Chemical modification of sarcoplasmic reticulum with methylbenzimidate

Stimulation of Ca^{2+} efflux

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Treatment of sarcoplasmic reticulum membranes with 12 mM-methylbenzimidate (MBI) for 5 min, in the presence of 5 mM-ATP at pH 8.5, resulted in a 2–3-fold stimulation of ATP hydrolysis and over 90% inhibition of Ca^{2+} accumulation. This phenomenon was strictly dependent upon the presence of nucleotides with the following order of effectiveness: adenosine 5'-[β,γ -imido]triphosphate $\geq$ ATP > UTP > ADP > AMP. Divalent cations such as Ca^{2+} , Mg^{2+} and Mn^{2+} , when present during the MBI treatment, prevented both the stimulation of ATPase activity and the inhibition of Ca^{2+} accumulation. Modification with MBI had no effect on E-P formation from ATP, ADP-ATP exchange, Ca^{2+} binding or ATP- P_i exchange catalysed by the membranes. Membranes modified with MBI in the presence of ATP and then passively loaded with Ca^{2+} released about 80% of their Ca^{2+} content within 3 s. Control membranes released only 3% of their Ca^{2+} during the same time period. MBI modification inhibited Ca^{2+} accumulation by proteoliposomes reconstituted with the partially purified ATPase but not with the purified ATPase fraction. These results suggest that MBI in the presence of ATP stimulates Ca^{2+} release by modifying a protein factor(s) other than the (Ca^{2+} + Mg^{2+})-ATPase.

INTRODUCTION

The sarcoplasmic reticulum regulates myoplasmic Ca^{2+} concentration, thereby regulating the contraction-relaxation cycle of skeletal muscle (Ebashi *et al.*, 1969). The functional unit associated with Ca^{2+} transport is the (Ca^{2+} + Mg^{2+})-ATPase (Racker, 1972). The reaction mechanism for Ca^{2+} uptake is well defined and the following sequence of reaction has been suggested (De Meis & Vianna, 1979). Ca^{2+} is bound to two high-affinity sites in the ATPase that face the cytoplasm. When ATP is hydrolysed, the ATPase is phosphorylated and the $\text{Ca}_2 \cdot \text{E} \cdot \text{P}$ complex undergoes a conformational change resulting in exposure of the bound Ca^{2+} to the luminal space. In this configuration the Ca^{2+} -binding affinity is diminished and Ca^{2+} is released. Dephosphorylation of the enzyme accompanies the transition to a form with high-affinity cytoplasmically exposed Ca^{2+} -binding sites. Thus, Ca^{2+} transport is tightly coupled to ATP hydrolysis, where two calcium ions are translocated per molecule of ATP hydrolysed.

Uncoupling of Ca^{2+} transport from ATPase activity has been obtained by incubation of sarcoplasmic reticulum membranes in mild acid conditions or in the presence of EGTA at neutral pH (Berman *et al.*, 1977; McIntosh & Berman, 1978). It has been suggested that this uncoupling is due to a minor conformational change in the ATPase, which prevents energy transduction (McIntosh & Berman, 1978).

Chemical modifications of sarcoplasmic reticulum membranes by several reagents generally results in the inhibition of Ca^{2+} -dependent ATPase activity and coupled Ca^{2+} transport (Panet & Selinger, 1970; Dupont

& Hasselbach, 1973; Martonosi, 1976; Murphy, 1976, 1978; Andersen & Moller, 1977; Yamada & Ikemoto, 1978; Pick & Racker, 1979; Bailin, 1980; Hidalgo, 1980; Hidalgo *et al.*, 1982). In some modifications, such as those induced by thiol reagents, ATP protects against the inhibition of both the ATPase activity and coupled Ca^{2+} transport (Martonosi, 1976; Murphy, 1976, 1978; Andersen & Moller, 1977). However, in the case of modification of sarcoplasmic reticulum with reagents that react principally with amino groups, the presence of ATP during chemical modification protects against the inhibition of ATPase activity but does not prevent the inhibition of Ca^{2+} transport (Hidalgo, 1980; Hidalgo *et al.*, 1982). It has been suggested that this 'uncoupling' is due to a specific defect in the Ca^{2+} -translocation reaction (Hidalgo, 1980; Hidalgo *et al.*, 1982). On the other hand, my results on chemical modification of sarcoplasmic reticulum with acetic or maleic anhydrides (Shoshan-Barmatz, 1986) suggest that the 'uncoupling' of Ca^{2+} transport from ATP hydrolysis, obtained upon modification in the presence of ATP, is due to stimulation of Ca^{2+} release.

The chemical modification of amino groups described in a number of studies causes a drastic change in the physicochemical state of lipids and proteins by eliminating the positive charge of the amino group at neutral pH. Amidation, on the other hand, leaves the net charge of the protein or lipid unaltered (Hunter & Ludwing, 1962; Wofsy & Singer, 1963).

