THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 8, Issue of February 23, pp. 5563–5569, 2001

Printed in U.S.A.

Leishmania Plasma Membrane Mg^{2+}-ATPase Is a H^+/K^+-Antiporter Involved in Glucose Symport

STUDIES WITH SEALED GHOSTS AND VESICLES OF OPPOSITE POLARITY*

Received for publication, September 15, 2000, and in revised form, November 21, 2000
Published, JBC Papers in Press, November 21, 2000, DOI 10.1074/jbc.M008469200

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Experiments from other laboratories conducted with *Leishmania donovani* promastigote cells had earlier indicated that the plasma membrane Mg^{2+}-ATPase of the parasite is an extrusion pump for H^+. Taking advantage of the pellicular microtubular structure of the plasma membrane of the organism, we report procedures for obtaining sealed ghost and sealed everted vesicle of defined polarity. Rapid influx of H^+ into everted vesicles was found to be dependent on the simultaneous presence of ATP (1 mM) and Mg^{2+} (1 mM). Excellent correspondence between rate of H^+ entry and the enzyme activity clearly demonstrated the Mg^{2+}-ATPase to be a true H^+ pump. H^+ entry into everted vesicle was strongly inhibited by SCH28080 (IC_{50} = ~40 μM) and by omeprazole (IC_{50} = ~50 μM), both of which are characteristic inhibitors of mammalian gastric H^+,K^+-ATPase. H^+ influx was completely insensitive to ouabain (250 μM), the typical inhibitor of Na^+,K^+-ATPase. Mg^{2+}-ATPase activity could be partially stimulated with K^+ (20 mM) that was inhibitable (>85%) with SCH28080 (50 μM). ATP-dependent rapid efflux of $^{86}Rb^+$ from preloaded vesicles was completely inhibited by preincubation with omeprazole (150 μM) and by 5,5′-dithiobis-(2-nitrobenzoic acid) (1 mM), an inhibitor of the enzyme. Assuming Rb^+ to be a true surrogate for K^+, an ATP-dependent, electroneutral stoichiometric exchange of H^+ and K^+ (1:1) was established. Rapid and 10-fold active accumulation of [U-^{14}C]2-deoxyglucose in sealed ghosts could be observed when an artificial pH gradient (interior alkaline) was imposed. Rapid efflux of [U-^{14}C]D-glucose from preloaded everted vesicles could also be initiated by activating the enzyme, with ATP. Taken together, the plasma membrane Mg^{2+}-ATPase has been identified as an electroneutral H^+/K^+ antiporter with some properties reminiscent of the gastric H^+,K^+-ATPase. This enzyme is possibly involved in active accumulation of glucose via a H^+-glucose symport system and in K^+ accumulation.

Kala-azar or visceral leishmaniasis is a major public health problem in many parts of the tropical and subtropical world including eastern regions of India (1). *Leishmania donovani* is the established etiological agent for Kala-azar (2). This protozoal pathogen belongs to the kinetoplastida group of parasites. The organism has a digenic life cycle. The flagellated vector or promastigote form normally resides and divides in the alimentary tract of phlebatomine sandflies. Following a bite by the sandfly, the parasite is rapidly internalized in human liver macrophages where it overcomes the host defense mechanism and undergoes a facile morphogenetic transformation to oval, aflagellated amastigote form in the phagolysosomal complex of the macrophages. Rapid proliferation and invasion of neighboring macrophages initiates pathogenesis (3). The commonly available laboratory cultural form corresponds to the vector or promastigote form of the organism.

Ion translocation and energetics of metabolite accumulation across the plasma membrane are important aspects in the physiology of any organism. Although some work has been done in this area for the kinetoplastida group of pathogens, a reasonably clear picture is still not available. *L. donovani* is known to have a Ca^{2+}-ATPase (4, 5) and a Mg^{2+}-ATPase (6, 7) in its plasma membrane. Catalytic sites of both these transmembrane proteins are oriented toward the cytosol (5, 7). Using sealed, everted vesicles we had earlier unambiguously demonstrated the Ca^{2+}-ATPase to be a true extrusion pump for cytosolic free Ca^{2+} (8). The specific function of the Mg^{2+}-ATPase, however, remains to be elucidated. Using vesicles of undefined and mixed polarity and employing acridine orange as the probe, Zilberstein and Dwyer (7) had earlier suggested that the Mg^{2+}-ATPase may be a H^+ translocator. More recently, using BCECF-loaded[1] *L. donovani* promastigote cells, Jiang *et al.* (9) have indicated the presence of a H^+/K^+ exchanger system in the surface of the parasite that is closely linked to the functioning of this Mg^{2+}-ATPase. The functional capability, if any, of the Mg^{2+}-ATPase to act as an ion translocator needs to be demonstrated clearly and unambiguously. This is also necessary to analyze the related problem of energy coupling in glucose transport across the plasma membrane (10). Somewhat contradictory results are available in the literature. Based on the effect of ionophores and protonophores on 2-deoxyglucose uptake in whole cells and also of inward flow of H^+ across the plasma membrane in de-energized cells on addition of glucose, Zilberstein and Dwyer (11) suggested a glucose/H^+ symport mechanism for glucose entry and accumulation. Experiments in chemostat with varying concentrations of glucose, on the other hand, indicate operation of a facilitated diffusion mech-

* This work was supported in part by Council of Scientific and Industrial Research and Department of Biotechnology (India). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: BCECF, 2′,7-bis(carboxyethyl)-5(6)-carboxyfluorescein; bis-oxanol, bis-(1, 3-diethylthiobarbituric. acid) trimethine oxanol; calcein, 2′,7′-{[bis(carboxymethyl) amino] methyl}-fluorescein; FCCP, Carbonylcyanide p-(trifluoromethoxy) phenylhydrazone; CDTA, transcyclohexane-1,2-diamine-N, N,N′,N′-tetraacetic acid; AMP-PNP, β,γ-imidoadenosine 5′-triphosphate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

anism for glucose entry independent of proton gradient in *L. donovani* (12). A promastigote stage-specific glucose transporter showing significant homology with mammalian facilitative glucose transporter has also been cloned (13) and functionally expressed in *Xenopus* oocytes (14). Analysis of 2-deoxyglucose entry process showed it to be a carrier-mediated process, but the requirement for H^+ could not be clearly established (14).

