ml of ice-cold 0.025 M EDTA, dried, and counted by liquid scintillation. To measure Ca\textsuperscript{2+}-ATPase activities in the presence of oxalate at 22 °C, the same reaction conditions as in the Ca\textsuperscript{2+} uptake measurements were used except that the solution contained 5 mM \[\gamma\textsuperscript{32P}\]ATP and 0.1 mM Ca\textsuperscript{2+} instead of Ca\textsuperscript{2+}. The amount of DOPC-ATPase was determined by extracting a fraction of the filtrate as described above.

To measure Ca\textsuperscript{2+} efflux, 8 mM EGTA was added to the SR vesicles at different times after starting the Ca\textsuperscript{2+} uptake reaction as described above.
RESULTS

It has been shown previously (13) that fluorescamine reacts quantitatively with SR amino groups at pH 8.3, provided the protein concentration is 2 mg/ml or more and the reagent concentration is in the range of 0.04-0.2 mM. Using this concentration of SR, we found that addition of 0.04-0.16 mM fluorescamine (2 to 8 mol of fluorescamine/10^5 g of SR protein) at pH 7.2 results in a linear increase in fluorescence intensity (Fig. 1); the slope decreased when the concentration of fluorescamine was increased to 0.2 mM (10 mol/10^5 g). Reagent concentrations are expressed in terms of moles/10^5 g of SR protein in Fig. 1 due to the fact that the molecular weight of the Ca^{2+}-ATPase, which represents 75-80% of the total SR protein, is about 1.1 x 10^6, so that the unit of moles/10^5 g of SR protein is roughly equivalent to moles of reagent/mol of enzyme. Control experiments indicate that labeling at pH 7.2 results in the same fluorescence intensities as those obtained by labeling at pH 8.3.

After separating the SR lipid and protein components as described under "Experimental Procedures," 80% of the total fluorescence of SR is present in the lipids and 20% in the protein (Fig. 1). The fluorescence in the lipids is due to labeling of PE, whereas the fluorescence in the protein is due to labeling of the Ca^{2+}-ATPase polypeptide, as evidenced by the fluorescence pattern of SDS-containing polyacrylamide gels of the protein extract (not shown).

It has been shown previously that 75-80% of the total PE present in SR is labeled with CFC (4). Since the SR vesicles used in these studies contain on the average 0.55 µmol of phosphorus/mg of protein and 13-14% PE (65 mol of phospholipids and 9 mol of PE/10^5 g of protein, respectively), this corresponds to a total of 7 mol of PE/10^5 g of protein that are labeled with CFC. It is interesting to note that in the present work the fluorescence intensity of the SR vesicles increases linearly up to 8 mol of fluorescamine/10^5 g of protein. Above 8 mol of fluorescamine, lipid labeling levels off, while protein labeling continues to increase (Fig. 1). Analysis of the lipid extract of SR vesicles labeled with 10 mol of fluorescamine/10^5 g of protein indicates that 78% of the PE present in SR, corresponding to 7 mol of PE/10^5 g of protein, has reacted with the label (Fig. 1). This clearly shows that the reaction of PE with fluorescamine proceeds to the same extent as the reaction of PE with CFC (7). Furthermore, from the fluorescence intensity of the lipid extract and from the number of moles of PE labeled, it is possible to calculate the fluorescence intensity/mol of amino group labeled. Using this number and the fact that the total fluorescence of SR in SDS is the sum of the fluorescence of its lipid and protein components (Fig. 1), the total number of lysine residues labeled can be calculated. As shown in Fig. 1, a maximum number of 7 mol of PE and 2 mol of lysine/10^5 g of protein is labeled when a concentration of 10 mol of fluorescamine/10^5 g of protein was used. This degree of lipid labeling is in agreement with previous reports by Hasselbach et al. (13), who also found a plateau of lipid labeling at a concentration of fluorescamine of 0.1 µmol/mg of protein (10 mol/10^5 g).

