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A HIGH-AFFINITY (Ca^{2+} + Mg^{2+})-ATPase IN PLASMA MEMBRANES OF RAT ASCITES HEPATOMA AH109A CELLS

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The activity of calcium-stimulated and magnesium-dependent adenosinetriphosphatase which possesses a high affinity for free calcium (high-affinity (Ca^{2+} + Mg^{2+})-ATPase, EC 3.6.1.3) has been detected in rat ascites hepatoma AH109A cell plasma membranes. The high-affinity (Ca^{2+} + Mg^{2+})-ATPase had an apparent half saturation constant of 77 ± 31 nM for free calcium, a maximum reaction velocity of 9.9 ± 3.5 nmol ATP hydrolyzed/mg protein per min, and a Hill number of 0.8. Maximum activity was obtained at 0.2 μM free calcium. The high-affinity (Ca^{2+} + Mg^{2+})-ATPase was absolutely dependent on 3–10 mM magnesium and the pH optimum was within physiological range (pH 7.2–7.5). Among the nucleoside trisphosphates tested, ATP was the best substrate, with an apparent K_m of 30 μM. The distribution pattern of this enzyme in the subcellular fractions of the ascites hepatoma cell homogenate (as shown by the linear sucrose density gradient ultracentrifugation method) was similar to that of the known plasma membrane marker enzyme alkaline phosphatase (EC 3.1.3.1), indicating that the ATPase was located in the plasma membrane. Various agents, such as K^+, Na^+, ouabain, KCN, dicyclohexylcarbodiimide and NaN_3, had no significant effect on the activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase. Orthovanadate inhibited this enzyme activity with an apparent half-maximal inhibition constant of 40 μM. The high-affinity (Ca^{2+} + Mg^{2+})-ATPase was neither inhibited by trifluoperazine, a calmodulin-antagonist, nor stimulated by bovine brain calmodulin, whether the plasma membranes were prepared with or without ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid. Since the kinetic properties of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase showed a close resemblance to those of erythrocyte plasma membrane (Ca^{2+} + Mg^{2+})-ATPase, the high-affinity (Ca^{2+} + Mg^{2+})-ATPase of rat ascites hepatoma cell plasma membrane is proposed to be a calcium-pumping ATPase of these cells.

Introduction

The electrochemical gradient of ionized free calcium (Ca^{2+}) between extracellular fluid and cytoplasm is maintained by the plasma membrane. It is widely accepted that the maintenance of cytoplasmic Ca^{2+} concentration in the submicromolar range against continuous influx of the cation from extracellular fluid is performed by Na^+/Ca^{2+} exchange and/or an ATP-driven Ca^{2+}-extruding pump. The kinetic properties of the ATP-driven Ca^{2+}-extruding pump of erythrocyte plasma membranes are similar to those of the calcium-stimulated and magnesium-dependent ATPase, (Ca^{2+} +

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DCCD, dicyclohexylcarbodiimide; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid).

Mg^{2+})-ATPase (EC 3.6.1.3) [1–4]. This enzyme is stimulated by submicromolar concentrations of Ca^{2+} and calmodulin in the presence of millimolar concentrations of Mg^{2+}. Similar (Ca^{2+} + Mg^{2+})-ATPases have been demonstrated in many mammalian cell plasma membranes [5–9]. We have previously reported the presence in the rat liver plasma membranes of a high-affinity Ca^{2+}-dependent ATPase which requires neither Mg^{2+} nor calmodulin for its activity [10]. Lotersztajn et al. [11] have also observed the presence of (Ca^{2+} + Mg^{2+})-ATPase in these membranes. This ATPase is dependent on submicromolar concentrations of Mg^{2+} and a proteinaceous activating factor distinct from calmodulin. Both of the ATPases, reported by us [10] and Lotersztajn et al., do not require millimolar concentrations of Mg^{2+}, the concentration generally required for ATPase-mediated cation-transport systems [5]. The problem of the Mg^{2+}-sensitivity of calcium-stimulated ATPases which have been proposed as Ca^{2+}-pumping ATPases is observed not only in plasma membranes of rat liver but also in those of alveolar macrophage, adipocyte, corpus luteum and C57 black mouse liver [12–15]. (Ca^{2+} + Mg^{2+})-ATPases observed in plasma membranes of erythrocyte, lymphocyte and heart sarcolemma are all dependent on a millimolar concentration range of Mg^{2+}, and their kinetic properties are very similar to those of the ATP-driven Ca^{2+}-extruding activity [2,3,7,8]. Therefore, these (Ca^{2+} + Mg^{2+})-ATPases are likely to play the role of Ca^{2+}-pumping ATPases. In this communication, we report the characterization of a high-affinity (Ca^{2+} + Mg^{2+})-ATPase present in the plasma membranes of a neoplastic liver cell line, rat ascites hepatoma AH109A. It is suggested that the high-affinity (Ca^{2+} + Mg^{2+})-ATPase is a Ca^{2+}-extruding pump in AH109A cells.