In general sealed plasma membrane ghosts or vesicles of defined polarity have been extremely valuable tools for transport studies in many systems. In this paper, using sealed ghosts and vesicles of opposite polarity, we demonstrate that the plasma membrane Mg^{2+}-ATPase of Leishmania is indeed an electroneutral H^+/K^+ antiporter. Further, we observe that imposition of an artificial pH gradient can drive glucose entry and accumulation in the sealed ghost. It is therefore likely that the major physiological roles for this enzyme is in generation of a pH gradient across the plasma membrane that in turn helps in active accumulation of metabolites like glucose. The enzyme is also probably involved in accumulation of K^+ inside the cell. The second role is particularly important in view of the uncertain status of Na^+,K^+-ATPase in this organism, as will be discussed later.

MATERIALS AND METHODS

Reagents

All biochemicals were purchased from Sigma, unless otherwise mentioned. Bis-oxanol and BCECF were from Molecular Probes. [γ-^{32}P]ATP (5000 Ci mmol^{-1}), $^{45}Ca^{2+}$ (39 mCi mg^{-1}), $^{86}Rb^+$ (10 mCi mg^{-1}), [^{3}H]Inulin (1 Ci mmol^{-1}), 2-deoxy-D-[U-^{14}C]glucose, and D-[U-^{14}C]glucose (300 mCi mmol^{-1}) were obtained from Amersham Pharmacia Biotech. Omeprazole and SCH28080 were kindly provided by Dr. Pratap Das (IICB), who got these chemicals as gift from Astra Hassle AB and Schering Corporation, respectively.

Organism

The organism used is a clinical isolate from a confirmed Kala-azar patient, and the strain is designated as MHOM/IN/1978/UR6 (15). The UR6 cells were grown and maintained on a solid blood-agar medium as described earlier (16). Only the highly motile promastigote cells were used for all experiments.

Preparation of sealed ghost and everted vesicles: *L. donovani* promastigote plasma membrane has a characteristic pellicular microtubular structure that provides strength to the membrane against hypotonic shock. Unsealed ghosts devoid of flagella were first prepared by exposing the promastigotes to controlled hypotonic shock (4, 8). These unsealed aflagellated ghosts were then used for preparation of both sealed ghosts and everted vesicles. The methods are briefly as follows. 2 gm of washed cells, collected at mid-log phase, were suspended in 100 ml of 5 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM phenylmethylsulfonyl fluoride that were kept in four long glass tubes at 4 °C. The tubes were mixed by mild vortexing in a cyclomixer 10 times for a total period of 90 min with occasional stirring. The hypotonic shock followed by gentle vortexing resulted in detachment of flagella from the cell body. Formation of unsealed ghosts was confirmed by total leakage of the marker cytoplasmic enzymes (8). $CaCl_2$ was added at this stage to a final concentration of 2 mM. The suspension was then centrifuged at 3300 × *g* for 20 min at 4 °C, and the pellet was resuspended in 5% sucrose in 50 mM Tris-HCl buffer, pH 7.4. 5 ml each of this suspension was layered on to a 10-ml cushion of 20% sucrose in the same buffer in a corex tube. These were centrifuged in a Sorvall HB4 rotor at 1500 × *g* for 30 min. The band at the interface of the two layers was highly enriched in flagella, whereas the pellet formed consisted largely of unsealed ghosts. The process was repeated twice, and the purified unsealed ghosts were utilized for the preparation of both sealed ghosts and everted vesicles.

For preparation of sealed ghosts, unsealed ghost pellets were resuspended in 30 ml of a medium containing 20 mM Hepes-Tris buffer, pH 7.5, 140 mM KCl, and 10 mM $CaCl_2$ at 35 °C for 40 min with gentle but continuous shaking. The suspension was then immediately cooled to 4 °C and was incubated with gentle shaking for another 30 min. The sealed ghosts obtained at this stage were pelleted by centrifugation at 3500 × *g* and resuspended in appropriate buffer for a particular experiment as described in the text. An electron microscopy picture of the sealed ghost from a typical preparation following this method is presented in Fig. 1*A*. Sealing could also be achieved by replacing KCl (140 mM) with sucrose (250 mM). Presence of Ca^{2+} (10 mM) was, however, essential for proper sealing.

Inside-out or everted vesicles were also prepared from unsealed ghosts. For this purpose, the unsealed ghosts were sheared into smaller membrane fragments, purified on a discontinuous sucrose gradient, washed, and finally sealed in presence of 25 mM Hepes-Tris buffer, pH 7.4, 140 mM KCl, and 4 mM Ca^{2+} or Mg^{2+} chloride. Everted vesicles were then pelleted, washed, and resuspended in appropriate buffer for subsequent experiments. Details of this procedure for preparing everted vesicles have been recently published (8). An electron microscopy picture of the everted vesicle is provided in Fig. 1*B*. Whenever necessary, everted vesicle could also be prepared in presence of sucrose (250 mM), Hepes-Tris buffer, pH 7.4 (20 mM), and Ca^{2+} (4 mM). This has been mentioned in the appropriate places in the text. Sealing of ghosts and of everted vesicles was confirmed with the help of fluorescent dye calcein by exploiting its self-quenching property at high concentrations (17) and also by $^{86}Rb^+$ trapping (18).

Electron Microscopy

Samples of sealed ghosts and everted vesicles were fixed with 3% gluteraldehyde for 15–20 h at 4 °C. After a wash with the respective buffers, the samples were post-fixed with 1% osmium tetroxide in Kellenburger buffer for 16–20 h at room temperature. The pellets were then washed again and dehydrated with a graded series of ethanol solutions and finally embedded in SPURRT resin. Thin sections were collected using an ultrathin microtome and picked up on copper grids. Observations were made in a transmission electron microscope (JEOL IN ex) at different magnifications.

