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INHIBITION OF CALCIUM TRANSPORT IN SARCOPLASMIC RETICULUM AFTER MODIFICATION OF HIGHLY REACTIVE AMINO GROUPS[1]

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Summary: Partial labeling of the amino groups of sarcoplasmic reticulum with a complex of fluorescamine with cycloheptaamylose in the presence of ATP results in marked inhibition of Ca^{2+} transport without affecting the enzyme phosphorylation or the Ca^{2+}-ATPase activity. Fast labeling, which parallels the time course of inhibition of Ca^{2+} transport, takes place into phosphatidylethanolamine; a slower labeling of the Ca^{2+}-ATPase polypeptide was observed. Vesicles in which mainly phosphatidylethanolamine has reacted with the label retain their impermeability barrier to Ca^{2+}, as judged by Ca^{2+} efflux measurements and by the stimulation of Ca^{2+}-ATPase activity produced by the ionophore A23187. These results suggest that modification of fast-reacting amino groups interferes specifically with the calcium translocation reaction.

The disposition of various proteins (1-4) and of the amino phospholipids (4,5) of sarcoplasmic reticulum (SR)[2] has been studied by several investigators by means of chemical labeling with a variety of probes. There seems to be general agreement that a considerable fraction of the Ca^{2+}-ATPase polypeptide is exposed to the outside of the SR vesicles, and that calsequestrin is buried in the vesicular interior. Our studies using both a complex of fluorescamine with cycloheptaamylose (CFC) and diazotized [^{35}S] sulfanilic acid (4) are consistent with the above view and, in addition, indicate that 70-80% of the phosphatidylethanolamine (PE) present in SR is located at the external side of the membrane bilayer, while phosphatidylserine is enriched at the inner side. The same asymmetric disposition of the amino phospholipids of SR has been observed with two other amino-labeling reagents (5).

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[1] Part of this work has been reported previously (Hidalgo, C., and Tong, S. (1978) Biophys. J. 21, 46a).

[2] Abbreviations used: SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethylether)-N,N'-tetraacetic acid; CFC, complex of fluorescamine with cycloheptaamylose; SDS, sodium dodecyl sulfate.

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However, after labeling SR with CFC both the Ca^{2+}-ATPase activity and the Ca^{2+} transport are abolished, although the labeled vesicles retain their structural integrity as judged by their impermeability to inulin (4). Since the analysis of the extent of labeling of both proteins and lipids in an inactive preparation is always open to the criticism that the labeling process has drastically altered the configuration of the membrane, it is desirable to obtain labeled membranes retaining their original activities.

In the present study it was found that the inhibition of Ca^{2+}-ATPase activity observed after labeling SR with CFC is due to inhibition of the phosphorylation reaction, and that this can be prevented by adding ATP prior to labeling. In contrast, Ca^{2+} transport was progressively inhibited by labeling; the inhibition was not produced by an increase in the membrane permeability to Ca^{2+}. The possibility that lipid or protein modification produces a specific defect in Ca^{2+} translocation is discussed.

MATERIALS AND METHODS

SR was prepared as described previously (6). Fresh SR vesicles were labeled with CFC for variable lengths of time (4) with or without ATP. Labeling was carried out at 37° in a solution containing 1 mg/ml of SR, 0.5 mg/ml CFC, 5 mM $MgCl_2$, 0.1M KCl, 20 mM Hepes buffer, pH 7.2, plus or minus 5 mM ATP. Since CFC has limited water solubility, lower CFC and protein concentrations than before (4) were used to ensure that all the label was in solution. The reaction was started by adding the SR vesicles, equilibrated at 37° for two minutes, to the labeling solution. If ATP was used, the vesicles were first mixed with 5 mM ATP, 5 mM $MgCl_2$, final concentration, just before addition to the labeling medium. The reaction was stopped by 10-fold dilution with ice-cold 0.3M sucrose, 20 mM Tris maleate buffer, pH 7.0 (SR buffer). After extensive washing by repeating sedimentation and homogenization, the labeled SR was resuspended in SR buffer at a final concentration of 5 mg of protein per ml. To measure fluorescence, the vesicles were diluted to 0.05 mg/ml in the same buffer as above. Ca^{2+}-ATPase was calculated as the difference between the ATPase activities measured in a solution containing 0.1M KCl, 5 mM $MgCl_2$, 2 mM ATP, 50 mM Tris maleate, pH 7.0, 0.1 mg of protein per ml, and either 0.1 mM $CaCl_2$ or 1 mM EGTA, at 32°. The amount of P_i liberated was determined colorimetrically (7). To measure phosphoenzyme formation, the procedure described previously was used (8) with [γ-^{32}P] ATP obtained from Amersham Corp.

