Stimulation of Ca^{2+} efflux from sarcoplasmic reticulum by preincubation with ATP and inorganic phosphate

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Preincubation of sarcoplasmic reticulum with 1 mM-ATP completely inhibits Ca^{2+} accumulation and stimulates ATPase activity by over 2-fold. This effect of ATP is obtained only when the preincubation is carried out in the presence of P_i , but not with arsenate, chloride or sulphate. The inhibition by ATP of Ca^{2+} accumulation is pH-dependent, increasing as the pH is increased above 7.5. Inhibition of Ca^{2+} accumulation is observed on preincubation with ATP, but not with CTP, UTP, GTP, ADP, adenosine 5'-[$\beta\gamma$ -methylene]triphosphate or adenosine 5'-[$\beta\gamma$ -imido]triphosphate. The presence of Ca^{2+} , but not Mg^{2+} , during the preincubation, prevents the effect of ATP + P_i on Ca^{2+} accumulation. The ATP + P_i inhibition of Ca^{2+} accumulation is not due to modification of the ATPase catalytic cycle, but rather to stimulation of a rapid Ca^{2+} efflux from actively or passively loaded vesicles. This Ca^{2+} efflux is inhibited by dicyclohexylcarbodi-imide. Photoaffinity labelling of sarcoplasmic-reticulum membranes with 8-azido-[α - ^{32}P]ATP resulted in specific labelling of two proteins, of approx. 160 and 44 kDa. These proteins were labelled in the presence of P_i , but not other anions.

INTRODUCTION

Muscle contraction and relaxation are controlled by the intra-fibre free Ca^{2+} concentration, which, in turn, is regulated by the sarcoplasmic reticulum (Endo, 1977; Tada *et al.*, 1978; De Meis & Vianna, 1979; Ikemoto, 1982; Martonosi, 1984). Thus the major functions of this membrane are Ca^{2+} uptake, storage and release. Ca^{2+} uptake occurs through the (Ca^{2+} + Mg^{2+})-dependent ATPase (Racker, 1972), and its enzymic mechanism is understood in rather detailed terms (De Meis & Vianna, 1979). The steps that link depolarization of the sarcolemma and transverse tubular membranes to the release of Ca^{2+} ions from the sarcoplasmic reticulum are poorly understood (Endo, 1977). Several mechanisms have been proposed for the physiological release of Ca^{2+} , including activation by external Ca^{2+} , 'depolarization' of the membrane, a change in membrane surface charge and/or a pH gradient, or inositol trisphosphate (Ebashi, 1976; Endo, 1977; Shoshan *et al.*, 1981, 1983; Winegrad, 1982; Ikemoto *et al.*, 1984; Volpe *et al.*, 1985; Vergara *et al.*, 1985).

The involvement of nucleotides in the Ca^{2+} -release process has been previously reported (Ogawa & Ebashi, 1976; Endo, 1981; Endo *et al.*, 1981; Morii & Tonomura, 1983; Meissner, 1984). Adenine nucleotides, in the millimolar range, enhance the rate of Ca^{2+} -induced Ca^{2+} release more than 1000-fold, without causing a major change in the Ca^{2+} affinity of the gate (Endo, 1981). Morii & Tonomura (1983) proposed that the Ca^{2+} -release channel may exist in inactive and active forms and that adenine nucleotides shift the equilibrium towards the active form. However, it seems that the nucleotide-binding site involved in Ca^{2+} -induced Ca^{2+} release also can bind CTP, because CTP inhibits the accelerating effect of p[NH]ppA (Morii & Tonomura, 1983). Several reports have suggested that ATP may

affect the membrane permeability for Ca^{2+} through a mechanism which is different from the Ca^{2+} -induced Ca^{2+} release. Millman & Azari (1977) showed that the exposure of Ca^{2+} -loaded vesicles to Mg^{2+} -free ATP induces a rapid transient release of Ca^{2+} ions. Chiesi & Wen (1983) observed that the rapid phase of ATP-induced Ca^{2+} release from vesicles passively loaded with Ca^{2+} is composed of two components, one involving the phosphorylated intermediate of the Ca^{2+} -ATPase, and the other (which can be induced by p[CH₂]ppA, as well as ATP) apparently requiring only binding of the nucleotide. Thus the effect of ATP on membrane permeability is complex, and probably is a result of interaction with different proteins. In the present study, the stimulation of Ca^{2+} efflux from sarcoplasmic-reticulum vesicles by preincubation with ATP in the presence of P_i , and a specific labelling of 160 kDa protein by 8-N₃-[α - ^{32}P]ATP, are described.

