The (Ca²⁺ + Mg²⁺)-stimulated ATPase of the rat parotid endoplasmic reticulum

Pangajavalli THIYAGARAJAH and Soh Chan LIM

Department of Biochemistry, Faculty of Medicine, National University of Singapore, Lower Kent Ridge Road, Singapore 0511

A membrane fraction enriched in endoplasmic reticulum was prepared from rat parotid glands by using sucrose-gradient centrifugation. The fraction showed a 10-fold increase in specific activity of NADPH: cytochrome *c* reductase activity over that of tissue homogenates and minimal contamination with plasma membranes or mitochondria. The endoplasmic reticulum fraction possessed both Mg²⁺-stimulated ATPase as well as Ca²⁺,Mg²⁺-ATPase [(Ca²⁺ + Mg²⁺)-stimulated ATPase] activity. The Ca²⁺,Mg²⁺-ATPase required 2–5 mM-Mg²⁺ for optimal activity and was stimulated by submicromolar concentrations of free Ca²⁺. The *K_m* for free Ca²⁺ was 0.55 μM and the average *V_{max}* was 60 nmol/min per mg of protein. The *K_m* for ATP was 0.11 mM. Other nucleotides, such as GTP, CTP or ADP, could not substitute for ATP in supporting the Ca²⁺-activated nucleotidase activity. Increasing the K⁺ concentration from 0 to 100 mM caused a 2-fold activation of the Ca²⁺,Mg²⁺-ATPase. Trifluoperazine, W₇, [*N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide] and vanadate inhibited the enzyme. The concentration of trifluoperazine and vanadate required for 50% inhibition of the ATPase were 52 μM and 28 μM respectively. Calmodulin, cyclic AMP, cyclic AMP-dependent protein kinase and inositol 1,4,5-trisphosphate had no effect on the ATPase. The properties of the Ca²⁺,Mg²⁺-ATPase were distinct from those of the Mg²⁺-ATPase, but comparable with those reported for the parotid endoplasmic-reticulum Ca²⁺-transport system [Kanagasuntheram & Teo (1982) *Biochem. J.* **208**, 789–794]. The results suggest that the Ca²⁺,Mg²⁺-ATPase is responsible for driving the ATP-dependent Ca²⁺ accumulation by this membrane.

INTRODUCTION

In recent years, ATP-driven Ca²⁺-transport systems located on the plasma membrane or the endoplasmic reticulum have been identified in a variety of tissues, including the liver (Moore *et al.*, 1975; Dawson, 1982; Lotersztajn *et al.*, 1984), adipocyte (Bruns *et al.*, 1976; Pershadsingh *et al.*, 1980a), pancreatic islet (Pershadsingh *et al.*, 1980b; Kotagal *et al.*, 1982), parotid (Selinger *et al.*, 1970; Kanagasuntheram & Teo, 1982a,b) and submaxillary (Terman & Gunter, 1983). These Ca²⁺ pumps are believed to function in conjunction with the Ca²⁺-transport system of the mitochondrial inner membrane in maintaining intracellular free Ca²⁺ concentrations at submicromolar levels. In addition, they also participate in the redistribution of intracellular Ca²⁺ that often accompanies hormonal or neural stimulation of tissues. Recent studies on liver (Burgess *et al.*, 1984; Joseph *et al.*, 1984) and pancreatic acinar (Streb *et al.*, 1983) cells have shown that the endoplasmic-reticular Ca²⁺ pool is the major source of Ca²⁺ mobilized by *myo*-inositol 1,4,5-trisphosphate (IP₃) and that IP₃ can induce rapid release of Ca²⁺ from isolated microsomes (Dawson & Irvine, 1984; Prentki *et al.*, 1984). Whereas these effects of IP₃ on endoplasmic-reticulum Ca²⁺ flux are transient, more prolonged effects of hormonal stimulation have also been reported. In the rat liver, for example, Ca²⁺ accumulation by the microsomal fraction is increased after stimulation of the liver cells with

glucagon (Waltenbaugh *et al.*, 1980) and decreased after exposure to insulin (Taylor *et al.*, 1979).

In common with the Ca²⁺-pumping ATPase of the sarcoplasmic reticulum (see review by De Meis & Inesi, 1982) the translocation of Ca²⁺ across the endoplasmic reticulum of cells is probably driven by Ca²⁺-activated ATPases. In some tissues, such as liver and adipose tissue (Black *et al.*, 1980; Dawson & Fulton, 1983), endoplasmic-reticulum- or microsomal-membrane-associated Ca²⁺-activated ATPases have been identified and shown to have properties similar to those of the corresponding Ca²⁺ pump. Further purification and reconstitution of these transport systems are, however, necessary before the identity of the Ca²⁺-activated ATPases and the Ca²⁺ pumps can be unequivocally demonstrated.