Effect of Labeling on Ca^{2+}-ATPase Activity—Unlabeled SR vesicles display at 22 °C Ca^{2+}-ATPase activities in the range of 0.6-0.7 µmol of P/mg/min. Increasing the Ca^{2+} permeability of the SR vesicles by addition of the ionophore A23187 results in 2.5- to 3-fold stimulation of Ca^{2+}-ATPase activity. As observed previously with CFC-labeled SR (7), labeling of SR vesicles with fluorescamine results in inhibition of Ca^{2+}-ATPase activity, measured either with or without A23187 in the reaction solution (Fig. 2). At low concentrations of fluorescamine, the percentage of inhibition of Ca^{2+}-ATPase activity in the absence of A23187 is comparable with the percentage of inhibition in the presence of the ionophore (Fig. 2). This suggests that the vesicles labeled with low reagent-to-protein ratios retain their permeability barrier to Ca^{2+} after labeling, since the above results indicate that A23187 stimulates the Ca^{2+}-ATPase activity in the labeled vesicles to the same extent as the control. ATP added prior to labeling protects Ca^{2+}-ATPase activity (Fig. 2). For protection of activity, it is required only that ATP be present during labeling, since no inhibition of Ca^{2+}-ATPase was observed in vesicles labeled in the presence of ATP, then sedimented and resuspended in ATP-free solution prior to the Ca^{2+}-ATPase assay. The same protective effect was observed by addition prior to labeling.

FIG. 1. Labeling of SR vesicles with increasing concentrations of fluorescamine. The labeling conditions are described in detail in the text. △, fluorescence of the SR vesicles; ◻, fluorescence of the lipid extract; ○, fluorescence of the protein residue. To measure fluorescence of the SR vesicles and of the protein residue, a concentration of 25 µg/ml of protein in 1% SDS was used; the resulting fluorescence values were multiplied by 40 to represent the fluorescence of a solution containing 1 mg of protein/ml. The fluorescence of the lipid extracts, dried and redissolved in 1% SDS as described in the text, was multiplied by 8 to obtain the fluorescence of a solution containing 1 mg of protein/ml. The moles of NH_{2} labeled were calculated from the fluorescence intensities as described in the text.

FIG. 2. Inhibition of Ca^{2+}-ATPase activity produced by labeling SR vesicles with fluorescamine. Ca^{2+}-ATPase activities were measured as described in the text. Ca^{2+}-ATPase activities measured with (○) or without (◆) A23187 present in the reaction solution. △, Ca^{2+}-ATPase activity of SR vesicles labeled with fluorescamine in the presence of 5 mM ATP, measured either with or without A23187. The moles of NH_{2} labeled were calculated as described in the text.
labeling of ATP alone or of ATP plus 5 mM MgCl₂. No protection was observed by labeling in the presence of 5 mM MgCl₂, nor by Ca²⁺ concentrations from 10⁻² to 10⁻⁴ M.

As seen previously with CFC (7), labeling of SR with fluorescamine produces inhibition of phosphoenzyme formation (Table I). Addition of ATP prior to labeling prevents the inhibition in both cases (Table I, Ref. 7).

Most likely, the inhibition of Ca²⁺-ATPase is due to labeling of lysine residues of the enzyme polypeptide. It has been shown recently that a large fraction of the total lysine residues present in the Ca²⁺-ATPase is contained in hydrophilic sequences (16), which makes it likely that they are exposed to the outside of the SR vesicles. To test whether labeling lysine residues with fluorescamine results in inhibition of Ca²⁺-ATPase activity, a purified ATPase preparation devoid of PE, DOPC-ATPase, was used. It was found that increasing label incorporation, as revealed by increasing fluorescence, results in progressive inhibition of both steady state phosphoenzyme levels and Pi liberation rates (Fig. 3). This clearly shows that labeling of lysines results in inhibition of Ca²⁺-ATPase. It is interesting to note that at the same extent of labeling the inhibition of the rate of Pi liberation is more pronounced than the reduction of steady state phosphoenzyme levels (Fig. 3).