EXPERIMENTAL

Materials

ATP, ADP, p[NH]ppA, p[CH₂]ppA, CTP, UTP, GTP, EGTA and Tricine were obtained from Sigma Chemical Co. $^{45}\text{CaCl}_2$ was from The Radiochemical Centre (Amersham, Bucks., U.K.), and [^{32}P]P_i from Nuclear Research Center, Negev, Israel. 8-N₃-[α - ^{32}P]ATP (6.6 Ci/mmol) was obtained from ICN. [γ - ^{32}P]ATP was synthesized from [^{32}P]P_i and ADP by phosphorylation with lettuce chloroplasts (Avron, 1960) and was purified on Dowex 1-X8 with NaCl as the eluent.

Membrane preparation

Sarcoplasmic-reticulum vesicles were prepared from rabbit fast-twitch skeletal muscle as described by Camp-

Abbreviations used: p[CH₂]ppA, adenosine 5'-[$\beta\gamma$ -methylene]triphosphate; p[NH]ppA, adenosine 5'-[$\beta\gamma$ -imido]triphosphate; DDCD, dicyclohexylcarbodi-imide; 8-N₃-ATP, 8-azido-ATP; FITC, fluorescein isothiocyanate.

bell & MacLennan (1981). This preparation contains a mixture of light, heavy and intermediate vesicles. Heavy and light sarcoplasmic-reticulum vesicles were isolated as described by Meissner (1984). The protein concentration was determined by the method of Lowry *et al.* (1951).

Modification of sarcoplasmic reticulum with ATP

Sarcoplasmic-reticulum membranes (1 mg/ml) were incubated with ATP (0.5–2.0 mM) in 0.1 M-potassium phosphate, pH 8.0. The mixture was incubated for 10 min at 24–28 °C, then the samples were transferred to ice. Samples were diluted 10-fold with the reaction mixture for Ca^{2+} transport or ATPase activity and assayed as described below.

In some experiments as indicated in the Table or Figure legends, the modified membranes were separated from the unchanged reagent by centrifugation-chromatography (Penefsky, 1977) on a column of Sephadex G-50 (fine grade) equilibrated with 20 mM-Tricine/100 mM-NaCl, pH 7.2. The protein concentration in each sample was determined (Lowry *et al.*, 1951).

Assays

Active Ca^{2+} uptake by sarcoplasmic-reticulum vesicles was determined by Millipore filtration with $^{45}\text{CaCl}_2$. The basic reaction mixture contained 20 mM-Mops, pH 6.8, 100 mM-KCl, 3 mM- MgCl_2 , 3 mM-ATP, 0.5 mM- CaCl_2 (containing ^{45}Ca , about 10^6 c.p.m./ μmol), 0.5 mM-EGTA and 50 mM-potassium phosphate. The uptake was started by the addition of sarcoplasmic reticulum (40–80 $\mu\text{g}/\text{ml}$). After incubation for 1 or 2 min at room temperature, 0.18 ml samples were filtered through nitrocellulose filters (0.3 μm pore), and washed with 5 ml of 0.15 M-KCl. Radioactivity on the filters was measured in a liquid-scintillation counter. ATPase activity was assayed under conditions similar to those used for Ca^{2+} uptake, except that $^{45}\text{CaCl}_2$ was omitted and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to the reaction mixture. Ca^{2+} efflux from passively or actively loaded vesicles, and E-P (phosphoenzyme) formation, were assayed as described in Table or Figure legends.

RESULTS

The effect of preincubation of sarcoplasmic-reticulum vesicles with ATP and P_i on their Ca^{2+} accumulation and ATPase activities is shown in Fig. 1. More than 2-fold stimulation of ATPase activity and about 90% inhibition of Ca^{2+} accumulation is obtained on preincubation of the membranes with 1 mM-ATP for 10 min. A half-maximal effect is obtained with 0.3–0.4 mM-ATP for both the inhibition of Ca^{2+} accumulation and the stimulation of ATPase activity, under the conditions of this experiment.

As shown in Fig. 2(a), the inhibition of Ca^{2+} accumulation by preincubation of sarcoplasmic-reticulum vesicles with P_i +ATP is strongly pH-dependent, increasing as the pH is increased above 7.5. In the presence of 100 mM- P_i and 1 mM-ATP and at pH 8.0, maximal inhibition of Ca^{2+} accumulation is obtained after about 10 min of incubation (Fig. 2b). In Fig. 3, the P_i -concentration-dependence of the inhibition of Ca^{2+} accumulation by preincubation of the membranes with ATP is shown. In the presence of 1 mM-ATP, and after 10 min preincubation, half-maximal inhibition of Ca^{2+} accumulation is obtained at P_i concentrations between 30 and 40 mM. On the other hand, preincubation of the

Fig. 1. Effect of preincubation of sarcoplasmic-reticulum vesicles with ATP, in the presence of P_i , on Ca^{2+} accumulation and ATPase activity

Sarcoplasmic-reticulum membranes (1 mg/ml) were incubated with the indicated concentration of ATP in the presence of 0.1 M-potassium phosphate, pH 8.0. After incubation for 10 min at room temperature, samples (20 μg of protein) were assayed for Ca^{2+} uptake and ATPase activity as described in the Experimental section.

membranes with 100 mM- P_i without ATP stimulates Ca^{2+} accumulation by about 1.5-fold. This is probably because of its accumulation in the vesicles during the preincubation time, which leads to increased Ca^{2+} accumulation.