The rat parotid acinar cell possesses an extensive endoplasmic-reticular network containing a relatively active Ca²⁺-transporting system (Kanagasuntheram & Teo, 1982a). Ca²⁺ transport by this membrane is likely to be important not only in maintaining intracellular free Ca²⁺ at submicromolar levels, but also in releasing Ca²⁺ in response to Ca²⁺-mobilizing agonists. The mechanism by which this pump translocates Ca²⁺ has not been investigated, and the Ca²⁺-dependent ATPase that presumably catalyses this translocation has not been characterized. We present here some results of our studies aimed at identifying and characterizing the Ca²⁺-activated ATPase of this membrane.

Abbreviations used: Mg²⁺-ATPase, Mg²⁺-stimulated ATPase; Ca²⁺,Mg²⁺-ATPase, (Ca²⁺ + Mg²⁺)-stimulated ATPase; W₇, *N*-(6-aminohexyl)-5-chloronaphthalene-1-sulphonamide; IP₃, *myo*-inositol 1,4,5-trisphosphate; CDTA, *trans*-1,2-diaminocyclohexane-*NNNN*'-tetra-acetic acid.

EXPERIMENTAL

Materials

$^{45}\text{CaCl}_2$ was obtained from New England Nuclear, Boston, MA, U.S.A. All biochemicals were from the Sigma Chemical Co., St. Louis, MO, U.S.A. Calmodulin was a gift from Dr. J. Wang. IP_3 was prepared from human erythrocytes as described by Downes *et al.* (1982).

Methods

Subcellular fractionation. A parotid membrane fraction enriched in endoplasmic reticulum was prepared by using a sucrose density gradient. Parotid glands were dissected from four to eight male Wistar rats that had been starved overnight, cleaned and homogenized in 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.4)/0.1 mM-dithiothreitol as described previously (Kanagasuntheram & Teo, 1982b). The homogenate was centrifuged at 10000 *g* for 10 min and the supernatant removed carefully and re-centrifuged at 30000 *g* for 20 min. The microsomal pellet was suspended in 2 ml of the homogenizing buffer and layered over a step gradient consisting of 2 ml of 1.5 M-sucrose/10 mM-Tris/HCl (pH 7.4)/0.1 mM-dithiothreitol overlaid with similar buffer containing 0.5 M-sucrose. After centrifuging at 200000 *g* for 1 h in a Beckman SW 41 rotor, the pellet containing the endoplasmic-reticulum membrane was resuspended by gentle homogenization in 1–2 ml of the homogenizing buffer and used immediately for enzyme and other assays. All stages of the isolation procedure were carried out at 4 °C.

ATPase assay. The Ca^{2+} , Mg^{2+} -ATPase was determined by measuring the difference in the rate of release of P_i from ATP by membranes incubated in assay buffer containing 0.2 mM- CaCl_2 and 0.2 mM-EGTA (8.6 μM free Ca^{2+}) or 0.2 mM-EGTA alone. The free Ca^{2+} concentration was calculated by a computer program using stability constants as given by Sillén & Martell (1971). Unless otherwise stated, all assays were carried out at 37 °C in 0.5 ml of assay buffer of the following composition: 100 mM-KCl, 20 mM-Hepes, pH 6.8, 2 mM- MgCl_2 , 1 mM-ATP, 1 μg of oligomycin, the appropriate concentrations of CaCl_2 and EGTA and about 30 μg of endoplasmic-reticulum protein. The membranes were preincubated for 10 min in assay buffer lacking ATP. The reaction was initiated by addition of

ATP and after incubating for 30 min, 0.25 ml of 5% (w/v) trichloroacetic acid was added to terminate the reaction. The tubes were chilled and centrifuged to remove precipitated proteins. About 100–500 μl of the supernatant fraction was used for the determination of P_i released by a modification of the method of Baginski *et al.* (1967). All the ATPase assays were carried out in triplicate and ATP hydrolysis was limited to less than 20% of the amount added. When required, blanks to which the trichloroacetic acid solution was added before addition of ATP were used to correct for endogenous P_i present in the assay system.

Enzyme and other assays. $^{45}\text{Ca}^{2+}$ uptake was determined as described previously (Kanagasuntheram & Teo, 1982a) in medium containing 20 μM - $^{45}\text{CaCl}_2$. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. 5'-Nucleotidase activity was measured as described by Widnell & Unkeless (1968), and NADPH:cytochrome *c* reductase by the method of Strobel & Dignan (1978). One unit of enzyme activity is defined as the amount of enzyme required to convert 1 μmol of substrate into product in 1 min under the stated assay conditions.

