Chemical modification of sarcoplasmic reticulum

Stimulation of Ca^{2+} release

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Pretreatment of sarcoplasmic membranes with acetic or maleic anhydrides, which interact principally with amino groups, resulted in an inhibition of Ca^{2+} accumulation and ATPase activity. The presence of ATP, ADP or adenosine 5'-[β,γ -imido]triphosphate in the modification medium selectively protected against the inactivation of ATPase activity by the anhydride but did not protect against the inhibition of Ca^{2+} accumulation. Acetic anhydride modification in the presence of ATP appeared to increase specifically the permeability of the sarcoplasmic reticulum membrane to Ca^{2+} but not to sucrose, Tris, Na^+ or P_i . The chemical modification stimulated a rapid release of Ca^{2+} from sarcoplasmic reticulum vesicles passively or actively loaded with calcium, from liposomes reconstituted with the partially purified ATPase fraction but not from those reconstituted with the purified ATPase. The inactivation of Ca^{2+} accumulation by acetic anhydride (in the presence of ATP) was rapid and strongly pH-dependent with an estimated pK value above 8.3 for the reactive group(s). The negatively charged reagents pyridoxal 5-phosphate and trinitrobenzenesulphonate, which also interact with amino groups, did not stimulate Ca^{2+} release. Since these reagents do not penetrate the sarcoplasmic reticulum membranes, it is proposed that Ca^{2+} release is promoted by modification of internally located, positively charged amino group(s).

INTRODUCTION

In recent years several mechanisms have been proposed for the link between the depolarization of the transverse tubular system of skeletal muscle and the release of Ca^{2+} from the sarcoplasmic reticulum (Endo, 1977; Ford & Podolsky, 1970; Nakajima & Endo, 1973; Schneider & Chandler, 1973; Volpe *et al.*, 1985; Vergara *et al.*, 1985). Under physiological conditions, Ca^{2+} release from the sarcoplasmic reticulum into the sarcoplasm is 100–1000-fold faster than the reverse process of Ca^{2+} uptake (Martonosi, 1972). The differential rates suggest that Ca^{2+} is released through a gated channel rather than through an enzymic process.

Chemical modifications of isolated sarcoplasmic reticulum vesicles have been reported to 'uncouple' Ca^{2+} transport from ATPase activity (Berman, 1983). It is not clear, however, whether the apparent uncoupling is due to a specific modification of the Ca^{2+} -ATPase which leads to intramolecular uncoupling, or whether it is the result of changes in the permeability of the sarcoplasmic reticulum so that internalized Ca^{2+} is rapidly released.

We have described several conditions which cause an increase in the permeability of sarcoplasmic reticulum to Ca^{2+} (MacLennan *et al.*, 1982; Shoshan *et al.*, 1981, 1983). We found that increasing the pH of the bathing solution led to Ca^{2+} release from Ca^{2+} -loaded sarcoplasmic reticulum in skinned muscle fibres. We also found that the lipophilic anions tetraphenylboron (TPB^-) and trinitrophenol stimulated Ca^{2+} release from both fragmented sarcoplasmic reticulum and from the sarcoplasmic reticulum in skinned muscle fibre. In contrast, the cation tetraphenylarsonium was ineffective in stimulating Ca^{2+} release. More recently, Taguchi & Kasai (1984)

showed that TPB^- activates pre-existing Ca^{2+} release channels in sarcoplasmic reticulum membranes. Their studies also showed that these channels are different from those involved in Ca^{2+} -induced Ca^{2+} release.

On the basis of these observations, we proposed (Shoshan *et al.*, 1983) that at least one form of Ca^{2+} release channel was controlled by either a membrane potential or membrane surface charges. In these studies we have examined Ca^{2+} permeability of sarcoplasmic reticulum vesicles after chemical modifications which affect the net surface charge of the membrane. The finding that acetic anhydride and maleic anhydride, but not pyridoxal 5-phosphate or trinitrobenzenesulphonate, increased Ca^{2+} permeability indicates the involvement of internally located, positively charged amino group(s) in the activation of Ca^{2+} release.

MATERIALS AND METHODS

Materials

ATP, ADP, App[NH]p, AMP, EGTA, Tris, Tricine, TNBS and PLP were obtained from Sigma Chemical Co.; NaBH_4 , acetic anhydride and maleic anhydride from Merck; asolectin (crude soybean phospholipids) was from Associated Concentrations (Woodside, NY, U.S.A.). $^{45}\text{CaCl}_2$ was from The Radiochemical Centre, Amersham, and $^{32}\text{P}\text{P}_i$ from the Nuclear Research Center, Negev, Israel. [γ - ^{32}P]ATP was synthesized from $^{32}\text{P}\text{P}_i$ and ADP by photophosphorylation with lettuce chloroplasts (Avron, 1960) and was purified on Dowex 1-X8 with NaCl as the eluant. Acetic anhydride was dissolved in anhydrous diethyl ether, and maleic

Abbreviations used: App[NH]p, adenosine 5'-[β,γ -imido]triphosphate; PLP, pyridoxal 5-phosphate; TNBS, 2,4,6-trinitrobenzenesulphonate; TPB^- , tetraphenylboron.