To further analyze this, the percentage of steady state phosphoenzyme levels and the percentage rate of Pi liberation observed after labeling DOPC-ATPase with fluorescamine were plotted as a function of the number of lysine residues modified (Fig. 4). Although there is some deviation at the lower end due to the low levels of phosphoenzyme formed, the values for percentage of steady state phosphoenzyme fit a straight line that intercepts the abscissa (100% inhibition) at 7 mol of lysine labeled/10⁵ g of enzyme. These results suggest that modification of 1 out of 7 lysine residues, all with equal reactivity to fluorescamine, results in 100% inhibition of phosphoenzyme formation. If we assume that labeling of a different lysine of the 7 with equal reactivity produces inhibition of phosphoenzyme decomposition but not of phosphoenzyme formation, at a given number of lysine residues modified, the rate of Pi liberation should be inhibited more than the steady state phosphoenzyme levels (since phosphoenzyme decomposition cannot take place in the absence of phosphoenzyme formation, labeling of either the lysine required for phosphoenzyme formation or for decomposition would result in inhibition of Pi liberation). The predicted curves for the cases in which labeling of the lysine residue involved in phosphoenzyme decomposition causes either 50 or 100% inhibition of Pi liberation rates are shown in Fig. 4. The lines were drawn assuming that there are seven lysines with equal reactivity to fluorescamine and that labeling of one of them causes 100% inhibition of phosphoenzyme formation (solid line) and of a different one causes either 100% (broken line, small traces) or 50% (broken line, long traces) inhibition of Pi liberation rates. The data represent values from four different preparations.

### Table I

**Effect of labeling SR vesicles with fluorescamine on steady state phosphoenzyme levels**

To measure steady state phosphoenzyme levels, the reaction solution contained 0.1 M KCl, 5 mM MgCl₂, 0.1 mM CaCl₂, 20 mM Tris-maleate, pH 7.0, 10 μM [γ-³²P]ATP and 0.1 mg of protein/ml. The reaction was carried out at 0 °C and it was stopped by addition of 7% trichloroacetic acid at 10, 20, and 30 s. The phosphoenzyme values represent the mean value ± S.D.

| Fluorescamine | Phosphoenzyme | Per cent |
|---------------|---------------|----------|
| mol/10⁵ g protein | nmol/mg |          |
| 0             | 2.12 ± 0.03 | 100      |
| 2             | 2.03 ± 0.02 | 96       |
| 4             | 1.75 ± 0.02 | 90       |
| 6             | 1.39 ± 0.10 | 66       |
| 8             | 1.16 ± 0.06 | 55       |
| 10            | 1.08 ± 0.03 | 51       |

**Labeling in the presence of 5 mM ATP**

|         | nmol/mg |          |
|---------|---------|----------|
| 0       | 2.18 ± 0.02 | 100      |
| 4       | 2.11 ± 0.06 | 97       |
| 8       | 2.04 ± 0.16 | 110      |

*At a concentration of 10 mol of fluorescamine/10⁵ g of SR protein, 7 mol of PE and 2 mol of lysine/10⁵ g of protein have been labeled.*

**Fig. 3.** Effect of labeling DOPC-ATPase with fluorescamine on phosphoenzyme formation and Pi liberation rates. Phosphoenzyme formation and Pi liberation were measured as described in the text. •, phosphoenzyme formation; O, Pi liberation rates; △, fluorescence.

**Fig. 4.** Steady state phosphoenzyme levels (solid symbols) and Pi liberation rates (open symbols) for DOPC-ATPase labeled with increasing amounts of fluorescamine. The lines were drawn assuming that there are seven lysines with equal reactivity to fluorescamine and that labeling of one of them causes 100% inhibition of phosphoenzyme formation (solid line) and of a different one causes either 100% (broken line, small traces) or 50% (broken line, long traces) inhibition of Pi liberation rates. The data represent values from four different preparations.
phosphoenzyme levels (Fig. 2 and Table I). ATP added prior to labeling DOPC-ATPase with fluorescamine also protects steady state phosphoenzyme levels and Pi liberation rates, which reflects the Ca\(^{2+}\)-ATPase activity of the DOPC-ATPase preparation (Table II). A concentration of ATP as low as 0.05 mM still affords partial protection.