RESULTS

Purification of endoplasmic-reticulum membranes

The use of a sucrose step gradient enabled us to obtain a parotid membrane fraction that was highly enriched in NADPH:cytochrome *c* reductase activity (see Table 1). Plasma-membrane-associated 5'-nucleotidase, which was a major contaminant in our previous preparation (Kanagasuntheram & Teo, 1982a), was effectively removed from the endoplasmic-reticulum pellet, since most of this activity was retained at the interface between 0.5 M- and 1.5 M-sucrose solutions. About 30% of the NADPH:cytochrome *c* reductase activity of the 30000 *g* pellet, representing about 11% of the total activity present in the homogenate, was recovered in the purified endoplasmic-reticulum fraction. This fraction showed a 10-fold increase in the specific activity of NADPH:cytochrome *c* reductase activity compared with that of tissue homogenates and a 1.6-fold increase compared with that of the 30000 *g* fraction. Corresponding to the

Table 1. Distribution of enzyme markers and $^{45}\text{Ca}^{2+}$ -transport activity in parotid subcellular fractions

Parotid glands were homogenized in 0.25 M-sucrose/10 mM-Tris/HCl/0.1 mM-dithiothreitol, pH 7.4, and fractionated as described under 'Methods'. The activities of NADPH:cytochrome *c* reductase, 5'-nucleotidase and $^{45}\text{Ca}^{2+}$ uptake by the 30000 *g* microsomal pellet and the purified endoplasmic-reticulum fraction were determined. Results shown are means $\pm$ S.E.M. for the numbers of preparations shown in parentheses. Abbreviation: N.D., not determined.

Parameter	Value		
	Subcellular fraction ...	30000 <i>g</i> pellet	Endoplasmic reticulum
	Tissue homogenate		
NADPH:cytochrome <i>c</i> reductase (munit/mg of protein)	2.96 $\pm$ 0.1 (4)	18.3 $\pm$ 1.1 (3)	28.3 $\pm$ 1.7 (3)
5'-Nucleotidase (munit/mg of protein)	22.4 $\pm$ 1.7 (3)	64.8 $\pm$ 7.2 (3)	7.8 $\pm$ 1.6 (3)
$^{45}\text{Ca}^{2+}$ uptake (nmol/min of protein)	N.D.	10.2 $\pm$ 1.4 (4)	18.2 $\pm$ 1.6 (14)

Fig. 1. Effect of Mg^{2+} concentration on Mg^{2+} -ATPase and $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase activities of parotid endoplasmic-reticulum membranes

Endoplasmic-reticulum membranes were incubated in ATPase assay buffer containing 0.2 mM-EGTA or 0.2 mM-EGTA and 0.2 mM- CaCl_2 and various amounts of Mg^{2+} as indicated. P_i released after 30 min was determined as described under 'Methods'. The $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase was calculated as the difference in the rate of P_i release in medium containing Ca^{2+} and that lacking it. (a): ○, Mg^{2+} -ATPase; ●, $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase and Mg^{2+} -ATPase. (b): ●, $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase. Results shown are the means of triplicate determinations in a representative experiment.

increase in activity of this enzyme, $^{45}\text{Ca}^{2+}$ uptake by the purified endoplasmic reticulum was also increased by about 1.8-fold compared with that of the 30000 g fraction. For reasons that are unclear at present, the rate of $^{45}\text{Ca}^{2+}$ uptake by endoplasmic-reticulum vesicles isolated by this procedure was initially less than 6 nmol/min per mg of protein. Preincubation in the assay buffer (lacking $^{45}\text{Ca}^{2+}$), however, resulted in rapid activation of the uptake mechanism until a constant rate of about 18 nmol/min per mg of protein was achieved after 20–30 min. The values quoted in Table 1 are the rates of $^{45}\text{Ca}^{2+}$ uptake by membrane vesicles that had been activated by prior incubation in assay buffer for 20 min. There was no effect of preincubation on the rate of $^{45}\text{Ca}^{2+}$ uptake by the 30000 g pellet.

Fig. 2. Time course of Mg^{2+} -ATPase and $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase activities

Parotid endoplasmic-reticulum membranes were incubated at 37 °C in assay buffer containing 0.2 mM-EGTA or 0.2 mM-EGTA and 0.2 mM- CaCl_2 . At various times, the reaction was terminated and ATPase activity determined. Results are the means $\pm$ S.E.M. of three determinations of a representative experiment. ○, Mg^{2+} -ATPase; ●, $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase.

Characterization of the Mg^{2+} -ATPase and $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase

Fig. 1 shows the effect of various amounts of added Mg^{2+} on the basal and Ca^{2+} -activated ATPase activities. In assay buffer containing 0.2 mM-EGTA, some hydrolysis of ATP occurred even when no added Mg^{2+} or Ca^{2+} was present. This residual activity probably represents ATPase activity that can be sustained by the small amount of endogenous Mg^{2+} present on the membrane, since it was completely abolished in the presence of 0.2 mM-CDTA. Addition of Mg^{2+} resulted in a steady increase in the rate of ATP hydrolysis, with maximal ATPase activity occurring at 0.5 mM or higher concentration of Mg^{2+} . The mean rate of ATP hydrolysis by membranes incubated with 2 mM- Mg^{2+} was 1.53 ± 0.21 μmol/30 min per mg of protein (mean $\pm$ S.E.M., $n = 7$). This activity is referred to as the 'basal' or ' Mg^{2+} -ATPase' activity. Addition of CaCl_2 to give a free Ca^{2+} concentration of 8.6 μM stimulated the ATPase activity. Although some Ca^{2+} -activated ATPase activity was detectable even in the absence of added Mg^{2+} , optimal $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase activity required 2–5 mM- Mg^{2+} . In medium containing 2 mM- Mg^{2+} , the mean $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase activity of the parotid endoplasmic-reticulum fraction was 1.23 ± 0.09 μmol/30 min per mg of protein (mean $\pm$ S.E.M., $n = 26$); the activities of different membrane preparations ranged from 0.54 μmol/30 min per mg of protein to 1.91 μmol/30 min per mg of protein.

