Journal of Bioenergetics and Biomembranes, Vol. 16, Nos. 5/6, 1984

Effects of Affinity-Purified Antibodies on the Ca^{2+} Pumping ATPase of Erythrocyte Membranes

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Received April 16, 1984; revised June 22, 1984

Abstract

Antibodies raised in rabbits against the purified erythrocyte membrane Ca^{2+} pumping ATPase were affinity-purified using an ATPase-Sepharose column. Addition of a few molecules of the purified antibody per molecule of ATPase was sufficient to inhibit the ATPase activity. Extensively washed ghosts or preincubated pure ATPase sometimes develop an appreciable Mg^{2+}-ATPase activity. In such cases, the antibodies inhibited the Mg^{2+}-ATPase as well as the Ca^{2+}-ATPase. This is consistent with the hypothesis that a portion of the Mg^{2+}-ATPase activity of ghosts is derived from the Ca^{2+}-ATPase. When nitrophenylphosphatase activity was observed, both Mg^{2+}- and Ca^{2+}-stimulated activities were observed. Only the Ca^{2+} activity was inhibited by the antibodies, confirming that this activity is due to the Ca^{2+} pump, and suggesting that the Mg^{2+}-nitrophenylphosphatase is due to a separate enzyme. Amounts of antibody comparable to those which inhibited the Ca^{2+}-ATPases had no effect on the Na^+-K^+-ATPase; 4-fold higher amounts of antibody significantly stimulted the Na^+-K^+-ATPase, but this effect of the antibody was not specific: Immunoglobulins from the nonimmune serum also significantly stimulated the Na^+-K^+-ATPase.

In resealed erythrocyte membranes, antibodies incorporated into the ghosts inactivated the Ca^{2+}-ATPase, while antibodies added to the outside had no significant effect.

Key Words: Ca^{2+}-ATPase; affinity-purification; antibodies; sidedness; erythrocyte; membrane; nitrophenylphosphatase.

Introduction

The Ca^{2+}-ATPase from human erythrocyte plasma membranes has been purified and characterized in our laboratory (Niggli *et al.*, 1979, 1981; Graf *et al.*, 1982; Zurini *et al.*, 1982). Antibodies against this pure Ca^{2+}-ATPase were raised in rabbits (Verma *et al.*, 1982). These antibodies were found to be

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0145-479X/84/1200-0365$03.50/0

specific against the Ca^{2+}-ATPase of the human erythrocytes and also had an inhibitory affect on the enzyme functions (Verma *et al.*, 1982).

In this paper, we describe a procedure to purify anti-ATPase immunoglobulin G by a Ca^{2+}-ATPase-Sepharose 4B affinity chromatography. We have studied the effect of anti-ATPase IgG on various enzyme functions. We have also carried out experiments to check the effect of sidedness of the antibodies on the inhibition of the enzyme activity of the resealed erythrocyte vesicles.

Materials and Methods

Chemicals

[γ-^{32}P]ATP (2–10 Ci/mmol) was obtained from New England Nuclear Boston, Massachusetts. Freund's adjuvants (complete and incomplete) were purchased from Miles Laboratories, Elkhart, Indiana. L-α-phosphatidylcholine (type 1X-E from egg yolk) and cyanogen bromide-activated Sepharose 4B were obtained from Sigma Chemical Company, St. Louis, Missouri. Bovine brain calmodulin was prepared according to the method of Watterson *et al.* (1976) and stored at −20°C. All other chemicals were the best available.

Ca^{2+} Pumping ATPase

The Ca^{2+}-ATPase was purified from the human erythrocyte membranes essentially according to Graf *et al.* (1982).

Antisera

Rabbits were immunized against the Ca^{2+}-ATPase and sera obtained as described by Verma *et al.* (1982).

Coupling of Ca^{2+}-ATPase to Sepharose 4B

Highly purified Ca^{2+}-ATPase preparations were pooled and concentrated using a YM5 Amicon membrane (molecular weight cut off 5,000). Finally, about 4 mg of Ca^{2+}-ATPase in 15 ml was dialyzed extensively against 10 mM phosphate buffer, pH 7.4, containing 0.05% Triton X-100. Because of an increase in the concentration of phosphatidylcholine due to the reduction in volume, the Ca^{2+}-ATPase solution became turbid. Enough 10% Triton X-100 was added to make the solution clear.

