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The Plasma Membrane (Mg^{2+})-Dependent Adenosine Triphosphatase from the Human Erythrocyte Is Not an Ion Pump

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Summary. The plasma membrane (Mg^{2+})-dependent adenosine triphosphatase ((Mg^{2+})-ATPase) from human erythrocytes has been tested for its ability to transport ions. Using a preparation of inside-out vesicles loaded with the pH-sensitive fluorescence probe 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS), we have demonstrated the absence of proton movement during (Mg^{2+})-ATPase activity. From the rate of ATP hydrolysis and the passive proton permeability of these vesicles, an upper limit of 0.03 H^+ transported per ATP hydrolyzed was calculated. To verify that proton pumping could be detected in this system, the intravesicular pH was monitored during (Ca^{2+})-dependent adenosine triphosphatase ((Ca^{2+})-ATPase) activity. Proton efflux associated with (Ca^{2+})-ATPase activity was observed (in agreement with a recent report of proton pumping by a reconstituted erythrocyte (Ca^{2+})-ATPase (Niggli, V., Sigel, E., Carafoli, E. (1982) *J. Biol. Chem.* **257**:2350–2356)) and was shown to be stimulated by calmodulin. The ability of the (Mg^{2+})-ATPase to pump $^{28}Mg^{2+}$, $^{35}SO_4^{2-}$ and $^{86}Rb^+$ was also tested, with the results leading to the conclusion that the human erythrocyte enzyme does not function as an ion transport system.

Key Words plasma membrane (Mg^{2+})-ATPase · human erythrocyte · proton transport · proton permeability · ion fluxes · plasma membrane (Ca^{2+})-ATPase

Introduction

A (Mg^{2+})-ATPase[1] activity has been detected in the plasma membranes derived from a variety of eukaryotic cell types [21, 29, 37] and has been partially purified from sheep kidney medulla [14]. This activity can be distinguished from the (Na^+,K^+)-ATPase and the (Ca^{2+})-ATPase by its insensitivity to Na^+, K^+, Ca^{2+} and ouabain. It can also be distinguished from the mitochondrial F_1 ATPase by its sensitivity to inhibition by vanadate. Although it has not been demonstrated that this (Mg^{2+})-ATPase activity results from a single enzyme in all of these cell types, its ubiquitous presence at high levels, often constituting over 50% of the plasma membrane-bound ATPase activity [21, 29, 37], suggests that it serves an important function.

The human red cell (Mg^{2+})-ATPase is an intrinsic membrane protein of molecular weight approximately 150,000 [10], which can be phosphorylated by [γ-^{32}P]ATP and can be resolved from the (Na^+,K^+) and (Ca^{2+})-ATPase by gel electrophoresis [10]. That this 150,000-dalton peptide is responsible for most of the (Mg^{2+})-ATPase activity of the red cell plasma membrane is supported by the observation that vanadate, which preferentially inhibits ATPases which form phosphorylated intermediates, inhibits greater than 70% of the (Mg^{2+})-ATPase activity with a K_i of 0.12 μM and hyperbolic saturation (L. Cantley, *unpublished observations*). Knauf et al. [20] have demonstrated that the phosphorylated form of the (Mg^{2+})-ATPase, like that of the (Na^+,K^+) and (Ca^{2+})-ATPase, is sensitive to hydroxylamine, suggesting that the site of phosphorylation in all three cases is a carboxyl group. In addition, the phosphorylated peptide obtained by peptic digestion of the (Mg^{2+})-ATPase comigrated on paper electrophoresis with that obtained from the (Na^+,K^+)-ATPase [19], suggesting that it has the same amino acid sequence around the active site as the (Na^+,K^+) and (Ca^{2+})-ATPases [2].

The similarity of the (Mg^{2+})-ATPase in size, localization, and phosphorylation properties to the (Na^+,K^+) and (Ca^{2+})-ATPases [17], enzymes known to be involved in the transport of Na^+/K^+ and Ca^{2+} ions, respectively [18, 33], suggests that the (Mg^{2+})-ATPase may function as an ion transport system. This view is supported by the observation

[1] *Abbreviations:* (Mg^{2+})-ATPase, (Mg^{2+})-dependent adenosine triphosphatase; (Ca^{2+})-ATPase, (Ca^{2+})-dependent adenosine triphosphatase; (Na^+,K^+)-ATPase, (Na^+ and K^+)-dependent adenosine triphosphatase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HPTS, 1-hydroxypyrene-3,6,8-trisulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate.