Fig. 1. Inactivation of Ca^{2+} -ATPase and Ca^{2+} accumulation by acetic anhydride treatment of sarcoplasmic reticulum

Ca^{2+} uptake and ATPase activity were measured for 2 min and acetic anhydride treatment was performed as described under 'Material and methods'. Sarcoplasmic reticulum membranes (0.9 mg/ml) were incubated with the indicated concentrations of acetic anhydride in the presence (■) or absence (□) of 5 mM-ATP.

anhydride was dissolved in dry acetone and used immediately. The final concentration of ether or acetone in all samples was 2% (v/v).

Membrane and protein preparation

Sarcoplasmic reticulum vesicles were prepared from rabbit fast twitch skeletal muscle as described by Campbell & MacLennan (1981). The purified and partially purified ATPase fractions were obtained as described by MacLennan (1970). The protein concentration was determined by the Lowry *et al.* (1951) method.

Reconstitution of Ca^{2+} -ATPase vesicles

The freeze-thaw-sonication method (Kasahara & Hinkle, 1977) was used. Phospholipids were sonicated to clarity at 40 mg/ml in 0.4 M-sodium phosphate, pH 7.4, in a bath-type sonicator (Ladd). Portions were mixed with the purified or partially purified Ca^{2+} -ATPase at a concentration of 20 mg of asolectin/mg of protein. The mixture was quickly frozen in liquid N_2 slowly thawed at room temperature, sonicated for 30 s, and used immediately.

Modification of sarcoplasmic reticulum membranes with chemical probes for amino groups

Acetic anhydride and maleic anhydride solutions were added to sarcoplasmic reticulum vesicles (1 mg/ml in 20 mM-Tricine, pH 7.0) to a final concentration ranging between 0.02 and 1.2 mM. The mixture was incubated for 5 min at 22 °C, then the reaction was stopped by the addition of lysine solution at pH 7.0, to a final concentration of 20 mM, and transferred to ice. The pH of the modified samples did not change during the incubation with the anhydrides. Portions were diluted 10-fold with the reaction mixture for Ca^{2+} transport or ATPase activity and assayed as described below. About

20–30% of the total amino groups of sarcoplasmic reticulum membranes had reacted with 1 mM-acetic anhydride after 5 min of incubation. This was measured by the binding of TNBS to SDS-solubilized membranes. Sarcoplasmic reticulum membranes (1 mg/ml in 20 mM-Tricine, pH 8.0) were also incubated with 5–100 μM -PLP for 10 min in the dark. The Schiff's base complex was then reduced with NaBH_4 at a final concentration of 10 mg/ml. The modified membranes were separated from the unreacted reagent by centrifugation-chromatography (Penefsky, 1977) on a column of Sephadex G-50 equilibrated with 20 mM-Tricine, pH 8.0.

Assays

Active Ca^{2+} uptake by sarcoplasmic reticulum vesicles was determined by Millipore filtration (Martonosi & Feretos, 1964) with $^{45}\text{CaCl}_2$. The basic reaction mixture contained 20 mM-Tris/maleate, pH 6.8, 100 mM-NaCl, 5 mM- MgCl_2 , 5 mM-ATP, 0.5 mM- CaCl_2 (containing ^{45}Ca at about 10^6 c.p.m./ μmol), 0.5 mM-EGTA, and 50 mM- P_i . The uptake was started by the addition of sarcoplasmic reticulum, 40–80 μg /ml in reaction mixture and, after 1 or 2 min of incubation at room temperature, 0.18 ml samples were filtered through 0.3 μm nitrocellulose filters, and washed with 5 ml of 0.15 M-NaCl. Radioactivity in the filters was measured in a liquid-scintillation counter. The ATPase activity was tested at room temperature in an assay medium identical with that described for Ca^{2+} uptake except that $^{45}\text{CaCl}_2$ was omitted and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2×10^5 c.p.m./ μmol) was used. The $^{32}\text{P}\text{P}_i$ released from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was extracted as the phosphomolybdate complex into butan-2-ol/xylene (1:1, v/v) according to Avron (1960) and was measured by liquid-scintillation counting. The Ca^{2+} -independent ATPase activity was measured without Ca^{2+} and in the presence of 1 mM-EGTA, and values corresponding to 2–8% of the total ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase were obtained.