Cyanogen bromide-activated Sepharose 4B (4 g) was swollen for 15 min in 1 mM hydrochloride acid and the excess hydrochloric acid was removed by filtration. The wet resin was washed quickly with 100 mM sodium bicarbon-

ate buffer, pH 8.3, containing 500 mM sodium chloride and 0.05% Triton X-100 (coupling buffer). Twice-concentrated coupling buffer (15 ml) and 4 mg of the Ca^{2+}-ATPase in 15 ml were added to the resin. This was mixed gently at 4°C for 22 hr. The resin was separated by filtration and suspended in 100 ml of ethanolamine, pH 8.0, containing 0.05% Triton X-100. This was allowed to mix gently at 4°C for 16 hr. The resin was then washed with several volumes of 100 mM acetate buffer, pH 4.0, containing 500 mM sodium chloride and 0.05% Triton X-100. Finally, the resin was poured into a column and washed with 10 mM Tes-TEA buffer, pH 7.4, containing 150 mM sodium chloride, 0.05% Triton X-100, and 0.02% sodium azide (storage buffer).

Affinity Chromatography

The Ca^{2+}-ATPase-Sepharose 4B column was washed with several volumes of 10 mM borate buffer, pH 8.4, containing 156 mM sodium chloride (BBS). To 20 ml of antiserum enough concentrated borate buffer, pH 8.4, was added to bring the final borate concentration to 10 mM. Also sodium chloride was added to a final concentration of 156 mM. This was passed through the Ca^{2+}-ATPase-Sepharose column at the rate of 0.5 ml/min. The column was washed with about 100 ml of BBS until no further protein was eluted as monitored by recording transmittance at 280 nm. The anti-ATPase IgG was eluted with a solution of 4 M urea in BBS. Finally, the column was extensively washed with the storage buffer. The fractions obtained on elution with 4 M urea were pooled, dialyzed extensively to remove 4 M urea, and concentrated.

Preparation of the Erythrocyte Membranes, Extensively Washed Ghosts

Human erythrocyte plasma membranes were prepared according to the method of Dodge *et al.* (1963), and used for the estimation of the Ca^{2+}-ATPase activity.

Preincubation of Anti-ATPase IgG with the Pure Ca^{2+}-ATPase or Ghosts

Aliquots of the pure Ca^{2+}-ATPase or ghosts were incubated with the anti-ATPase IgG at 0°C for the desired period of time in Tes-TEA buffer, pH 7.4. Suitable controls were run in which IgG from nonimmune serum was taken.

ATPase Assay

ATPase activity was quantitated by measuring the release of inorganic phosphate from [γ-^{32}P]ATP at 37°C. (Graf *et al.*, 1982). An appropriate basal activity was subtracted to determine stimulation due to a particular addition; in the case of Ca^{2+}-ATPase activity the activity in the presence of Mg^{2+} was subtracted.

To quantitate the Ca^{2+}-ATPase activity of the membranes, the reaction mixture (in a final volume of 0.5 ml) contained 50 mM Tes-TEA, pH 7.4, 2.02 mM EDTA-TEA, pH 7.4, 6 mM magnesium chloride, 2 mM calcium chloride, 10 μg calmodulin, 6 mM [γ-^{32}P]ATP (0.1 nCi/nmole), and 150–200 μg membrane protein. This resulted in a free calcium ion concentration of 67.6 μM and a free magnesium ion concentration of 0.45 mM. Incubation was at 37°C for 30 min. The same conditions were followed when measuring activity of Triton X-100 solubilized ghosts.

To quantitate the Ca^{2+}-ATPase activity of the pure enzyme, the reaction mixture (in a final volume of 0.5 ml) contained 50 mM Tes-TEA, pH 7.4, 10 mM EGTA-TEA, pH 7.4, 0.79 mM EDTA-TEA, pH 7.4, 6 mM magnesium chloride, 10.22 mM calcium chloride, 10 μg calmodulin, 6 mM [γ-^{32}P]ATP (0.1 nCi/nmol), and 1 μg of pure Ca^{2+}ATPase. This resulted in a free calcium ion concentration of 5.8 μM and a free magnesium ion concentration of 0.41 mM. Incubation was at 37°C for 30 min.