that (Mg^{2+})-ATPases present in the plasma membrane of simple eukaryotes, such as *Neurospora* [31] and *Saccharomyces* [38], which are also phosphorylated by ATP at a hydroxylamine-sensitive site [1, 8] and are of similar molecular weight [7, 40], have been shown to function as ATP-dependent proton pumps. We have also identified an ATP-dependent proton pump in clathrin-coated vesicles isolated from bovine brain [11]. We have suggested that this pump, which is presumably derived from the plasma membrane during pinching off of the coated vesicles, is responsible for acidification of the endosome that appears to be required for receptor recycling during receptor-mediated endocytosis [4, 24, 36]. ATP-dependent proton pumping has also been demonstrated in various intracellular organelles, such as chromaffin granules [15] and lysosomes [27, 32], and serves in acidification of gastric fluid, where proton movement is coupled to K^+ [30]. More importantly, Blostein and Harvey [3] have recently reported that the (Mg^{2+})-ATPase activity of inside-out human erythrocyte vesicles can be stimulated by proton ionophores (such as FCCP), suggesting that a proton gradient is established during turnover of the enzyme.

In light of these observations, we decided to investigate the transport properties of the plasma membrane (Mg^{2+})-ATPase from human erythrocytes. Although ATP-dependent proton pumping could be observed during (Ca^{2+})-ATPase activity, no proton movement was associated with turnover of the (Mg^{2+})-ATPase, despite the sufficiently low proton permeability of this system to have made such proton movement observable. Additional experiments measuring the distribution of $^{28}Mg^{2+}$, $^{35}SO_4^{2-}$ and $^{86}Rb^+$ have led to the conclusion that the (Mg^{2+})-ATPase from human erythrocytes is not an ion pump.

Materials and Methods

Inside-out vesicles loaded with the pH-sensitive fluorescence probe HPTS [6] were prepared from human erythrocytes by a modification of the procedure of Steck and Kant [35]. Cells (either fresh or within two weeks of drawing) were washed with 5.0 mM sodium phosphate (pH 8.0), 150 mM NaCl as described and then lysed and washed in 10 mM HEPES (pH 8.0). The resulting unsealed ghosts were then incubated in 0.50 mM HEPES (pH 8.5), 0.20 mM HPTS, 0.10 mM ouabain, and 0.05 mM EGTA overnight at 4°C. Vesiculation was completed by passage through a 27-gauge needle and sealed inside-out vesicles were isolated as the band remaining on top of a 10% Dextran T-70 cushion after sedimentation for 1.25 hr at 215,000 × *g*. Vesicles were then washed in 0.50 mM HEPES (pH 7.5), 0.05 mM EGTA, 10 mM KCL and 10 mM glucose to remove external HPTS by sedimentation or by passage through a Sephadex G-50 column (the dye is readily removed by such procedures because of its high water solubility and lack of sticking to membranes containing a small percentage of negatively charged phospholipid [6]). Vesicles prepared by this procedure appeared to be sealed since addition of external ouabain caused inhibition of only 4–5% of the total (Na^+,K^+)-ATPase activity, indicating the virtual absence of enzyme molecules with both the cytoplasmic ATP site and the extracellular ouabain site exposed. In addition, vesicles appeared to be inside out since no greater activation of (Mg^{2+})-ATPase activity was observed on addition of low levels of detergent (0.05% Triton) to vesicles than was observed for unsealed ghosts[2], indicating the absence of ATPase sites sealed in right-side-out vesicles. Except where indicated, vesicles were prepared and assayed in 0.10 mM ouabain and 0.05 mM EGTA to eliminate ATPase activity associated with the (Na^+,K^+) and (Ca^{2+})-ATPases, respectively. Buffers were deaerated and saturated with nitrogen to remove CO_2, which is capable of increasing the proton permeability of phospholipid bilayers [9]. In addition, except where indicated, vesicles were prepared from cells treated with the anion exchange protein inhibitor DIDS [5], to reduce the rate of transport of any residual HCO_3^-. Cells were treated with 100 μM DIDS in 150 mM NaCl, 5.0 mM sodium phosphate (pH 8.0) for 1 hr at 37°C followed by washing in the same buffer (minus DIDS) containing 0.50% bovine serum albumin (to remove unreacted DIDS) and then the original buffer alone. This procedure resulted in inhibition of less than 10% of the (Mg^{2+})-ATPase activity. Vesicles were stored at 4°C and were used within a week of preparation.