In the experiments presented in the present paper, the effect of amidation of sarcoplasmic reticulum on the coupling between ATP hydrolysis and Ca^{2+} transport was studied. It will be demonstrated that modification of

Abbreviations used: App[NH]P, adenosine 5'-[β,γ -imido]triphosphate; MBI, methylbenzimidate; App[CH₂]p, adenosine 5'-[β,γ -methylene]triphosphate.

sarcoplasmic reticulum with MBI stimulates Ca^{2+} efflux only when ATP is present during the modification procedure. The results suggest the involvement of an amino group(s) and an ATP-binding site in the control of Ca^{2+} release.

EXPERIMENTAL PROCEDURES

Materials

ATP, ADP, App[NH]p, EGTA and Tricine were obtained from Sigma Chemical Co. MBI was obtained from Aldrich. Asolectin (crude soybean phospholipids) was obtained from Associated Concentrates (Woodside, NY, U.S.A.). $^{45}\text{CaCl}_2$ was from Amersham International and $^{32}\text{P}]\text{P}_i$ from the Nuclear Research Center, Negev, Israel. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ 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 eluent.

Protein preparation

Sarcoplasmic reticulum vesicles were prepared from rabbit fast twitch skeletal muscle as described by Campbell & MacLennan (1981). This preparation contains a mixture of light, heavy and intermediate vesicles. The purified (R_3) and partially purified (R_2) ATPase fractions were obtained as described by MacLennan (1970). The protein concentration was determined by the Lowry *et al.* (1951) method.

Amidination of sarcoplasmic reticulum membranes with MBI

MBI was dissolved in 0.1 M-sodium borate, pH 8.5, the pH of the solution was adjusted to about 6.5 and it was used immediately. Aliquots of MBI solution (5–20 μl) were added to 0.2 ml of 0.1 M-sodium borate, pH 8.5, containing 1.0–1.2 mg of sarcoplasmic reticulum and other reagents as indicated in Figure and Table legends. The reaction of the reagent with the membranes was stopped after 5–10 min at 22 °C by the addition of lysine solution, at pH 7.0, to a final concentration of 30 mM and transfer of the samples to ice. In some experiments, 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/100 mM-NaCl, pH 7.2. The protein concentration in each sample was determined.

The degree of amidination was measured by the binding of trinitrobenzenesulphonate to SDS-solubilized membranes. After 10 min of incubation, about 30% and 13% of the amino groups of sarcoplasmic reticulum membranes had reacted with 10 mM-MBI in the presence and the absence of ATP, respectively.

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). Aliquots were mixed with the purified (R_3) or partially purified (R_2) Ca^{2+} -ATPase at a concentration of 20 mg of asolectin/mg of protein. The mixture was frozen in liquid N_2 , slowly thawed at room temperature, sonicated for 30 s and used immediately.

Assays

Ca^{2+} uptake, ATPase activity, ADP-ATP exchange, E-P formation and ATP- P_i exchange were assayed as described in Table or Figure legends or as described previously (Shoshan & MacLennan, 1981).

RESULTS

Modification of sarcoplasmic reticulum with MBI

Amidination of a protein specifically modifies the ϵ -amino group of lysine residues (Hunter & Ludwig, 1962). The benzamidinium group increases the size of the lysine side chain but does not change the net charge through most of the pH region (pK about 12).

Fig. 1 shows the effect on Ca^{2+} transport and ATPase activity of pretreating sarcoplasmic reticulum with MBI. When modification of the membranes was carried out in the absence of ATP, both activities were affected only slightly at high concentrations of the reagent. However, the presence of ATP in the modification medium caused 'uncoupling' of Ca^{2+} transport from ATPase activity. A 2-fold stimulation of ATP hydrolysis and 92% inhibition of Ca^{2+} uptake were obtained with membranes modified with 12 mM-MBI in the presence of 5 mM-ATP. The extent of the 'uncoupling' was dependent on the time and the pH of the incubation of sarcoplasmic reticulum with the reagent. In the presence of 5 mM-ATP and 12 mM-MBI at pH 8.5, a maximal inhibition of Ca^{2+} accumulation and stimulation of ATPase activity was obtained after 5 min of incubation (results not shown). The inhibition of Ca^{2+} accumulation and stimulation of ATPase activity by MBI in the presence of ATP was strongly pH-dependent, increasing dramatically as the pH was increased above 8.0 (Table 1).