The Na^{+}-K^{+}-ATPase activity of membranes was measured in a reaction mixture (0.5 ml) containing 50 mM Tes-TEA, pH 7.4, 6 mM magnesium chloride, 100 mM sodium chloride, 20 mM potassium chloride, 6 mM [γ-^{32}P]ATP (0.2 nCi/nmole), and 100 μg of membrane protein. Incubation was at 37°C for 30 min.

Preparation of the Erythrocyte Membranes for Measuring Nitrophenylphosphatase Activity

One-week-old human erythrocytes obtained from the blood bank were washed three times with a solution containing 75 mM potassium chloride, 75 mM sodium chloride, 0.2 mM magnesium chloride, 0.1 mM EGTA, and 10 mM Tris-HCl, pH 7.5. About 4 ml of the packed cells (packed to 80% hematocrit) were lysed into 40 ml ice-cold medium containing 2.5 mM HEPES-sodium, pH 7.5, 1 mM magnesium chloride, and 0.1 mM EGTA and kept in ice for 10 min. The membranes were washed twice by centrifugation with 1 mM sodium phosphate buffer, pH 7.4, and 10 mM EGTA-sodium to remove endogenous calmodulin. This was followed by two washings with a medium containing 1 mM HEPES-sodium, pH 7.4, and 1 mM magnesium chloride. The membranes were frozen (in liquid nitrogen) and thawed (at room temperature) three times. This was followed by two additional washings in the latter medium. The membranes were stored at 4°C until used.

Effect of Anti-ATPase IgG on the Nitrophenylphosphatase Activity of the Broken Membranes

Membranes (0.5 ml) were incubated with 100 μl (13.5 μg) anti-ATPase IgG for 20 min at 37°C. Parallel controls were run in which the membranes

were incubated with IgG isolated from nonimmune sera. At the end of this incubation, the *p*-nitrophenylphosphatase activity was measured in 1 ml reaction mixture containing 18.3 mM HEPES-sodium, 18.3 mM EGTA-sodium, 0.09 mM ouabain, 9.1 mM sodium chloride, 91.5 mM potassium chloride, 4.57 mM magnesium chloride with and without 50 μM free $CaCl_2$, 1.8 mM ATP, 4.58 mM *p*-nitrophenylphosphate, and membranes. The pH of the reaction mixture was 7.4 and incubation was at 37°C for 2 hr.

To stop the reaction, the tubes were chilled and 0.11 ml of solution containing 55% TCA and 0.8 mM phosphoric acid was added. The clear supernatants after centrifugation (0.9 mL) were mixed with 2 ml of 1 M NaOH containing 20 mM EDTA-sodium. The absorbance was monitored at 410 nm and the amount of nitrophenol released was determined from a standard curve.

Experimental Methods for Observing the Sidedness of the Antibody Inhibition

An ATP regeneration system in chemical and isotopic equilibrium was prepared by 1-hr incubation at 37°C of a solution containing 1.5 mM ATP (containing [γ-^{32}P]ATP), 100 mM creatine phosphate, 1 mM creatine, 20 mM $MgCl_2$, 50 mM inosine, 1 mM ouabain, 50 mM HEPES-Na, pH 7.4, 1 mM EGTA, 50 units/ml creatine kinase, and 100 units/ml hexokinase. Then the solution was diluted five times with water and kept at 0°C until used. For the preparation of resealed ghosts, human erythrocytes were washed three times (5000 *g*, 10 min centrifugation) with a medium containing 10 mM Tris-HCl, pH 7.5, 75 mM sodium chloride, 75 mM potassium chloride, and 0.1 mM EGTA. The cells were suspended at 10% hematocrit in the same medium containing 5 mM sodium iodoacetate and 5 mM inosine and incubated at 37°C for 1 hr. At the end of incubation, the cells were washed three times with the same medium. Then 1 ml of packed, washed cells were lysed in 9 ml of 2 mM Na-HEPES, pH 7.4, containing 0.5 mM $MgCl_2$. After 5 min at 0°C, membranes were recovered by 10 min centrifugation at 50,000 *g* and resuspended in the medium used for the lysis.