Materials and Methods

Chemicals

ATP (disodium salt, vanadate free) was a product of Sigma (U.S.A.). EGTA, Pipes and orthovanadate were purchased from Wako (Japan). Other chemicals used were of highest purity.

Preparation of plasma membranes

AH109A, a rat ascites hepatoma cell line obtained as a gift from the Sasaki Institute, Tokyo, was employed in the studies reported here. The ascites hepatoma cells do not form 'islets', but grow as free cells. The ascites hepatoma cells were inoculated intraperitoneally into female Donryu rats weighing 100–150 g ($1 \cdot 10^7$ cells/rat). After 6–9 days, the cells from 12 rats were harvested with a heparinized syringe, and washed five times with cold Dulbecco's phosphate-buffered saline (pH 7.1) by centrifugation at $150 \times g$ for 2 min. The following procedures were done at 0–4°C unless otherwise stated. Contaminating erythrocytes were removed by suspending the cells in a hypotonic medium (10 vol. 5 mM Tris-HCl, pH 7.5), which ruptured the erythrocytes but not the ascites hepatoma cells. The cells were then washed with saline five times as above. Light microscopic examination of an aliquot confirmed the absence of erythrocytes. The ascites hepatoma cells were suspended in 9 vol. 20 mM Tris-HCl (pH 8), and homogenized in a tight-fitting Potter-Elvehjem Teflon-glass homogenizer (0.1 mm clearance) with 15 strokes at 1400 rpm. In the experiments designed to test the calmodulin effect, the homogenizing solution contained 5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). About one-half of the ascites hepatoma cells were ruptured by this homogenization, as checked by phase-contrast microscopy. The homogenate was mixed with an equal volume of 60% sucrose (w/v), and then centrifuged for 15 min at $800 \times g$. The supernatant was centrifuged for 20 min at $6000 \times g$. The resultant pellet was suspended in 120 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 15 mM NaCl and 60% sucrose. 20 ml of the suspension were deposited at the bottom of a centrifuge tube, and sucrose density gradients consisting of 10 ml of 45% sucrose and 5 ml of 30% sucrose were layered onto the suspension, successively. This method was proposed by Shimizu and Fanakoshi [16]. The gradients were centrifuged for 2 h in a RPS27.2 rotor (Hitachi) at $100\,000 \times g$. The plasma membranes at the interface of 30–45% sucrose were collected, and subsequently used or stored at 4°C overnight. The plasma membranes obtained by this method were used in all experiments except that of Fig. 1. To characterize the subcellular fractions, the activities of alkaline phosphatase (EC 3.1.3.1), glucose-

6-phosphatase (EC 3.1.3.9) and succinic cytochrome *c* reductase (EC 1.3.99.1), which are the marker enzymes for plasma membranes, microsomes and mitochondria, respectively, were assayed.

Enzyme assay

The high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase was assayed in a reaction mixture (0.5 ml) which contained 10 μmol Tris-Pipes buffer, pH 7.5, 5 μmol of NaN_3, 1.5 μmol $MgCl_2$, 0.5 μmol ATP, the desired concentration of Ca^{2+} adjusted with EGTA (calcium-EGTA buffer) and plasma membranes (60–300 μg protein). The reaction was started by adding ATP, and carried out at 37°C. After 15–30 min, depending on the activity, the reaction was stopped by adding 1 N H_2SO_4 and 1% SDS. The released inorganic phosphate was determined colorimetrically [17]. The activity of high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase was determined by subtracting values obtained with EGTA plus Mg^{2+} (Mg^{2+}-ATPase activity) from those with calcium-EGTA buffer plus Mg^{2+}, and is expressed as nmol ATP hydrolyzed/mg protein per min unless otherwise stated.

Alkaline phosphatase, glucose-6-phosphatase and succinic cytochrome *c* reductase were assayed by the methods of Ikehara et al. [18], Morré [19] and Tisdale [20], respectively.