Fig. 2. Protection by ATP against the inactivation of ATPase activity but not of Ca²⁺ accumulation by acetic anhydride

Sarcoplasmic reticulum membranes were modified with acetic anhydride (0.58 mM) as described under 'Materials and methods', except that ATP at the indicated concentrations was included in the incubation medium. Ca²⁺ uptake and ATPase activity were assayed as described under 'Materials and methods'. Control activity were 0.85 and 0.46 μ mol/min per mg of protein of Ca²⁺ uptake and ATPase activity, respectively.

RESULTS

Modification of sarcoplasmic reticulum with acetic anhydride

Fig. 1 shows the effect on ATPase activity and Ca²⁺ accumulation of pretreatment of sarcoplasmic reticulum vesicles with acetic anhydride. Both activities were inhibited, with half-maximal inhibition occurring after incubation with about 0.1–0.2 mM-acetic anhydride. The presence of 5 mM-ATP in the modification medium protected against the loss of ATPase activity by acetic anhydride but did not protect against the inhibition of

Ca²⁺ accumulation (Fig. 1). CaCl₂ (5–100 μ M), MgCl₂ (1–5 mM) and NaCl (up to 100 mM) had no effect on either the degree of inhibition of Ca²⁺ accumulation or ATPase activity by acetic anhydride or on the protection by ATP against the inhibition of ATPase activity (results not shown). Inhibition of net Ca²⁺ accumulation by acetic anhydride modification in the presence of ATP was also observed when Ca²⁺ accumulation was assayed in the absence of P_i, suggesting that the inhibition is not due to inhibition of P_i entry.

In the presence of 0.58 mM-acetic anhydride, the protection against the loss of ATPase activity was maximal at about 5 mM-ATP (Fig. 2). The inhibition of Ca²⁺ accumulation by acetic anhydride was only slightly affected by alterations in ATP concentration. While ATP completely prevented the inhibition of ATPase activity by acetic anhydride, App[NH]p and ADP were only partially effective and AMP was ineffective (Table 1). Since the order of nucleotide effectiveness was the same as the order in which these nucleotides inhibited ATP hydrolysis, the inactivation of ATPase activity by acetic anhydride may have been due to a modification of amino group(s) involved in the binding site for ATP.

Increasing the Ca²⁺ permeability of the sarcoplasmic reticulum vesicles by adding the ionophore A23187 had no effect on either the inhibition of ATPase activity by acetic anhydride or on the protection against inhibition by ATP. Moreover, A23187 stimulated the ATPase activity of both unmodified and modified membranes (result not shown). This suggests that the modification does not alter the enzyme activity.

Effect of preincubation time and pH on the inactivation of Ca²⁺ accumulation and ATPase activity by acetic anhydride: the nature of the reactive groups

The rate of inactivation of Ca²⁺ uptake and ATPase activity by acetic anhydride at 22 °C and pH 7.0 or pH 8.0, in the presence and the absence of ATP, is shown in Fig. 3. ATP prevented the inactivation of ATPase activity for the complete time of treatment (Fig. 3a). In the absence of ATP, and at pH 8.0 with a short period of preincubation of sarcoplasmic reticulum with acetic anhydride, an initial monophasic inactivation process was observed for inactivation of both Ca²⁺ uptake and ATPase activity (Fig. 3b). This process represented the

Table 1. Effect of adenine nucleotides on the inhibition of Ca²⁺ uptake and ATPase activity by acetic anhydride

Experiments were done as in Fig. 3, except that adenine nucleotides, as indicated, were included in the modification medium for control and treated membranes. The control and treated membranes were subjected to centrifugation-chromatography (Penefsky, 1977) to remove unbound nucleotides and reagents. Control ATPase and Ca²⁺ uptake activities were 0.407 and 0.718 μ mol/min per mg of protein, respectively.

Preincubation with acetic anhydride (0.58 mM)	Nucleotide concentration (mM)	ATPase activity (% of control)	Ca ²⁺ uptake (% of control)
No addition		35	22
ATP	0.07	86	30
ATP	4.4	100	11
App[NH]p	0.07	60	31
App[NH]p	4.4	72	22
ADP	0.07	34	16
ADP	4.4	57	18
AMP	4.4	36	27

Fig. 3. Time course of the inactivation of Ca^{2+} accumulation and ATPase activity by acetic anhydride