The effect of preincubation of the membranes with ATP+ P_i on their Ca^{2+} accumulation or ATPase activity was obtained with the heavy fraction of sarcoplasmic reticulum (Meissner, 1984) and with membranes previously washed with 0.6 M-KCl or washed twice with 1 mM-EGTA (results not shown), as well as with the membranes used in most of this study (see the Experimental section). In addition, the presence of quercetin, an inhibitor of Ca^{2+} -ATPase (Shoshan & MacLennan, 1981), during the preincubation of the membranes with ATP+ P_i has no effect on the inhibition of Ca^{2+} accumulation (results not shown).

A number of experiments demonstrate that the effect of preincubation of the membranes with ATP+ P_i on their Ca^{2+} accumulation and ATPase activity is not rapidly reversible, and is not due to the presence of nucleotides bound to the membrane at the time of the determination of Ca^{2+} uptake. Table 1 shows that the effect of the preincubation of the membranes with ATP+ P_i on their Ca^{2+} -accumulation capability is retained after the removal of free ATP and P_i by the centrifugation-chromatography (Penefsky, 1977). After preincubation of the membrane with 0.15 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and then passing the membranes through Sephadex G-50 (Penefsky, 1977), 0.25 nmol of $^{32}\text{P}/\text{mg}$ of protein is incorporated into the membranes.

The nucleotide specificity of the inhibition of Ca^{2+}

Fig. 2. Effect of pH and time of incubation on the inhibition of Ca²⁺ accumulation by preincubation with ATP + P_i

Sarcoplasmic-reticulum vesicles were incubated with 100 mM-potassium phosphate in the absence (○) or presence (●) of 3 mM-ATP at the indicated pH (a). In (b), the membranes were incubated at pH 8.0 for the indicated time. After incubation for 10 min in (a), or the indicated time in (b), samples were assayed for Ca²⁺ accumulation as described in the Experimental section.

Fig. 3. P_i-dependence of ATP inhibition of Ca²⁺ accumulation by sarcoplasmic-reticulum vesicles

Preincubation of sarcoplasmic-reticulum membranes without (○) or with (●) 1 mM-ATP was carried out as described in Fig. 1, except that potassium phosphate was present at the indicated concentration. At potassium phosphate concentrations below 50 mM, KCl (50 mM) was also added to the incubation medium. Ca²⁺ uptake was assayed as described in the Experimental section.

accumulation is shown in Table 2. Inhibition of Ca²⁺ accumulation was obtained by preincubation with adenine nucleotide, but not with CTP, UTP or GTP. AMP and the ATP analogues p[NH]ppA and p[CH₂]-ppA have almost no activity in all the sarcoplasmic-reticulum preparations tested. However, ADP was partially effective in some preparations, as shown in Table 2, but in other preparations was completely

Table 1. ATP inhibition of Ca²⁺ accumulation remains after centrifugation-chromatography of the ATP-modified membranes

Sarcoplasmic reticulum was incubated with ATP as described in Fig. 1. After 10 min, samples were assayed for Ca²⁺-uptake activity, and 0.12 ml portions were subjected to rapid chromatography (Penefsky, 1977) on a Sephadex G-50 column equilibrated with 0.1 M-NaCl/20 mM-Tricine, pH 7.2. Samples were assayed for protein concentration and Ca²⁺-uptake activity as described in the Experimental section.

Preincubation with:	Ca ²⁺ uptake (μmol/min per mg of protein)	
	Before G-50	After G-50
P _i (100 mM)	1.11	0.85
P _i + 0.5 mM-ATP	0.17	0.18
P _i + 2.0 mM-ATP	0.05	0.04

ineffective. This observation may suggest that its activity is due to its conversion into ATP by adenylate kinase, which contaminated some sarcoplasmic-reticulum preparations. Table 2 also shows that the presence of CTP or UTP together with ATP in the preincubation has no significant effect on the degree of ATP inhibition of Ca²⁺ accumulation. This suggests that the ATP-binding site responsible for the effect described here is very specific for ATP. This nucleotide specificity is different from that obtained for the (Ca²⁺ + Mg²⁺)-ATPase activity (Makinose & The, 1965).