Effect of nucleotides on modification by MBI of sarcoplasmic reticulum membranes

The ability of MBI to produce 'uncoupling' of Ca^{2+} transport from ATPase activity only in the presence of ATP was examined further to define the ATP concentration-dependence of this effect and the nucleotide specificity. Maximal 'uncoupling' between Ca^{2+} transport and ATP hydrolysis was produced by modification of sarcoplasmic reticulum with 12 mM-MBI in the presence of 3 mM-ATP (Fig. 2). The 'uncoupling' by the reagent can also be obtained in the presence of other adenine nucleotides (Table 2). However, while a stimulation of ATPase activity of about 3-fold and inhibition of Ca^{2+} accumulation by about 90% were obtained when the modification was carried out in the presence of 5 mM-ATP, stimulation of only about 1.3-fold and inhibition by 43% of ATPase activity and Ca^{2+} accumulation, respectively, were obtained with 5 mM-ADP. The ATP analogue App[NH]p was even more effective than ATP. At a concentration of 3 mM, UTP was almost as effective as ATP, while CTP was only half as effective and AMP was not effective at all (results not shown). This nucleotide specificity is different from that observed for (Ca^{2+} + Mg^{2+})-ATPase activity (Makinose & The, 1965).

Effect of divalent cations on the 'uncoupling' produced by modification of sarcoplasmic reticulum with MBI in the presence of ATP

The ability of MBI, in the presence of ATP, to inhibit Ca^{2+} accumulation and to stimulate ATPase activity

Fig. 1. Effect of modification of sarcoplasmic reticulum with MBI on Ca²⁺ uptake and ATPase activity

Sarcoplasmic reticulum membranes were incubated with the indicated concentration of MBI, in the presence (●) or absence (○) of 5 mM-ATP. Ca²⁺ uptake and ATPase activity were assayed for 2 min as described under 'Experimental procedures'.

Table 1. Dependence of the modification of sarcoplasmic reticulum activities with MBI on the incubation pH

Sarcoplasmic reticulum membranes were incubated at the indicated pH with or without ATP (5 mM) and/or MBI (10 mM) for 5 min as described under 'Experimental procedures'. The buffers used were 0.1 M-sodium phosphate for pH 7.0 and 0.1 M-sodium borate for pH 8.0, 8.5 and 9.0. Ca²⁺ uptake and ATPase activity were assayed for 2 min.

Incubation conditions	Membrane preparation . . .	Ca ²⁺ uptake (μmol/mg of protein)		ATPase activity (μmol/mg of protein)	
		Control	MBI-treated	Control	MBI-treated
pH, 7.0		1.64	1.48	0.90	0.90
pH, 7.0+ATP		1.48	1.45	0.79	—
pH, 8.0		1.51	1.37	1.14	0.98
pH, 8.0+ATP		1.22	1.12	0.90	1.36
pH, 8.5		1.43	1.26	0.95	1.04
pH, 8.5+ATP		1.00	0.62	1.15	2.59
pH, 9.0		1.27	1.02	1.14	1.20
pH, 9.0+ATP		1.06	0.15	1.37	3.36

could be prevented by the presence of divalent cations in the treatment medium (Fig. 3). MgCl₂ at 7.5 mM completely prevented both the stimulation and the inhibition of ATPase activity and Ca²⁺ accumulation, respectively. The effect of MBI modification on sarcoplasmic reticulum activities could also be prevented by other divalent cations, as shown in Table 3. CaCl₂ was more effective than MgCl₂ and MnCl₂ was less effective than the other two cations. The free ATP concentrations in the presence of 2 mM-CaCl₂, 5 mM-MgCl₂ or 5 mM-MnCl₂ were expected to be 1.06 mM, 40 μM and 20 μM, respectively. CaCl₂ also prevented the inhibition by MBI of Ca²⁺ accumulation when the membranes were modified in the presence of App[NH]p and where the free

App[NH]p concentration was in the mM range (results not shown). Thus, the divalent cations apparently prevented the effect of the nucleotide not by decreasing their free concentrations, but by a direct interaction with the protein(s) modified by MBI.

Effect of modification by MBI of sarcoplasmic reticulum, in the presence of ATP, on the partial reactions of ATP-dependent Ca²⁺ transport

In order to clarify whether the effect of MBI on Ca²⁺ accumulation and ATPase activity was due to the modification of the ATPase molecule itself, the effect of the modification on several partial reactions of the transport cycle was tested.

Fig. 2. ATP-dependence of modification by MBI of Ca^{2+} accumulation and ATPase activity

Modification of sarcoplasmic reticulum by MBI (12 mM) was carried out for 5 min as described under 'Experimental procedures' except that ATP was present at the indicated concentration in the incubation medium of the control or MBI-treated samples. Control activity (100%) for Ca^{2+} uptake (●) was between 0.902 and 0.875 $\mu\text{mol}/\text{min}$ per mg of protein, and for ATPase activity (○) was 0.54–0.575 $\mu\text{mol}/\text{min}$ per mg of protein, in the absence or presence of 5 mM-ATP in the incubation, respectively. The activities at zero ATP correspond to the activities of membranes modified with 12 mM-MBI.