Oligomycin at a concentration of 2 μg/ml had no effect on the endoplasmic reticulum $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase. This inhibitor of the mitochondrial ATPase was, however,

Table 2. Nucleotidase activity of endoplasmic-reticulum membranes

Parotid membranes were preincubated for 10 min in assay buffer containing all additions except ATP. Nucleotidase activity was initiated by addition of the nucleotide as indicated above, at a final concentration of 1 mM. P_i released during 30 min of incubation was determined. Appropriate blanks in which the reaction was terminated with trichloroacetic acid before addition of nucleotide were used to correct for endogenous P_i . Results are means $\pm$ S.E.M. of six determinations.

	Nucleotidase activity (μ mol of P_i /30 min per mg of protein)	
	Mg ²⁺ -stimulated	(Ca ²⁺ -Mg ²⁺)-stimulated
1 mM-AMP	0.33 $\pm$ 0.03	0
1 mM-ADP	0.64 $\pm$ 0.03	0.03 $\pm$ 0.02
1 mM-ATP	1.80 $\pm$ 0.12	0.64 $\pm$ 0.05
1 mM-CTP	1.87 $\pm$ 0.14	0.05 $\pm$ 0.01
1 mM-GTP	2.29 $\pm$ 0.16	0.03 $\pm$ 0.01
1 mM-GTP + 1 mM-ATP	2.43 $\pm$ 0.12	0.41 $\pm$ 0.08

routinely included in the assay buffer to eliminate any interference from mitochondrial ATPase activity; 2 mM-Ruthenium Red, an inhibitor of mitochondrial Ca²⁺ transport, also had no effect on the ATPase. In studies on the liver microsomal Ca²⁺-ATPase, Dawson & Fulton (1983) noted that the presence of a Ca²⁺ ionophore or a Ca²⁺-precipitating agent such as oxalate was necessary for maximal expression of ATPase activity. The parotid endoplasmic-reticulum Ca²⁺,Mg²⁺-ATPase activity, however, was not affected by the addition of either 2 or 8 μ M-ionophore A23187 or 5 or 10 mM-oxalate.

The Ca²⁺-activated-ATPase activity of the endoplasmic-reticulum membrane fraction remained stable when membranes were stored in ice for up to 4 h. Freezing and storage overnight at -70°C , however, resulted in a 15–20% loss of activity. Most of the experiments were therefore done on freshly isolated membranes.

Time course of ATP hydrolysis

Fig. 2 shows the time course of ATP hydrolysis by membranes incubated in the presence and in the absence of added Ca²⁺. ATP hydrolysis in assay buffer lacking Ca²⁺ proceeded at a rapid rate during the initial 10 min of incubation. Thereafter, hydrolysis proceeded at a lower, but steady, rate of about 21 nmol/min per mg of protein for up to 40 min. In contrast, Ca²⁺-activated ATP hydrolysis proceeded at a linear rate of 24 nmol/min per mg of protein throughout the first 40 min. After 40 min, both the basal Mg²⁺-ATPase as well as the Ca²⁺,Mg²⁺-ATPase activities showed a decline in their rates due to the depletion of ATP in the assay medium.

Nucleotide specificity

The ability of the parotid endoplasmic-reticulum fraction to hydrolyse nucleotides other than ATP is shown in Table 2. All the nucleotides tested were hydrolysed to various extents when incubated with membranes in buffer containing 2 mM-Mg²⁺. Nucleotidase activities using AMP or ADP as substrates were relatively low compared with Mg²⁺-ATPase activity. CTP and GTP were hydrolysed at rates comparable with, or slightly greater than, that of ATP hydrolysis. When Ca²⁺ was also included in the assay buffer, there was no significant increase in the rate of hydrolysis of any of these nucleotides, indicating that none of these could

substitute for ATP in driving the Ca²⁺,Mg²⁺-ATPase. Addition of 1 mM-GTP together with 1 mM-ATP inhibited the Ca²⁺-activated hydrolysis of ATP by about 43%. When both nucleotides were present, P_i released in the absence of added Ca²⁺ (presumably owing to the Mg²⁺-dependent hydrolysis of ATP as well as GTP) was also less than the sum of the rates of hydrolysis of ATP and GTP determined individually.