In (a), sarcoplasmic reticulum membranes were incubated without or with acetic anhydride (0.7 mM) and/or 5 mM-ATP at pH 7.0 for the indicated periods of time and Ca^{2+} uptake and ATPase activity also were assayed as described under 'Materials and methods'. ● and ○, Ca^{2+} uptake by sarcoplasmic reticulum membranes modified by acetic anhydride in the presence or absence of ATP, respectively; △ and ▲, ATPase activity of the membranes modified in the presence or absence of ATP, respectively. Control activity was 1.0 $\mu\text{mol}/\text{min}$ per mg of protein for both Ca^{2+} uptake and ATPase activity. In (b), sarcoplasmic reticulum membranes were incubated with acetic anhydride (0.7 mM) for a given period of time at pH 8.0. Reactions were started by injecting 5 μl of acetic anhydride or ether and terminated by injecting 10 μl of 0.2 M-lysine, pH 7.0 (final concentration, 10 mM). The effective reaction time following mixing was determined from the response of a photodiode connected to a storage oscilloscope. Aliquots were assayed for Ca^{2+} uptake and ATPase activity as described under 'Materials and methods'. Control activities were 1.16 and 0.79 $\mu\text{mol}/\text{min}$ per mg of protein for Ca^{2+} uptake and ATPase activity, respectively.

Fig. 4. pH-dependence of acetic anhydride inactivation of Ca^{2+} accumulation and ATPase activity

The experimental conditions were as described under 'Materials and methods', except that the pH of the incubation medium was varied. ▲ and ■ indicate the presence or absence, respectively, of 5 mM-ATP during preincubation of the membranes with acetic anhydride (0.38 mM). Control activities were 1.02 and 0.69 $\mu\text{mol}/\text{min}$ per mg of protein for Ca^{2+} uptake and ATPase activity, respectively.

main effect of acetic anhydride on both activities. The rate and the extent of this inactivation phase were both relative higher for Ca^{2+} accumulation than for ATPase activity.

The inactivation of ATPase activity and of Ca^{2+} transport by acetic anhydride was found to be strongly pH-dependent (Fig. 4). Half-maximal inactivation was obtained in a 5 min incubation at about pH 6.5, and

maximal inactivation at pH values about 7.0. The presence of ATP during the treatment of the sarcoplasmic reticulum with acetic anhydride protected against the inactivation of ATPase activity at all pH values tested but was more effective at alkaline pH (Fig. 4). A similar pH profile for the activation of Ca^{2+} accumulation by acetic anhydride was obtained in the presence or absence of ATP.

Fig. 5. Estimation of the pK of the groups reacting with acetic anhydride

Sarcoplasmic reticulum membranes were modified by acetic anhydride (0.6 mM) for 15 s in the presence of 5 mM-ATP at the indicated pH. Control Ca²⁺ uptake activities of membranes incubated without acetic anhydride at different pH values were between 1.82 and 1.70 $\mu\text{mol}/2 \text{ min per mg}$ of protein.

In order to estimate the apparent pK of the reactive group, the rate of inactivation of Ca²⁺ accumulation by acetic anhydride, in the presence of ATP, as a function of the pH of the modification medium was determined (Fig. 5). High pH alone increases membrane permeability for Ca²⁺; therefore, it is difficult to determine the inactivation rate of Ca²⁺ accumulation at pH values above 8.7. If the inactivation were exponential, then $\log(\text{remaining activity})$ should be proportional to $-k_{\text{in}}$ (rate constant for inactivation) in such experiments. Because the inactivation increased more than 2-fold between pH 8.3 and 8.7, the pK of the reactive group must have been above 8.3. Although the pK value obtained was lower than that reported for the ϵ -amino groups of polypeptide chains (Segel, 1975) it may have corresponded to that of a highly reactive amino group (Schmidt & Westheimer, 1971).

Acetic anhydride could also react with SH groups or with the imidazole ring of histidine. The possible involvement of SH groups in the modification by acetic anhydride was tested by the titration of free thiol groups of unmodified and anhydride-modified membranes with 5,5'-dithiobis-(2-nitrobenzoic acid) (Ikemoto *et al.*, 1978). The binding of this reagent to the unmodified and modified membranes was the same either before or after solubilization of the membranes with SDS (results not shown). Histidine can be more specifically modified by diethylpyrocarbamate (Schindler *et al.*, 1976), and the effect of this reagent on the Ca²⁺ uptake and ATPase activity of sarcoplasmic reticulum was tested. Chemical modification of the membrane by diethylpyrocarbamate in the presence of ATP had no effect on either ATPase activity or coupled Ca²⁺ transport (results not shown). These findings do not support the possibility that thiol or histidine groups reacted with acetic anhydride to inhibit Ca²⁺ accumulation.