The pre-steady-state phosphoenzyme (E-P) formation from ATP by control and MBI-modified membranes is shown in Fig. 4. The same rate of E-P formation and steady state level of E-P were obtained with the unmodified or MBI-modified membranes. The following reactions also were not affected by the modification of the membranes with MBI in the presence of ATP: the rate of decay of E-P formed from ATP, induced by the addition of ADP or EGTA; the phosphate transfer from ATP to ADP (ADP-ATP exchange) catalysed by the Ca^{2+} -dependent ATPase; phosphatase activity (the

Fig. 3. MgCl_2 prevents the 'uncoupling' between Ca^{2+} accumulation and ATPase activity obtained by modification with MBI in the presence of ATP

Sarcoplasmic reticulum membranes were modified for 5 min with MBI (12 mM) in the presence of 5 mM-ATP and the indicated concentration of MgCl_2 as described under 'Experimental procedures'. Control activity (100%) for Ca^{2+} uptake (●) was 0.85 $\mu\text{mol}/\text{min}$ per mg of protein and for ATPase activity (○) was 0.507 $\mu\text{mol}/\text{min}$ per mg of protein in the absence or presence of ATP and/or MgCl_2 .

hydrolysis of dinitrophenyl phosphate); the high- or the low-affinity Ca^{2+} binding sites.

The effect of the modification on ATP- P_i exchange was tested under two different conditions; at optimal Ca^{2+} concentration (100 μM) and high Ca^{2+} concentration (4 mM). As shown in Table 4, modification of the membranes with MBI inhibited both Ca^{2+} accumulation and ATP- P_i exchange, measured at the low Ca^{2+} concentration, only if ATP were present during the modification. However, the modified membranes displayed a similar rate of ATP- P_i exchange as the unmodified membranes when the exchange was assayed at the high Ca^{2+} concentration. ATP- P_i exchange reflects the reversal of the Ca^{2+} pump and it is coupled to Ca^{2+} exchange between the pool of Ca^{2+} located separately in the vesicles and in the assay medium

Table 2. Effect of adenine nucleotides on the modification of sarcoplasmic reticulum activities by MEI

Sarcoplasmic reticulum membranes were modified at pH 8.5 for 5 min, with MBI (15 mM) in the presence of the indicated nucleotide (5 mM). Ca^{2+} uptake and ATPase activity were assayed for 2 min as described under 'Experimental procedures'.

Sarcoplasmic reticulum treated with:	Ca^{2+} uptake ($\mu\text{mol}/\text{mg}$ of protein)	ATPase activity ($\mu\text{mol}/\text{mg}$ of protein)
ATP	1.77	0.96
ATP + MBI	0.19	2.81
App[NH]p	1.78	1.32
App[NH]p + MBI	0.14	3.23
ADP	2.25	1.16
ADP + MBI	1.29	1.52

Table 3. Effect of divalent cations on the inhibition of Ca²⁺ accumulation by modification of sarcoplasmic reticulum membranes with MBI

Modification of sarcoplasmic reticulum with MBI (16 mM) in the presence of 3 mM-ATP was carried out for 5 min as described under 'Experimental procedures', except that the indicated divalent cation was added to the incubation medium. The free ATP concentrations were calculated with a computer program using an apparent binding constant (K_a) of 2.8×10^4 , 3.8×10^4 and 7.6×10^4 M⁻¹ for Ca-ATP, Mg-ATP and Mn-ATP, respectively, at pH 8.5 (Shikama & Nakamura, 1973).

Divalent cation present during the modification (mM)	Free [ATP] (mM)	Ca ²⁺ uptake (μ mol/min per mg of protein)	
		Control membranes	MBI-modified membranes
None	3.0	0.74	0.08
MgCl ₂ (2.0)	1.09	1.02	0.20
MgCl ₂ (5.0)	0.04	1.02	0.82
MnCl ₂ (0.2)	2.81	0.94	0.08
MnCl ₂ (2.0)	1.12	1.06	0.24
MgCl ₂ (5.0)	0.04	1.18	0.54
CaCl ₂ (0.2)	2.80	1.18	0.10
CaCl ₂ (1.0)	2.05	1.16	0.86
CaCl ₂ (2.0)	1.06	1.00	0.86

(Makinose, 1973; Carvalho *et al.*, 1976). The low exchange activity of the modified membranes, measured in the presence of the relatively low Ca²⁺ concentration, was a result of the low Ca²⁺ accumulated in the vesicles. When this condition was overcome by increasing the Ca²⁺ concentration, the modified membranes catalysed ATP-P_i exchange at rates similar or higher to those obtained with the unmodified membranes. Similar results were obtained with leaky vesicles (De Meis & Carvalho, 1974).