Functional Measurements for Sealed Ghosts and Everted Vesicles

Internal pH—Both the unsealed ghost and the membrane fragment were sealed as described above in presence of BCECF at a concentration of 4 μg ml^{-1}. For fluorescence measurements, the dye-loaded and washed everted vesicles (500 μg protein ml^{-1}) were placed in a 1-ml fluorescence cuvette. A Hitachi spectrofluorimeter model F4010 was used to monitor the readings of BCECF at 505 nm excitation and at 525 nm emission wavelengths (19). No appreciable leakage of the dye was observed during the period in which experiments were completed. Calibration of the internal pH of the everted vesicle was done by incubating the BCECF-loaded vesicle in a buffer containing 140 mM KCl, 4 mM $CaCl_2$, and 25 mM Hepes-Tris buffer with varying pH (6.5–7.6) in presence of valinomycin (1 μg ml^{-1}) and FCCP (5 μM). FCCP and valinomycin equilibrate the intracellular and extracellular proton and K^+, respectively (20). The pH_i of the vesicle is assumed to be the same as the extracellular pH as the pH_i and pH_o become equal in presence of FCCP. A calibration curve was made by plotting the ratio of the fluorescence at 535 nm when the samples were excited at 503 and 440 nm, respectively, at various pH buffers (21) and found to be linear between pH 6.5 and 7.6 (data not shown). For stoichiometric calculations, the H^+ influx in the everted vesicle was calculated according to the method of Levy *et al.* (22). In this case, H^+ influx = Buffering capacity × ΔpH × internal volume.

Transmembrane Potential—The development of transmembrane potential was measured fluorimetrically in the same instrument with bis-oxanol at λ_{ex}535 nm and at λ_{em} at 559 mm (23). For this purpose, a final concentration of 80 nM dye was added to the cuvette containing vesicle suspension (400 μg protein ml^{-1}) in sealing buffer pH 7.4.

Internal Volume—The average internal volumes of the sealed ghost and everted vesicle were determined by loading either with ^{86}RbCl or with [^{3}H]inulin during the sealing process. These were then washed and counted. Internal volumes were calculated according to Rottenberg (18).

Enzyme Assays

Mg^{2+}-ATPase activity was determined either colorimetrically by measuring the Mg^{2+}-dependent release of inorganic phosphate or by measuring the liberation of ^{32}P from [λ-^{32}P]ATP. Whenever required, the concentration of free Mg^{2+} was controlled by complexation with CDTA. For colorimetric assay, the incubation medium contained in a total volume of 1 ml, 100 mM Tris-HCl, pH 7.4, 500 μM CDTA with or without $MgCl_2$, and 0.5 mM ATP. The reaction was initiated by addition of everted vesicle (200 μg protein ml^{-1}) as the source of enzyme. $MgCl_2$ concentration was varied to give the required free Mg^{2+} concentration that was calculated according to Parshadsingh and McDonald (24). Incubation was for 30 min at 28 °C. The reaction was terminated by addition of 50 μl of 20% trichloroacetic acid. After removal of the

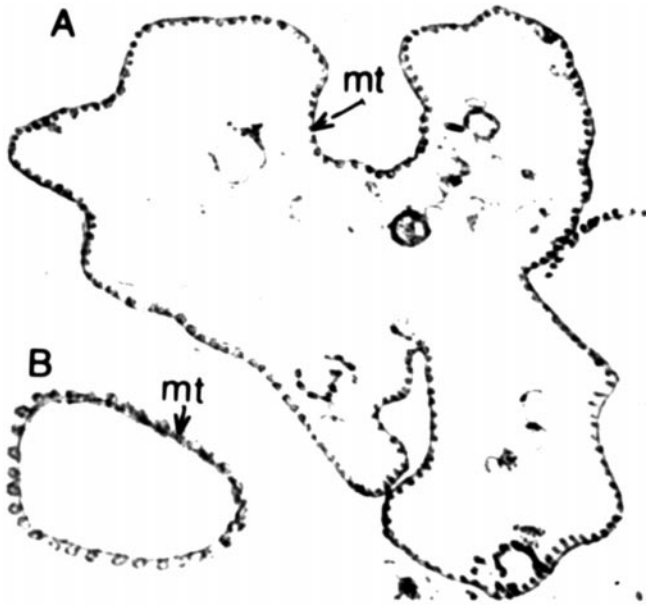

FIG. 1. **Thin section electron micrograph of a single sealed ghost with right-side-out orientation (×22,000)** *A*, an everted plasma membrane vesicle (×45,000) *B*, *mt* indicates microtubules.

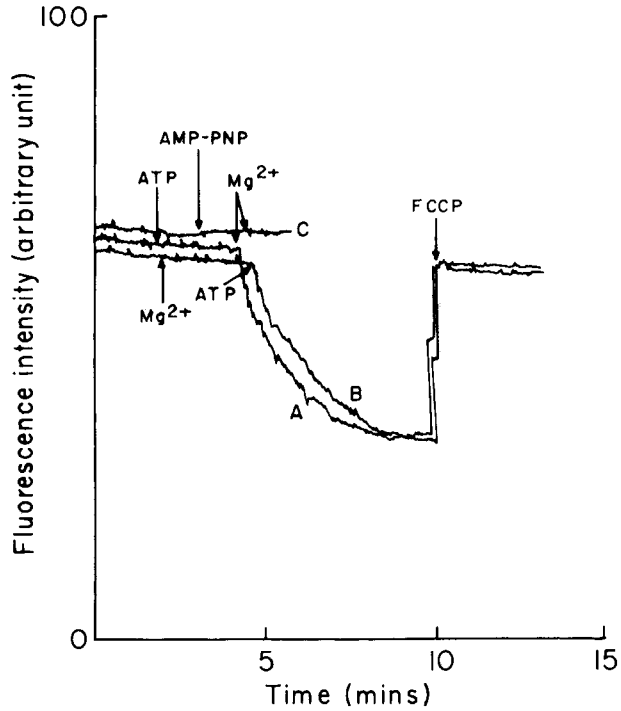

FIG. 2. **Mg^{2+}-ATPase is a proton translocator in everted vesicles.** The BCECF-loaded vesicles each containing 250 μg of protein were placed in 1-ml cuvettes, and fluorescence intensities were measured as described under "Materials and Methods." Each of the reaction mixture contained in a total volume of 500 μl, 20 mM Tris-Hepes buffer, pH 7.4, 125 mM KCl, 15 mM NaCl, 4 mM $MgCl_2$, and 5 mM CDTA. In tube *A*, ATP (1 mM), $MgCl_2$ (2 mM), and FCCP (10 μM) were added sequentially at indicated times. In tube *B*, order of addition of ATP and Mg^{2+} was reversed. In tube *C*, AMP-PNP (1 mM) replaced ATP.