The inhibition of Ca²⁺ accumulation and stimulation of ATPase activity by preincubation of the membranes with ATP were obtained only if the incubation was carried out in the presence of P_i, but not in the presence of other anions such as chloride, arsenate or sulphate

Table 2. Effect on Ca^{2+} uptake of preincubation of sarcoplasmic-reticulum vesicles with different nucleotides

Sarcoplasmic-reticulum membranes (1 mg/ml) were incubated with 0.1 M-potassium phosphate and the indicated nucleotide at pH 8.0 for 10 min. Then the membranes were subjected to centrifugation-chromatography as described in the Experimental section. Ca^{2+} uptake was assayed as described in Fig. 1.

Expt. no.	Nucleotides present in preincubation	Concn. (mM)	Ca^{2+} uptake ($\mu\text{mol}/\text{min}$ per mg of protein)
I	None	—	1.12
	AMP	1.0	0.89
		2.0	1.02
	ADP	1.0	0.90
		2.0	0.63
	ATP	1.0	0.32
		2.0	0.23
	p[NH]ppA	1.0	0.95
		2.0	0.90
	p[CH ₂]ppA	1.0	1.09
		2.0	1.07
	UTP	1.0	1.01
		2.0	0.95
	CTP	1.0	0.93
		2.0	0.96
	GTP	0.5	1.03
		1.0	0.98
II	None	—	1.12
	ATP	2.0	0.13
	CTP	2.0	0.96
	UTP	2.0	1.35
	ATP + CTP	2.0 + 2.0	0.16
	ATP + UTP	2.0 + 2.0	0.24

(Table 3). Oxalate (5 mM) also did not replace P_i in this ATP-dependent effect (results not shown).

The effect of the bivalent cations Ca^{2+} and Mg^{2+} on the inhibition of Ca^{2+} accumulation by preincubation of the membranes with ATP + P_i is shown in Table 4. As shown, inhibition of Ca^{2+} accumulation is also obtained when the membranes were preincubated with ATP in the presence of Mg^{2+} , but not of Ca^{2+} . The effect of Ca^{2+} in preventing the modification by ATP could be a result of a decrease in ATP concentration, due to its hydrolysis. However, the presence of quercetin, which inhibits the Ca^{2+} -ATPase activity of sarcoplasmic reticulum (Shoshan & MacLennan, 1981), during the preincubation did not interfere with the effect of Ca^{2+} in preventing the effect of ATP (results not shown). Thus it is possible that the Ca^{2+} effect is due to its interaction with a specific site and preventing the modification by ATP.

Several partial reactions of the Ca^{2+} -dependent ATPase were not changed by the modification of the membranes with ATP. Similar steady-state amounts of E-P formed from ATP are obtained with membrane preincubated with P_i or with P_i plus 0.5 mM-ATP, although the rate of Ca^{2+} accumulation by the ATP-modified membranes is about 18% of that of the unmodified membranes (Table 5). Preincubation of the membranes with ATP in the absence of P_i has no effect on Ca^{2+} accumulation (as shown in Table 3) or on E-P

Table 3. Effect of preincubation of sarcoplasmic reticulum with ATP in the presence of different anions on Ca^{2+} uptake

Sarcoplasmic-reticulum vesicles (1 mg/ml) were incubated with 100 mM of the indicated salt, in the absence or presence of 2 mM-ATP, and at pH 8.0 for 10 min. Ca^{2+} uptake was assayed as described in the Experimental section.

Preincubation with:	Ca^{2+} uptake ($\mu\text{mol}/\text{min}$ per mg of protein)	ATPase activity ($\mu\text{mol}/\text{min}$ per mg of protein)
NaCl (100 mM)/ Tricine (20 mM)	0.73	0.80
NaCl, Tricine + ATP	0.64	0.74
Potassium phosphate	0.82	0.82
Potassium phosphate + ATP	0.19	1.72
Potassium arsenate	0.87	0.85
Potassium arsenate + ATP	0.62	0.76
K_2SO_4	0.53	0.79
K_2SO_4 + ATP	0.58	0.79

Table 4. Effect of Ca^{2+} and Mg^{2+} on the inhibition of Ca^{2+} accumulation by preincubation of sarcoplasmic reticulum with ATP and P_i

Membranes were incubated with 100 mM-potassium phosphate in the absence or the presence of the indicated compounds as described in the Experimental section. After 10 min, samples were assayed for Ca^{2+} -accumulation capacity as described in Fig. 1. The numbers in parentheses indicate percentage inhibition.