The results described in this section suggest that the modification of the membranes by MBI did not affect the catalytic cycle of the Ca²⁺-transport system.

Ca²⁺ efflux in MBI-modified sarcoplasmic reticulum vesicles

Fig. 5 shows the Ca²⁺ efflux from unmodified and MBI-modified sarcoplasmic reticulum vesicles passively loaded with Ca²⁺. As shown, the modified membranes released about 80% of their trapped Ca²⁺ within 3 s. In contrast, the unmodified membranes released only about 3% of their Ca²⁺ content during the same period of time. The increased efflux from MBI-treated membranes was Ca²⁺-specific and was not linked to a general increase of membrane permeability, since the efflux kinetics of [³²P]P_i or [¹⁴C]sucrose from passively loaded vesicles were not affected by the modification (results not shown).

These observations suggest that MBI, in the presence of ATP, stimulates Ca²⁺ release (leading to lowered accumulation), rather than uncouples Ca²⁺ transport from ATP hydrolysis during the catalytic cycle of the Ca²⁺-ATPase.

Ca²⁺ accumulation by proteoliposomes reconstituted with partially purified and more purified ATPase fractions isolated from native and MBI-modified membranes

To test whether the Ca²⁺-ATPase molecule is involved in the increased Ca²⁺ efflux produced by the modification of the membrane with MBI in the presence of ATP,

experiments were conducted on the purified enzyme in a reconstituted system. Table 5 shows the Ca²⁺ uptake by proteoliposomes reconstituted with the partially purified (R₂) or more purified (R₃) ATPase fractions isolated from native or from MBI-modified sarcoplasmic reticulum membranes. The ability of proteoliposomes reconstituted with R₂ isolated from MBI-modified membranes to accumulate Ca²⁺ was about 30% of that of proteoliposomes reconstituted with R₂ isolated from unmodified membranes. However, proteoliposomes reconstituted with the more purified ATPase fraction (R₃) derived from the unmodified or MBI-modified membranes showed no difference in their Ca²⁺ accumulation ability. The ATPase activity of sarcoplasmic reticulum modified with MBI and of R₂ isolated from these membranes was about 2- and 1.5-fold that of the unmodified preparations, respectively. The ATPase activity of R₃ isolated from control and MBI-modified membranes was the same (result not shown). It should be noted, since the purified ATPase fraction was obtained at similar yield from the unmodified and MBI-modified membranes, that there is no preferential purification of the enzyme from the MBI-modified membranes and also that the electrophoretic patterns of the proteins in unmodified or MBI-modified membranes were identical (results not shown).

Table 6 shows the effect of MBI modification subsequent to reconstitution of native R₂ or R₃ into proteoliposomes. Treatment of R₂ proteoliposomes resulted in 54% inhibition of Ca²⁺ accumulation, while R₃ proteoliposomes were inhibited only 11%. It should be noted that, before reconstitution, both R₂ and R₃ preparations were passed through a Sephadex-G-50 column (Penefsky, 1977) in order to remove NH₄⁺ ions present in the storage solution of R₃, which interfere with MBI's action on sarcoplasmic reticulum, probably by reacting with the reagent. Thus, it is clear that as the purity of the Ca²⁺-ATPase increased, the effect of modification with MBI (in the presence of ATP) on Ca²⁺ accumulation and ATPase activity diminished.

Fig. 4. Pre-steady-state phosphoenzyme formation rate in control and MBI-modified sarcoplasmic reticulum membranes

Sarcoplasmic reticulum membranes were modified with MBI (15 mM) in the presence of 3 mM-ATP (●) as described under 'Experimental procedures'. Ca^{2+} uptake activity of the unmodified and MBI-modified membranes was 1.29 and 0.17 $\mu\text{mol}/\text{min}$ per mg of protein, respectively. ATPase phosphorylation was obtained in the presence of 30 mM-Tris/maleate, pH 6.8, containing 100 mM-KCl, 20 mM-MgCl₂, 0.1 mM-CaCl₂, 10 μM -[γ -³²P]-ATP (8×10^4 c.p.m./nmol) and 0.75 mg of protein/ml. Rapid mixing and quenching were carried out with a Durrum Dionex D-133 multimixer which permits reaction times varying between 15 ms and 10 s through electronically controlled flow and aging procedures. Standardization of the instrument with regards to mixing and reaction times was as described (Verjovski-Almeida *et al.*, 1978). Quenching was obtained in 3.5% (w/v) trichloroacetic acid and samples were filtered through Millipore filters (0.3 μm) and washed five times with 5 ml of 5% (w/v) trichloroacetic acid/0.1 mM-ATP/2 mM-P_i, and the filters were counted in a scintillation counter.