precipitate, the released inorganic phosphate was measured following the method of Lowry and Lopez (25). To determine free Mg^{2+} concentration, Mg^{2+} from the vesicle (10 μM) and from other reagents (4 μM) were included in the calculations. Mg^{2+}-stimulated ATPase activity was determined by subtracting the values with chelator alone from the values with Mg^{2+} and chelator. For the radioactive assay, the method of Bais (26) was followed with few modifications. In this case, the final volume of the assay mixture was reduced to 0.1 ml. The composition and concentration of all the ingredients of the assay mixture remained the same as before. In this assay, 50 nmol of [γ-^{32}P]ATP (0.1 μCi) was added. After 10 min of incubation at 28 °C, the reaction was stopped by addition of 5 μl of 20% trichloroacetic acid. To this 10 μl of 100 mM KH_2PO_4 and 0.1 ml of suspension of 50% activated charcoal in water were added consecutively. After mild agitation for 10 min, charcoal was precipitated by centrifugation. The process was repeated once more, and finally 100 μl of the supernatant was transferred to a scintillation vial.

Generation of pH Gradient and [^{14}C]2-Deoxyglucose Uptake

pH gradient was imposed by using "pH jumps" (27). All assays were performed at 25 °C. To initiate the reaction, everted vesicles or sealed ghosts at pH 8.0 (protein concentration about 15 mg ml^{-1}) were diluted 50-fold into mixtures containing sealing buffer of either pH 5.5 or 8 and 50 μM of [^{14}C]2-deoxyglucose (1 μCi ml^{-1}) or other additions as required. Each tube containing 500 μl of mixture was filtered at different time intervals through millipore filters (pore size, 0.8 μm) and washed with 10 ml of cold resuspension buffer, and trapped radiolabeled count was measured as described earlier (8).

RESULTS

Characterization of Ghost and Vesicle Preparations—Determination of polarity or sidedness of sealed vesicles or ghosts in the absence of a marker for plasma membrane often poses some problems. All flagellated trypanosomatids, including Leishmania have an unique cytoskeletal microtubular arrangement that can serve as an excellent marker for this purpose. Extensive cytological studies had earlier shown that these microtubular beads are closely associated with the inner lamina of the plasma membrane of intact cells (28). This arrangement provides scaffolding and rigidity for the membrane structure and can be very conveniently used for determining polarity of closed structures. This is evident from the thin section electron photomicrograph of a typical sealed ghost that has an array of microtubules at regular intervals in the inner side of the ghost (Fig. 1*A*). For this purpose, unsealed ghosts were sealed in presence of Ca^{2+} (10 mM). The presence of Ca^{2+} was found to be an obligatory requirement, and it could not be replaced by other divalent cations. Mild hypotonic shock in the absence of drastic homogenization resulted in the preparation of deflagellated unsealed ghosts that retain most of the microtubular structure but lose all internal structures and cytoplasmic marker enzymes. In contrast to the sealed ghost preparation, the everted vesicles that were prepared from sheared plasma membrane were of smaller dimension and clearly displayed the marker microtubules on the outside (Fig. 1*B*). The lesser number of microtubules were probably due to the shearing during the membrane preparation.

Sealing was initially confirmed by calcein loading. Both the ghost and the everted vesicle are effectively impermeable within experimental time to cations such as $^{45}Ca^{2+}$ and $^{86}Rb^{+}$. These ions could be rapidly released only in presence of A23187 and valinomycin, respectively. The average internal volume of sealed ghost was calculated to be 3.6 μl mg^{-1} protein (n = 4), and everted vesicle was 1.4 μl mg^{-1} protein (n = 4) (see "Materials and Methods").

H^+ Entry and Accumulation in Everted Vesicles—The plasma membrane Mg^{2+}-ATPase, which is known to have its catalytic site on the cytoplasmic side, will display its catalytic site to the outer mileau in the everted vesicle. Rapid entry of H^+ in the everted vesicle could be demonstrated only when both ATP and Mg^{2+} were added, indicating the Mg^{2+}-ATPase to be responsible for H^+ uptake. Addition of FCCP resulted in an immediate release of accumulated H^+. ATP hydrolysis was obligatory for H^+ entry as nonhydrolyzable ATP analogue AMP-PNP completely failed to replace ATP (Fig. 2). No H^+ uptake was observed on addition of Ca^{2+} and ATP, thus excluding any role for the plasma membrane Ca^{2+}-ATPase in the process (data not shown). In a separate control experiment, we

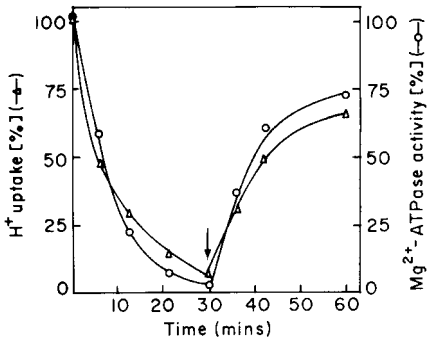

FIG. 3. **Correspondence between Mg^{2+}-ATPase activity and H^+ uptake.** Each tube contained in a total volume of 500 μl, BCECF-loaded everted vesicle (250 μg ml^{-1} protein), 20 mM Tris-Hepes buffer, pH 7.4, 125 mM KCl, 15 mM NaCl, 4 mM $MgCl_2$, and DTNB (1 mM) except in zero time control where DTNB was omitted. In all cases, reactions were initiated with ATP (1 mM). For reversal of enzyme activity and of H^+ uptake capacity, dithiothreitol (10 mM) was added to a few tubes as indicated ($\downarrow$). Enzyme activity was measured by removing suitable aliquots. H^+ influx was measured as percent fluorescence quench at the end of 5 min.

observed that unlike the everted vesicle, the sealed ghost with original polarity failed to show any significant uptake of H^+ on addition of ATP and Mg^{2+} (data not shown). Clearly, ATP was not available to the catalytic site of Mg^{2+}-ATPase in this case.