Preincubation with potassium phosphate and:	Ca^{2+} uptake ($\mu\text{mol}/\text{min}$ per mg of protein)
—	1.29
ATP (0.5 mM)	0.38 (71)
Mg^{2+} (5 mM)	1.29
Mg^{2+} + ATP	0.26 (80)
Ca^{2+} (0.2 mM)	1.23
Ca^{2+} + ATP	1.15 (7)

formation. Similar results were obtained for the E-P formed from P_i (results not shown). The Ca^{2+} -dependence of the ATPase activity is similar for the unmodified and the ATP-modified membrane (results not shown). This result indicates that the ATPase activity remains dependent on Ca^{2+} and, therefore, that the decrease in Ca^{2+} accumulation observed in ATP-modified membranes is not due to a change in the Ca^{2+} -binding properties of the (Ca^{2+} + Mg^{2+})-ATPase.

Since it appears that the modification of the membrane by ATP in the presence of P_i does not affect the catalytic cycle of the Ca^{2+} -transport system, its effect on the

Table 5. Steady-state amounts of phosphoprotein (E-P) formed from ATP by unmodified and ATP-modified membranes

Sarcoplasmic-reticulum vesicles were incubated with 100 mM-potassium phosphate, pH 8.0, or with 20 mM-Tricine/0.1 M-NaCl, pH 8.0, in the absence or the presence of the indicated concentration of ATP, as described in the Experimental section. After 10 min of incubation, the membranes were passed through Sephadex G-50 (Penefsky, 1977), and samples were assayed for Ca²⁺ uptake and E-P formation. ATPase phosphorylation was obtained in the presence of 30 mM-Tris/maleate (pH 6.8), 100 mM-KCl, 20 mM-MgCl₂, 0.1 mM-CaCl₂, 10 μM-[γ-³²P]ATP (8 × 10⁴ c.p.m./nmol) and 0.75 mg of protein/ml. The reaction was stopped after 30 s by the addition of cold trichloroacetic acid (final concn. 5%). Samples were filtered through nitrocellulose filters (0.3 μm) and washed with 5 × 5 ml of 5% trichloroacetic acid/0.1 mM-ATP/2 mM-P_i, and the filters were counted for radioactivity in a scintillation counter.

Incubation conditions	Ca ²⁺ uptake (μmol/min per mg of protein)	E-P formed (nmol/mg of protein)
Potassium phosphate	1.2	4.97
Potassium phosphate	0.22	5.05
+ ATP (0.5 mM)		
Potassium phosphate	0.14	4.18
+ ATP (1 mM)		
Tricine/NaCl	0.94	3.86
Tricine/NaCl	0.89	3.80
+ ATP (0.5 mM)		
Tricine/NaCl	0.62	3.17
+ ATP (1 mM)		

membrane permeability for Ca²⁺ was tested. Fig. 4 shows the Ca²⁺ efflux from unmodified or ATP-modified membranes, passively or actively loaded with Ca²⁺. In Fig. 4(a), Ca²⁺ efflux from vesicles actively loaded with calcium phosphate is shown. A rapid Ca²⁺ efflux is obtained from vesicles preincubated with ATP + P_i upon 20-fold dilution of the vesicles into the efflux medium. It should be noted that, under the conditions used (pH 7.0, 100 mM-P_i and 1 mg of protein/ml), the Ca²⁺ content of the vesicles preincubated with P_i or with ATP + P_i was identical after the incubation period. As shown in Fig. 4(b), the membranes modified with ATP and then passively loaded with 5 mM-Ca²⁺ release about 60% of their trapped Ca²⁺ within 5 s. In contrast, the unmodified membranes release less than 5% of their Ca²⁺ content during the same period of time. The rate of Ca²⁺ efflux from ATP-modified membranes shows two phases, a rapid one completed in less than 5 s, and a slow phase. The slow phase could be due to the slow dissociation of the Ca-P_i complex, which may be formed because of the consecutive incubations with ATP + P_i and with Ca²⁺; although the membranes were passed through a Sephadex column after the preincubation with ATP + P_i, some P_i may be trapped in the vesicles. Fig. 4(b) also shows that in sarcoplasmic-reticulum membranes treated with fluorescein isothiocyanate (FITC), in which the ATPase activity and the coupled Ca²⁺ transport were inhibited by

about 70%, the membrane permeability for Ca²⁺ is the same as in untreated membranes. These results suggest that modification of the ATP-binding site of the Ca²⁺-ATPase with FITC (Pick & Karlsh, 1980; Mitchinson *et al.*, 1982) has no effect on the membrane permeability to Ca²⁺. Therefore the (ATP + P_i)-induced Ca²⁺ efflux is due to modification of an ATP-binding site other than that of the Ca²⁺-ATPase. The increased efflux from ATP-modified membranes was Ca²⁺-specific and was not linked to a general increase of membrane permeability, since the efflux kinetics of [³²P]phosphate or [¹⁴C]sucrose from passively loaded vesicles are not significantly affected by the modification (results not shown).