Ca²⁺ efflux stimulated by acetic anhydride modification of Ca²⁺-loaded sarcoplasmic reticulum vesicles

The ability of acetic anhydride (in the presence of ATP) to inactivate Ca²⁺ accumulation without affecting Ca²⁺-dependent ATPase activity suggests that the modification caused either uncoupling of the inward movement of Ca²⁺ from the ATP hydrolytic activity or a stimulation of Ca²⁺ release. Therefore, the effect of acetic anhydride on Ca²⁺ efflux from sarcoplasmic reticulum was tested. If sarcoplasmic reticulum actively loaded with calcium phosphate were incubated for 3 min with 0.4 mM-acetic anhydride, followed by a 10-fold dilution of the vesicles, a rapid release of Ca²⁺ was observed (Fig. 6). Fig. 6 also shows that passive Ca²⁺ efflux from identical vesicles treated only with diethyl ether was at least 10-fold lower.

Rapid Ca²⁺ efflux was also obtained from vesicles treated with acetic anhydride in the presence of ATP and then passively loaded with 5 mM-Ca²⁺ (Table 2). The Ca²⁺ release from acetic anhydride-modified vesicles was probably not due to an increase in the permeability of the lipid bilayer to Ca²⁺, because acetic anhydride treatment

Fig. 6. Ca²⁺ release stimulated by acetic anhydride modification of Ca²⁺-loaded vesicles

Vesicles were loaded with Ca²⁺ by incubation of sarcoplasmic reticulum (100 $\mu\text{g}/\text{ml}$) in a reaction mixture for Ca²⁺ uptake, as described under 'Materials and methods', except that Mops buffer was used instead of Tris/maleate. After 30 min, the membranes were placed on ice and collected by centrifugation at 80000 g for 20 min. The vesicles were resuspended in 20 mM-Tricine (pH 7.0)/100 mM-NaCl at a protein concentration of 3.6 mg/ml. The vesicles were diluted 5-fold in 20 mM-Tricine (pH 7.0)/100 mM-NaCl/5 mM-ATP and were incubated with 0.4 mM-acetic anhydride or with an equivalent volume of ether as a control. After 3 min, aliquots were diluted 10-fold in 20 mM-Mops (pH 7.0)/100 mM-NaCl/1 mM-EGTA to start the assay for Ca²⁺ release. At the indicated times, samples (20 μg of protein) were assayed for ⁴⁵Ca content by filtration through 0.3 μm nitrocellulose filters, ○ and ■, Ca²⁺ content of unmodified and acetic anhydride-modified membranes, respectively.

Table 2. Effect of acetic anhydride modification of sarcoplasmic reticulum membranes on Ca^{2+} efflux from passively loaded vesicles

Acetic anhydride-modified and control membranes were obtained as described in Fig. 1 and were concentrated by centrifugation at 100 000 *g* for 20 min. The membranes (5 mg/ml) were loaded with Ca^{2+} by incubation at 0 °C in 20 mM-Mops (pH 6.8)/100 mM NaCl/5 mM- $^{45}\text{CaCl}_2$ (10^6 c.p.m./ μmol) for 20 h. Passive Ca^{2+} efflux was initiated by 25-fold dilution into buffer containing 20 mM-Mops (pH 6.8), 100 mM-NaCl, 5 mM- MgCl_2 and 2 mM-EGTA. After 10, 30 and 60 s, aliquots (0.13 mg of protein) were assayed for Ca^{2+} content as described under 'Materials and methods'. Initial Ca^{2+} content of the loaded vesicles (100%) was 73.2 nmol/mg of protein.

Membrane preparation	Ca^{2+} released/30 s	
	(nmol/mg of protein)	(% of control)
Control	19.8	27
Acetic anhydride-modified (0.5 mM)	50.5	69
Acetic anhydride-modified (1.0 mM)	54.2	74

Fig. 7. Effect of maleic anhydride modification of sarcoplasmic reticulum membranes on Ca^{2+} accumulation

Acetic anhydride- and maleic anhydride-modified membranes were obtained as described under 'Materials and methods', except that ATP (5 mM) was included in the incubation medium. ■ and □, Ca^{2+} uptake by maleic anhydride- and acetic anhydride-modified membranes, respectively. Control activity was 0.8 $\mu\text{mol}/\text{min}$ per mg of protein.

of Ca^{2+} -loaded phospholipid liposomes had no effect on their Ca^{2+} content (results not shown). We have found (V. Shoshan-Barmatz & G. Meissner, unpublished work) that the membrane permeability for Ca^{2+} , but not for sucrose, Tris, Na^+ or P_i , was increased by

Table 3. Effect of ATP on the inhibition of ATPase activity by maleic anhydride

Sarcoplasmic reticulum membranes were incubated with maleic anhydride as described under 'Materials and methods', in the presence or absence of 5 mM-ATP. Control ATPase activity was 1.06 μmol of P_i released/min per mg of protein.