At this point, the membranes were divided into those which were to be treated with internal antibody and those which were not, and one volume of membranes was diluted into three volumes of the ATP regenerating system plus two volumes of either phosphate buffer, pH 8.0, or antibody. When antibody was used, 13.5 μg of the affinity-purified antibody was added to 50 μl of membranes. After 5 min at 0°C with the regeneration medium and with and without the antibody, the resealing was initiated by adding 2 M NaCl to give a final concentration of 154 mM; the ghosts were then resealed by further incubation for 20 min at 37°C. The resealed ghosts were then washed three

times with the same medium as the original red cells. The final intracellular concentrations of the various reagents in the resealed ghosts were 154 mM NaCl, 0.15 mM ATP, 10 mM creatine phosphate, 0.1 mM creatine, 2 mM $MgCl_2$, 5 mM inosine, 0.1 mM ouabain, 5 mM HEPES-Na, 0.1 mM EGTA, 5 units/ml creatine kinase, and 10 units/ml hexokinase. The final pellet of washed, resealed ghosts was resuspended in 80 volumes of a medium which was 150 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 10 mM Na-HEPES, pH 7.4, 0.1 mM ouabain, 0.1 mM EGTA, and 0.15 mM $CaCl_2$. At this point, antibody (the same amount as was used above) was added to those samples which were to contain external antibody. Enough of the divalent cation ionophore A23187 was then added to each sample to make the medium 10 μM in the ionophore.

The measurement of the ATPase was then carried out in tubes containing a total volume of 0.2 ml. To the 0 time tubes and the Mg^{2+} only tubes, concentrated EGTA was added to a final concentration of 0.5 mM. After each incubation time, the reaction was stopped by the addition of enough 0.2 M glucose to obtain a final concentration of 10 mM glucose; this penetrated the cells and converted the remaining ATP and creatine phosphate to glucose-6-phosphate, which is acid stable. For the 0 time measurement, the glucose was added before incubation, and incubation was carried out for 10 min. The total radioactivity was also measured by making the suspension 1 M in HCl and boiling for 20 min. For the 5–15 min times, these were incubated for an additional 10 min after the addition of glucose and then chilled. Finally, 0.1 ml of a solution which was 4% ammonium molybdate in sulfuric acid, 15% perchloric acid, and 1.5 mM sodium phosphate was added to each tube. This was followed by 0.8 ml of isobutanol, and the tubes were then mixed and the layers allowed to separate. This was repeated twice more; the tubes were then centrifuged for 15 sec in an Eppendorf centrifuge and 0.5 ml of the upper phase transferred to scintillation vials for counting the radioactive inorganic phosphate extracted into the upper phase.

Protein Determination

Protein concentrations were determined according to the method of Lowry *et al.* (1951) modified by Bensadoun and Weinstein (1976) with bovine serum albumin as standard.

Free Calcium and Magnesium Ion Concentrations

The concentrations of free calcium and magnesium ions were controlled using EDTA and EGTA. These were calculated using computer programs (Perrin and Sayce, 1967) which took into account all complexes involving Mg^{2+}, Ca^{2+}, EGTA, and EDTA, and ATP. These computations also took into

account the effects of temperature and ionic strength on the formation of the above complexes. Standard solutions of calcium chloride, magnesium chloride, EDTA, and EGTA were employed.

Plotting of Figures

Figures were plotted using a Houston Instrument plotter, using a program which plots error brackets only when errors are more than 3% of the total y-axis values.

Results

Affinity Chromatography

The results of a typical purification experiment are shown in Fig. 1. The anti-ATPase immunoglobulin-G (IgG) was eluted from the ATPase affinity column by 4 M urea. No Triton X-100 was added to the buffers used in the purification experiments. This was done to avoid any possible interference due to detergent in the later experiments. It was considered beneficial to store the ATPase-Sepharose 4B in 0.05% Triton X-100 containing buffer. Typically 160–200 μg anti-ATPase IgG was obtained from 10 ml of the antiserum. This may or may not represent the concentration of anti-ATPase IgG in the antiserum as we did not determine the capacity of the ATPase-affinity column.