These observations suggest that modification with ATP decreases the Ca²⁺ accumulation by sarcoplasmic-reticulum vesicles owing to stimulation of Ca²⁺ release, rather than by uncoupling Ca²⁺ transport from ATP hydrolysis during the catalytic cycle of the Ca²⁺-ATPase.

The enhanced Ca²⁺ efflux from sarcoplasmic-reticulum vesicles, caused by preincubation with ATP in the presence of P_i, was completely prevented by dicyclohexylcarbodi-imide (DCCD) (Table 6). Because of the short time of incubation of the loaded vesicles with DCCD, a relatively high concentration of it was used. It has been shown previously (Shoshan *et al.*, 1981; Yamamoto & Kasai, 1982) that DCCD blocks Ca²⁺ release induced by elevated pH or external Ca²⁺. The results in Table 6 suggest that ATP increases membrane permeability for Ca²⁺ by modification of a specific protein(s) involved in Ca²⁺ release.

In an attempt to identify possible proteins(s) which were modified with ATP in the presence of P_i, 8-N₃-[α-³²P]ATP was used as a photoaffinity label for ATP-binding sites. Fig. 5 shows the results of labelling sarcoplasmic-reticulum membranes with 8-N₃-[α-³²P]-ATP in the presence of 100 mM-P_i, pH 8.0, or 100 mM-NaCl/20 mM-Tricine, pH 8.0. Under both conditions, incorporation of 8-N₃-[α-³²P]ATP into 220, 85 and 53 kDa proteins is observed. However, specific labelling of two additional proteins, of 160 kDa and 44 kDa, was obtained only when the labelling with 8-N₃-[α-³²P]ATP was carried out in the presence of P_i. The increase in membrane permeability produced by incubation with ATP + P_i may be related to modification of one or both of the proteins specifically labelled with 8-N₃-[α-³²P]-ATP.

DISCUSSION

The main findings in this study are that preincubation of sarcoplasmic-reticulum membranes with ATP in the presence of P_i at alkaline pH increases membrane permeability for Ca²⁺, and that under these conditions two proteins, of 160 kDa and 44 kDa, are specifically labelled by the photoaffinity probe 8-N₃-[α-³²P]ATP. The first question is whether the (ATP + P_i)-induced decrease in Ca²⁺ accumulation is due to the interaction of ATP with the (Ca²⁺ + Mg²⁺)-ATPase, and modification of the Ca²⁺-ATPase catalytic cycle, or alternatively to binding and modification of different protein(s). Several observations suggest that it is unlikely that the ATP effect results from its binding to the Ca²⁺-ATPase. (a) Modification of the ATP-binding site of the Ca²⁺-ATPase by FITC (Pick & Karlsh, 1980; Mitchinson *et al.*, 1982) has no effect on the membrane permeability (Fig. 4b). (b) The nucleotide

Fig. 4. Ca^{2+} efflux from unmodified and (ATP + P_i)-modified sarcoplasmic-reticulum membranes

In (a), the vesicles were actively loaded with Ca^{2+} by incubation of sarcoplasmic reticulum (100 $\mu\text{g}/\text{ml}$) in the reaction mixture for Ca^{2+} uptake as described in the Experimental section. After 15 min the vesicles were collected by centrifugation at 100 000 g for 20 min and resuspended in 0.1 M-potassium phosphate, pH 7.0, at a protein concentration of 1 mg/ml. The vesicles actively loaded with calcium phosphate were incubated without (○) or with (●) 1 mM-ATP for 10 min and then diluted 20-fold in 20 mM-Mops (pH 6.8)/100 mM-KCl/1 mM-EGTA to start the assay for Ca^{2+} efflux. At the indicated time, samples were filtered and washed as described for determination of Ca^{2+} content in the Experimental section. Ca^{2+} content of unmodified or (ATP + P_i)-modified membranes was determined by the filtration and washing of 20 μl of membranes with no previous dilution, and it was the same, 0.38 $\mu\text{mol}/\text{mg}$ of protein. In (b), for the passively loaded sarcoplasmic-reticulum vesicles, the membranes (1 mg/ml) were incubated for 10 min with 0.1 M-potassium phosphate, pH 8.0, without (○) or with (●) 2 mM-ATP or with 10 μM -FITC (▲). Ca^{2+} uptake activity was 1.25, 0.21 and 0.41 $\mu\text{mol}/\text{min}$ per mg of protein for control, (ATP + P_i)-modified and FITC-modified membranes respectively. The treated vesicles were centrifuged at 80 000 g for 30 min and resuspended (1.8 mg/ml) in 20 mM-Mops (pH 6.8)/100 mM-KCl/5 mM- $^{45}\text{CaCl}_2$ (1.5×10^7 c.p.m./ml) and were incubated for 2 h at 22 °C. For Ca^{2+} efflux, samples (30 μl) were rapidly filtered and washed for the indicated time with a solution identical with the loading solution, except that CaCl_2 was omitted. The washing rate was approx. 1 ml/s.