ADP and AMP also protect the enzyme against inhibition of both activities although less efficiently than ATP does (Table II). However, addition of AMP-PNP before labeling results in protection only of phosphoenzyme formation since the inhibition of Pi liberation rates is the same as that observed after labeling without AMP-PNP (Table II). This finding supports the proposed model for there being two classes of lysine residues in the enzyme, so that modification of one class of lysine would result in inhibition of phosphoenzyme formation and of the other, in inhibition of Pi liberation. It is important to note at this point that the inhibition of Pi liberation does not result in a significant increase in the levels of the steady state phosphoenzyme intermediate. The reason for this is that at 0.01 mM ATP and at 0 °C, the apparent rate of liberation does not result in a significant increase in the levels of the steady state phosphoenzyme intermediate. The reason for this is that at 0.01 mM ATP and at 0 °C, the apparent rate constant for phosphoenzyme formation (17 s\(^{-1}\); Table III) is much higher than the rate of phosphoenzyme decomposition (0.09 s\(^{-1}\), calculated as the ratio between the rate of Pi liberation and the maximum value in each case.

**Table II**

| Addition prior to labeling | Phosphoenzyme | Pi liberation rate |
|---------------------------|---------------|--------------------|
|                           | Phosphoenzyme | %                  |
|                           | nmol mg\(^{-1}\) | nmol mg\(^{-1}\) min\(^{-1}\) |
| Control                   | 2.11 ± 0.06   | 100                |
| Fluorescamine-labeled\(^a\) | 0.95 ± 0.01   | 45.0               |
|                           | 0.91 ± 0.01   | 43.1               |
|                           | 1.30 ± 0.01   | 61.6               |
|                           | 1.88 ± 0.15   | 89.1               |
|                           | 2.13 ± 0.02   | 100.9              |
|                           | 1.51 ± 0.06   | 71.6               |
|                           | 1.56 ± 0.04   | 73.5               |
|                           | 1.96 ± 0.11   | 92.9               |

\(^a\) To label DOPC-ATPase, a concentration of 6 mol of fluorescamine/10\(^5\) g of protein was used. This resulted in 4 mol of lysine labeled/mol of enzyme.

**Table III**

| Substrate concentration | Rate constant | Final phosphoenzyme |
|-------------------------|---------------|---------------------|
|                         | SR | Labeled SR \(^b\) | Per cent | SR | Labeled SR \(^b\) | Per cent |
| 1 μM ATP                | 4.47 | 3.47 | 72.8 | 0.94 | 0.52 | 55.7 |
| 2.5 μM ATP              | 5.78 | 5.13 | 88.8 | 1.81 | 0.83 | 45.7 |
| 10 μM ATP               | 17.3 | 12.6 | 77.6 | 2.26 | 1.00 | 44.3 |

\(^b\) A concentration of fluorescamine of 6 mol/10\(^5\) g of SR protein was used.

It is important to resolve whether the selective inhibition of Ca\(^{2+}\) uptake rates is due to labeling of amino groups of the protein, of the lipid, or of both. Although DOPC-ATPase would represent a suitable system to test the effect of protein labeling on Ca\(^{2+}\) uptake, under the conditions used in the present work to prepare DOPC-ATPase, no Ca\(^{2+}\) uptake can be measured, presumably due to leakage of the replaced enzyme vesicles. Looking for conditions to label SR lipids only, SR was labeled in the presence of bovine serum albumin (BSA). The rationale behind this experiment is that if an exogenous protein such as BSA, which contains a large number of lysine residues, was added to SR prior to labeling, fluorescamine would react preferentially with the lysines of the exogenous protein and would not label the Ca\(^{2+}\)-ATPase polypeptide. Furthermore, since fluorescamine reacts preferentially with PE, it was likely that even if a large number of exogenous lysines were present some labeling of PE would still take place. That this is indeed the case is illustrated in the experiment depicted in Fig. 5.

When SR was labeled in the presence of BSA, and then separated from BSA by two cycles of sedimentation, it was found that all the label was covalently bound to PE and none to the Ca\(^{2+}\)-ATPase polypeptide. No inhibition of phosphoenzyme formation rates (not shown), steady state phosphoenzyme levels (Table IV), or Ca\(^{2+}\)-ATPase activity (Fig. 5) was observed in these conditions, conclusively showing that these are due to protein labeling. In contrast, the inhibition of Ca\(^{2+}\) uptake rates persisted (Table IV). The labeled vesicles accumulate as much Ca\(^{2+}\) as the unlabeled control (Table IV). This finding makes unlikely the possibility that the inhibition of Ca\(^{2+}\) uptake rates is due to the presence of vesicles that have become fully permeable to Ca\(^{2+}\) as a result of labeling. However, experiments that conclusively rule out the possibility that labeling produces the inhibition of Ca\(^{2+}\) uptake rates
The SR vesicles used in this experiment contained PE. This gives a total of phosphoenzyme and Ca\(^{2+}\) uptake rates, see text. Ca\(^{2+}\) uptake was measured in the presence of BSA as described in Fig. 5. Lipids were extracted and analyzed as described in detail in the text.