Maleic anhydride concentration (μM)	Preincubation . . .	ATPase activity (% of control)	
		With ATP	Without ATP
30		96.6	73.0
60		94.0	67.5
155		102.0	60.0
310		97.0	43.6

modification with acetic anhydride. The results suggest that, in the presence of ATP, modification of sarcoplasmic reticulum by acetic anhydride stimulates specifically Ca^{2+} efflux through protein modification.

Maleic anhydride inactivation of Ca^{2+} accumulation and ATPase activity

Maleic anhydride also reacts specifically with amino groups of proteins at alkaline pH (Butler *et al.*, 1969). The products, maleylated proteins, have an increased negative charge at neutral pH. Like acetic anhydride, maleic anhydride also inactivated Ca^{2+} accumulation when it was incubated with sarcoplasmic reticulum in the presence of ATP (Fig. 7). Half-maximal inhibition of Ca^{2+} accumulation was obtained at about 120 μM -maleic anhydride, compared with 230 μM for acetic anhydride. Similar to acetic anhydride, maleic anhydride inactivated the Ca^{2+} -ATPase activity of the membranes and ATP prevented the inhibition of ATPase activity but not that of Ca^{2+} accumulation (Table 3).

The pH-dependence of the inactivation of Ca^{2+} accumulation by maleic anhydride was similar to that obtained with acetic anhydride (results not shown). Maleic anhydride treatment of Ca^{2+} -loaded vesicles also stimulated Ca^{2+} release from the vesicles as did acetic anhydride (results not shown). Thus, it seems that the interaction of maleic and acetic anhydride with the sarcoplasmic reticulum membrane has similar effects and properties

Modification of sarcoplasmic reticulum membranes by PLP and TNBS

PLP interacts specifically with ϵ -amino groups of lysyl residues (Glazer, 1976). Modification of sarcoplasmic reticulum membranes by PLP resulted in inactivation of Ca^{2+} transport and ATPase activity, with half-maximal inhibition at about 25 μM , and maximal inhibition at about 60 μM -PLP for both activities (Fig. 8). The presence of ATP prevented the inactivation of both Ca^{2+} -ATPase and coupled Ca^{2+} transport by PLP. Murphy (1977) reported that 8 mM- MgATP or CaATP protected against the inactivation of Ca^{2+} transport and ATPase activity by 1 mM-PLP. In our hands, protection was obtained at relatively low concentrations (100 μM) of ATP (result not shown). PLP did not stimulate Ca^{2+}

Fig. 8. Effect of PLP modification of sarcoplasmic reticulum on Ca²⁺ uptake and ATPase activity

PLP modification of sarcoplasmic reticulum membranes was carried out as described under 'Materials and methods', except that ATP was included in the incubation medium (■). Ca²⁺ accumulation and ATPase activity were assayed for 1 min as described under 'Materials and methods'.

Table 4. Effect of acetic anhydride treatment on Ca²⁺ accumulation by PLP-modified sarcoplasmic reticulum vesicles

PLP-modified membranes were prepared as described under 'Materials and methods'. PLP concentration was 70 μM. The presence of 5 mM-ATP in the incubation medium is indicated by '+ ATP'. The treated membranes (a) were subjected to centrifugation-chromatography as described under 'Materials and methods' before aliquots were assayed for Ca²⁺ uptake activity. Other samples were subsequently incubated without (b) or with (c) 0.5 mM-acetic anhydride for 5 min. After incubation, aliquots were assayed for Ca²⁺-uptake activity.

Membrane preparations	Ca ²⁺ uptake (μmol/min per mg of protein)		
	(a)	(b)	(c)
Control	0.836	—	—
PLP-modified	0.275	—	—
Control + ATP	0.892	0.686	0.164
PLP-modified + ATP	0.909	0.708	0.192

efflux from vesicles previously loaded with Ca²⁺ (result not shown). These results suggest that a reactive lysyl residue is essential for the catalytic activity of the (Ca²⁺ + Mg²⁺)-ATPase, and that PLP reacts close to or at the binding site for ATP.