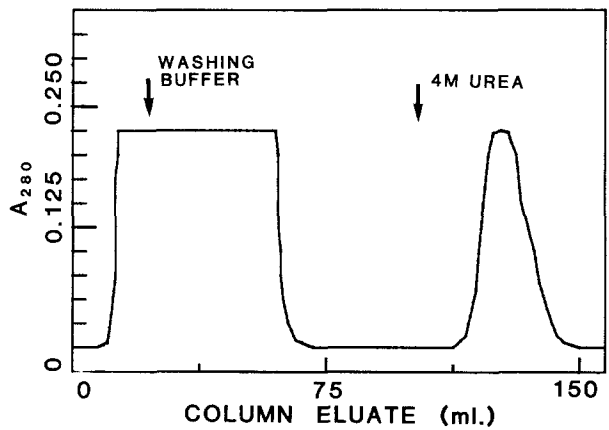

Fig. 1. Affinity chromatography purification of anti-Ca^{2+}-ATPase IgG. A column of Ca^{2+}-ATPase bound to Sepharose 4B was used.

Effect of the Anti-ATPase IgG on Ca^{2+}-ATPase Activity

Figure 2 shows the results of an experiment in which time of preincubation of the membranes with anti-ATPase IgG was varied. Only the total activity due to magnesium, calcium, and calmodulin was monitored in this experiment. These results demonstrate that under the conditions of preincubation (0°C), 4-hr preincubation inhibits more than 90% of the activity of the membranes. Four hours preincubation time was selected for the remaining experiments.

Figure 3 shows the results of an experiment in which the ATPase activities of the membranes were measured at increasing concentrations of anti-ATPase IgG. The results shown in Fig. 3 demonstrate that the calmodulin stimulation of the Ca^{2+}-ATPase could be completely inhibited at 1 μg anti-ATPase IgG per 100 μg ghost protein, while about 15% of the basal part of the ATPase was still expressed at the highest concentration of anti-ATPase IgG used.

The magnesium and calcium ATPase activities of 0.5% Triton X-100 solubilized human erythrocyte membranes could be completely inhibited by 1 μg of anti-ATPase IgG (Fig. 4). The conditions of preincubation and assays were the same as described for the experiment of Fig. 3. The complete

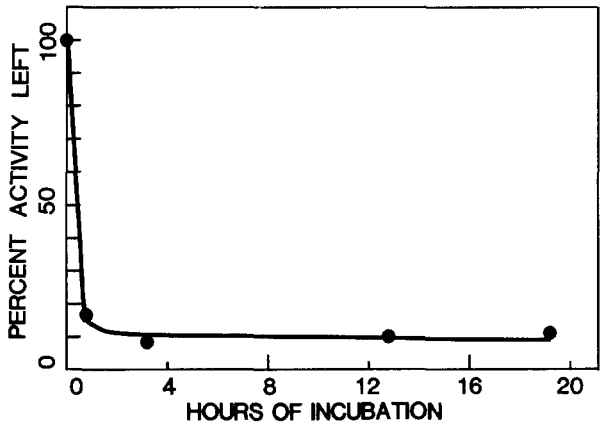

Fig. 2. Effect of the anti-Ca^{2+}-ATPase IgG on Ca^{2+}-ATPase activity. On the x axis is shown the time of preincubation of 2 μg anti-Ca^{2+}-ATPase IgG with 100 μg ghosts at 0°C before the enzyme activity was measured. The ordinate represents the percent enzyme activity (activity due to magnesium, calcium, and calmodulin minus no enzyme blank) remaining as determined by:

$$\frac{\text{Activity in the presence of anti-Ca}^{2+}\text{-ATPase IgG}}{\text{Activity in the presence of same amount of IgG from nonimmune serum}} \times 100$$

