Journal of Bioenergetics and Biomembranes, Vol. 28, No. 6, 1996

Reconstituted Cl^- Pump Protein: A Novel Ion(Cl^-)-Motive ATPase

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Received January 2, 1996; accepted June 10, 1996

Cl^- absorption by the *Aplysia californica* foregut is effected through an active Cl^- transport mechanism located in the basolateral membrane of the epithelial absorptive cells. These basolateral membranes contain both Cl^--stimulated ATPase and ATP-dependent Cl^- transport activities which can be incorporated into liposomes via reconstitution. Utilizing the proteoliposomal preparation, it was demonstrated that ATP, and its subsequent hydrolysis, Mg^{2+}, Cl^-, and a pH optimum of 7.8 were required to generate maximal intraliposomal Cl^- accumulation, electrical negativity, and ATPase activity. Additionally, an inwardly-directed valinomycin-induced K^+ diffusion potential, making the liposome interior electrically positive, enhanced both ATP-driven Cl^- accumulation and electrical potential while an outwardly-directed valinomycin-induced K^+ diffusion potential, making the liposome interior electrically negative, decreased both ATP-driven Cl^- accumulation and electrical potential compared with proteoliposomes lacking the ionophore. Either orthovanadate or *p*-chloromercurobenzene sulfonate inhibited both the ATP-dependent intraliposomal Cl^- accumulation, intraliposomal negative potential difference, and also Cl^--stimulated ATPase activity. Both aspects of Cl^- pump transport kinetics and its associated catalytic component kinetics were the first obtained utilizing a reconstituted transporter protein. These results strongly support the hypothesis that Cl^--ATPase actively transports Cl^- by an electrogenic process.

KEY WORDS: Ion-motive ATPase; chloride pump; catalytic and transport kinetics; electrogenic.

INTRODUCTION

Most cation-motive primary active transport proteins have been extensively studied and well characterized (Pedersen and Carafoli, 1987). However, there is a scarcity of knowledge about anion transport proteins, with the exception of halorhodopsin in *Halobacterium halobium* (Blanck and Oesterhelt, 1987) and band 3 in erythrocytes (Lukacovic *et al.*, 1981). Neither of these proteins, however, is an ATPase as are most of the cation-motive primary active transporters (Pedersen and Carafoli, 1987). The few studies of Cl^--stimulated ATPase in eukaryotes (Inagaki and Shiroya, 1988; Ikeda *et al.*, 1990) demonstrated transport activities in native membrane vesicles; however, active Cl^- transport had not yet been demonstrated in reconstituted systems using purified or semipurified transporter proteins.

Transepithelial Cl^- flux studies (Gerencser, 1981, 1984) across *Aplysia californica* gut have shown that the short-circuit current is carried by a net active Cl^- absorptive flux. It was hypothesized that Cl^- absorption across the *Aplysia* gut is mediated by a primary active transport process (i.e., an ATPase) (Gerencser and White, 1980; Gerencser, 1983) located in the basolateral membrane (BLM) by means of thermodynamic determinations (Gerencser, 1983). Lending support to this idea were the subsequent demonstrations of: (1) the existence of Cl^--stimulated ATPase activity in *Aplysia* foregut absorptive cell BLMs (Gerencser and Lee, 1985a), and (2) the existence of an ATP-dependent

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0145-479X/96/1200-0459$09.50/0

Cl^- uptake in these same *Aplysia* foregut absorptive cell BLM vesicles (Gerencser and Lee, 1985b; Gerencser, 1988). Furthermore, both Cl^--stimulated ATPase and ATP-dependent Cl^- transport activities were reconstituted into artificial liposomes (Gerencser, 1990), and the ATPase's type, molecular weight, reaction sequence (Gerencser and Zelezna, 1993), and stoichiometry (Gerencser, 1993a) were subsequently delineated.

In the present study, our aims were to delineate the catalytic and transport kinetics of this reconstituted transporter protein (Gerencser and Zelezna, 1993) and further characterize the transporter under these highly rigorous conditions. The unusual significance of this work is that it is one of the few studies of any anion pumps' transport and catalytic kinetics (Gerencser and Lee, 1985a; Shiroya *et al.*, 1989) and properties (Gerencser and Zelezna, 1992) and the first and only study at the reconstitution level to simultaneously analyze all of the manifestations of the Cl^- pump (ATP hydrolysis, active transport of chloride, and generation of a membrane-potential change).