Kinetic parameters

The K_m for ATP was determined by measuring the Mg²⁺-ATPase and the Ca²⁺,Mg²⁺-ATPase activities in the presence of ATP concentrations ranging from 0.05 to 2 mM. In these experiments, the incubation time was decreased to 10 min and the protein concentration lowered to about 20 μ g/0.5 ml of assay mixture to ensure that ATP hydrolysis during incubation did not lead to significant changes in the concentration of the substrate. A Lineweaver–Burk transformation of the data points gave linear plots from which the K_m for ATP was calculated to be 0.11 ± 0.2 mM (mean $\pm$ S.E.M., $n = 3$) for the Ca²⁺,Mg²⁺-ATPase and 0.06 ± 0.01 mM for the Mg²⁺-ATPase. Fig. 3 shows the effect of varying the free Ca²⁺ concentration of the medium from 0.2 to 20 μ M on the Ca²⁺,Mg²⁺-ATPase activity. The Ca²⁺-activated ATPase activity increased as a function of free Ca²⁺ concentration until maximal rates were achieved at free Ca²⁺ concentrations greater than 5 μ M. From kinetic analysis of three different membrane preparations, the K_m for Ca²⁺ was found to be 0.55 ± 0.02 μ M; V_{max} for the same preparations ranged from 45 to 74 nmol/min per mg of protein, and the average V_{max} was 60 ± 8 nmol/min per mg of protein.

Effects of K⁺ and vanadate

In previous studies on the parotid endoplasmic-reticulum Ca²⁺-transport system (Kanagasuntheram & Teo, 1982a), we observed that ⁴⁵Ca²⁺ uptake was markedly stimulated by K⁺. To determine whether the Ca²⁺,Mg²⁺-ATPase was similarly influenced by K⁺, Ca²⁺-activated-ATPase activity was determined in the presence of various concentrations of added K⁺. As shown in Fig. 4, increasing the K⁺ concentration from 0 to 100 mM stimulated the Ca²⁺,Mg²⁺-ATPase in a concentration-dependent manner. The activation profile was similar to that observed for the Ca²⁺-transport

Fig. 3. Ca^{2+} -dependence of the $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase activity

Endoplasmic-reticulum membranes were incubated in assay buffer containing 0.2 mM-EGTA and various concentrations of CaCl_2 to give the free Ca^{2+} concentrations indicated. P_i released after 30 min of incubation was determined as described under 'Methods'. Results are the means $\pm$ S.E.M. of three determinations on a representative preparation.

Fig. 5. Effect of vanadate on the Mg^{2+} -ATPase and $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase activities

Endoplasmic-reticulum membranes were preincubated for 10 min in assay buffer containing various concentrations of potassium orthovanadate. The reaction was initiated by addition of ATP, and P_i released after 30 min of incubation was determined. ATPase activity is expressed as a percentage of the activity in the absence of vanadate. Results are means $\pm$ S.E.M. of three different experiments. $\circ$, Mg^{2+} -ATPase; $\bullet$, $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase.

Fig. 4. Effect of K^+ concentration on the Mg^{2+} -ATPase and $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase activities

ATPase activity was determined in assay medium buffered with Tris/HCl instead of Hepes/KOH. The KCl concentration was varied from 0 to 100 mM and iso-osmolality maintained by addition of compensating amounts of Tris/HCl. Results, which are the means $\pm$ S.E.M. of three separate experiments, are expressed as a percentage of the ATPase activity in medium containing 100 mM-KCl. $\circ$, Mg^{2+} -ATPase; $\bullet$, $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase.

system, and the ATPase activity in medium containing 100 mM- K^+ was about twice as great as that in medium lacking K^+ . When tested under similar conditions, K^+ failed to have any effect on the Mg^{2+} -ATPase activity.

Vanadate inhibited the $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase, but not the Mg^{2+} -ATPase (Fig. 5). The sensitivity to inhibition by vanadate displayed by the $\text{Ca}^{2+}, \text{Mg}^{2+}$ ATPase was slightly greater than that reported for the corresponding Ca^{2+} -transport system (Kanagasuntheram & Teo, 1982a), particularly at higher concentrations of vanadate. Vanadate at 60 μM , for example, inhibited the $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase by 72%, compared with only 50% inhibition of the Ca^{2+} -transport system. The IC_{50} (concentration of vanadate required for 50% inhibition of the $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase) was 28 μM , which is about one order of magnitude greater than the IC_{50} quoted for plasma-membrane-associated Ca^{2+} -activated ATPases, but is comparable with that of other endoplasmic-reticulum or sarcoplasmic-reticulum-associated $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPases (Caroni & Carofoli, 1981a).