DISCUSSION

The study has shown that chemical modification of sarcoplasmic reticulum membranes with MBI yielded a stimulated ATPase activity (up to 3-fold) and a lowered Ca^{2+} accumulation capability (Fig. 1). This 'uncoupling' phenomenon was obtained only if the modification with MBI were carried out in the presence of ATP. MBI reacts almost exclusively with the ϵ -amino group of lysine (Hunter & Ludwing, 1962). The pH profile of the modification (Table 1) suggests that the lysyl groups are, indeed, targets of MBI and, moreover, suggests that a lysyl group is essential for maintaining the integrity of the Ca^{2+} -accumulation system. It is possible that the MBI reactive group(s) is the same group which interacted with acetic anhydride and stimulated Ca^{2+} release (Shoshan-Barmatz, 1986).

The observed 'uncoupling' could be due to a modification of the Ca^{2+} -ATPase catalytic cycle or, alternatively, to modification of the membrane at 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.

Modification of the membranes with MBI, in the presence of ATP, had no effect on partial reactions of the transport cycle such as E-P formation (Fig. 3), ADP-ATP exchange and Ca^{2+} binding. Moreover, the ATP-P_i exchange, which reflects the forward and backward reactions of the Ca^{2+} pump (Carvalho *et al.*, 1976), was also not affected by MBI modification (Table 4). These results suggest that the action of the (Ca^{2+} + Mg^{2+})-ATPase as an enzyme which catalyses the transport of Ca^{2+} coupled to ATP hydrolysis was not affected by the modification. Moreover a rapid Ca^{2+} efflux was obtained from MBI-modified, passively loaded vesicles (Fig. 5). This suggests that MBI, in the presence of ATP, modified an amino residue(s) involved in the control of Ca^{2+} release.

Stimulation of ATPase activity and inhibition of Ca^{2+} accumulation were also obtained by MBI modifications of heavy sarcoplasmic reticulum isolated according to Meissner (1984).

It has been suggested (Cheisi & Carafoli, 1982; Leonards & Kutchai, 1985) that the 53 kDa glycoprotein

Table 4. ATP-P_i exchange at two different Ca^{2+} concentrations by unmodified and MBI-modified sarcoplasmic reticulum

Sarcoplasmic reticulum membranes were incubated with MBI (16 mM), in the presence or absence of ATP (3 mM), for 5 min as described under 'Experimental procedures'. Aliquots were assayed for Ca^{2+} uptake and ATP-P_i exchange activity at two different Ca^{2+} concentrations. The assay medium with low Ca^{2+} concentration contained 30 mM-Mops, pH 6.8, 20 mM-MgCl₂, 10 mM-ATP, 10 mM-[³²P]P_i (containing 5×10^6 c.p.m./ μmol) and 0.1 mM-CaCl₂. The composition of the assay medium with the high Ca^{2+} concentration was identical to that with the low Ca^{2+} concentration, except that 4 mM-CaCl₂ was used instead of 0.1 mM. The reaction was started by the addition of sarcoplasmic reticulum membranes (125 $\mu\text{g}/\text{ml}$) and after 2 or 10 min at 22 °C, for the 0.1 mM- or 4 mM-CaCl₂, respectively, the reaction was stopped by addition of trichloroacetic acid to a final concentration of 5%. The [γ -³²P]ATP formed was measured as described (Avron, 1960).

Sarcoplasmic reticulum treated with:	Ca^{2+} uptake ($\mu\text{mol}/\text{min}$ per mg)	ATP-P _i exchange (nmol of ATP/min per mg)	
		0.1 mM- Ca^{2+}	4 mM- Ca^{2+}
None	0.437	82.3	35.5
ATP	0.366	84.4	41.2
MBI	0.478	109.1	48.2
MBI + ATP	0.019	8.3	54.7

Fig. 5. Ca²⁺ efflux from unmodified and MBI-modified sarcoplasmic reticulum membranes

Sarcoplasmic reticulum vesicles were incubated with 3 mM-ATP, with (●) or without (○) 15 mM-MBI as described under 'Experimental procedures'. After 10 min of incubation, lysine was added to a final concentration of 30 mM. Vesicles were centrifuged at 80000 *g* for 30 min and resuspended (3 mg/ml) in 20 mM-Mops (pH 6.8)/100 mM-KCl/5 mM-⁴⁵CaCl₂ (1.5 × 10⁷ c.p.m./μmol) and were incubated overnight at 0 °C. For Ca²⁺ efflux, aliquots (20 μl) were rapidly filtered and washed for the indicated time with a solution identical with the loading solution, except that CaCl₂ was omitted. The washing rate was approx. 1 ml/s. The zero time points were determined by washing with a solution containing 5 mM-LaCl₃. Ca²⁺ uptake activity was 0.775 and 0.025 μmol/min per mg of protein for control and MBI-modified membranes, respectively.