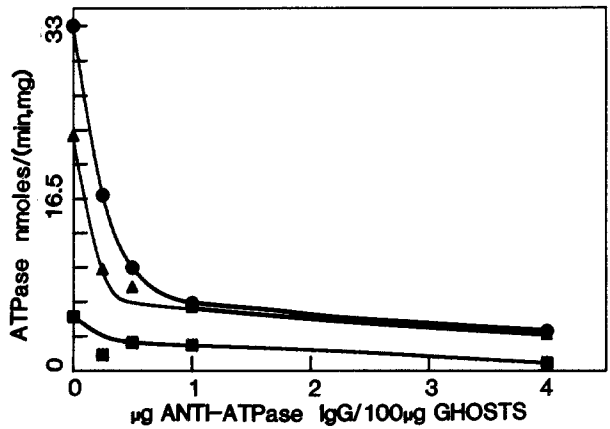

Fig. 3. Effect of increasing concentrations of anti-Ca^{2+}-ATPase IgG on the Mg^{2+}-ATPase (■) (Mg^{2+} + Ca^{2+})-ATPase (▲), and (Mg^{2+} + Ca^{2+} + calmodulin)-ATPase (●) activities of the ghosts. 100-μg fractions of the ghost protein were incubated with increasing amounts of anti-ATPase IgG. Preincubation with the IgG was 4 hr at 0°C.

inhibition shown here could be because of complete accessibility of ATPase to the antibody molecules.

Figure 5 shows the results of an experiment which was done to compare the inhibition pattern of Mg^{2+}-ATPase and Ca^{2+}-ATPase. The activities of the extensively washed human erythrocyte membranes were measured in the presence of increasing amounts of the anti-ATPase IgG. The results demon-

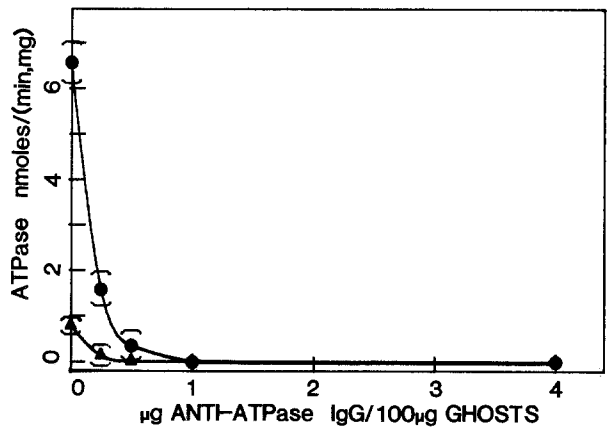

Fig. 4. Effect of anti-Ca^{2+}-ATPase IgG on the Ca^{2+}-ATPase activity of Triton X-100 solubilized ghosts. (▲) Mg^{2+}-ATPase activity; (●) (Mg^{2+} + Ca^{2+})-ATPase activity. Preincubation period was 6 hr at 0°C.

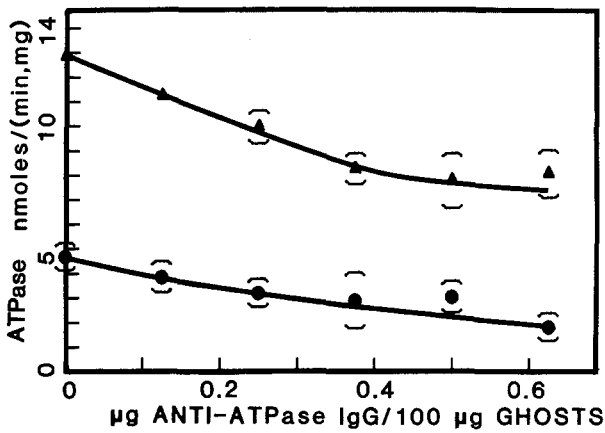

Fig. 5. Inhibition of the Ca^{2+}-ATPase and Mg^{2+}-ATPase activities as a result of preincubation with 0–0.6 μg anti-Ca^{2+}-ATPase IgG per 100 μg ghosts. (▲) (ΔCa^{2+})-ATPase; (●) Mg^{2+}-ATPase activity.

strate approximately similar patterns of inhibition of the basal (magnesium) and calium-stimulated ATPase activities.

Table I shows the effect of anti-ATPase IgG on the Ca^{2+}-ATPase activity of the pure enzyme. All three types of ATPase activities were sensitive to the presence of anti-ATPase IgG.

Table II shows the effect of anti-ATPase IgG on the nitrophenyl phosphatase activity of the erythrocyte membranes. The inhibition of background magnesium-dependent nitrophenyl phosphatase was minimal under conditions when the calcium-stimulated activity was inhibited by 79%.