Effects of trifluoperazine, W_7 and calmodulin

The calmodulin antagonists trifluoperazine and W_7 both inhibited the $\text{Ca}^{2+}, \text{Mg}^{2+}$ -ATPase activity of the endoplasmic-reticulum membranes. Increasing the concentration of trifluoperazine from 10 μM to 100 μM caused progressively greater inhibition of the ATPase, and 52 μM -trifluoperazine inhibited the enzyme by 50%

Fig. 6. Inhibition of Mg^{2+} -ATPase and Ca^{2+} , Mg^{2+} -ATPases by trifluoperazine

Parotid membranes were pre-incubated in assay buffer containing 0 to 100 μM -trifluoperazine, and ATPase activity was determined as described under 'Methods'. Results are expressed as a percentage of the activity obtained in the absence of trifluoperazine, ▲, Mg^{2+} -ATPase; ●, Ca^{2+} , Mg^{2+} -ATPase.

(Fig. 6). At concentrations greater than 10 μM , trifluoperazine also caused some inhibition of the basal Mg^{2+} -ATPase activity, although the magnitude of the inhibition was always substantially lower than that of the Ca^{2+} -activated ATPase. These effects of trifluoperazine on the Mg^{2+} -ATPase suggest that, at high concentrations, trifluoperazine may have non-specific effects on membrane ATPases that are not necessarily linked to its ability to antagonize calmodulin function.

W_7 also inhibited the Ca^{2+} , Mg^{2+} -ATPase, but was less effective than trifluoperazine (results not shown). When added at a concentration of 50 μM , W_7 inhibited the Ca^{2+} , Mg^{2+} -ATPase by 19%.

Addition of pure calmodulin at a concentration of 2 $\mu\text{g}/\text{ml}$ had no effect on the Ca^{2+} -activated ATPase. In previous studies (Kanagasuntheram & Teo, 1982b) it was found that calmodulin is tightly associated with the endoplasmic-reticulum-containing fraction. When this endogenous calmodulin was depleted by hypo-osmotic washing, the Ca^{2+} -transporting activity of the membrane was lowered by about 70%. Re-addition of calmodulin to the calmodulin-depleted membranes resulted in partial re-activation of the Ca^{2+} -transport activity. When a similar protocol of hypo-osmotic washing (twice with 0.1 mM-EGTA/10 mM-Tris/HCl, pH 7.4) was adopted in the present studies, the Ca^{2+} , Mg^{2+} -ATPase activity of the purified endoplasmic-reticulum membrane was decreased by 36%. Addition of exogenous calmodulin at a concentration of 2 $\mu\text{g}/\text{ml}$, however, failed to restore the ATPase activity.

Other properties

Some Ca^{2+} -pumping ATPases may be stimulated by phosphorylation by cyclic AMP-dependent protein

kinase (Tada *et al.*, 1974; Caroni & Carafoli, 1981b) or by hormones that elevate intracellular cyclic AMP (Walton *et al.*, 1980). The effects of cyclic AMP and cyclic AMP-dependent protein kinase on the activities of the parotid endoplasmic-reticulum ATPases were therefore investigated. Incubation of the membranes in assay buffer containing 5 μM -cyclic AMP alone or 5 μM -cyclic AMP and cyclic AMP-dependent protein kinase (10 $\mu\text{g}/\text{ml}$) for up to 30 min had no significant effect on Mg^{2+} -ATPase or Ca^{2+} , Mg^{2+} -ATPase activities. IP_3 , a potent stimulator of Ca^{2+} efflux from the endoplasmic reticulum of a variety of cell types (Prentki *et al.*, 1984; Berridge & Irvine, 1984), also had no effect on either ATPase over a 10 min incubation period when added at a concentration at 10 μM .

DISCUSSION

A Ca^{2+} , Mg^{2+} -ATPase has been identified in a parotid membrane fraction highly enriched in endoplasmic-reticulum membrane. Its properties were similar to those reported for the parotid endoplasmic-reticulum ATP-dependent Ca^{2+} pump (Kanagasuntheram & Teo, 1982a,b), indicating that this ATPase is likely to be responsible for driving the Ca^{2+} pump. In addition to the Ca^{2+} , Mg^{2+} -ATPase, the membrane fraction also possessed Mg^{2+} -ATPase activity that did not require Ca^{2+} for activity. The Mg^{2+} -ATPase displayed different sensitivities to K^+ , vanadate and trifluoperazine and had a higher affinity for ATP compared with the Ca^{2+} -activated ATPase. These distinguishing properties suggest that the Mg^{2+} -ATPase and the Ca^{2+} , Mg^{2+} -ATPase are two distinct enzymes. The nature and function of the Mg^{2+} -ATPase are at present unclear.

Like the Ca^{2+} , Mg^{2+} -ATPases of other tissues, the parotid endoplasmic-reticulum ATPase displayed a high affinity for Ca^{2+} . The K_m for Ca^{2+} of 0.55 μM is comparable with the value of 0.5 μM reported for the sarcoplasmic reticulum (see review by De Meis & Inesi, 1982) and 0.6–1.2 μM reported for adipocyte endoplasmic reticulum (Black *et al.*, 1980). In studies on the ATP-dependent Ca^{2+} pump of the parotid endoplasmic reticulum (Kanagasuntheram & Teo, 1982a), the K_m for Ca^{2+} was found to be 3.7 μM . The discrepancy between this value and that obtained in the present study may be attributed to the different experimental conditions used in the two studies. Ca^{2+} /EGTA buffers were not employed in the studies on Ca^{2+} transport, and this may have led to an overestimation of the free Ca^{2+} concentration. Moreover, as reported for the erythrocyte membrane Ca^{2+} pump, EGTA itself may increase the Ca^{2+} -binding affinity of transport ATPases (see review by Schatzmann, 1982).