Correlation between Mg^{2+}-ATPase Activity and H^+ Translocation—Role of Mg^{2+}-ATPase in H^+ entry could be further confirmed when an excellent correlation between activity of the enzyme and translocation of H^+ could be demonstrated in the everted vesicle with the help of a reversible thiol-modifying reagent. We found that the enzyme could be completely inactivated (>95%) by *p*-chloromercaribenzoate (0.5 mM) in 20 min, DTNB (1 mM) in 30 min, and *N*-ethylmealamide (1 mM) in 40 min. Subsequent incubation with excess dithiothreitol (10 mM) could reactivate (>60%) both the *p*-chloromercuribenzoic acid and DTNB-inactivated enzymes (data not shown). Based on these observations, an experiment was set up to monitor the possible correlation between enzyme activity and capacity for H^+ uptake. Fig. 3 shows that an excellent correlation exist between Mg^{2+}-ATPase activity and the initial rate of H^+ transport (first 2 min) in the everted vesicle, leaving little doubt that the plasma membrane Mg^{2+}-ATPase is exclusively responsible for H^+ translocation across the plasma membrane.

Effect of Metabolites and Inhibitors on H^+ Transport—Table I summarizes the results of an experiment in which H^+ accumulation in the everted vesicle at the end of 5 min was monitored in presence of some nucleotide triphosphates and inhibitors. ATP could not be replaced to any significant extent by any of the other triphosphates tried. Strong inhibition by orthovanadate suggests the H^+ translocator to be a P-type ATPase. Inhibition of this enzyme activity by vanadate (29) and formation of β-aspartyl phosphate intermediate (30) were earlier shown by other workers. Most importantly, omeprazole and SCH28080 both specific inhibitors for gastric H^+,K^+-ATPase (31) showed strong inhibitory influence on H^+ accumulation. Direct participation of Na^+,K^+-ATPase or of a V-type ATPase in H^+ pumping could be ruled out because neither ouabain nor bafilomycin A_1 showed any effect on H^+ accumulation (32, 33). To analyze more carefully the extent of inhibition that can be achieved by omeprazole and by SCH28080, the initial rate of H^+ entry (first 2 min) was followed with increasing concentrations of these two inhibitors and vanadate. The experimental protocol was the same as in Table I. Both the drugs inhibited the entry process (up to 95 and 75%, respectively) in a dose-dependent manner. IC_{50} for omeprazole and SCH28080 were 50 and 40 μM, respectively. Omeprazole could

TABLE I
Effect of energy sources and potential inhibitors on H^+ transport in everted vesicles

For this experiment vesicles were preincubated as described in the legend to Fig. 2. ATP and other nucleotides were added after 5 min. For monitoring the effect of inhibitors, vesicles were separately preincubated for 10 min with indicated inhibitors, and then ATP (1 mM) was added. Each reaction was started with $MgCl_2$ (2 mM), and respective fluorescence quenching was measured. H^+ uptake was expressed as the percentage of fluorescence quench at the end of 5 min. DCCD, *N*,*N*-dicyclohexylcarbodi-imide.

Addition	H^+ pumping activity
	%
ATP (1 mM)	100
CTP (1 mM)	05
GTP (1 mM)	03
UTP (1 mM)	20
AMP-PNP (1 mM)	03
DCCD (100 μM)	50
Oligomycin (5 μg ml^{-1})	96
Vanadate (100 μM)	18
Ouabain (250 μM)	90
Bafilomycin A_1 (5 μM)	98
Omeprazole (50 μM)	51
SCH28080 (50 μM)	55

effectively abolish H^+ influx (>90%) at 150 μM, whereas SCH28080 at that concentration had a less pronounced effect. The inhibitory effect of omeprazole compared favorably well with that of vanadate (data not shown). These results suggested that the parasite H^+ translocating plasma membrane Mg^{2+}-ATPase may have some enzymological and physiological properties that may be similar to the mammalian gastric H^+,K^+-ATPase (34).

Stimulation of Mg^{2+}-ATPase Activity with K^+—The strong inhibition of H^+ translocating capacity of Mg^{2+}-ATPase by omeprazole and SCH28080 prompted us to see whether as in the case of mammalian gastric H^+,K^+-ATPase, the parasite enzyme can also be activated by K^+. For this purpose, we used unsealed ghost preparation as our source of enzyme. This preparation provides free access for ions and nucleotides to the cytosolic face of the enzyme. Further, the membrane-bound enzyme represents physiologically a more authentic situation than the enzyme solubilized with detergents (5). The ATP hydrolytic activity was found to be only moderately but systematically stimulated by K^+ ion in all the preparations tested so far. At the saturating concentration of free Mg^{2+} that was controlled by CDTA-Mg^{2+} buffering system (24), activation by K^+ (20 mM) was 50%. More importantly, the stimulated activity could be abolished in presence of SCH28080, a specific competitor for K^+ binding site of H^+,K^+-ATPase (31, 34) (Fig. 4). We also observed that (*a*) further increase in K^+ concentration did not result in any additional stimulation, (*b*) Na^+ (upto 100 mM) had no stimulatory or inhibitory effect, and (*c*) ouabin had no inhibitory effect on the K^+-stimulated activity (data not shown).

ATP-dependent $^{86}Rb^+$ Extrusion—To ascertain whether the parasite plasma membrane Mg^{2+}-ATPase can translocate K^+ as a counterion to H^+, everted vesicles were sealed with $^{86}Rb^+$, and then the reaction was initiated by adding ATP. For this experiment, vesicles were sealed in presence of 25 mM Hepes-Tris buffer, pH 7.4, containing 100 mM RbCl, 40 mM NaCl, 4 mM $MgCl_2$, and $^{86}Rb^+$ (20 μCi ml^{-1}). Vesicles (200 μg protein ml^{-1}) were washed twice and finally suspended in a medium containing 25 mM Hepes-Tris buffer, pH 7.4, 250 mM sucrose, and 4 mM $MgCl_2$. On addition of ATP (1 mM), an immediate and rapid extrusion of $^{86}Rb^+$ could be observed (Fig. 5). When ATP was replaced with AMP-PNP, no outward movement of $^{86}Rb^+$ could be observed, clearly implicating Mg^{2+}-dependent ATP hydrolysis to be essential for $^{86}Rb^+$ extrusion. Preincubation with