Other determinations

Ca^{2+} concentrations in the reaction mixture were determined by the calculation method reported by Katz et al. [21], using logarithms of apparent stability constants of 7.66, 3.93 and 4.61 for [Ca · EGTA], [Ca · ATP] and [Mg · ATP], respectively. Ca^{2+} concentrations were also measured directly by a calcium-selective electrode (Radiometer F2112Ca, Copenhagen) as reported by Al-Jobore and Roufogalis [22]. The calculated and observed Ca^{2+} values were similar (Table I), in agreement with the data of Al-Jobore and Roufogalis [22]. Calculated values of Ca^{2+} are plotted in the figures. When the effect of pH on the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase was studied, the apparent stability constants of EGTA and ATP for Ca^{2+} and Mg^{2+} were calculated as reported previously [10].

Total calcium concentrations were determined by atomic absorption spectroscopy (Perkin-Elmer, model 403). Protein concentration was determined by the method of Lowry et al. [23] with bovine serum albumin as reference protein. Calmodulin was purified from bovine brain by the method of Yazawa et al. [24].

TABLE I

COMPARISON OF CALCULATED AND MEASURED Ca^{2+} CONCENTRATIONS IN THE REACTION MIXTURE

The reaction mixture contained 118 μM EGTA and 985 μM $Mg \cdot ATP^{2-}$.

Total $CaCl_2$ (μM)	Free calcium	
	Calculated (μM)	Measured (μM)
90.3	0.071	0.05
95.2	0.091	0.09
98.9	0.112	0.11
115	0.669	0.67
120	2.68	2.7
125	6.62	6.8
130	11.1	12
150	29.1	32
175	51.7	56

Fractionation of the ascites hepatoma cell homogenate into subcellular particulate fractions

Erythrocyte-free ascites hepatoma cells obtained from three rats were homogenized with 17 ml of 0.25 M sucrose using a Potter-Elvehjem Teflon-glass homogenizer (clearance of 0.1 mm) with 30 strokes at 1400 rpm. The homogenate was centrifuged for 10 min at $1000 \times g$. The supernatant was dialyzed against 200 vol. 0.25 M sucrose for 3 h. Dialyzed homogenate (4 ml) was layered onto a linear concentration gradient of sucrose (0.303–1.140 M at 0°C, 26 ml). The sucrose gradient was centrifuged at $100\,000 \times g$ using an RPS27.2 rotor at 0°C. After 16 h, the sucrose gradient was fractionated into 30 fractions, 1 ml each.

Results

Characterization of plasma membrane fraction

Plasma membranes of rat ascites hepatoma cells were successfully enriched using the discontinuous

TABLE II

ACTIVITIES OF MARKER ENZYMES IN THE PLASMA MEMBRANE FRACTION

Data represent the mean ± S.D. of four determinations each. The activities of high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase were obtained with 0.4% Triton X-100-solubilized preparations in the presence of 5.3 μM Ca^{2+}.

Enzyme	Spec. act. (nmol/mg per min)	Purification over homogenate (fold)	Recovery from homogenate (%)
Alkaline phosphatase	1196 ± 54	7.3 ± 1.0	5.8 ± 0.8
Glucose-6-phosphatase	0.524 ± 0.11	0.53 ± 0.1	0.17 ± 0.03
Succinic cytochrome *c* reductase	55.5 ± 18	2.1 ± 0.2	3.0 ± 1.7
$(Ca^{2+} + Mg^{2+})$-ATPase	9.81 ± 5.6	7.2 ± 2.3	3.9 ± 1.5

sucrose gradient ultracentrifugation method reported by Shimizu and Funakoshi [16]. Table II shows the extent of purification and recovery of marker enzymes from homogenates. Purification of alkaline phosphatase indicated the enrichment of the plasma membranes. The membrane fraction was considered to contain only a small amount of microsomes because of the decrease in the specific activity of glucose-6-phosphatase. However, the mitochondrial marker enzyme, succinic cytochrome *c* reductase, was also enriched 2-fold, suggesting a slight contamination with mitochondria. For further characterization of the membrane preparation, the preparation was examined by electron microscopy using uranyl acetate and lead hydroxide staining as well as alkaline phosphatase staining. Plasma membrane vesicles of various sizes (diameter 0.16–0.3 μm) were noted in the high-power field. Almost all vesicles were stained with alkaline phosphatase staining. On an average only one fragment of a mitochondrion could be observed for every 5–6 high-power fields examined. Therefore, NaN_3, a strong mitochondrial ATPase blocker, was routinely added to the ATPase assay reaction mixture. As shown in Table II, the high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase copurified with alkaline phosphatase.