### Table IV

Inhibition of Ca\(^{2+}\) uptake rates in SR by labeling phosphatidylethanolamine with fluorescamine in the presence of BSA

| SR protein | Ca\(^{2+}\) uptake | Ca\(^{2+}\)-ATPase | Ca\(^{2+}\) influx |
|-----------|------------------|--------------------|-----------------|
| 25 mg/ml | 1827 ± 146 (6) | 1557 ± 132 (6) | 1246 ± 79 (9) |
| 30 mg/ml | 1627 ± 146 (6) | 1557 ± 132 (6) | 1246 ± 79 (9) |
| 35 mg/ml | 1427 ± 146 (6) | 1557 ± 132 (6) | 1246 ± 79 (9) |

### Table V

Effect of labeling phosphatidylethanolamine with fluorescamine on Ca\(^{2+}\)-ATPase activity, Ca\(^{2+}\) uptake, ATP-dependent Ca\(^{2+}\) influx, and Ca\(^{2+}\) efflux, all measured in the presence of oxalate

| Experiment I | Experiment II |
|--------------|--------------|
| Ca\(^{2+}\) uptake | Ca\(^{2+}\)-ATPase | ATP-dependent Ca\(^{2+}\) influx | Ca\(^{2+}\) efflux |
| nmol mg\(^{-1}\) min\(^{-1}\) | nmol mg\(^{-1}\) min\(^{-1}\) | nmol mg\(^{-1}\) min\(^{-1}\) | nmol mg\(^{-1}\) min\(^{-1}\) |
| 29.3 ± 10.5 (6) | 42.4 ± 4.6 (6) | 47.4 ± 17.6 (6) | 47.4 ± 17.6 (6) |

### Fig. 5

Labeling of SR with fluorescamine in the presence of BSA. To a solution containing SR vesicles (2 mg/ml) and BSA (5 mg/ml) in 0.1 M KCl, 20 mM Hepes, pH 7.2, increasing amounts of fluorescamine as indicated were added. The labeled SR vesicles were separated from the labeled BSA by sedimentation at 150,000 × g for 30 min, followed by resuspension of the sedimented vesicles in 0.3 M sucrose, 20 mM Tris-maleate, pH 7.0. Over 95% of the BSA fluorescence was present in the supernatant. After two cycles of sedimentation to remove all traces of BSA, it was found that the fluorescence present in SR originated from the SR lipids; less than 2% of the total fluorescence of SR was present in the protein extracts. To compare the fluorescence incorporation into SR and BSA, all fluorescence measurements were corrected to a protein concentration of 1 mg/ml for SR and 2.5 mg/ml for BSA (half the concentrations present during labeling). 0, fluorescence intensity of the initial solution containing SR and BSA; △, fluorescence intensity of BSA; ○, fluorescence intensity of the protein residue after extracting the lipids. The upper part shows the effect of labeling in the presence of BSA on Ca\(^{2+}\)-ATPase activity (○) and on the initial rate of Ca\(^{2+}\) uptake in the presence of oxalate (●).