Figure 6 shows the results of an experiment to determine from which side of the membrane the antibodies acted. The presence of the anti-ATPase IgG inside the resealed ghosts caused complete inhibition of enzyme activity. The specific activity shown on the *y* axis is the increment in ATPase activity due to the addition of calcium. When the anti-ATPase IgG was present only on the outer side of the resealed ghosts, the enzyme activity was not significantly inhibited.

The effect of anti-ATPase IgG on the Na^+-K^+-ATPase of the human

Table I. Inhibition of Purified Ca^{2+}-ATPase Activity by Anti-ATPase IgG

μg Anti-ATPase IgG / μg Ca^{2+}-ATPase	Activity (nmol/mg min)		
	Mg^{2+}	Ca^{2+}	Calmodulin
None	73	151	240
1	73	86	180
2	39	64	78

Table II.

Additions	Nitrophenylphosphatase activity of the broken membranes	
	Specific activity μmol/(ml cells hr)	Percent inhibition
Mg^{2+}-Nitrophenylphosphatase		
Without anti-ATPase IgG	1.26	2%
With anti-ATPase IgG	1.23	
Ca^{2+}-Stimulated nitrophenylphosphatase		
Without anti-ATPase IgG	0.91	79%
With anti-ATPase IgG	0.19	

erythrocyte membranes is shown in Fig. 7. Higher concentrations of the anti-ATPase IgG stimulated the Na^+-K^+-ATPase, but this effect was not specific for anti-ATPase IgG. The IgG (4 μg) from nonimmune serum also stimulated the Na^+-K^+-ATPase activity to 26.5 nmol per milligram protein per minute (not shown).

Discussion

On the basis of these results and results published earlier (Verma *et al.*, 1982), it is clear that the antibodies raised against the human erythrocyte Ca^{2+}-ATPase are very specific and have a high affinity for the enzyme. On the

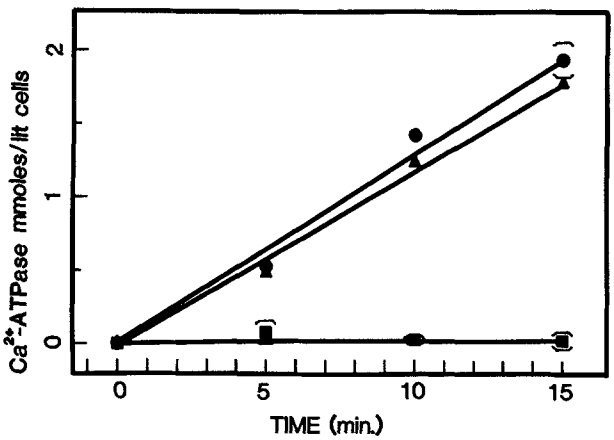

Fig. 6. Effect of sidedness of the antibody on the (Mg^{2+} + Ca^{2+})-ATPase activity of the resealed erythrocyte vesicles. This figure represents the enzyme activities (mmoles ATP split/liter cells) versus the incubation time of resealed vesicles with ATP at 37°C. Results of control (●), antibodies outside the vesicles (▲), and antibodies inside the vesicles (■) are shown.

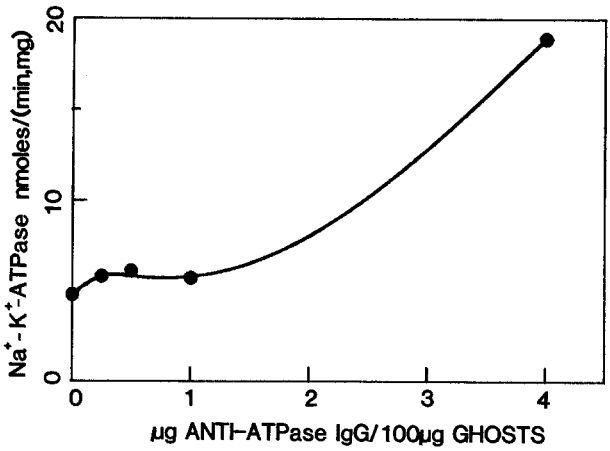

Fig. 7. The effect of anti-Ca^{2+}-ATPase IgG on the (Na^+-K^+)–ATPase of the human erythrocyte membranes. The figure simply shows the effect of preincubation (2 hr, 0°C) of varying amounts of anti-Ca^{2+}-ATPase IgG with 100 μg membranes. It does not show the effect of IgG from nonimmune serum, which also stimulated the enzyme activity.