Very little activity of the high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase could be detected in the plasma membrane fraction, due to a large activity of Mg^{2+}-ATPase. Treatment of the plasma membranes with Triton X-100 selectively reduced the activity of Mg^{2+}-ATPase, resulting in an increase in the activity of high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase. The optimal concentration of Triton X-100 required to reduce the activity of Mg^{2+}-ATPase was 0.4%. Higher concentrations had the same effect as 0.4%. As shown in Table III, an approx. 19-fold increase in the activity of high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase was obtained by the treatment of the plasma membranes with 0.4% Triton X-100, probably due to the lowering of the Mg^{2+}-ATPase activity and/or an unmasking effect of the detergent on the high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase. A similar effect of SDS on the $(Na^+ + K^+)$-ATPase of cardiac sarcolemmal vesicles has been reported [25]. In all the subsequent experiments, 0.4% Triton X-100-solubilized plasma membranes were employed without clarify-

TABLE III

COMPARISON OF THE ACTIVITIES OF $(Ca^{2+} + Mg^{2+})$-ATPase BETWEEN NATIVE AND TRITON X-100-SOLUBILIZED PLASMA MEMBRANES

Total ATPase activity represents the sum of the values obtained with Mg^{2+}-ATPase and $(Ca^{2+} + Mg^{2+})$-ATPase assays. The concentration of Triton X-100 used was 0.4%. The high-affinity $(Ca^{2+} + Mg^{2+})$-ATPase was assayed in the presence of 5.3 μM Ca^{2+}.

	$(Ca^{2+} + Mg^{2+})$-ATPase (nmol/mg per min)	Mg^{2+}-ATPase (nmol/mg per min)	$(Ca^{2+} + Mg^{2+})$-ATPase/total ATPase
Native membranes	3.0	131	2.3%
Solubilized membranes	17.9	24	42.7%
Recovery from native membranes	591%	18%	19-fold

ing the solubilizate by centrifugation, since the turbidity of the reaction mixture was greatly reduced by the addition of the stop solution (1 N H_2SO_4 and 1% SDS) and it did not interfere with the colorimetric assay.

The activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase was linear with plasma membrane concentration (60–300 μg protein) and with time up to 30 min when ATP concentration was not rate-limiting (data not shown).

Localization of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase in particulate fractions of ascites hepatoma cell homogenates

Although subfractionation methods for tumor cells have been reported, there was no convenient method for preparing three particulate fractions such as microsomes, mitochondria and plasma membranes of rat ascites hepatoma cell at the same time [26–28]. In order to examine the subcellular localization of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase in the ascites hepatoma cells, the distribution of marker enzymes for these organelles and that of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase were tested in the fractions obtained after sucrose density gradient ultracentrifugation of the homogenates. The distribution pattern of the activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase was similar to that of the plasma membrane marker enzyme alkaline phosphatase (Fig. 1). One peak of the activity of glucose-6-phosphatase, a marker enzyme for microsomes, and that of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase overlapped. However, the other peaks of both enzymes did not. The overlapping peaks of the activities of high-affinity (Ca^{2+} + Mg^{2+})-ATPase, alkaline phosphatase and succinic cytochrome *c* reductase noticeable at the bottom of the tube were probably due to the presence of large fragments of plasma membranes and mitochondria which could not be separated by the sucrose gradient ultracentrifugation. The data strongly suggested that the high-affinity (Ca^{2+} + Mg^{2+})-ATPase was located in the plasma membranes.

Kinetic properties of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase

The calcium-stimulated ATP hydrolysis in Triton X-100-treated plasma membranes required the

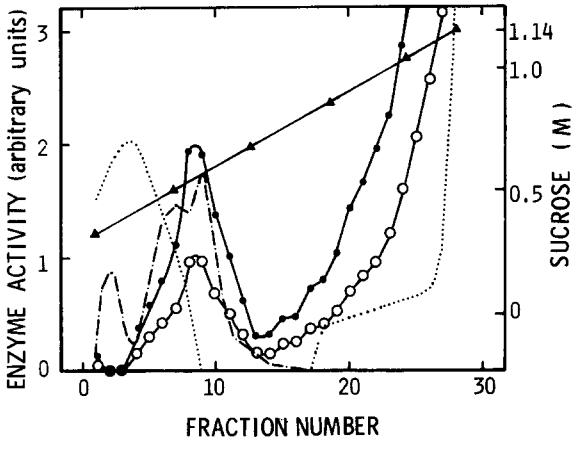

Fig. 1. Distribution of marker enzymes and the high-affinity (Ca^{2+} + Mg^{2+})-ATPase among fractions obtained from linear sucrose gradient ultracentrifugation. AH109A cell homogenates were subjected to ultracentrifugation as described under Experimental procedures. An aliquot of each fraction was assayed for the high-affinity (Ca^{2+} + Mg^{2+})-ATPase (●), alkaline phosphatase (○), glucose-6-phosphatase (·–·–·), or succinic cytochrome *c* reductase (·····). For the assay of high-affinity (Ca^{2+} + Mg^{2+})-ATPase, 0.4% Triton X-100-solubilized preparations were employed (see Table III). ▲———▲, sucrose concentration.