The maximal rates of ATP hydrolysis by the parotid Ca^{2+} , Mg^{2+} -ATPase ranged from 45 to 74 nmol/min per mg of protein. These values are about 3–5 times higher than those reported for the Ca^{2+} -activated ATPases of the endoplasmic reticulum of liver and adipose tissue (Black *et al.*, 1980; Dawson & Fulton, 1983). Muscle sarcoplasmic reticulum in which the Ca^{2+} -pumping ATPase constitutes the major protein component, however, has a V_{max} for Ca^{2+} , Mg^{2+} -ATPase that is one to two orders of magnitude greater (Meissner, 1974).

Unlike the sarcoplasmic-reticulum Ca^{2+} pump, many non-muscle Ca^{2+} -transport systems display a strict requirement for ATP (Moore *et al.*, 1975; Penniston,

1983). Other nucleotides, such as GTP, CTP or ITP, support little or no Ca^{2+} -pumping activity. The parotid endoplasmic-reticulum Ca^{2+} , Mg^{2+} -ATPase showed a similar narrow specificity with respect to nucleotide requirement, a property shared by the Ca^{2+} pump located on this membrane (P. Thiyagarajah & S. C. Lim, unpublished work). The ability of added GTP to inhibit ATP hydrolysis by the Ca^{2+} , Mg^{2+} -ATPase suggested that a GTP-binding protein may regulate its activity, since there is increasing evidence that GTP-binding proteins similar to the regulatory proteins of the adenylate cyclase complex are involved in the action of Ca^{2+} -mobilizing receptors (Berridge & Irvine, 1984). We were, however, unable to detect any inhibitory effect of GTP or its analogue, guanosine 5'-[β -imidol]triphosphate, on the Ca^{2+} , Mg^{2+} -ATPase at concentrations lower than 0.2 mM (P. Thiyagarajah & S. C. Lim, unpublished work), indicating that the inhibitory effect observed with 1 mM-GTP may be due to direct antagonism of ATP interaction with the ATPase rather than an effect that is mediated through a GTP-binding regulatory protein.

The Ca^{2+} , Mg^{2+} -ATPase, but not the Mg^{2+} -ATPase, was activated when the K^{+} concentration in the assay buffer was increased from 0 to 100 mM. Sensitivity to activation by K^{+} is shared by other intracellular-membrane-associated Ca^{2+} -transport systems such as smooth muscle (Wibo *et al.*, 1981), liver (Moore *et al.*, 1975) and adipocyte (Pershad Singh & McDonald, 1980), and it has been suggested that this property may be used to distinguish these Ca^{2+} -transport systems from those which are located on the plasma membrane, since the latter generally show little or no sensitivity to K^{+} . Another criterion that is often used to distinguish plasma-membrane Ca^{2+} pumps from those on the endoplasmic reticulum is their differential sensitivity to inhibition by vanadate. The plasma-membrane Ca^{2+} -pumping ATPases are inhibited by more than 50% by a vanadate concentration of 2 μM or less (Caroni & Carafoli, 1981a; Michaelis *et al.*, 1983), whereas those associated with intracellular membranes, such as the endoplasmic reticulum, require 10–100 times higher concentrations of vanadate. In the present studies, the parotid endoplasmic-reticulum Ca^{2+} , Mg^{2+} -ATPase required a relatively high concentration of 28 μM -vanadate for 50% inhibition of activity.

The physiological mechanisms responsible for the regulation of the Ca^{2+} , Mg^{2+} -ATPase remain to be elucidated. Inhibition of the ATPase by the calmodulin antagonists trifluoperazine and W, suggest that calmodulin may regulate its activity. However, the concentration of trifluoperazine required for 50% inhibition of the ATPase was slightly higher than that required for inhibition of the parotid endoplasmic-reticulum Ca^{2+} pump (Kanagasuntheram & Teo, 1982b) and some other calmodulin-regulated Ca^{2+} -transport systems (Caroni & Carafoli, 1981a; Terman & Gunter, 1983). IP_3 , at concentrations sufficient to cause maximal release of Ca^{2+} from the endoplasmic reticulum of cells, failed to affect the activity of the Ca^{2+} , Mg^{2+} -ATPase. These observations suggest that the Ca^{2+} -mobilizing second messenger probably acts on the Ca^{2+} -efflux mechanism rather than the ATP-dependent uptake process. In a separate paper (Thiyagarajah & Lim, 1984) we presented evidence that phosphatidylinositol 4,5-bisphosphate stimulates the Ca^{2+} , Mg^{2+} -ATPase of the parotid endoplasmic reticulum. Further studies are, however, necessary to establish

clearly whether changes in the membrane content of this phospholipid serve as a physiological mechanism for regulating its activity.