MATERIALS AND METHODS

Materials

Seahares *(Aplysia californica)* were obtained from Marinus, Inc. (Long Beach, California) and were maintained at 25°C in circulating filtered seawater. Adult *Aplysia* (600–1000 g), were used in these experiments. Tris (hydroxymethyl) aminomethane, (Tris)-ATP, and all other nucleotides, phenylmethylsulfonylfluoride (PMSF), *p*-trifluoromethoxyphenylhydrazone (FCCP), digitonin, Triton-X, 5′-adenylylimidodiphosphate [AdoP(NH)P], triphenylmethylphosphonium ($TPMP^+$) bromide, 1,4-dithiothreitol (DTT), valinomycin, N,N′-dicyclohexylcarbodiimide (DCCD), and oligomycin were purchased from Sigma. All other reagent-grade purity chemicals, including sodium orthovanadate, were purchased from Fisher Scientific. *p*-Chloromercurobenzene sulfonate (PCMBS) was always made with equimolar EDTA to scavenge any free mercury. [Methyl-^{3}H]triphenylphosphonium bromide ([^{3}H]$TPMP^+$), $^{36}Cl^-$ and [carboxyl-^{14}C] inulin were obtained from New England Nuclear.

Preparation of BLM and Proteoliposomes

The inside-out BLM vesicles were prepared from *Aplysia* foregut epithelial cells by homogenization and differential and discontinuous sucrose density-gradient centrifugation techniques as described previously (Gerencser and Lee, 1985a, b; Gerencser 1988). Briefly, the membrane layer at the 40–50% sucrose interface was removed (BLM), suspended in a choline chloride medium [10 mM imidazole-HCl (pH 7.8), 250 mM sucrose, 3 mM $MgSO_4$ and 25 mM choline chloride], and centrifuged at 200,000 *g* for 60 min to obtain a pellet that was then homogenized in a choline chloride medium (above) with a glass-Teflon homogenizer and recentrifuged at 200,000 *g* for 60 min. The final membrane pellet was suspended in the same buffer at 2 mg protein/ml.

Digitonin extraction of BLM and digitonin-induced reconstituted proteoliposomes were prepared as previously detailed (Gerencser, 1990; Gerencser and Zelezna, 1993). In short, Cl^- pump activity was solubilized by a modification of the method of McCormick *et. al.* (1985). BLM were diluted (1:2, v/v) with solubilization buffers (10 mM imidazole-HCl (pH 7.8), 25 mM choline chloride, 250 mM sucrose, 3 mM $MgSO_4$, 1 mM Tris ATP, 1 μM phenylmethylsulfonyl fluoride, and 2% digitonin to give a final protein concentration of about 3 mg/ml. After incubation for 35 min at 4°C, the insoluble membrane fragments were removed by centrifugation at 100,000 *g* for 60 min. Moist copolymer beads (0.3 g) (BioBeads SM-2: Bio-Rad, Richmond, California) were added to 1.2-ml protein samples containing digitonin to remove the detergent by adsorption. Samples were then gently agitated at 4°C on a blood tube rotator for 45 min. Supernatants from each sample were withdrawn manually via syringe and needle at 4°C. The basic reconstitution protocol was adapted from that described by McCormick *et. al.* (1985) for their studies on transport in the Ehrlich ascites cell membrane. Reconstitution of Cl^- pump activity was performed by mixing 0.5 mg of solubilized membrane protein with 10 mg of sonicated asolectin and 1% digitonin in a total volume of about 1 ml. The solution was diluted with 5–10 vol. of imidazole buffer, and then sonicated for 20 s. The proteoliposomes were pelleted by centrifugation at 200,000 *g* for 60 min and then resuspended in 350 μl of imidazole buffer for immediate use. The orientation of the transporter in the liposome approximated random distribution; that is, the ratio of right-side-out to inside-out

proteoliposomes approached unity by determination with and without Triton-X and the transport assay.