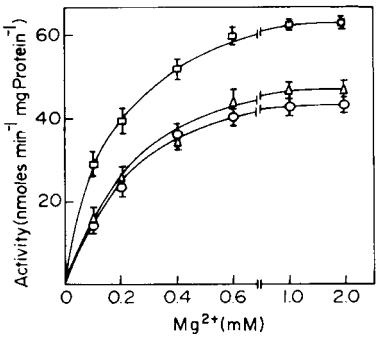

FIG. 4. **Stimulation of Mg^{2+}-ATPase activity with K^+ and inhibition with SCH28080.** The rate of Mg^{2+}-dependent ATP hydrolysis was estimated as [^{32}P]P_i release (see "Materials and Methods"). An appropriate control was run in the absence of Mg^{2+}. Each assay mixture contained in a total volume of 100 μl, 100 mM Tris-HCl, pH 7.4, 0.5 mM ATP (Tris-salt), 500 μM CDTA, and varying amounts of $MgCl_2$ with other additions as indicated. The reaction was started by the addition of a requisite amount of unsealed membrane ghost preparation (~5 μg of protein). After 10 min of incubation at 30 °C, the reaction was terminated by the addition of 5 μl of 20% trichloroacetic acid, and liberated $^{32}P_i$ from [γ-^{32}P]ATP was measured as Mg^{2+}-ATPase activity. Additions, without KCl (*open circle*), 20 mM KCl (*open square*), and 20 mM KCl + 50 μM SCH28080 (*open triangle*). Mean ± S.D. values are plotted from three separate experiments.

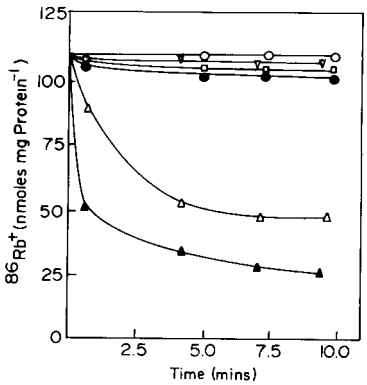

FIG. 5. **ATP-dependent $^{86}Rb^+$ extrusion from preloaded everted vesicles.** Everted vesicles (200 μg of protein) preloaded with $^{86}Rb^+$ were suspended in 1.0 ml of 25 mM Hepes-Tris buffer, pH 7.4, 250 mM sucrose, and 4 mM $MgCl_2$ at 30 °C. Extrusion of $^{86}Rb^+$ was measured for each individual tube at indicated time after following additions: control without ATP (*open circle*), immediately after addition of 1 mM ATP (*open triangle*), immediately after addition of 5 μM valinomycin (*close triangle*), immediately after addition of 1 mM AMP-PNP (*reverse open triangle*), preincubation with 150 μM omeprazole for 15 min and then 1 mM ATP (*close circle*), and preincubation with 1 mM DTNB for 30 min and then 1 mM ATP (*open square*).

omeprazole (100 μM) for 10 min and DTNB (1 mM) for 30 min resulted in total blocked of $^{86}Rb^+$ extrusion, providing strong evidence that the Mg^{2+}-ATPase is indeed a H^+/K^+ antiporter. Valinomycin (5 μM)-dependent $^{86}Rb^+$ release served as the control (Fig. 5). As with omeprazole, preincubation with SCH28080 could also completely inhibit the Rb^+ extrusion process (data not shown). We failed to detect the presence of any H^+/K^+ exchanger in the plasma membrane of the parasite. For this purpose, $^{86}Rb^+$-loaded everted vesicle (internal pH 7.6) was diluted 50-fold in a medium containing Hepes-Tris buffer, pH 6.5. No efflux of $^{86}Rb^+$ above the control (dilution in pH 7.6 of Hepes-Tris buffer) could be observed.

Stoichiometry—Ion-translocating ATPases can either be electrogenic or electroneutral depending upon the stoichiometry of the overall ion movement. Rapid accumulation of H^+ inside the everted vesicle and simultaneous release of $^{86}Rb^+$ as a surrogate for K^+ from the vesicle provided us with an opportunity to calculate the stoichiometry of ion movement during ATP hydrolysis. Table II summarizes results of such an experiment. For this experiment, a calibration curve for the internal pH of the everted vesicle was first drawn according to the method of Negulescu and Machen (21) and as described under "Materials and Methods." The internal volume was calculated following the method of Rottenberg (18), and with these two values the total influx of H^+ into the everted vesicle was calculated by multiplying buffering capacity with change in pH according to Leavy *et al.* (22). For calculation of K^+ extrusion, kinetics of the outward flow of $^{86}Rb^+$ and K^+ were assumed to be identical (35). Average internal volume of the everted vesicle had earlier been calculated to be 1.4 μl mg^{-1} protein. For accurate determination of the extent of ATP hydrolysis, [γ-^{32}P]ATP was used as the substrate. Detailed procedures are provided under "Materials and Methods."

Table II shows that both for the initial rate (1.5 min) and for the average rate over 3 min when the rates of both H^+ influx and Rb^+ extrusion considerably slowed down, hydrolysis of one molecule of ATP can maximally be related to the movements of one H^+ and one Rb^+ across the plasma membrane of the everted vesicle. Extrapolation of the rates to zero time of ATP addition are expected to give the true initial values for these ion movements, although no experiment was set up for this purpose. Higher values for ATP hydrolytic activity were probably due to the presence of some unsealed vesicles and contaminating membrane fragments. More importantly, at both stages of measurement the ratio between H^+ influx and Rb^+ extrusion remains very close to one, strongly indicating the process to be an electroneutral one. In separate experiments, when such Rb^+ or K^+-loaded everted vesicles were suspended in the same medium that also contained bis-oxanol to record changes in membrane potential, activation of Mg^+-ATPase by addition of ATP brought about no change in bis-oxanol fluorescence (data not shown), confirming once again the electroneutral character of this ion-translocating ATPase. Assuming Rb^+ to be a faithful replacement for K^+ for this enzyme, the plasma membrane Mg^{2+}-ATPase should therefore be regarded as an electroneutral H^+/K^+ antiporter.