may be involved in regulation of the coupling between Ca²⁺ transport and ATP hydrolysis. However, as discussed above, it seems that the catalytic cycle of the Ca²⁺ transport system was not affected by the modification. Moreover, the 53 kDa glycoprotein is almost completely absent from the partially purified ATPase

Table 5. Ca²⁺ accumulation by proteoliposomes reconstituted with R₂ or R₃ isolated from native or MBI-modified sarcoplasmic reticulum membranes

Sarcoplasmic reticulum membranes were incubated with 3 mM-ATP and in the absence or the presence of MBI (12 mM) as described under 'Experimental procedures'. The MBI-treated and untreated membranes were collected by centrifugation at 80000 *g* for 30 min, then R₂ and R₃ were isolated from both preparations and reconstituted by freeze-thaw-sonication as described under 'Experimental procedures'. Ca²⁺ uptake was assayed for 1 min.

Vesicle preparations	Ca ²⁺ uptake	
	(μmol/mg of protein)	Inhibition (%)
Sarcoplasmic reticulum	0.525	—
MBI-modified sarcoplasmic reticulum	0.131	75
R ₂	0.301	—
MBI-modified R ₂	0.109	64
R ₃	0.175	—
MBI-modified R ₃	0.173	1

fraction (R₂) which was sensitive to the modification by MBI. Thus, although the 53 kDa glycoprotein has an ATP-binding site (Campbell & MacLennan, 1983) it is unlikely that the effect of MBI in the presence of ATP is due to modification of this protein.

Two proteins are generally considered to be capable of mediating rapid Ca²⁺ efflux from sarcoplasmic reticulum membranes: the Ca²⁺-ATPase and the putative Ca²⁺ channels active during Ca²⁺ release *in vivo*. A phosphorylated intermediate of the Ca²⁺-ATPase (Takenaka *et al.*, 1982; Chiesi & Wen, 1983) as well as the non-phosphorylated Ca²⁺-ATPase (Chiu & Haynes, 1980) have been reported to mediate rapid Ca²⁺ release from sarcoplasmic reticulum. We observed that the purified (Ca²⁺ + Mg²⁺)-ATPase isolated from sarcoplasmic membranes previously modified with MBI in the presence of ATP, upon reconstitution into liposomes, catalysed Ca²⁺ transport at a rate similar to or even higher than the enzymes isolated from membranes treated with MBI or ATP alone (Table 5). Moreover, modification of liposomes reconstituted with the more purified ATPase fraction had no effect on their

Table 6. Effect of treatment of proteoliposomes, reconstituted with R₂ or R₃, with MBI on their Ca²⁺-accumulation ability

Proteoliposomes reconstituted with R₂ or R₃ were prepared and incubated with 3 mM-ATP in the presence or absence of MBI as described under 'Experimental procedures'. After 5 min of incubation aliquots were assayed for Ca²⁺ accumulation ability (2 min).

Proteoliposomes reconstituted with:	Treatment	Ca ²⁺ uptake	
		(μmol/mg of protein)	Inhibition (%)
R ₂	None	0.277	—
R ₂	MBI (7 mM)	0.186	33
R ₂	MBI (15 mM)	0.130	53
R ₃	None	0.396	—
R ₃	MBI (7 mM)	0.354	11
R ₃	MBI (15 mM)	0.343	13

Ca^{2+} -accumulation capacity (Table 6). We have obtained similar results with membranes modified with acetic anhydride (Shoshan-Barmatz, 1986), or digested by trypsin (V. Shoshan-Barmatz, N. Oziel & D. M. Chipman, unpublished work). These data suggest that MBI in the presence of ATP modified a protein factor(s) which is not the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase. The involvement of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase in Ca^{2+} release has also been ruled out by Meissner (1984) on the basis of his observations that $\text{App}[\text{CH}_2]\text{p}$ stimulated Ca^{2+} efflux from vesicles capable of Ca^{2+} -induced Ca^{2+} release but not from vesicles that lacked this mechanism but nevertheless contained the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase. However, it cannot be excluded that the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase is required in conjunction with another membrane component for the activation of Ca^{2+} release by modification of the membranes with MBI, in the presence of ATP.

The effect of nucleotides on the modification by MBI of the membrane permeability for Ca^{2+} is probably not due to their binding to the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase. The nucleotide specificity of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase and that of the system studied here are different (Makinose & The, 1965; Table 2). The velocity of nucleotide hydrolysis by the ATPase is graded in the following order: $\text{ATP} > \text{ITP} > \text{GTP} > \text{CTP} > \text{UTP}$ (Makinose & The, 1965). In our system however, the order of nucleotide effectiveness was as follows: $\text{App}[\text{NH}]\text{p} > \text{ATP} > \text{UTP} > \text{ADP} > \text{CTP} > \text{AMP}$. Moreover, Mg^{2+} prevented the effect of the nucleotide on the modification by MBI, but it had no effect on the protection by ATP against modification of the ATPase activity by acetic anhydride (Shoshan-Barmatz, 1986), pyridoxal phosphate (Murphy, 1978), fluorescein isothiocyanate or thiol reagents (V. Shoshan-Barmatz, unpublished work). These reagents modify the Ca^{2+} -ATPase, probably at the ATP-binding site (Murphy, 1978; Hidalgo, 1980; Pick & Karlsh, 1980; Pick & Bassilian, 1981; Mitchinson *et al.*, 1982). These data suggest that the ATP-binding site involved in the modification with MBI is distinct from the NTP-binding site of the Ca^{2+} -ATPase.