In order to test whether PLP also interacted with the reactive amino groups involved in Ca²⁺ release, the effect of additional modification by acetic anhydride on Ca²⁺ accumulation by PLP-modified membranes was examined. The modifications by PLP and by acetic anhydride were performed in the presence of ATP. Modification of both PLP-modified and unmodified membranes with acetic anhydride resulted in inhibition of Ca²⁺ accumulation (Table 4). These results suggest that PLP did not interact with the same amino groups which reacted with acetic anhydride and, therefore, it did not stimulate Ca²⁺

release. Because PLP is a negatively charged compound, the effect of the negatively charged reagent TNBS on Ca²⁺ uptake and ATPase activity was tested. It has been shown that amino groups are the most reactive towards TNBS (Freedman & Radda, 1968), that TNBS is a non-penetrating reagent in sarcoplasmic reticulum (Vale, 1977) and that it inhibits Ca²⁺ uptake (Vale, 1970). Table 5 shows that TNBS inhibited both Ca²⁺ accumulation and ATPase activity. At 0.5 mM-TNBS, inhibition of about 88% and 57% was obtained for Ca²⁺ uptake and ATPase activity, respectively. The presence of ATP partially protected against the inactivation both of the Ca²⁺-ATPase and Ca²⁺ accumulation (Expt. I). Table 5 (Expt. II) also shows that acetic anhydride modification of TNBS-modified membranes (in the presence of ATP) resulted in a further inhibition of Ca²⁺ accumulation.

On the basis of the results reported in Fig. 8 and in Tables 4 and 5, and considering that TNBS does not penetrate the sarcoplasmic reticulum membranes (Vale, 1977), it is reasonable to conclude that the reactive group(s) involved in Ca²⁺ release are not accessible to the negatively charged reagents.

Effect of acetic anhydride modification on Ca²⁺ efflux from proteoliposomes reconstituted with the partially purified or the more purified ATPase fraction

Table 6 shows the effect of acetic anhydride modification of proteoliposomes reconstituted with the partially purified or with the more purified ATPase fraction, actively loaded with calcium phosphate, on their Ca²⁺-efflux properties. As shown, treatment with acetic anhydride stimulated Ca²⁺ efflux from sarcoplasmic reticulum vesicles and from proteoliposomes reconstituted with the partially purified ATPase fraction but not from proteoliposomes reconstituted with the more purified ATPase fraction. The increase in rate of Ca²⁺ efflux from 3% to 5% was also obtained with liposomes free of the Ca²⁺-ATPase (result not shown). Thus, it is clear that as the purity of the Ca²⁺-ATPase increases, the stimulation of Ca²⁺ efflux by the modification with acetic anhydride diminishes.

Table 5. Effect of TNBS modification of sarcoplasmic reticulum on Ca^{2+} uptake and on the inhibition of Ca^{2+} accumulation by acetic anhydride

In Expt. I, the modification of sarcoplasmic reticulum was carried out at 20 °C, pH 8.0, with 20 mM-Tricine. The membrane (1 mg/ml) were incubated for 10 min with the concentrations of TNBS indicated in the presence or absence of 5 mM-ATP. Lysine, pH 7.0, was added to a final concentration of 14 mM to stop the reaction and samples were diluted 10-fold with the reaction mixtures for Ca^{2+} uptake or ATPase activity. In Expt. II, TNBS-modified membranes were prepared as described above for Expt. I, except that the modification was carried out in the presence of 5 mM-ATP. The treated membranes were subjected to centrifugation-chromatography as described under 'Materials and methods'. Samples were incubated with diethyl ether (control) or with 0.5 mM-acetic anhydride for 3 min, then aliquots were assayed for Ca^{2+} uptake activity.

Membranes pretreated with:	Ca^{2+} uptake ($\mu\text{mol}/2 \text{ min per mg of protein}$)	ATPase activity ($\mu\text{mol}/2 \text{ min per mg of protein}$)
Expt. I		
None	1.58	1.42
ATP	1.50	1.35
TNBS, 0.2 mM	0.75	0.85
TNBS, 0.2 mM + ATP	1.40	1.42
TNBS, 0.3 mM	0.32	0.71
TNBS, 0.3 mM + ATP	1.20	1.32
TNBS, 0.5 mM	0.20	0.65
TNBS, 0.5 mM + ATP	1.05	1.28
Ca^{2+} uptake ($\mu\text{mol}/2 \text{ min per mg of protein}$)		
	Control	Acetic-anhydride treated
Expt. II		
None	1.55	0.40
TNBS, 0.3 mM	1.13	0.35
TNBS, 0.5 mM	1.03	0.33

DISCUSSION

Modification of sarcoplasmic reticulum with acetic anhydride, maleic anhydride, PLP or TNBS resulted in inhibition of ATPase activity and of coupled Ca^{2+} transport, indicating that specific amino groups in the ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase are crucial for these activities. ATP protected the ATPase activity against the various modifications. This may suggest that ATPase inactivation was due to modification of amino groups that are involved in an ATP binding site. Similar results were obtained with other reagents (Murphy, 1977; Pick & Karlsh, 1980; Hidalgo *et al.*, 1982), where investigators have proposed that a lysyl residue constitutes part of the active site. However, it is not clear whether this lysyl residue constitutes part of the ATP-binding site or of the phosphorylation site. Mitchinson *et al.* (1982) demonstrated the fluorescein isothiocyanate labelling of a single highly reactive lysine residue at the nucleotide-binding domain of the enzyme, which is not the site of phosphorylation.