H^+-driven 2-Deoxyglucose Accumulation in Sealed Ghosts—Results presented so far indicate that the operation of the plasma membrane Mg^{2+}-ATPase in a respiring *L. donovani* cell should generate a H^+ gradient across the membrane with interior alkaline. Can this proton gradient be involved in the active accumulation of important metabolites? Earlier, contradictory results were reported for glucose transport with resting whole cell (11) or with growing whole cells in a chemostat (12). When a pH gradient of 2.5 was imposed across the plasma membrane of sealed ghost of original polarity (pH 5.5 and 8.0 for outside and inside, respectively), a rapid accumulation of ^{14}C-2-deoxyglucose could be demonstrated (Fig. 6). In absence of an imposed proton gradient or when a powerful protonophore like FCCP (10 μM) was present, no such accumulation could be observed. More importantly, based on an approximate internal volume of 3.6 μl mg^{-1} protein (see above), the ghost accumulates more than 0.5 mM of deoxyglucose, which is at least 10 times more than its external concentration. We could also show similar uptake and accumulation with isotopic glucose and competition between the two sugars (data not shown). Clearly, a H^+-glucose symport system is operative in the plasma membrane of Leishmania promastigote cells.

Direct Correlation between Plasma Membrane Mg^{2+}-ATPase Activity and Glucose Movement across the Membrane—Active accumulation of glucose in sealed ghost on imposition of a pH gradient does not necessarily signify the involvement of the plasma membrane Mg^{2+}-ATPase in the process. To find out whether H^+ movement initiated by activation of Mg^{2+}-ATPase

TABLE II

Stoichiometry of ATP hydrolysis, H^+ influx, and Rb^+ extrusion in everted vesicle

For the determination of H^+ influx and Rb^+ extrusion, everted vesicles were first preloaded with BCECF and $^{86}Rb^+$ (see "Materials and Methods"). Each tube contained in a total volume of 500 μl an aliquot of everted vesicle (400 μg of protein ml^{-1}), 20 mM Tris-Hepes buffer, pH 7.4, 250 mM sucrose, and 4 mM $MgCl_2$. For one set of tubes quenching of BCECF fluorescence (ΔpH from standard pH calibration curve, see methods) was determined at 1.5 and 3.0 min. For the second set of tubes $^{86}Rb^+$ extrusion was determined at also 1.5 and 3.0 min. For ATP hydrolysis data, vesicles were scaled under identical conditions but in presence of cold Rb^+. After addition of 1 mM [λ-^{32}P]ATP (0.5 μCi), appropriate aliquots were withdrawn for estimation of liberated radiolabeled phosphate as described under "Materials and Methods." Details of calculation procedures have been described in the text. All tubes were in triplicate, and the mean average was taken for calculation.

	1.5 min		3.0 min	
	Total	Rate	Total	Rate
	nmol mg protein^{-1}	*nmol min^{-1} mg protein^{-1}*	*nmol mg protein^{-1}*	*nmol min^{-1} mg protein^{-1}*
ATP hydrolysis [A]	36.0	24.0	69.1	23.0
H^+ influx [B]	29.1	19.4	48.7	16.2
Rb^+ efflux [C]	30.6	20.4	52.4	17.5
Ratio [A]:[B]:[C]		1:0.81:0.85		1:0.70:0.76
Ratio [B]:[C]		1:1.05		1:1.09

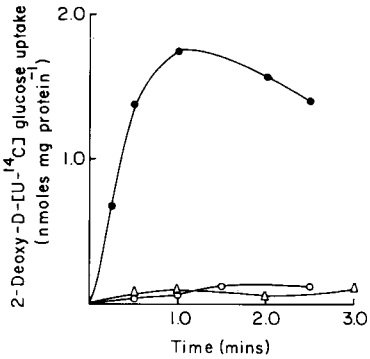

FIG. 6. **Imposed pH gradient drives 2-deoxyglucose influx and accumulation in sealed ghost.** Sealed ghost was prepared as described under "Materials and Methods." In the sealing medium 20 mM Tris-HCl buffer, pH 7.5, was replaced by the same buffer of pH 8.0. Pelleted sealed ghost was resuspended (~15 mg ml^{-1}) in a incubation medium containing 20 mM Tris-Hepes buffer, pH 8.0, 250 mM sucrose, and 10 mM $CaCl_2$ for 10 min at 30 °C. Appropriate aliquots of sealed ghost (~150 μg of protein) were rapidly diluted 50 times into 0.5 ml of medium containing 250 mM sucrose, 10 mM $CaCl_2$ 50 μM [U-^{14}C]2-deoxyglucose (0.8 μCi) with other ingredients as described below and were rapidly filtered at indicated time intervals. Additions, 50 mM Hepes-Tris buffer, pH 8.0 (*open triangle*), 50 mM Mes-Tris buffer, pH 5.5 (*close circle*), and 50 mM Mes-Tris buffer, pH 5.5, and 10 μM FCCP (*open circle*).

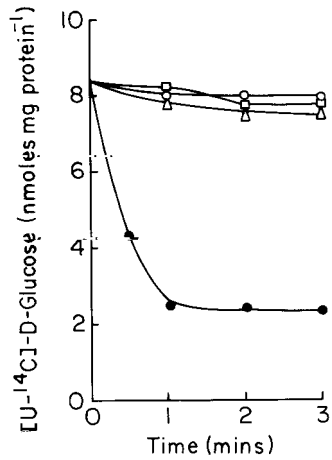

FIG. 7. **Mg^{2+}-ATPase dependent [U-^{14}C]D-glucose extrusion from preloaded everted vesicles.** For this experiment, everted sealed ghosts were prepared in presence of 25 mM Tris-Hepes buffer, pH 7.4, 140 mM KCl,10 mM $CaCl_2$, and 7.5 mM [U-^{14}C]D-glucose (20 μCi ml^{-1}). Washed and preloaded sealed ghosts (200 μg protein ml^{-1}) were suspended in separate tubes, each containing in 500 μl, 25 mM Tris-Hepes buffer, pH 7.4, 250 mM sucrose, and 4 mM $MgCl_2$. Other additions, if made, are indicated below. Incubation time was for 5 min at 30 °C. Reactions were initiated by additions as indicated below. Released counts in the incubation medium were recorded at indicated times by rapid filtration as described under "Materials and Methods." *Open circle*, control without any addition; *closed circle*, with 1 mM ATP; *open square*, preincubated with 10 μM FCCP and then 1 mM ATP; *open triangle*, with 1 mM AMP-PNP.