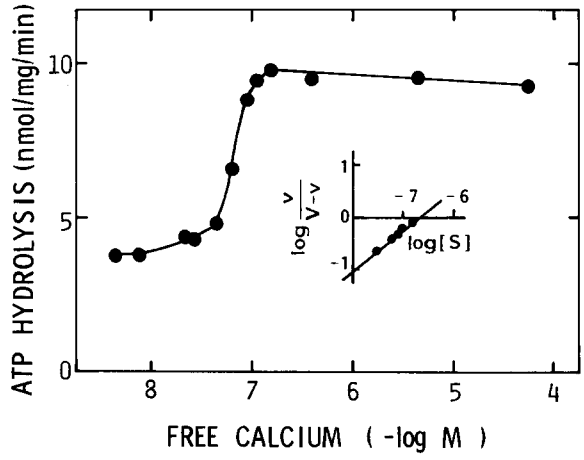

Fig. 2. Effect of Ca^{2+} concentration on the activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase. ATPase activity was assayed under standard conditions described under Experimental procedures in the presence of various concentrations of Ca^{2+}. Ca^{2+} concentrations in the reaction medium were determined as described under Experimental procedures. The inset represents the best-fitting linear form of the Hill equation using datum points between 30–160 nM Ca^{2+}. Results are from a typical experiment. Triton X-100-solubilized membranes were employed in this and all subsequent experiments.

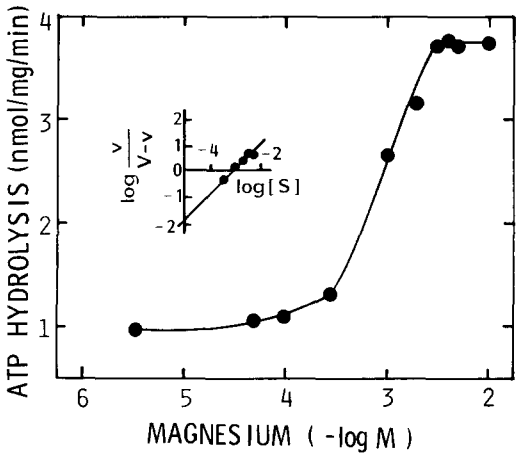

Fig. 3. Dependence of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase on $MgCl_2$ concentration. ATPase activity was assayed in the presence of fixed concentrations of $CaCl_2$ (106 μM) and EGTA (100 μM) with varying concentrations of $MgCl_2$ as indicated. Under these conditions, Ca^{2+} concentrations varied very little (3 to 21 μM) over the entire range of Mg^{2+} concentration employed (3.6 μM–10 mM). However, the ATPase activity did not change over this range of Ca^{2+} concentration (see Fig. 2). The inset represents a Hill plot using points between 0.35–4 mM $MgCl_2$. A Hill number of 1.4 with a correlation coefficient of 0.98 was obtained. Triton X-100-solubilized plasma membranes were used.

presence of both Ca^{2+} and Mg^{2+}. In the presence of 3 mM Mg^{2+}, the activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase was dependent on Ca^{2+} concentration with an apparent half-saturation constant ($K_{0.5}$) of 77 ± 31 nM ($n = 3$) for Ca^{2+}. The maximum stimulation of the ATPase by Ca^{2+} was obtained at 0.2 μM. The activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase did not change significantly up to a concentration of 50 μM Ca^{2+} (Fig. 2). Fig. 2 shows the relationship between the rate of ATP hydrolysis and Ca^{2+} concentration. A Hill number of 0.8 yielded the best-fit linear form of the Hill equation (Fig. 2 inset), giving a correlation coefficient of 0.99, a maximum reaction velocity of 9.9 ± 3.5 nmol ATP hydrolyzed/mg protein per min. An apparent K of 223 nM for Ca^{2+} (over the concentration range of 30–160 nM Ca^{2+}) was also obtained from Fig. 2 (inset).