Ca^{2+} uptake in the presence of oxalate was measured at 22° in a solution containing 0.1M KCl, 5 mM $MgCl_2$, 4 mM ATP, 50 mM Tris-maleate, pH 7.0, 5 mM K^+-oxalate, 0.1 mM $^{45}CaCl_2$, and 0.05 mg of SR protein per ml. The reaction was stopped at different times by filtering 1.0 ml of the reaction solution through Millipore filters (HA 0.45μ pore size). A 0.2 ml fraction of the filtrate was placed on filter paper strips, dried and counted in a liquid scintillation counter (8).

Ca^{2+} transport in the absence of precipitating anions was measured in a solution containing 0.1M KCl, 4 mM ATP, 5 mM $MgCl_2$, 100 mM Hepes buffer, pH 6.8, 0.1 mM $^{45}CaCl_2$, and 0.1 mg of protein per ml, at 22°. The amount of Ca^{2+} taken up by the vesicles was measured by the filtration procedure described above.

A fraction of each labeled SR preparation was used to determine the amount of label incorporation into PE. Lipid extracts and phospholipid analysis were done as described (8).

Protein was determined by the method of Lowry et al. (9) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

As reported in our previous work (4), labeling of the free amino groups of SR with CFC results in marked inhibition of Ca^{2+}-ATPase activity. However, addition of 5 mM ATP to the vesicles prior to the addition of CFC results in significant protection of the enzymatic activity (Fig. 1). No protection against inactivation was observed if labeling was carried out in the presence of 10 mM $CaCl_2$ without ATP. The total amount of label incorporated into the vesicles, as measured by the increase in fluorescence intensity, was decreased by the presence of ATP during labeling; no label incorporation into ATP itself takes place in the conditions used.

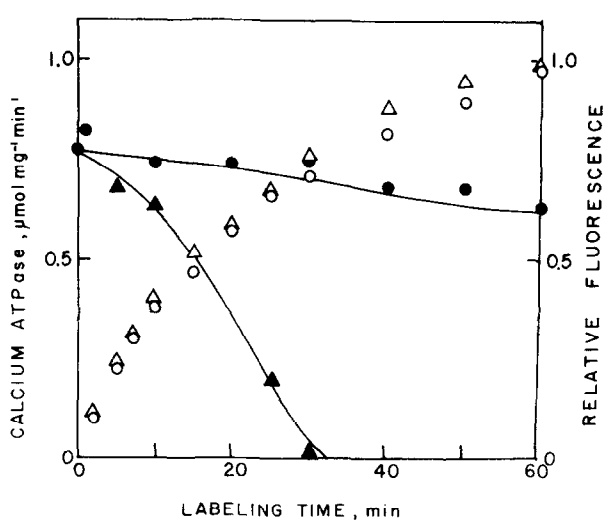

Fig. 1: Key: Ca^{2+}-ATPase activities (●, ▲) and relative fluorescence (○, △) of SR vesicles labeled with CFC in the presence (●, ○) or in the absence (▲, △) of 5 mM ATP. Ca^{2+}-ATPase activities were measured as described in the text and fluorescence as in reference (4). Relative fluorescence was calculated as the ratio of each value to the maximum fluorescence obtained under the same labeling conditions. The maximum fluorescence of SR vesicles labeled in the presence of 5 mM ATP is 70-80% of the maximum fluorescence of vesicles labeled in the absence of ATP.

TABLE I.

Phosphoenzyme Formation and Ca^{2+}-ATPase Activity of SR and CFC-labeled SR

	Phosphoenzyme (nmol mg^{-1})	Ca^{2+}-ATPase Activity (μmol P_i mg^{-1} min^{-1})	
		Control	+ A23187
SR	2.05 ± 0.12 (4)	0.98 ± 0.09 (2)	1.66 ± 0.02 (2)
CFC-SR, no ATP	1.31 ± 0.06 (2)	0.59 ± 0.04 (2)	
CFC-SR, plus ATP	2.01 ± 0.08 (4)	0.89 ± 0.08 (2)	1.64 ± 0.08 (2)

SR was labeled with CFC for 5 min as described in the text, with or without ATP present as indicated above. Phosphoenzyme formation and Ca^{2+}-ATPase activities were measured as described in 'Materials and Methods'. The number of determinations is in parentheses. The concentration of A23187 was 2 μM.