can directly be correlated to a simultaneous movement of glucose across the plasma membrane, the following experiment was set up with the everted vesicle. In this case, although the catalytic site of the enzyme is exposed outside, the binding sites of the putative H^+-glucose symporter are presumed to be oriented inside the vesicle. Everted vesicles containing Tris-HCl buffer, pH 7.4 (50 mM), KCl (120 mM), NaCl (10 mM), and $MgCl_2$ (4 mM) were sealed in presence of [U-^{14}C]D-glucose (20 μCi ml^{-1}; 7.5 mM). Everted vesicles loaded with labeled glucose were washed twice and then suspended in the same Tris-HCl buffer containing sucrose (250 mM) and $MgCl_2$ (4 mM). Fig. 7 shows an immediate and rapid efflux of glucose on addition of ATP in the incubation mixture. When ATP was replaced with AMP-PNP or the Mg^{2+}-ATPase was inactivated by pretreatment with DTNB, no efflux of glucose could be detected. Finally, preincubation with FCCP (10 μM) also resulted in total abolition of the efflux. Clearly, influx of H^+ on activation of Mg^{2+}-ATPase results in the parallel activation of the H^+-glucose symport system that initiates the rapid efflux of glucose from the preloaded vesicles.

DISCUSSION

Ghosts and vesicles of defined polarity have proven to be extremely powerful tools in many studies involving bioenergetics, ion translocation, and active accumulation of metabolites (31, 36). Taking advantage of the comparative rigidity provided by the pellicular microtubular structure to the plasma membrane of *L. donovani*, we could quite conveniently prepare sealed ghosts and everted vesicles of defined polarity that are free from cytoplasmic marker enzymes and internal metabolites (Fig. 1 and Methods). Everted vesicles provide free access to ATP and other charged molecules to the catalytic site of the plasma membrane Mg^{2+}-ATPase, which now becomes exposed to the suspending medium. Mg^{2+}-dependent ATP hydrolysis led to rapid accumulation of H^+ in everted vesicles (Fig. 2) and to almost equally rapid depletion of loaded $^{86}Rb^+$ (Fig. 5) from these vesicles. Direct involvement of the Mg^{2+}-ATPase in these ion movements was further confirmed when an excellent correspondence between the rate of H^+ influx and enzyme activity could be demonstrated (Fig. 3) or when Rb^+ efflux could be completely prevented by inactivating the enzyme with DTNB (Fig. 5). Thus, in terms of its ion-translocating capacity this Mg^{2+}-ATPase can be considered as a H^+,K^+-ATPase. Assuming Rb^+ to be a true surrogate for K^+, we also calculated the stoichiometry between H^+ and K^+ exchange. This appears to be an electroneutral process giving a stoichiometry of 1:1 (Ta-

ble II and text). The electroneutral character of the antiporter is very similar to the gastric mucosal H^+,K^+-ATPase from different mammalian sources (31, 37, 38).

Can the Leishmania plasma membrane Mg^{2+}-ATPase be regarded as identical to the gastric H^+,K^+-ATPase? In absence of any significant biochemical, mechanistic, and molecular data, this remains an open question at the moment. In our initial efforts we have failed to purify and biochemically characterize this enzyme because of its severe aggregation problems. However, complete abolition of the K^+- stimulated activity by SCH28080 (Fig. 4) and also the strong inhibition of H^+ uptake in and Rb^+ efflux from the everted vesicle by inhibitors of H^+,K^+-ATPase (Table I, text, and Fig. 5) clearly indicate that this enzyme has some characteristic properties that are very similar to those of the gastric H^+,K^+ATPase. The K^+ translocating activity and sensitivity to inhibitors of gastric H^+,K^+-ATPase also set this enzyme apart from the well characterized plasma membrane H^+-ATPase of lower eucaryotes such as yeast and other fungi (39, 40). Thus, we conclude that in terms of its functional capability this plasma membrane enzyme is very similar to gastric H^+,K^+-ATPase.

What are the possible physiological roles of this Mg^{2+}-ATPase that we have identified as a H^+,K^+ antiporter? The electroneutral but energy-dependent extrusion of H^+ from the cytosol and uptake of K^+ into the cytosol will result in setting up of a pH gradient across the plasma membrane with interior alkaline and in accumulation of K^+ in the cytosol. The pH gradient generated by this enzyme is fully capable of driving active accumulation of metabolites. This has been exemplified by the demonstration of rapid uptake and accumulation of glucose in sealed ghosts (Fig. 6) and by the presence of a H^+-glucose symporter in the plasma membrane of the parasite that is functionally closely coupled to this ATPase (Fig. 7). This is the first clear demonstration of a H^+-glucose symport mechanism for glucose accumulation operative in Leishmania parasites. It is important to note that developmentally regulated glucose transporter genes having significant homology with such genes of higher eucaryotes were earlier cloned from Leishmania, although their relationship to energy coupling remained uncertain (14, 41).

Membrane potential of Leishmania was determined earlier to be around −100mV by two independent methods (42, 43). The concentration of cytosolic K^+ also appears to be typical of eucaryotic cells (9). How is the potential built up and maintained? Several laboratories have failed to detect the presence of a plasma membrane Na^+,K^+-ATPase in *L. donovani* (7, 42, 44), although its occurence has been claimed in a different species of Leishmania by Felibertt *et al.* (45). Whatever be the status of Na^+,K^+-ATPase in this parasite, it is clear that operation of this H^+,K^+-ATPase will significantly help in the accumulation of K^+ in the cytosol. In fact, this process may be critical if Na^+,K^+-ATPase is truly absent in *L. donovani*. Electroneutral accumulation of K^+, mediated by this enzyme, is obviously incapable of generating the desired membrane potential. Very little is known about other ion translocators, anion conducting pathways, and channels that may be involved in generating this potential. So far, only a Cl^- conducting pathway has been demonstrated in this parasite (43), and a putative Ca^{2+}-activated K^+ channel gene has been uncovered in *Leishmania major* as part of the genome sequencing program (46). Needless to say, much more work needs to be done in this area before a comprehensive picture emerges. Further work on this putative H^+,K^+-ATPase is expected to throw considerable light on the enzymology and function of this important and interesting enzyme in the life cycle and physiology of this dreaded protozoal pathogen. This enzyme may also be explored as a potential target for drug development.

Acknowledgments—We thank Sailen Dey for assistance with electron microscopic photography and Suprabhat Bhattacharyya for typing the manuscript. We also thank Dr. Pratap Das for stimulating discussions.

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