Even though the ATPase was protected by ATP, a decrease in the rate of Ca^{2+} accumulation was still observed in anhydride-modified membranes. This could be due to a modification of the Ca^{2+} -ATPase catalytic cycle or, alternatively, to modification of the membrane at a different site(s) which leads to an increased permeability for Ca^{2+} in the vesicles. In the latter case, Ca^{2+} would be pumped inward by the ATPase, but its increased efflux would decrease the net accumulation. The second possibility is supported by: (a) the fact that

rapid Ca^{2+} release was obtained upon acetic anhydride modification of passively or actively loaded sarcoplasmic reticulum vesicles (Fig. 6 and Table 2), (b) acetic anhydride modification stimulated Ca^{2+} release from liposomes reconstituted with the partially purified ATPase fraction but not from liposomes reconstituted with the more purified ATPase fraction (Table 6) or from protein-free liposomes, and (c) modification of sarcoplasmic reticulum by acetic anhydride (in the presence of ATP) had no effect on the partial reactions of the transport cycle, such as E-P formation or ADP-ATP exchange, which should be affected if the modification caused enzymic uncoupling (V. Shoshan-Barmatz, unpublished work).

Hidalgo *et al.* (1982) suggested that uncoupling of Ca^{2+} transport from the ATPase occurs during modification of the lipids around the enzyme with fluorescamine. Our results (Table 5), however, showed that TNBS, which modifies about 70–90% of the amino groups in phosphatidylethanolamine (Vale, 1977), in the presence of ATP did not cause 'uncoupling' of Ca^{2+} transport. Moreover, acetic anhydride inhibited Ca^{2+} accumulation by TNBS-modified membranes (Table 5). Furthermore, acetic anhydride stimulated Ca^{2+} release from proteoliposomes reconstituted with the partially purified Ca^{2+} -ATPase but not from those reconstituted with the purified enzyme (which contained most of its original bound lipids) (Table 6). These results suggest that the Ca^{2+} release stimulated by chemical modification involves protein factors (other than the Ca^{2+} -ATPase) rather than lipids around the ATPase molecule or of the

Table 6. Effect of acetic anhydride modification on Ca²⁺ efflux from Ca²⁺-loaded liposomes reconstituted with partially purified or more purified ATPase fractions

Protein preparations and proteoliposomes were prepared as described under 'Materials and methods'. Vesicles (100 µg/ml) were loaded with Ca²⁺ and modified with acetic anhydride as described in Fig. 6, except that the vesicles actively loaded with calcium phosphate (1 mg/ml) were incubated with ether (control) or with 1.5 mM-acetic anhydride for 1 min. Ca²⁺ efflux from these vesicles was also assayed as in Fig. 6, except that 0.2 µm nitrocellulose filters were used. Ca²⁺ content was 1200, 500 and 350 nmol/mg protein for sarcoplasmic reticulum and liposomes reconstituted with the partially purified or with the more purified ATPase fraction, respectively.

Vesicle preparation	Ca ²⁺ released/min (% of control)	
	Control	Acetic anhydride modified
Sarcoplasmic reticulum	16	65
Partially purified ATPase liposomes	16	52
Purified ATPase liposomes	3	5

ATPase molecule itself. It is possible that acetic anhydride modifies highly reactive amino acid residues involved specifically in the control of Ca²⁺ release activity.

Since PLP or TNBS in the presence of ATP did not stimulate Ca²⁺ efflux, and because TNBS is a non-penetrating reagent in sarcoplasmic reticulum membranes (Vale, 1977), it seems probable that TNBS did not interact with the amino groups involved in Ca²⁺ release. These results also suggest that the active amino groups of the Ca²⁺ release system are buried within the protein or located on the internal surface of the membrane, so that they are not accessible to PLP or TNBS.

We would like to propose that Ca²⁺ might be released through a channel, consisting of 'gated' proteins, whose opening is controlled by the protonation/deprotonation of amino group(s) located in the gate. Deprotonation might be brought about by increased pH, by chemical modification, or by lipophilic anions such as TPB⁻, all of which trigger Ca²⁺ release (MacLennan *et al.*, 1982; Shoshan *et al.*, 1981, 1983). The eventual isolation of this Ca²⁺-release channel and its reconstitution into liposomes would be an important step in evaluating the control mechanism of Ca²⁺ release in the sarcoplasmic reticulum.

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