In Triton X-100-treated plasma membranes, only a small activity of Ca^{2+}-stimulated ATPase could be detected over the range 1–9 μM Ca^{2+} in the absence of added Mg^{2+}. When 1 mM $MgCl_2$ was added, the Ca^{2+}-stimulated ATPase activity became obvious. The Mg^{2+}-dependence of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase is illustrated in Fig. 3. In this study, Ca^{2+} concentrations were varied from 3 to 21 μM depending on added $MgCl_2$ concentrations, since the change of concentration of Mg · ATP resulted in a change in Ca^{2+} level [21]. However, it was obvious from Fig. 2 that this range of Ca^{2+} concentration did not cause any significant change in the activity of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase. In the presence of this range of Ca^{2+} concentration and 1 mM ATP, the maximum activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase (in Triton X-100-treated plasma membranes) was obtained at 3 mM $MgCl_2$. Higher $MgCl_2$ concentrations had the same effect as 3 mM $MgCl_2$. An apparent K_m of 0.91 mM for $MgCl_2$ with a maximum reaction velocity of 4.8 nmol/mg per min was observed (Fig. 3, inset). This requirement of Mg^{2+} for Ca^{2+}-stimulated ATPase activity of ascites hepatoma cell plasma membrane was in striking contrast to the non-requirement of the metal ion for the high-affinity Ca^{2+}-ATPase of normal rat liver plasma membrane [10].

The high-affinity (Ca^{2+} + Mg^{2+})-ATPase utilized ATP as the best substrate. Other nucleoside triphosphates such as GTP, UTP, ITP, CTP and TTP could not substitute for ATP (Table IV). The strict substrate specificity of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase is in good agreement with

TABLE IV

SUBSTRATE SPECIFICITY OF THE HIGH-AFFINITY (Ca^{2+} + Mg^{2+})-ATPase

ATPase was assayed in the presence of 5.3 μM Ca^{2+} using Triton X-100-solubilized plasma membranes. Data represent the mean ± S.D. of three determinations. n.d., not detectable.

Nucleoside triphosphate	(Ca^{2+} + Mg^{2+})-ATPase activity	
	nmol/mg per min	%
ATP	4.59 ± 0.41	(100.0)
UTP	0.51 ± 0.01	11.0
CTP	0.31 ± 0.03	6.8
GTP	0.08 ± 0.01	1.7
ITP	n.d.	—
TTP	n.d.	—

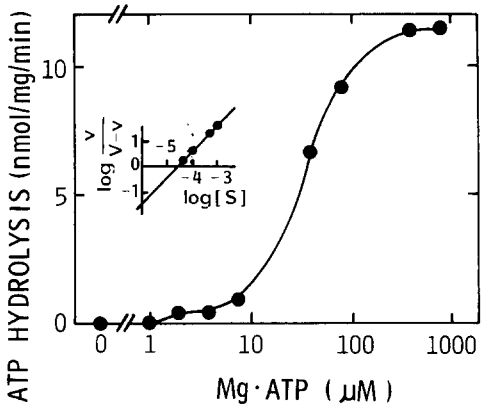

Fig. 4. Dependence of the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase activity on ATP concentration. Experimental conditions were similar to those given under Fig. 3 except that various concentrations of $MgCl_2$ and ATP were used such that the final Mg^{2+} concentration was always in excess over that of ATP by 2 mM. The inset represents a Hill plot using data points between 10–1000 μM Mg·ATP. Triton X-100-solubilized plasma membranes were used.

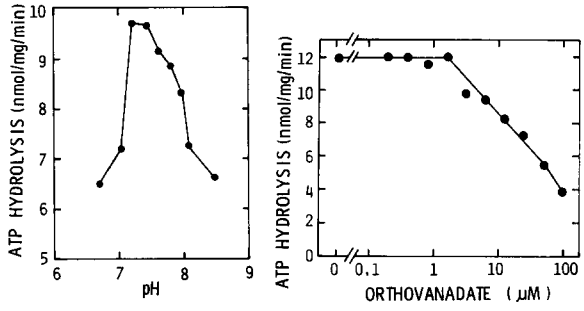

Fig. 5. (left-hand figure) Effect of pH on the activity of high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase. The ATPase was assayed in the presence of fixed concentrations of Ca^{2+} at 30 μM adjusted by calcium-EGTA buffer at various pH values as indicated. Apparent stability constants with ATP and EGTA for a given pH were calculated as reported previously [10]. Triton X-100-solubilized plasma membranes were used.

Fig. 6. Effect of orthovanadate on the activity of high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase. ATPase was assayed under standard conditions in the presence of 5.3 μM Ca^{2+} and various concentrations of orthovanadate as indicated. Triton X-100-solubilized plasma membranes were used.

that of Ca^{2+}-extruding pump ATPases in plasma membranes of various cells [5,7,29]. Fig. 4 shows the Mg · ATP concentration dependence of the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase in Triton X-100-treated plasma membranes in the presence of 2.85–20.8 μM Ca^{2+}, and 2 mM Mg^{2+} in excess over Mg · ATP concentration. The activity of the ATPase was a function of Mg · ATP concentration and saturable. Analysis of the data by double reciprocal plot yielded a broken line with two apparent components. An apparent K_m of 7.2 μM was found in the Mg · ATP concentration range 2.5–10 μM. On the other hand, the apparent K_m at higher substrate concentrations was 29 μM (data not shown). A Hill plot of the data (10–1000 μM Mg · ATP) showed a straight line with a Hill number of 1.36, giving a correlation coefficient of 0.99 (Fig. 4 inset). The Ca^{2+}-pumping ATPase of erythrocyte also has two kinds of affinity for ATP [30,31].