Table 6. Modification of sarcoplasmic reticulum with DCCD prevents the stimulation of Ca^{2+} efflux by preincubation with ATP + P_i

Sarcoplasmic-reticulum membranes were actively loaded with $^{45}\text{CaCl}_2$ as described in Fig. 4. The Ca^{2+} -loaded membranes were collected by centrifugation for 20 min at 100 000 g and resuspended in 0.1 M-potassium phosphate, pH 8.0. Samples (2 mg/ml) were incubated with or without 3 mM-ATP. After 10 min the membranes were diluted (1:1 v/v) with 100 mM-Mes (pH 5.0)/100 mM-NaCl. The final pH was 6.5. Then, the membranes were incubated for 3 min with or without 1 mM-DCCD. The final concentration of ethanol in control and DCCD-treated samples was 2%. Ca^{2+} efflux was assayed by 10-fold dilution of the treated membranes into 20 mM-Mops (pH 6.8)/100 mM-NaCl/1 mM-EGTA. Ca^{2+} content was assayed at the indicated times after dilution as described in the Experimental section.

Membrane treated with:	Ca ²⁺ content (μmol/mg of protein)			
	Time (s) ...	10	30	60
None		0.62	0.63	0.64
ATP		0.37	0.32	0.29
DCCD		0.59	0.63	0.61
ATP, then DCCD		0.64	0.60	0.55

specificity of the (Ca^{2+} + Mg^{2+})-ATPase activity and that of the system described here are different (Makinosi & The, 1965; Table 2). It seems that the latter site specifically binds ATP. (c) Preincubation of the membranes with

ATP in the presence of P_i has no effect on partial reactions of the transport cycle, such as E-P formation (Table 5). In addition, the dependence of Ca^{2+} -ATPase activity on Ca^{2+} concentration is similar for unmodified and (ATP + P_i)-modified membranes. 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 is not affected by the modification, and that the ATP-binding site involved in the modification of membrane permeability for Ca^{2+} is distinct from the NTP-binding site of the Ca^{2+} -ATPase.

The Ca^{2+} -efflux system activated here by ATP + P_i might be identical, at least in part, with other described Ca^{2+} -release systems. It has properties in common with several such systems. (a) Adenine nucleotide enhances Ca^{2+} -induced Ca^{2+} release (Ogawa & Ebashi, 1976; Ohnishi, 1979; Nagasaki & Kasai, 1981; Endo, 1981; Endo *et al.*, 1981; Morii & Tonomura, 1983; Meissner, 1984), and ATP is required for stimulation of Ca^{2+} efflux by chemical modification of sarcoplasmic reticulum with methylbenzimidate (Shoshan-Barmatz, 1987). (b) DCCD inhibits the Ca^{2+} efflux stimulated by ATP + P_i , as it does in the Ca^{2+} -induced Ca^{2+} release (Yamamoto & Kasai, 1982) and in the stimulation of Ca^{2+} release by elevated pH (Shoshan *et al.*, 1981).

A number of possible mechanisms may account for the effect of preincubation of sarcoplasmic-reticulum vesicles with ATP + P_i on the increased membrane permeability for Ca^{2+} . One of these is phosphorylation of protein(s) through protein kinase activity. This possibility is supported by the observations that the ATP analogues p[NH]ppA and p[CH₂]ppA are much less effective than ATP, and that P_i is required during the preincubation, conceivably as an inhibitor of the membrane-associated

Fig. 5. Photoaffinity labelling of sarcoplasmic-reticulum proteins with $8\text{-N}_3\text{-}[\alpha\text{-}^{32}\text{P}]\text{ATP}$

Sarcoplasmic-reticulum membranes (1 mg/ml) were incubated for 10 min at 22 °C in 0.1 M-sodium phosphate, pH 8.0 (1), or in 0.1 M-NaCl/20 mM-Tricine, pH 8.0 (2). Then MgCl_2 and $8\text{-N}_3\text{-}[\alpha\text{-}^{32}\text{P}]\text{ATP}$ were added (final concns. 5 mM and 1.4 μM respectively). After incubation in dark for 5 min at 4 °C, the samples were then irradiated for 60 s with a focused light beam from a 100 W high-pressure mercury lamp (PRA Photochemical Research Associates, London, Ontario, Canada). The labelled vesicles were analysed by SDS/polyacrylamide (10%) gel electrophoresis (Laemmli, 1970), stained with Coomassie Blue (a), and the dried gel was then subjected to autoradiography on Kodak X-Omat film (b); 50 μg of protein was applied to each lane. The positions of proteins that were labelled with $\text{N}_3\text{-}[\alpha\text{-}^{32}\text{P}]\text{ATP}$ are indicated; 'ATPase' is ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase. In (b), the proteins labelled with $\text{N}_3\text{-}[\alpha\text{-}^{32}\text{P}]\text{ATP}$, and the positions of M_r standards are indicated.