Fluorescence measurements were made on an SLM-4800 Spectrofluorometer using excitation and emission wavelengths of 460 and 520 nm, respectively. ATPase activity was quantitated by release of $^{32}P_i$ from [γ-^{32}P]ATP [16], while transport activity was measured by separation of vesicles from the external solution at 4°C by passage through a 10-ml Sephadex G-50 column [12]. Unless otherwise indicated, experiments were carried out in 0.50 mM HEPES/KOH (pH 7.5), 0.05 mM EGTA, 10 mM KCl and 10 mM glucose at 23°C. Protein concentrations were measured by the method of Lowry et al. [23] in the presence of 1.0% sodium dodecyl sulfate. HPTS was obtained from Molecular Probes, Inc., as the trisodium salt and was converted to the tripotassium salt as previously described [13]. ATP (the vanadate free dipotassium salt), bovine serum albumin (fatty acid free), ouabain, and valinomycin were purchased from Sigma Chemical Co., while DIDS, calmodulin (bovine), and FCCP were obtained from Pierce, Calbiochem and Dupont Chemical Co., respectively. $^{28}MgCl_2$ was obtained from Brookhaven National Laboratories while [γ-^{32}P]ATP (the triethanolamine salt), $H_2{}^{35}SO_4$ (neutralized with KOH) and ^{86}RbCl were purchased from New England Nuclear.

Results and Discussion

In light of the similarities of the erythrocyte (Mg^{2+})-ATPase to the ATP-dependent proton pumps

[2] Addition of 0.05% Triton to freshly prepared erythrocyte ghosts, previously shown to be unsealed [35], caused an approximately 30% increase in (Mg^{2+})-ATPase activity, presumably due to alterations in the bilayer structure of the membrane or to a direct effect on the enzyme. It is also possible that part of the stimulation observed on addition of Triton to inside-out vesicles is due to exposure of "ecto-ATPase" sites within the vesicles [33].

present in the plasma membranes of *Neurospora* [31] and *Saccharomyces* [28] (*see* Introduction), and because of the report that the red cell enzyme could be stimulated by proton ionophores [3], the most likely ion to be transported by the (Mg^{2+})-ATPase appeared to be protons. Figure 1 shows, however, that no proton movement could be detected during turnover of the (Mg^{2+})-ATPase (sp act 1.0–2.0 nmol ATP/min/mg protein) in inside-out vesicles loaded with the pH-sensitive fluorescence probe HPTS. This probe has a pK_a of approximately 7.5 [6], and a change in pH of 0.10 units (around pH 7.5) resulted in about a 10% change in fluorescence intensity at 520 nm (fluorescence intensity increasing with increasing pH). This experiment was repeated in the presence of valinomycin to eliminate any membrane potential built up during proton transport, which might impede further proton movement. No ATP-dependent change in intravesicular pH was observed (data not shown). Since these results are only significant if the proton permeability of the vesicles is sufficiently low to allow establishment of a pH gradient if protons are being pumped, the rate of passive proton flux in these vesicles was measured (Fig. 1). Because uncompensated proton movement will result in the generation of a membrane potential, which would tend to slow down the rate of equilibration, valinomycin (in the presence of K^+) was added to allow dissipation of this membrane potential. That sufficient valinomycin was added to prevent counterion movement from becoming rate limiting was demonstrated by the fact that proton equilibration was complete within the 50-sec mixing time when the proton ionophore FCCP was present in addition to valinomycin (Fig. 1). From these results, a halftime for proton equilibration in vesicles prepared from DIDS-treated cells in the presence of valinomycin of 1.8 min was obtained. If valinomycin was excluded, a halftime of 4.9 min was observed, while in vesicles prepared from cells not treated with DIDS[3], halftimes of 0.8 and 1.8 min were obtained in the presence and absence of valinomycin, respectively (data not shown).

The turnover number, k_{cat}, of the (Na^+,K^+)-ATPase is approximately 75 sec^{-1} at 23°C (reference [34] and *unpublished observations*). From the fact that there are approximately 1.5 times as many copies of the (Na^+,K^+)-ATPase as (Mg^{2+})-ATPase in the red blood cell membrane [10] and from the relative activities observed ((Na^+,K^+)-ATPase/