In the absence of ATP even limited labeling with CFC results in inhibition of phosphoenzyme formation (Table I), indicating that the inhibition of the Ca^{2+}-ATPase reaction is due to interference with the phosphorylation step. If labeling is carried out in the presence of ATP, no inhibition of phosphoenzyme formation is observed (Table I).

In contrast to its protective effect on Ca^{2+}-ATPase activity, ATP offered no protection against the progressive inhibition of Ca^{2+} transport produced by labeling (Fig. 2). It has been described that agents that increase the permeability of SR vesicles to Ca^{2+} produce inhibition of Ca^{2+} transport without concomitant inhibition or even with two to three-fold stimulation of the Ca^{2+}-ATPase activity. It was already shown that CFC-labeled SR vesicles retain their impermeability to inulin (4), thus ruling out major vesicular disruption by labeling. However, it is conceivable that labeling might have increased the Ca^{2+} permeability of the SR vesicles. This would result in a decreased rate of Ca^{2+} uptake with precipitating anions and in a decreased amount of Ca^{2+} transported without them. Following the procedure described by Hara and Kasai (10) to determine membrane leakiness, the time course of Ca^{2+} efflux produced by addition

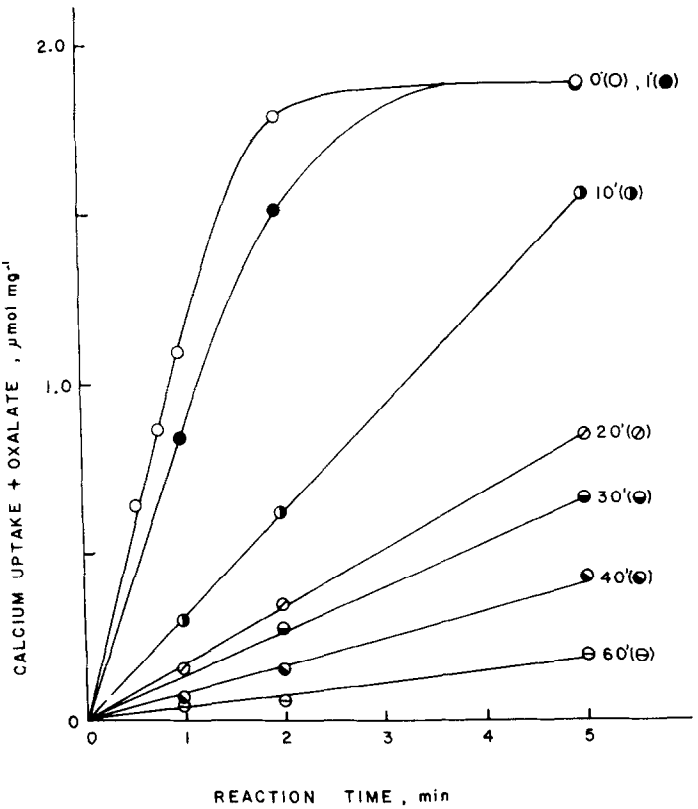

Fig. 2: Effect of CFC-labeling on Ca^{2+} transport in SR. Fresh SR vesicles in the presence of 5 mM ATP were labeled with CFC for variable lengths of time, as indicated in the right hand side of each curve. After extensive washing, the vesicles were resuspended in 0.3M sucrose, 20 mM Tris-maleate, pH 7.0. Ca^{2+} uptake in the presence of oxalate was measured at 22° as described in the text. The uptake reaction was stopped at the times indicated in the abscissa.

of EGTA to vesicles loaded with Ca^{2+} in the presence of ATP was investigated. In the absence of precipitating anions the vesicles labeled only partially with CFC (5 min incubation under the conditions described in Fig. 1) show 75% of the Ca^{2+} accumulating capacity of the unlabeled vesicles (Fig. 3). After addition of EGTA, the Ca^{2+} efflux rate from the partially labeled vesicles is not significantly different from the control, indicating that this limited extent of labeling has not increased appreciably the permeability of the membrane to Ca^{2+}. The fact that the ionophore A23187 produces the same stimulation of the Ca^{2+}-ATPase activities of control and of vesicles partially labeled with CFC in the presence of ATP (Table I), also indicates that limited labeling does not increase membrane leakiness.