### Fig. 6

Correlation between the extent of PE labeling and the inhibition of the rate of Ca\(^{2+}\) uptake in the presence of oxalate. The data represent values obtained from several different preparations. SR was labeled with fluorescamine in the presence of BSA as described in Fig. 5. Lipids were extracted and analyzed as described in detail in the text.
uptake reaction in the presence of oxalate. Since the observed low values for the efflux could conceivably be due to slow dissociation of the calcium oxalate precipitate inside the vesicles, and if so they would not reflect the true membrane permeability to Ca$^{2+}$, a control experiment in which EGTA and the ionophore A23187 were added at early times during the uptake reaction was carried out. Simultaneous addition of A23187 and EGTA 1 min after starting the uptake reaction with ATP stimulates the efflux rate about 50-fold over that seen with EGTA alone, from 40 to 2000 nmol/mg/min, showing that at the early phase of the uptake reaction the dissociation of calcium oxalate is not the rate-limiting step in the efflux of Ca$^{2+}$. This finding demonstrates conclusively that in the early phase of the uptake reaction the efflux measured after addition of EGTA is in fact controlled only by the membrane permeability to Ca$^{2+}$. It has been shown recently (17) that during the early phase of Ca$^{2+}$ uptake in the presence of oxalate, the crystallization of calcium oxalate does not reach equilibrium, so that addition of A23187 produces a marked increase in Ca$^{2+}$ efflux by competing with oxalate for intra-vesicular Ca$^{2+}$. In the present work, addition of A23187 in the absence of EGTA 1 min after the initiation of the reaction either stops Ca$^{2+}$ uptake or produces a small increase in Ca$^{2+}$ efflux (not shown), indicating that under these conditions the ATP-dependent Ca$^{2+}$ influx is approximately equal to the ionophore-induced Ca$^{2+}$ efflux.

As discussed above, after 10 min of starting the uptake reaction by addition of ATP, the vesicles containing fluorescamine-labeled PE contain the same amounts of calcium oxalate as the unlabeled vesicles (Table IV), indicating that although they take longer they eventually accumulate as much calcium oxalate as the unlabeled vesicles. To completely rule out the possibility that the labeled vesicles represent a leaky subfraction, both labeled and unlabeled vesicles were fractionated in sucrose gradients after loading with calcium oxalate as described in detail in Table VI. A relatively high concentration of fluorescamine that produced 55% inhibition of Ca$^{2+}$ uptake rates but no increase in Ca$^{2+}$ efflux (Table VI) was chosen to label the vesicles to be loaded with calcium oxalate and fractionated in sucrose gradients. The same fraction of the initial vesicular population was found loaded with calcium oxalate for both control and labeled vesicles (Table VI). If the vesicles had become progressively leaky as a function of the extent of labeling, a lower fraction of the initial vesicular population for the labeled vesicles should have been found loaded with calcium oxalate as compared to the unlabeled control vesicles. Furthermore, both the unfraccionated labeled vesicles as well as the calcium oxalate-loaded subfraction had the same content of fluorescamine-labeled PE (Table VI), showing conclusively that labeling does not produce a leaky membrane subfraction.

These results clearly indicate that the inhibition of Ca$^{2+}$ uptake rates produced by labeling PE is due to a defect in the transport process itself and it is not caused by membrane leakage or by inhibition of the Ca$^{2+}$-ATPase reaction.

**DISCUSSION**

Fluorescamine reacts with primary amino groups and yields highly fluorescent products (18), which makes it suitable as an amino-labeling reagent. However, labeling of SR with fluorescamine at pH 8.3 and at a reagent-to-protein ratio of 10 mol of fluorescamine/10$^5$ g of SR results in dissociation of calsequestrin from the SR membranes (4). This is presumably the cause of the significant extent of calsequestrin labeling observed under these conditions (4, 13). For these reasons, in previous experiments aimed at analyzing the disposition of proteins and lipids under conditions in which the vesicles retain their original structure even after extensive labeling, CFC, which is a water-soluble derivative of fluorescamine, was used (4, 7). Labeling of SR with CFC produces considerable inhibition of Ca$^{2+}$ transport and Ca$^{2+}$-ATPase activity (4, 7), indicating that there are some amino groups in SR crucial for these activities. Furthermore, under certain labeling conditions, such as limited labeling of SR with CFC in the presence of ATP, it was found that the labeled vesicles display normal Ca$^{2+}$-ATPase activity but decreased Ca$^{2+}$ transport rates (7), indicating that uncoupling of Ca$^{2+}$ transport has taken place. Since even limited labeling with CFC in the presence of ATP results in covalent labeling of both the Ca$^{2+}$-ATPase polypeptide chain and PE (7), it was not possible to ascertain unambiguously whether it is the modification of amino groups in the protein, or in the lipid, or maybe of both, which results in inhibition of Ca$^{2+}$ transport. In the present work, fluorescamine was chosen instead of CFC since fluorescamine, as opposed to CFC, reacts quantitatively and quickly with free amino groups (18), allowing a better control of the number of amino groups modified than CFC does. Also, since with CFC it was found that at the initial stages of the labeling reaction a larger fraction of the label was covalently bound to PE and much less to the Ca$^{2+}$-ATPase, it was hoped that by limiting the amount of fluorescamine to 1 or 2 mol/10$^5$ g of SR it would be possible to label only PE to analyze whether this had any effect on Ca$^{2+}$ transport.