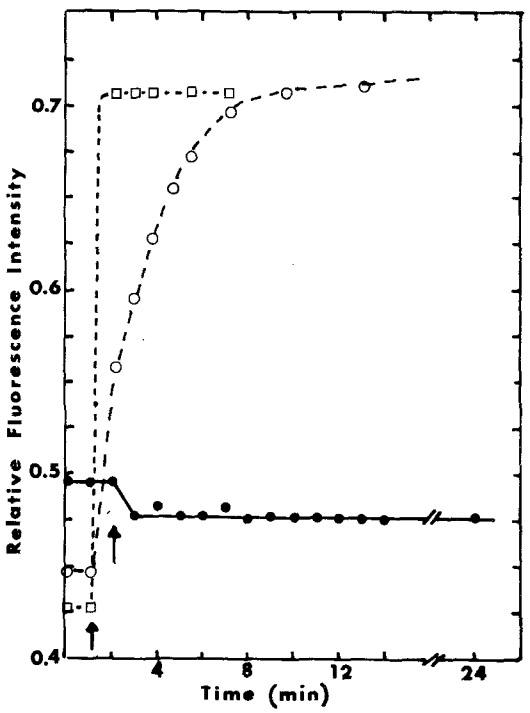

Fig. 1. Proton distribution during (Mg^{2+})-ATPase activity and proton permeability of inside-out vesicles. Inside-out vesicles were prepared as described under Materials and Methods and were diluted into 2.0 ml 0.50 mM HEPES (pH 7.5), 0.05 mM EGTA, 10 mM KCl, 10 mM glucose, 100 μM ouabain to a concentration of approximately 20 μg protein/ml. The permeability experiments (○,□) were performed in the presence of 1.5 μM valinomycin and in the absence (○) or presence (□) of 5.0 μg/ml FCCP. At the arrows were added either 100 μl 5.0 mM ATP, 10 mM $MgSO_4$ (pH 7.5) (●) or 3 μl 100 mM KOH (○,□), and the fluorescence intensity at 520 nm was measured at 23°C as described under Materials and Methods

(Mg^{2+})-ATPase = 2.9), a k_{cat} for the (Mg^{2+})-ATPase of approximately 40 sec^{-1} (at 23°C) was calculated. Since the concentration of ATP used (250 μM) was only sufficient for half-saturation, the effective turnover number of the (Mg^{2+})-ATPase under these conditions was approximately 20 sec^{-1}. From the observed specific activity of the (Mg^{2+})-ATPase at saturating ATP (2.7 nmol/min/mg protein), the above turnover number (40 sec^{-1}) and assuming 0.6 mg membrane protein per cell [10], a value of about 400 copies of (Mg^{2+})-ATPase per red cell was calculated[4]. From the size of the red cell (with a surface

[3] Proton transport was also not observed during (Mg^{2+})-ATPase activity in inside-out vesicles prepared from red cells not treated with DIDS (either in the presence or absence of valinomycin).

[4] This number is in good agreement with that calculated from the amount of $^{32}P_i$ incorporated into red cell ghosts on phosphorylation with [γ-^{32}P]ATP in the presence of Mg^{2+} (approximately 350 molecules per cell [20]), but is higher than that determined by phosphorylation followed by gel electrophoresis (approximately 100 molecules per cell [10]). The latter value is, however, an underestimate due to release of $^{32}P_i$ during electrophoresis [28].