Fig. 5 shows the pH profile of the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase at a fixed concentration of Ca^{2+} adjusted by calcium-EGTA buffer. Apparent stability constants with EGTA and ATP are strongly dependent on pH over the physiological pH range (pH 6–9). In this pH study, apparent stability constants with each ligand under a given pH were calculated as reported previously [10]. Under this condition, the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase showed an optimal pH of 7.2–7.5, a value similar to that reported for the erythrocyte plasma membrane Ca^{2+}-pumping ATPase.

TABLE VI

EFFECT OF CALMODULIN AND TRIFLUOPERAZINE ON THE ACTIVITY OF HIGH-AFFINITY ($Ca^{2+} + Mg^{2+}$)-ATPase

Data represent the mean ± S.D. of three determinations each. Triton X-100-solubilized plasma membranes were prepared in the presence of 5 mM EGTA as described in Experimental procedures. The ATPase was assayed in the presence of 5.3 μM Ca^{2+}.

Additions (μM)	($Ca^{2+} + Mg^{2+}$)-ATPase (nmol/mg per min)
None	7.87 ± 0.39
Calmodulin	
0.011	7.87 ± 0.40
0.11	7.87 ± 0.38
1.1	7.54 ± 0.59
12	7.50 ± 0.75
Trifluoperazine	
10	7.85 ± 0.70
100	7.70 ± 0.68
500	3.95 ± 0.21

Effects of various agents on the activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase

In order further to characterize the high-affinity (Ca^{2+} + Mg^{2+})-ATPase as a Ca^{2+}-pumping ATPase, we have examined the effects of various agents which have been previously tested on erythrocyte Ca^{2+}-pumping ATPase. As shown in Table V, mitochondrial ATPase inhibitors such as dicyclohexylcarbodiimide, KCN and NaN_3 showed no effect on the ATPase activity. More specifically, NaN_3 had no effect on the enzyme activity over a wide range of concentration (0.1–10 mM), indicating the absence of contaminated mitochondrial ATPase activity in the plasma membrane preparation. NaCl and KCl had no significant effect, in contrast to their stimulating effect on erythrocyte Ca^{2+}-pumping ATPase [32,33]. Also, ouabain did not alter the enzyme activity. Orthovanadate, a potent inhibitor of many ATPases [34], inhibited the activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase with a K_i (the concentration needed for half-maximal inhibition) of 40 μM (Fig. 6). This K_i value is equivalent to that observed with (Ca^{2+} + Mg^{2+})-ATPase of rabbit muscle sarcoplasmic reticulum, but higher than those reported for erythrocyte, heart and Ehrlich ascites cell plasma membranes [2,8,35].

Certain types of (Ca^{2+} + Mg^{2+})-ATPase are calmodulin-sensitive. Activities of such plasma membrane ATPases can be stimulated several-fold by a submicromolar concentration range of calmodulin [2,7,9]. Therefore the effect of calmodulin on the activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase of ascites hepatoma cell was examined. Calmodulin had no effect on the ATPase activity in Triton X-100-solubilized plasma membranes prepared in the presence of 5 mM EGTA over a wide concentration range (11 nM–12 μM). It was considered possible that the lack of sensitivity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase to externally added calmodulin might have been due to the enzyme's being fully activated by endogenous calmodulin which was not dissociated by the EGTA treatment and bound tightly to the membranes. Therefore, we examined the effect of trifluoperazine, a calmodulin-antagonist, on the activity of high-affinity (Ca^{2+} + Mg^{2+})-ATPase. Trifluoperazine, up to a concentration of 100 μM, did not inhibit the enzyme activity in Triton X-100-solubilized plasma membranes (Table VI). Although higher concentrations of the drug did inhibit the enzyme activity, this inhibition may be considered nonspecific [5]. It was possible that detergent activation of the high-affinity (Ca^{2+} + Mg^{2+})-ATPase abolished the sensitivity of the enzyme to calmodulin. However, calmodulin (0.01–12 μM) did not stimulate the ATPase activity even in the absence of Triton X-100 (data not presented). Also, it has been shown that the presence of Triton X-100 in the reaction medium does not interfere with the interaction between calmodulin and calmodulin-sensitive (Ca^{2+} + Mg^{2+})-ATPase [3]. Hence, the high-affinity (Ca^{2+} + Mg^{2+})-ATPase of rat ascites hepatoma cell plasma membranes may not be a calmodulin-sensitive enzyme.