Measurement of Membrane Electrical Potential

The transmembrane (liposome) electrical potential ($\Delta\psi$) was estimated from the steady-state distribution of the lipophilic cation $TPMP^+$ between the extra- and intravesicular space by ultrafiltration (Gerencser, 1988; Gerencser *et al.*, 1988). After the proteoliposomes were equilibrated with [^{3}H]$TPMP^+$ for 10 min at 25°C, a sample was removed, and filtered but not washed. The membrane filters were presoaked in choline chloride buffer containing 10 μM nonradioactive $TPMP^+$. The extravesicular water content of each filter was calculated from the [carboxyl-^{14}C] inulin space. The distribution ratio of $TPMP^+$ was estimated with a correction for the extravesicular contamination by [^{3}H]-$TPMP^+$. Nonspecific binding of $TPMP^+$ to the vesicular membranes (Gerencser, 1988; Gerencser *et al.*, 1988) was also corrected for by subtracting from the total $\Delta\psi$ and was obtained by using nonionic media in the membrane preparation, reaction mixture, and ultrafiltration stages of the $TPMP^+$ electrical potential difference assay. Thus, the control potential difference was always "corrected" to 0 mV, and any change in potential difference, and its associated polarity, was noted as a function of an experimental forcing. When used, inhibitors were preincubated with the proteoliposomes for 10 min, as was valinomycin, the K^+-ionophore (Gerencser, 1988; Gerencser *et al.*, 1988), and FCCP, the H^+ ionophore (Gerencser, 1988). $\Delta\psi$ was calculated from the Nernst equation (at 25°C): $\Delta\psi = -58.8 \log [TPMP^+]_{in}/[TPMP^+]_{out}$.

Analysis of Cl^--Dependent Catalytic and Transport Activities

Both Cl^--stimulated ATPase and ATP-dependent transport activities were measured as described previously (Gerencser and Lee, 1985a; Gerencser, 1988). When used, inhibitors were preincubated with the proteoliposomes for 10 min, as was valinomycin, the K^+-ionophore (Gerencser, 1988; Gerencser and Zelezna, 1992), and FCCP, the H^+ ionophore (Gerencser, 1988).

RESULTS

The first set of experiments examined the concentration dependence of Cl^- on both transport parameters, ATP-dependent $\Delta\psi$ and ATP-dependent Cl^- transport, and also on the Cl^--ATPase activity which is the catalytic component. ATP-dependent Cl^- transport is defined as [(ATP-dependent Cl^- transport + ATP-dependent Cl^- transport)—(ATP-independent Cl^- transport)]. The ATP-independent Cl^- transport component was experimentally determined to be 82.5 nmol/mg protein in the proteoliposomal preparation. Cl^--ATPase activity is defined as [(Mg^{2+} + Cl^-)-ATPase]—[Mg^{2+}-ATPase]. Mg^{2+}-ATPase activity was experimentally determined to be 2.6 μmol/15 min/mg protein in the proteoliposomal preparation. As seen in Fig. 1A, B, and C there are curvilinear increases in $\Delta\psi$, Cl^- transport, and Cl^--ATPase activity, respectively, as Cl^- concentrations increase. The apparent K_m's for ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--ATPase activity were 9.7, 9.6, and 9.6 mM Cl^-, respectively, and the V_{max}'s were 42 mV, 250 nmol/mg protein, and 10.4 μmol/15 min/mg protein respectively, as calculated from double-reciprocal plots of the data in Fig. 1A, B, and C. When NO_3^- or SO_4^{-2} totally replaced Cl^- in the reaction mixture, there was no change in $\Delta\psi$, in the presence of ATP, above that of control (0 mV) at any concentration (Fig. 1A), and, similarly, there was no change in Mg^{2+}-ATPase activity (control) when either of these ions replaced Cl^- in the reaction mixture (data not shown, $n = 3$). Also shown in Fig. 1A and B, both the ATP-dependent $\Delta\psi$ and ATP-dependent Cl^- transport were enhanced when the proteoliposomes were preincubated with 0.18 mM valinomycin which resulted in an initial K^+ concentration difference of 40 mM outside the vesicles to 0 mM inside. Conversely, the ATP-dependent $\Delta\psi$ and ATP-dependent Cl^- transport were reduced when the proteoliposomes were preincubated with 0.18 mM valinomycin plus K^+ which resulted in an initial K^+ concentration difference of 40 mM inside the vesicles to 0 mM outside (Fig. 1A and B).