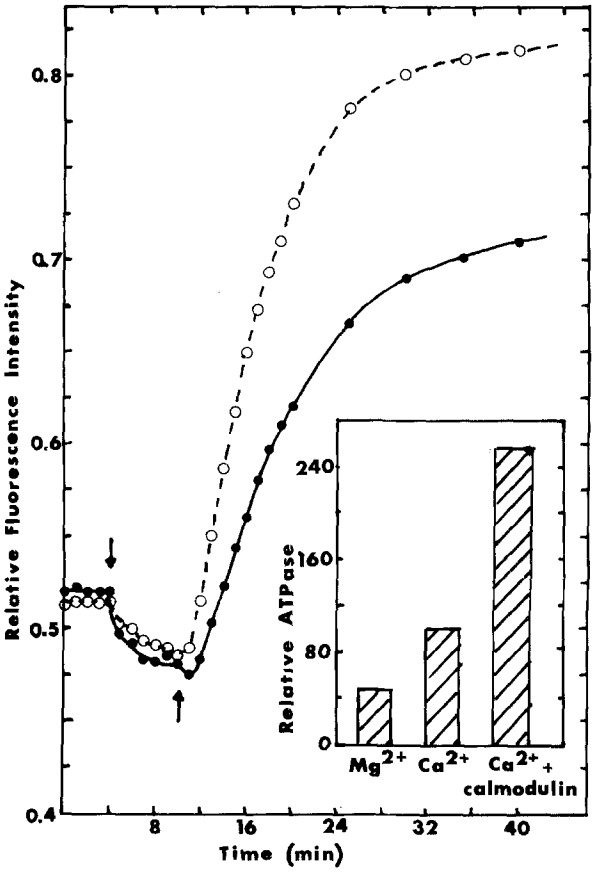

Fig. 2. Calmodulin sensitivity of proton transport and ATP hydrolysis by the erythrocyte (Ca^{2+})-ATPase. Inside-out vesicles were prepared and diluted as described in Fig. 1. Experiments were conducted in either the absence (●) or presence (○) of 1.5 μM calmodulin. At the left arrow was added 2 μl 100 mM $CaCl_2$ and 1 μl 100 mM KOH, while at the right arrow was added 100 μl 5.0 mM ATP, 10 mM $MgSO_4$ (pH 7.5), and the fluorescence intensity at 520 nm was measured at 23°C as described under Materials and Methods. *Inset:* ATPase activity was measured as described under Materials and Methods in the above buffer (at approximately 0.5 mg protein/ml) with 0.25 mM ATP, 0.50 mM $MgSO_4$ (Mg^{2+}) and either 100 μM $CaCl_2$ (Ca^{2+}) or 100 μM $CaCl_2$ and 1.5 μM calmodulin (Ca^{2+} + calmodulin) present. Values represent the average of 2 determinations

area of 1.4×10^2 μm^2 [39]) and the size of the inside-out vesicles (determined from trapping experiments to have a surface area of approximately 0.12 μm^2), it was estimated that each cell gave rise to about 1200 vesicles, so that approximately 30% of the vesicles should contain at least one active (Mg^{2+})-ATPase. Since the fluorescence change observed during (Mg^{2+})-ATPase activity was <3% (and thus the change in vesicles containing a (Mg^{2+})-ATPase would be <10%), the maximal pH gradient that could have been established in (Mg^{2+})-ATPase-containing vesicles was 0.10 units. From the volume of the vesicles (4.2×10^{-15} cm^3), the internal buffering capacity (0.50 mM HEPES, pK_a = 7.3), and a proton equilibration described by a single exponential decay with a $t_{1/2}$ of 1.8 min, it was calculated that a proton flux of about 40 H^+/min/vesicle must occur in order to maintain a 0.10-unit pH gradient. Since the turnover number of the (Mg^{2+})-ATPase under these conditions is about 20 ATP/sec, the stoichiometry of proton pumping by the (Mg^{2+})-ATPase must be less than 0.03 H^+/ATP. Thus the (Mg^{2+})-ATPase does not appear to function as a proton pump[5].

Because the calculation described above depends on many different measurements (and is hence subject to a variety of errors), we wished to confirm that it was possible to detect proton pumping in our system. Since a reconstituted preparation of the erythrocyte (Ca^{2+})-ATPase has recently been shown to catalyze an ATP-dependent proton movement [26], we decided to test whether the same activity could be observed in inside-out vesicles. As can be seen in Fig. 2, an increase in fluorescence intensity, corresponding to an efflux of protons, was observed on addition of Ca^{2+} and Mg^{2+}/ATP to HPTS loaded vesicles (the slight decrease in fluorescence observed on addition of Ca^{2+} alone was due to incomplete compensation for the protons released on chelation of Ca^{2+} by EGTA). From the initial rates of proton movement and (Ca^{2+})-ATPase activity, a stoichiometry of approximately 1.9 H^+/ATP was calculated (this value was corrected for the protons released on chelation of transported Ca^{2+} by intravesicular EGTA assuming a stoichiometry of 1 Ca^{2+}/ATP [26]). That proton movement was only partially (approximately 20%) reduced by addition of valinomycin (data not shown) indicated that protons were not simply diffusing in response to a membrane potential established by Ca^{2+} transport. In addition, the claim that the observed pH change was due to the (Ca^{2+})-ATPase was supported by the observation (Fig. 2) that both proton movement and ATPase activity were stimulated (in the expected proportions) by the addition of calmodulin, previously shown to directly stimulate (Ca^{2+})-ATPase activity in erythrocyte membranes [25]. Our results thus confirm that

[5] It is possible that the stimulation of (Mg^{2+})-ATPase activity by FCCP previously observed [3] is due to an alteration of some membrane property, such as fluidity, other than proton conductance. Alternatively, the presence of trace amounts of Ca^{2+} could result in proton pumping by the (Ca^{2+})-ATPase [26]. In our hands, no stimulation of (Mg^{2+})-ATPase activity was observed (using a freshly prepared solution) in the range of 1–10 μg/ml FCCP (5 μg/ml FCCP gave complete proton equilibration within the 50-sec mixing time in the presence of valinomycin). Some stimulation was observed using FCCP solutions stored at −20°C for longer than a month.