TABLE V

EFFECTS OF VARIOUS AGENTS ON THE ACTIVITY OF HIGH-AFFINITY (Ca^{2+} + Mg^{2+})-ATPase

ATPase was assayed in the presence of 5.3 μM Ca^{2+} using Triton X-100-solubilized membranes. Data represent the mean ± S.D. of three determinations each. DCCD, dicyclohexylcarbociimide.

Additions (mM)		(Ca^{2+}-Mg^{2+})-ATPase activity	
		nmol/mg per min	%
None		9.08 ± 0.58	(100)
DCCD	(0.1)	8.52 ± 0.22	94
KCN	(10)	8.35 ± 0.39	92
NaN_3	(0.1)	8.49 ± 0.18	94
NaN_3	(10)	9.57 ± 0.20	105
NaCl	(30)	8.35 ± 0.35	92
KCl	(30)	8.19 ± 0.43	90
Ouabain	(1)	8.11 ± 0.18	89

Discussion

The results obtained show the occurrence of a high-affinity (Ca^{2+} + Mg^{2+})-ATPase in plasma membrane fraction of rat ascites hepatoma AH109A cells. Continuous sucrose density gradient ultracentrifugation experiments indicate that the high-affinity (Ca^{2+} + Mg^{2+})-ATPase is located in plasma membranes of these cells. Parallel increments in the specific activities of the ATPase and alkaline phosphatase (a marker enzyme for plasma

membrane) in the same fraction and to the same degree support this conclusion.

Kinetic properties of the high-affinity ($Ca^{2+}Mg^{2+}$)-ATPase, including the affinity for Ca^{2+}, dependence on Mg^{2+}, substrate specificity, optimum pH and response to various agents, resemble those of the Ca^{2+}-pumping ATPases of plasma membranes of erythrocyte, lymphocyte and heart [1–4,7,8], except for the sensitivity to calmodulin (Table VI). Not all the Ca^{2+}-pumping ATPases such as ($Ca^{2+} + Mg^{2+}$)-ATPases of skeletal muscle and heart muscle sarcoplasmic reticulum are not regulated directly by calmodulin [36,37]. Therefore, calmodulin-insensitivity of the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase per se does not exclude the possibility that the hepatoma ATPase is a Ca^{2+}-pumping ATPase. Analogous characteristics of the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase to those of Ca^{2+}-pumping ATPases previously reported suggest that the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase reported in this paper may be a Ca^{2+}-pumping ATPase. However, it remains to be shown that the kinetic characteristics are same for the high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase and ATP-dependent transport of Ca^{2+} in the same plasma membrane preparation. In the course of this study, the activity of ATP-dependent Ca^{2+}-transport could be detected in the same membrane preparations used in ATPase experiments (results not shown). However, the activity of the ATP-dependent Ca^{2+}-transport (which was usually tested in membranes prepared in the absence of Triton X-100) was very small, less than one-tenth of the activity of high-affinity ($Ca^{2+} + Mg^{2+}$)-ATPase (which was assayed in Triton X-100-solubilized membranes) and precise characterization of the Ca^{2+}-transport was difficult. Procedures for preparing the plasma membrane vesicles in high yield and specific activity should be developed in order to study the kinetic properties of Ca^{2+}-transport. Currently this is being attempted in our laboratory. Preliminary experiments revealed that the Ca^{2+}-titration curve and the insensitivity to calmodulin of Ca^{2+}-transport activity were almost the same as those of the high-affinity Ca^{2+}-ATPase.

We have previously reported the occurrence of an Mg^{2+}-independent, high-affinity Ca^{2+}-ATPase in plasma membranes of normal rat liver [10]. An analogous Ca^{2+}-ATPase has also been demonstrated in C57 black mouse liver plasma membranes [15]. In these non-tumor tissues, no ($Ca^{2+} + Mg^{2+}$)-ATPase activity was detected in the plasma membrane fraction, in contrast to the tumor cells used in this study. AH109A cells do not form 'islets', but exist as free cells in the peritoneal cavity similarly to blood corpuscles. For this reason, it is not unlikely that AH109A cells possess a ($Ca^{2+} + Mg^{2+}$)-ATPase in their plasma membrane similar to that of blood cells such as erythrocytes and lymphocytes.

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