basis of saturation of inhibition by 0.5 μg of antibody per 100 μg ghost protein, it is possible to calculate the number of molecules of antibody required to achieve this inhibition. Assuming 8×10^{-13} g membrane protein per ghost and a molecular weight of 150,000 for the antibody, 0.5 μg per 100 μg ghost protein works out to be 15,000 molecules of IgG per ghost. Since there are about 4,000 Ca^{2+}-ATPase molecules per ghost (Graf *et al.*, 1980), this corresponds to four molecules of IgG binding to each molecule of calcium-pumping ATPase.

The results of Figs. 3 and 4 show that inhibition of the Ca^{2+}-ATPase was incomplete when assayed in ghosts, but was complete when assayed in solubilized ghosts. This suggests that, in ghosts, some molecules or portions of molecules were inaccessible to the antibodies.

The aim of the experiment shown in Fig. 5 was to see if the Mg^{2+}-ATPase and the Ca^{2+}-stimulated ATPase activity of extensively washed ghosts were located on the same enzyme molecule. Antibodies (0–0.6 μg per 100 μg ghosts) caused inhibition of the two activities to a similar extent, implying that the two activities are the functions of the same molecule.

When purified Ca^{2+}-ATPase is stored over long periods of time, its calcium-stimulated ATPase activity sometimes decreases with a corresponding increase in the basal Mg^{2+}-ATPase activity (unpublished observations from this laboratory). Table I shows that, when a purified Ca^{2+}-ATPase had developed such an Mg^{2+}-ATPase, both it and the Ca^{2+}-ATPase were inhibit-

ed. This behavior suggests that at least a part of the Mg^{2+}-ATPase seen in ghosts develops in a similar fashion from the Ca^{2+}-ATPase.

The Ca^{2+}-stimulated nitrophenylphosphatase of the erythrocyte membranes was also sensitive to the antibody (Table II). In contrast, Mg^{2+}-nitrophenylphosphatase failed to show any sensitivity to the antibody. These results confirm that the Ca^{2+} nitrophenylphosphatase is due to the Ca^{2+} pump as previously suggested (Caride *et al.*, 1983; Verma and Penniston, 1984). The insensitivity of the Mg^{2+}-nitrophenylphosphatase to antibody suggests that this activity may be due to some enzyme other than the Ca^{2+}-pumping ATPase. The possibility remains, however, that this could be an activity of the pump which is not blocked by antibody.

Previously, these antibodies had been found to inhibit the Ca^{2+}-ATPase activity and Ca^{2+} transport in inside-out vesicles prepared from human erythrocytes (Verma *et al.*, 1982). Antibodies were also found to inhibit the Ca^{2+}-ATPase activity when present inside resealed ghosts, but not when present only outside the resealed ghosts (Fig. 6). This failure to inhibit from the outside may occur because the rabbit's immune system did not recognize the outer portion of the Ca^{2+}-ATPase as foreign. This is likely, because a similar Ca^{2+}-ATPase exists in the rabbit's own erythrocytes, and its outer portion is continually exposed to the immune system. Other possible reasons for a failure to inhibit from outside include: little of the ATPase may be exposed to the outside, or binding of antibodies to the outside may occur, but not inhibit activity. Similar studies on the Na^+-K^+-ATPase of erythrocytes (Jorgensen *et al.*, 1973) also showed inhibition from the inside only.

Amounts of antibody comparable to that which inhibited the Mg^{2+} and Ca^{2+}-ATPase had no effect on the Na^+-K^+-ATPase, further illustrating the specificity of the antibodies. The stimulation of the Na^+-K^+-ATPase by IgG from both immune and nonimmune sera is due to unknown causes, but it seems unrelated to the presence of antibodies against the Ca^{2+}-ATPase.

Acknowledgements

This work was supported in part by grant GM28835 from the U.S. National Institutes of Health.

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