To avoid the problem of calsequestrin dissociation, the
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concentration of fluorescamine was kept below 10 mol/10^5 g of SR, and labeling was carried out at pH 7.2 instead of 8.3. No dissociation of calsequestrin was observed under these conditions, and the labeled vesicles retained their permeability barrier to Ca^{2+}. However, although at a concentration of 1 or 2 mol of fluorescamine/10^5 g of SR most of the label reacts with PE, still significant label incorporation in the Ca^{2+}-ATPase polypeptide chain was observed. This was avoided by labeling SR in the presence of BSA, as will be discussed below.

Labels to the Ca^{2+}-ATPase Polypeptide Chain and Inhibition of the Ca^{2+}-ATPase Activity—The results obtained with DOPC-ATPase conclusively show that modification of lysine residues results in inhibition of Ca^{2+}-ATPase activity. It is interesting to note in this regard that covalent labeling of SR amino groups with two or more reagents also results in varying degrees of inhibition of Ca^{2+}-ATPase activity (19, 20). Using pyridoxal-5-phosphate followed by reduction with NaBH₄, progressive loss of Ca^{2+}-ATPase activity was observed (19). Furthermore, Ca-ATP and Mg-ATP offer complete or partial protection, respectively, against this inactivation (19). It was proposed that modification of a single lysine residue, located in the ATPase fragment of M₄, 30,000, is responsible for the loss of ATPase activity (19). The results obtained in this work in addition to confirming that modification of lysine residues produces inhibition of Ca^{2+}-ATPase, clearly indicate that the inhibition of the Ca^{2+}-ATPase is due to inhibition of both phosphoenzyme formation and phosphoenzyme decomposition caused presumably by modification of two different lysine residues. Addition of ATP prior to labeling, and with less effectiveness ADP and AMP, preserves steady state phosphoenzyme levels and decomposition. It is likely that these nucleotides prevent the reaction of fluorescamine with lysine residues that are crucial for both phosphoenzyme formation and decomposition.

The results obtained with AMP-PNP indicate that there are lysine residues whose modification interferes only with phosphoenzyme decomposition and not with phosphoenzyme formation, since addition of AMP-PNP before labeling results in protection only of the lysine groups involved in phosphoenzyme formation.

The protective effect of ATP was interpreted by Murphy (19) as an indication of an active site location of one or more lysine residues, since lysines might bind to the anionic substrate Mg-ATP. Allen and Green (21) have reported most of the sequence of a 31-residue peptide containing the active site aspartyl residue that is phosphorylated by ATP. Of possible significance is the location of a lysyl residue adjacent to it. Although active site residues need not be near one another in the primary structure, it is likely that modification of this lysine residue could be blocked by ATP and by AMP-PNP, which would bind to the substrate site. Furthermore, this lysine residue could be involved in the phosphorylation reaction and a different lysine (one or more) in the phosphoenzyme decomposition steps. However, it is possible to visualize other protective mechanisms, such as allosteric effects, which might involve lysine residues other than those present in the active site.