The next group of experiments was designed to examine ATP-dependent $\Delta\psi$, ATP-dependent Cl^- accumulation, and Cl^--ATPase activity as a function of ATP concentrations in the proteoliposomal preparation. As seen in Fig. 2A, B, and C there are curvilinear increases in $\Delta\psi$, Cl^- transport, and Cl^--ATPase activity as extravesicular ATP concentra-

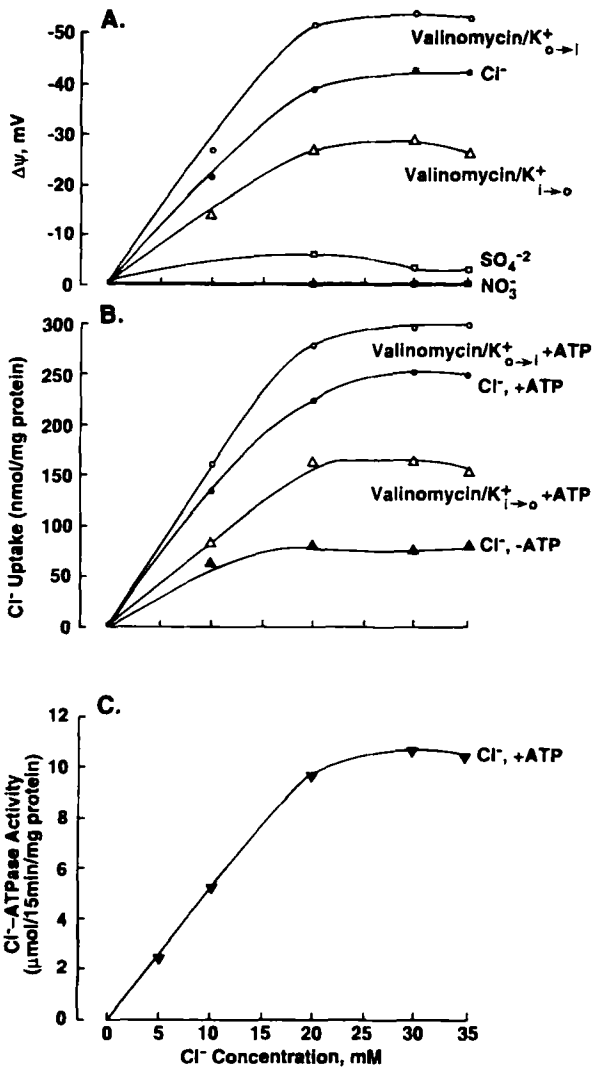

Fig. 1. Effect of valinomycin-induced transmembrane K^+ diffusion potential on Cl^- concentration dependence of ATP-dependent $\Delta\psi$ and Cl^- transport by *Aplysia* foregut basolateral membrane Cl^--ATPase reconstituted into proteoliposomal vesicles. ○, proteoliposomal vesicles whose reaction mixture contained 40 mM K^+ and 0.18 mM valinomycin but otherwise were under isosmotic equilibrium tracer exchange conditions. ●, Same as above except that no valinomycin or K^+ was present on either side of the vesicular membrane. △, Same as above except proteoliposomes were preincubated with 40 mM K^+ and 0.18 mM valinomycin for 10 min. at 25°C and pH 7.8. ATP-dependent Cl^- transport is defined as [(ATP-dependent Cl^- transport + ATP-independent Cl^- transport)—(ATP-independent Cl^- transport)]. The ATP-independent Cl^- transport component was experimentally determined to be 82.5 nmol/mg protein in the proteoliposomal preparation. □, Same as above except SO_4^{-2} replaced Cl^- in both intra- and extravesicular medium. ■, Same as above except NO_3^- replaced Cl^- in both intra- and extravesicular medium. ▲, Same as above except ATP was absent in the reaction mixture. ▼, Cl^--ATPase activity as a function of Cl^- concentration. Cl^--ATPase activity is defined as [(Mg^{2+} + Cl^-) - ATPase]—[Mg^{2+}-ATPase]. Mg^{2+}-ATPase activity was experimentally determined to be 2.6 μmol/15 min/mg protein. Extra- and intravesicular medium composition was as follows: 10 mM Tris-HEPES (pH 7.8), 5 mM ATP, 3 mM $MgSO_4$, variable concentrations of choline chloride, choline sulfate, or choline nitrate, and 200–250 mM sucrose in order to maintain constant osmolality. Values reported are means of 6 different experiments; each experiment had triplicate determinations.