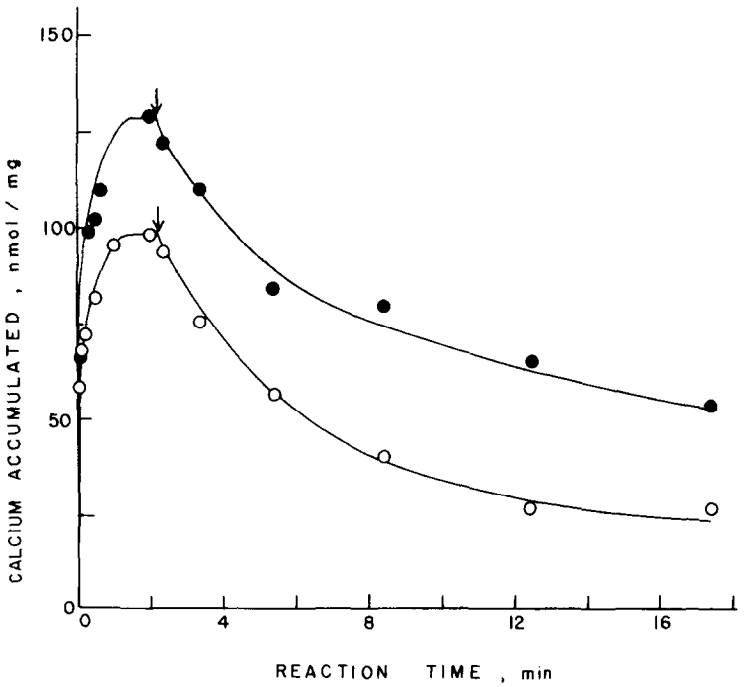

Fig. 3: Ca^{2+} transport in the absence of precipitating anions by control and by vesicles labeled with CFC for 5 minutes in the presence of 5 mM ATP. Ca^{2+} transport was measured as described in 'Materials and Methods'. After 2 min 10 s from the start of the reaction, 10 mM EGTA was added (arrow). Key: ●, SR; ○, CFC-SR.

It was not possible to measure by the above procedure the permeability to Ca^{2+} of vesicles extensively labeled with CFC, since extensive labeling resulted in total inhibition of Ca^{2+} transport. However, in some experiments extensive labeling in the presence of ATP produced significant increase in Ca^{2+}-ATPase activity relative to the unlabeled SR, suggesting increased leakiness to Ca^{2+}.

As determined previously (4), labeling with CFC results in a fast incorporation of label into the free amino group of 70-80% of the phosphatidylethanolamine present in SR, indicating that most of the PE is present in the outer half of the lipid bilayer. Since SR contains about 10 moles of PE per 10^5 g of protein (calculated from the phospholipid content and composition given in Ref. 8), 7 to 8 moles of PE per 10^5 g of protein react with CFC. Analysis of the time course of label incorporation into this phospholipid shows that the fast labeling of PE (Fig. 4b) parallels the time course of inhibition of Ca^{2+} uptake (Fig. 4a). The