As analyzed under "Results," the inhibition of steady state phosphoenzyme formation and of the rates of P_, liberation can be explained assuming that there are seven lysine residues in each enzyme molecule that have equal reactivity to fluorescamine. Labeling of one of them would result in total inhibition of phosphoenzyme formation (and hence of P_, liberation rates) and of another, in inhibition of P_, liberation rates without affecting steady state phosphoenzyme levels. However, it is important to note here that this model involving seven lysines with equal reactivity to fluorescamine is valid for the purified Ca^{2+}-ATPase only, since it is conceivable that after purification more lysine residues than in the original SR vesicles can react with the label. In fact, this was shown to be the case for SR and purified enzyme labeled with CFC (4). However, the data shown in Table I and in Fig. 2 indicate that labeling of 2 mol of lysine/10^5 g of protein in SR produces 50% inhibition of phosphoenzyme formation and 70% inhibition of Ca^{2+}-ATPase activity. Since the SR vesicles contain about 70% Ca^{2+}-ATPase and since this is the only protein that reacts with fluorescamine under the labeling conditions used in this work, it represents about 3 mol of lysine labeled/mol of enzyme. If SR had seven lysines of equal reactivity to fluorescamine, labeling of three should produce 43% inhibition of phosphoenzyme and 70% inhibition of Ca^{2+}-ATPase, which is within the range of the observed experimental values. So it is conceivable that both the purified enzyme system as well as the original vesicles have the same number of lysine residues of equal reactivity to fluorescamine and that blocking of one prevents phosphoenzyme formation and of the other, phosphoenzyme decomposition.

Uncoupling of Ca^{2+} Transport—As shown under "Results," labeling of SR with fluorescamine in the presence of BSA produces sealed vesicles in which only PE has reacted with the label. The fact that labeling of PE produces inhibition of the Ca^{2+} transport rates but does not affect the rate or the extent of phosphoenzyme formation, or the rate of phosphoenzyme decomposition, indicates that the transport reaction has been uncoupled from the hydrolysis of ATP. Although the intermediate reaction steps of the Ca^{2+}-ATPase reaction have been clarified in considerable detail (1, 2), very little is known concerning the actual molecular mechanisms involved in the translocation of Ca^{2+} from the outside to the inside of the SR vesicles. For this reason, it is difficult to visualize how the enzyme proceeds with the intermediate steps of the reaction at normal rates but transports Ca^{2+} to the vesicular interior at a fraction of the rate of the unlabeled vesicles. Since leakage has been ruled out, the present results suggest that there are membrane configurations in which the rate of ATP hydrolysis is not controlled by the rate at which Ca^{2+} is transported into the vesicular lumen. The uncoupling of Ca^{2+} transport from the Ca^{2+}-ATPase produced by limited tryptic digestion of SR (22) or by proton inactivation (23) has been explained by postulating selective defects in the enzyme polypeptide. The present findings would be the first example of uncoupling produced not by a change in the enzyme polypeptide itself but in the lipids around the enzyme.

As to the nature of the defect induced by PE modification, there is some indication that it might be due to a highly localized modification of the membrane in the immediate vicinity of the Ca^{2+}-ATPase. Modifications of the lipid environment that produce changes in membrane fluidity result in significant changes in Ca^{2+}-ATPase activity (11, 24-26). As expected from the lack of effect on Ca^{2+}-ATPase activity, no changes in membrane fluidity are observed after PE modification, since the same EPR spectra were detected with fatty acid spin probes added to the SR membranes with or without modified PE. In support of the view that the labeled phospholipid is in the close vicinity of the enzyme is the finding that modification of as little as 1 mol of PE/mol of enzyme results in significant quenching of intrinsic tryptophan fluorescence due to fluorescence energy transfer between the enzyme tryptophan residues as donors and the fluorescamine-labeled PE as accepter. Furthermore, another fluorescein PE analog, dansyl PE, when incorporated at low concentrations

\[ \text{C. Hidalgo, unpublished observations.} \]

\[ \text{C. Hidalgo, manuscript in preparation.} \]
into sealed SR vesicles also establishes fluorescence energy transfer with the Ca\textsuperscript{2+}-ATPase tryptophan residues (27), the interaction between these two chromophores persists even after the SR vesicles have been completely dissociated by SDS or deoxycholate. All these data suggest a strong and close range interaction between the enzyme and labeled PE analogs. It is conceivable that the modification of the polar head group of PE, a phospholipid which is externally located (4-6), causes a change in the immediate environment around the enzyme (such as a change in local charge in the external surface of the membrane) that uncouples the transport of Ca\textsuperscript{2+} from the Ca\textsuperscript{2+}-ATPase reaction.

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