Ca^{2+} -inhibited protein phosphatase activity present in sarcoplasmic reticulum (Varsanyi & Heilmeyer, 1979). The absence of a Mg^{2+} requirement for the $(\text{ATP} + \text{P}_i)$ -induced Ca^{2+} efflux might appear to exclude this possibility, since kinase activity is usually strictly dependent on Mg^{2+} . However, the fact that a significant amount of ATP is hydrolysed and phosphorylation of sarcoplasmic-reticulum proteins is obtained with no added Mg^{2+} suggests that the endogenous Mg^{2+} concentration is high enough for this activity. The finding that ADP was also effective only in some sarcoplasmic-reticulum preparations in the modification of membrane permeability (Table 2) may suggest that its effect is due to its conversion into ATP by an adenylate kinase activity present in some membrane preparations.

Another possibility is a direct binding of ATP to sarcoplasmic-reticulum protein(s). In this case ATP would affect the membrane permeability by binding to

specific protein(s), involved in Ca^{2+} release, causing reorganization of a protein complex or a conformational change within a protein(s), in a relatively irreversible manner, under the required specific conditions (e.g. high P_i and alkaline pH). In this case, the function of P_i in this modification is not clear. The observation that the effect of ATP on the membrane permeability remained after the removal of free ATP (Table 1) is in agreement with the phosphorylation rather than with the ATP-binding mechanism. Although the results favour the phosphorylation mechanism, we cannot exclude definitely the ATP-binding mechanism. The involvement of protein phosphorylation in Ca^{2+} release has been suggested previously (MacLennan *et al.*, 1984; Kim & Ikemoto, 1985). However, although inhibition of Ca^{2+} release by Ca^{2+} - and calmodulin-dependent phosphorylation of 60 kDa protein is suggested, the results presented here suggest activation of the Ca^{2+} -release system by phosphorylation of the 160 kDa protein (see below). It is not clear whether the protein is autophosphorylated or phosphorylated by another protein with protein kinase activity.

We suggest that $(\text{ATP} + \text{P}_i)$ -modification of membrane permeability for Ca^{2+} is a result of specific modification of a protein component of the Ca^{2+} -release channel, probably by its phosphorylation. The finding that the 160 kDa and the 44 kDa proteins were labelled with $8\text{-N}_3\text{-}[\alpha\text{-}^{32}\text{P}]\text{ATP}$ only under the specific conditions (e.g. high P_i concentration and alkaline pH) which lead to the $(\text{ATP} + \text{P}_i)$ -induced increase in membrane permeability for Ca^{2+} may imply that one or both proteins are involved in the modification by ATP. Campbell & MacLennan (1983) observed a binding of $8\text{-N}_3\text{-ATP}$ to two intrinsic glycoproteins, of 160 and 53 kDa. Also, phosphorylation of skeletal-muscle sarcoplasmic-reticulum proteins of 46 and 42 kDa was observed by Campbell & Shamoo (1980) in the absence of EGTA, cyclic AMP or exogenous protein kinase. More recently, photolabelling of junctional sarcoplasmic reticulum with [^{14}C]doxorubicin, which resulted in labelling mainly of two proteins of 350 and 170 kDa, has been reported (Zorzato *et al.*, 1986). At the moment, the relationship between these proteins and the 160 kDa protein that is labelled under our specific conditions is not clear. The presence of ATP-sensitive channels was suggested in other membranes, such as those of skeletal muscle (Spruce *et al.*, 1985) and heart muscle (Noma, 1983), and in β -cells of pancreatic islets (Cook & Hales, 1984; Ashcroft *et al.*, 1984). Further investigation of the specific binding and effects of ATP might lead to an understanding of the entire Ca^{2+} -release system in the sarcoplasmic reticulum.

This research was supported by a grant from the Chief Scientist's Office, Ministry of Health, Israel. I thank Dr. D. M. Chipman for reading the manuscript and offering valuable suggestions. The excellent technical assistance of Mrs. Y. Lichtenfeld is greatly appreciated.

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Received 20 January 1987/20 May 1987; accepted 14 July 1987