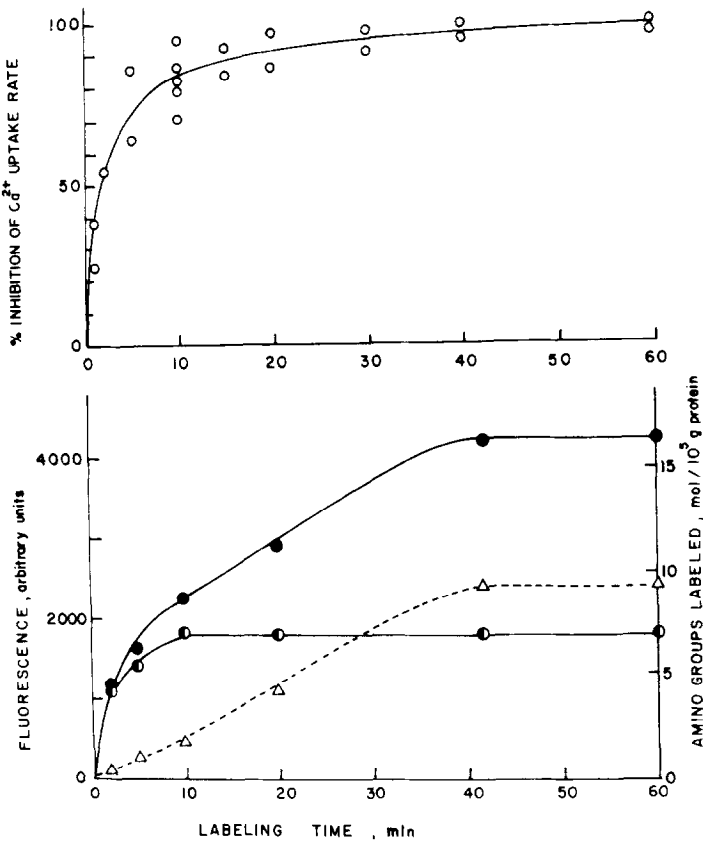

Fig. 4: Parallelism between CFC-labeling of phosphatidylethanolamine and Ca^{2+} uptake inhibition. a) The % inhibition of the initial rate of Ca^{2+} uptake of SR vesicles, labeled with CFC as described in the text, was calculated from Ca^{2+} uptake curves such as those shown in Fig. 2. Data from five different experiments are included. b) Fractions from one SR preparation, labeled for different lengths of time, were used to determine the amount of fluorescence incorporation into the SR vesicles after solubilization in 1% SDS. Lipids were extracted as described (8), and either a fraction of the lipid extract was evaporated to dryness and resuspended in 1% SDS to determine fluorescence, or the extent of PE labeling was measured by chemical analysis of the phospholipid composition (8). The fluorescence of the lipid extract corresponded precisely with the amount of PE labeled. The degree of protein labeling was calculated as the difference between the SR fluorescence and the lipid fluorescence, both measured in 1% SDS; controls in which the fluorescence incorporation into the protein residue obtained after lipid extraction was measured directly indicate that in SDS the fluorescence intensity of SR corresponds to the sum of protein plus lipid fluorescence. To calculate the amount of amino residues per 10^5 g of protein modified by CFC, the fluorescence of the lipid extract in 1% SDS was correlated with the mole of PE modified per 10^5 g of protein. Key: a) O , % inhibition of Ca^{2+} uptake rates. b) ● , fluorescence of SR vesicles; ◐ , fluorescence of lipid extract; Δ , calculated fluorescence of the protein fraction.

protein labeling with CFC takes place at a slower rate than PE labeling (Fig. 4b). In fact, after labeling SR with CFC for 2 min, which produces 40-50% inhibition of Ca^{2+}-uptake rates, 4 of the 10 moles of PE present per 10^5 g of SR

protein have reacted with CFC (40% PE labeling). On the same 10^5 daltons basis, only 0.4 amino residues in the protein have been labeled (Fig. 4b). Maximum inhibition of Ca^{2+} uptake as well as maximum PE labeling occurs before the labeling reaction levels off.

The above results indicate that, in the presence of ATP, modification of fast-reacting amino groups in SR results in Ca^{2+} transport inhibition, while the phosphorylation of the enzyme and the overall Ca^{2+}-ATPase reaction are not inhibited by labeling, nor is the membrane permeability to Ca^{2+} increased. This rules out both major defects in enzyme function and membrane leakiness as causes of the inhibition of Ca^{2+} transport and indicates that modification of fast-reacting amino residues produces a specific defect in the Ca^{2+} translocation reaction.

The correlation between the extent of PE labeling and the decrease in Ca^{2+} transport rates raises the possibility that PE modification causes the inhibition of Ca^{2+} translocation. Most of the PE of SR is present in the outer half of the lipid bilayer (4,5) where the enzyme phosphorylation takes place and where presumably the Ca^{2+} translocation reaction is initiated. It is likely that Ca^{2+} translocation involves a conformational change in the enzyme. By modifying the polar head group of PE, the outer membrane surface might change so that although the phosphorylation step and the subsequent hydrolysis of the phosphorylated intermediate proceed normally, the conformational change associated with translocation is inhibited and Ca^{2+} is not translocated to the inside of the vesicles as efficiently as in the unmodified SR. However, the results can be explained just as well by assuming that there is one highly reactive amino residue in the Ca^{2+}-ATPase polypeptide that is specifically involved in the translocation reaction. Partial modification of this residue would result in partial inhibition of Ca^{2+} uptake. Experiments are in progress to distinguish between these two alternatives.

Similar selective inhibition of Ca^{2+} transport without changes in the overall Ca^{2+}-ATPase activity and without increase in membrane leakiness has been

observed after limited tryptic digestion of SR (11), or after proton inactivation (12). These findings indicate that the translocation of Ca^{2+} to the vesicular interior has more stringent requirements than the mere existence of sealed vesicles displaying normal Ca^{2+}-ATPase activity.

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