REFERENCES

- Baginski, F., Foa, P. P. & Zak, B. (1967) *Clin. Chim. Acta* **15**, 155–158
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Black, B. L., McDonald, J. M. & Jarrett, L. (1980) *Arch. Biochem. Biophys.* **199**, 92–102
- Bruns, D. E., McDonald, J. M. & Jarrett, L. (1976) *J. Biol. Chem.* **251**, 7191–7197
- Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F. & Putney, J. W. (1984) *Nature (London)* **309**, 63–66
- Caroni, P. & Carafoli, E. (1981a) *J. Biol. Chem.* **256**, 3263–3270
- Caroni, P. & Carafoli, E. (1981b) *J. Biol. Chem.* **256**, 9371–9373
- Dawson, A. P. (1982) *Biochem. J.* **206**, 73–79
- Dawson, A. P. & Fulton, D. V. (1983) *Biochem. J.* **210**, 405–410
- Dawson, A. P. & Irvine, R. F. (1984) *Biochem. Biophys. Res. Commun.* **120**, 858–864
- De Meis, L. & Inesi, G. (1982) in *Membrane Transport of Calcium* (Carafoli, E., ed.), pp. 141–186, Academic Press, New York
- Downes, C. P., Mussat, M. C. & Michell, R. M. (1982) *Biochem. J.* **203**, 169–177
- Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 3077–3081
- Kanagasuntheram, P. & Teo, T. S. (1982a) *Biochem. J.* **208**, 789–794
- Kanagasuntheram, P. & Teo, T. S. (1982b) *FEBS Lett.* **141**, 233–236
- Kotagal, N., Patke, C., Landt, M., McDonald, J., Colca, J., Lacy, P. & McDaniel, M. (1982) *FEBS Lett.* **137**, 249–252
- Lotersztajn, S., Epand, R. M., Mallat, A. & Pecker, F. (1984) *J. Biol. Chem.* **259**, 8195–8201
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Meissner, G. (1974) *Methods Enzymol.* **31**, 238–246
- Michaelis, E. K., Michaelis, M. L., Chang, H. H. & Kitos, T. (1983) *J. Biol. Chem.* **258**, 6101–6108
- Moore, L., Chen, T., Knapp, H. R. & Landan, E. J. (1975) *J. Biol. Chem.* **250**, 4562–4568
- Penniston, J. J. (1983) in *Calcium and Cell Function* (Cheung, W. Y., ed.), pp. 100–149, Academic Press, New York
- Pershad Singh, H. A. & McDonald, J. M. (1980) *J. Biol. Chem.* **255**, 4087–4093
- Pershad Singh, H. A., Landt, M. & McDonald, J. M. (1980a) *J. Biol. Chem.* **255**, 8983–8986
- Pershad Singh, H. A., McDaniel, M. L., Landt, M., Bry, C. G., Lacy, P. E. & McDonald, J. M. (1980b) *Nature (London)* **288**, 492–495
- Prentki, M., Biden, T. J., Janic, D., Irvine, R. F., Berridge, M. J. & Wolheim, C. B. (1984) *Nature (London)* **309**, 562–564
- Schatzmann, H. J. (1982) in *Membrane Transport of Calcium* (Carafoli, E., ed.), pp. 41–108, Academic Press, New York
- Selinger, Z., Naim, E. & Lasser, M. (1970) *Biochim. Biophys. Acta* **203**, 326–334
- Sillén, L. G. & Martell, A. E. (1971) *Stability Constants of metal Ion Complexes*, 2nd edition, The Chemical Society, London
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–68
- Strobel, H. W. & Dignan, J. S. (1978) *Methods Enzymol.* **52**, 89–96
- Tada, M., Kirchberger, M. A., Repke, D. I. & Katz, A. M. (1974) *J. Biol. Chem.* **249**, 6174–6180

- Taylor, W. M., Bygrave, F. L., Blackmore, P. F. & Exton, J. H. (1979) FEBS Lett. **104**, 31–34
- Terman, B. I. & Gunter, T. E. (1983) Biochim. Biophys. Acta **730**, 151–160
- Thiyagarajah, P. & Lim, S. C. (1984) Biochem. Int. **9**, 625–630
- Waltenbaugh, A. A., Lam, A., Hummel, L. & Friedmann, N. (1980) Biochim. Biophys. Acta **630**, 165–175
- Wibo, M., Morel, N. & Godfraind, T. (1981) Biochim. Biophys. Acta **649**, 651–660
- Widnell, C. C. & Unkless, J. C. (1968) Proc. Natl. Acad. Sci. U.S.A. **61**, 1050–1057
-

Received 4 September 1985/14 November 1985; accepted 12 December 1985