Table. Ion transport by the red cell (Mg^{2+})-ATPase[a]

Isotope	Method of equilibration	% isotope trapped	
		+ATP	−ATP
$^{28}Mg^{2+}$	Sonication	1.10(±.01)	1.11(±.01)
	Incubation at 37°C	1.42(±.01)	1.42(±.01)
$^{35}SO_4^{2-}$	Sonication	0.31(±.01)	0.33(±.01)
	Incubation at 37°C	0.37(±.02)	0.37(±.01)
$^{86}Rb^{+}$	Incubation at 23°C	1.16(±.03)	1.14(±.05)

[a] Inside-out vesicles were prepared as described under Materials and Methods except that HPTS was not included, 5.0 mM HEPES replaced 0.50 mM HEPES in the final buffer, and in the case of the $^{35}SO_4^{2-}$ experiments 5.0 mM K_2SO_4 replaced 10 mM KCl. Vesicles were equilibrated with the indicated isotopes (either 2 μCi/ml $^{28}Mg^{2+}$ (1.4 mM $MgCl_2$), 8 μCi/ml $^{35}SO_4^{2-}$ or 1.2 μCi/ml $^{86}Rb^{+}$) by either sonication (two 10-sec bursts at a setting of 8 using an Ultrasonics Inc. bath sonicator, model W-220F), or incubation overnight at 37 or 23°C. Except in the $^{28}Mg^{2+}$ experiments (where Mg^{2+} was already present), 1.5 mM $MgSO_4$ was added, and the transport experiment was initiated by the addition of either 0.50 mM ATP (+ATP) or 0.50 mM EDTA (−ATP). Transport activity was measured as described under Materials and Methods with the values shown being the average of the percent isotope trapped at 20 and 40 min (values in parentheses represent average deviations from the means).

the (Ca^{2+})-ATPase is capable of proton pumping and demonstrate that this proton movement is also sensitive to calmodulin.

Since the red cell (Mg^{2+})-ATPase does not appear to function as a proton pump, although its similarity to other ion pumps has been noted (*see* Introduction), we wished to determine whether it might be capable of transporting some other ion. Because Mg^{2+} is the only ion known to be required for activity, we decided to test for ATP-dependent $^{28}Mg^{2+}$ transport in our system ($^{28}Mg^{2+}$ was used rather than $^{54}Mn^{2+}$ to avoid possible complications due to transport by the (Ca^{2+})-ATPase). In addition, although the (Mg^{2+})-ATPase appears to demonstrate little specificity for the anions and monovalent cations usually present during assay of its activity (*unpublished observations*), we also tested whether this enzyme was capable of ATP-dependent $^{35}SO_4^{2-}$ or $^{86}Rb^{+}$ transport. In order to be able to detect transport of ions out of the vesicles, they were equilibrated with the isotopes either by sonication or by incubation overnight at 37°C (the two procedures resulted in a loss of 30 and 40% of the (Mg^{2+})-ATPase activity, respectively). As can be seen from the results presented in the Table no significant active transport of any of the ions tested was observed to be associated with (Mg^{2+})-ATPase activity, despite the sufficiently low permeability of the vesicles to all of these ions (the halftime for equilibration in all cases being greater than 30 min). Thus the (Mg^{2+})-ATPase of the human erythrocyte appears not to function as an ion pump.

Although it is possible that the plasma membrane (Mg^{2+})-ATPase may serve as an ion pump in other animal cell types, other functions can be suggested. It may be involved in regulation of intracellular ATP levels or intracellular pH (since ATP hydrolysis would result in the release of an equivalent number of protons inside the cell). Although such a regulatory system might appear to be wasteful, having a membrane-embedded system that could respond rapidly to extracellular signals (such as the binding of a hormone to its receptor), might compensate for this disadvantage. Alternatively, the (Mg^{2+})-ATPase might be involved in thermogenesis (again presumably under hormonal control), providing an advantage over using a system such as the (Na^+,K^+)-ATPase [22] in that ATPase activity could be modulated without alteration of the cytoplasmic ion composition or the membrane potential. It should be possible to test the sensitivity of the (Mg^{2+})-ATPase to various hormonal signals by purification and coreconstitution with the appropriate hormone receptor.

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