In our experiments, there are two possibilities for the relationship between the modification of MBI and the requirement of ATP for the increase in membrane permeability for Ca^{2+} produced by the modification. The first is that ATP by its binding to the protein(s) produces conformational changes which change the reactivity of a specific ϵ -amino group of lysine and allow the modification by MBI, thereby leading to increased membrane permeability. The second possibility is that MBI modifies the specific ϵ -amino group and induces a conformational change in the protein which allows the binding of ATP to the modified membranes. This in turn leads to the change in membrane permeability. The following observations support the first possibility. In our experiments, the nucleotides had to be present during the modification by MBI and, after their removal, the permeability of the vesicles to Ca^{2+} remained high. The possibility that the ATP present in the Ca^{2+} -uptake assay medium acted as the activator of Ca^{2+} released was ruled out because the high Mg^{2+} concentration present in the assay medium would prevent the effect of ATP (Fig. 3). Moreover, MBI-modified membranes previously separated from ATP and MBI and then passively loaded with Ca^{2+} rapidly released their Ca^{2+} content in the absence of ATP (Fig. 5). Thus it seems that ATP, by its binding

to a specific protein(s), allows the activation of Ca^{2+} efflux by the chemical modification with MBI.

In both skinned muscle fibres (Endo, 1981; Martonosi, 1984) and fragmented sarcoplasmic reticulum vesicles (Ohnishi, 1979; Nagasaki & Kasai, 1981; Meissner, 1984) Mg^{2+} , at physiological levels, inhibited Ca^{2+} -induced Ca^{2+} release. However, under these conditions a marked Ca^{2+} release could still be induced from sarcoplasmic reticulum vesicles by external Ca^{2+} and $\text{App}[\text{CH}_2]\text{p}$. This could be prevented by further increasing the Mg^{2+} concentration to 10 mM (Meissner, 1984). The experiments described here (Fig. 3, Table 3) showed that either Mg^{2+} , Ca^{2+} or Mn^{2+} , when present during the modification, prevented the increase in the membrane permeability for Ca^{2+} produced by MBI modification. This effect was probably not due to the formation of a metal-ATP complex, and thus to a decrease in the concentration of free ATP, because the effect of the cation was obtained when the free ATP concentration was either 20 μM or 1 mM. Thus, the mechanism by which the divalent cations prevented the change in the membrane permeability is more complex, suggesting a direct binding to a protein(s), thereby preventing the effects of ATP and/or the modification by MBI. It is clear that the effect of the cations takes place during the modification because the addition of divalent cations after the modification had no effect on the properties of the modified membranes. These results suggest the involvement of a divalent-cation-binding site in the Ca^{2+} release system activated by chemical modification with MBI in the presence of ATP. A similar suggestion has been proposed for Ca^{2+} -induced Ca^{2+} release (Endo, 1981; Meissner, 1984).

There are several similarities between the enhancement of Ca^{2+} -induced Ca^{2+} release by adenine nucleotides and the effect of nucleotides in stimulating Ca^{2+} release by MBI-modification, which might indicate that a common release system might have been involved. (a) The concentration of the nucleotide required for the effects was in the millimolar range (Fig. 2; Ogawa & Ebashi, 1976; Meissner, 1984; Endo, 1981). (b) The nucleotides probably did not exert their effect through binding to the ATPase (Tables 5 and 6; Morii & Tonomura, 1983; Martonosi, 1984; Meissner, 1984). (c) High concentrations of divalent cations prevented the effect of the nucleotide (Table 3 and Fig. 3; Morii & Tonomura, 1983; Meissner, 1984). (d) The non-hydrolysable ATP analogues acted as well as ATP (Table 2; Ogawa & Ebashi, 1976; Ohnishi, 1979; Endo, 1981; Nagasaki & Kasai, 1981; Morii & Tonomura, 1983; Meissner, 1984).

The question of whether the effect of adenine nucleotide in stimulating Ca^{2+} -induced Ca^{2+} release and the effect of the nucleotide on the stimulation of Ca^{2+} release by modification with MBI, described here, are due to the interaction of the nucleotide with the same protein factor(s) remains to be clarified.

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