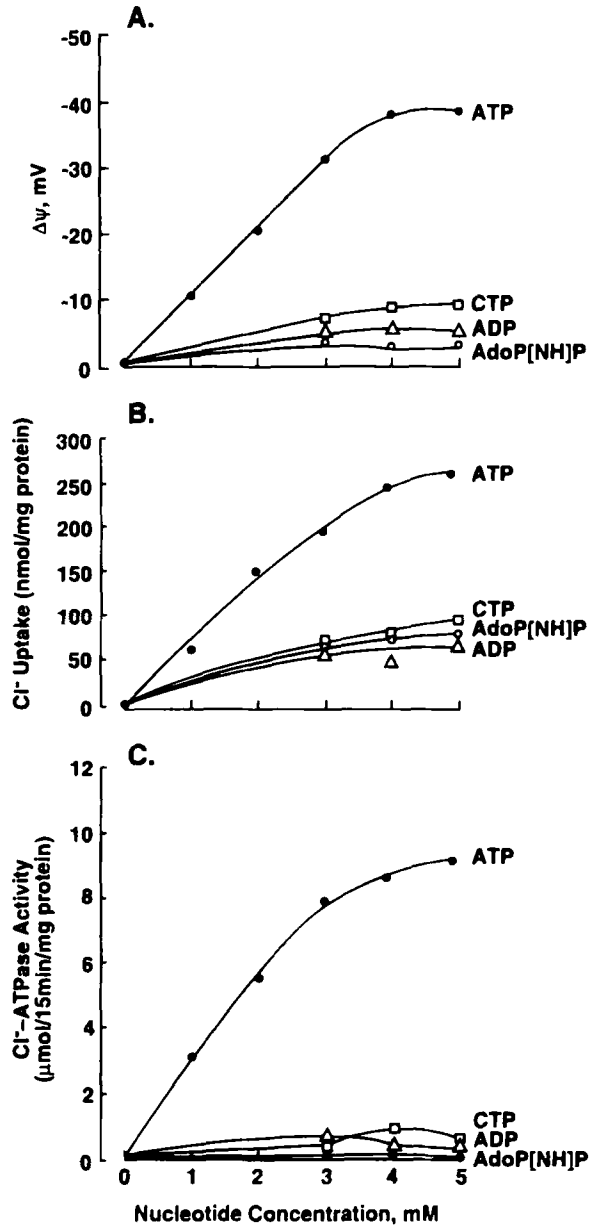

Fig. 2. Effect of nucleotide concentrations on $\Delta\psi$, Cl^- transport and Cl^--ATPase activity in proteoliposomes containing Cl^--ATPase generated from BLM of *Aplysia* gut. ●, ATP; ○, AdoP(NH)P; □, CTP; and △, ADP. Extra- and intravesicular medium composition was as follows: 10 mM Tris-HEPES (pH 7.8), 3 mM $MgSO_4$, 25 mM choline chloride, 250 mM sucrose, and variable concentrations of nucleotides. Values reported are means of 6 different experiments; each experiment had triplicate determinations.

tions increase. The apparent K_m's for $\Delta\psi$, Cl^- transport, and Cl^--ATPase activity were 2.0, 1.9, and 1.6 mM ATP, respectively, while the V_{max}'s were 39 mV, 258 nmol/mg protein and 9.3 μmol/15 min/mg protein, respectively, as calculated from the data derived from double-reciprocal plots for Fig. 2A, B, and C. Cytosine triphosphate (CTP), adenosine diphosphate (ADP), nor the nonhydrolyzable ATP analog, 5'-adenylimidodiphosphate (AdoP[NH]P) had no significant effect on $\Delta\psi$, Cl^- transport, or Cl^--ATPase activity above that of control.

The next series of experiments examined the effects of divalent cation concentrations on ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--ATPase activity in the proteoliposomal preparation. As shown in Fig. 3A, B, and C, the ATP-dependent $\Delta\psi$, ATP-dependent Cl^- accumulation, and Cl^--ATPase activity increased with increasing concentrations of Mg^{2+} and Mn^{2+} until a maximum was reached at both 3 mM Mg^{2+} and 3 mM Mn^{2+}. In the absence of Mg^{2+} (also Mg^{2+}-free ATP), the ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--ATPase activity were not significantly different from that determined for control (Fig. 3). When Mn^{2+} replaced Mg^{2+}, the ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--ATPase activity were significantly greater than control values; however, the Mn^{2+} stimulating ability was much less than that of Mg^{2+} at all concentrations (Fig. 3).

To further examine the factors responsible for ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--stimulated ATPase activity, the effect of bilateral iso-pHs (5.8, 6.8, 7.8, and 8.8) across proteoliposomal vesicles preincubated with the protonophore FCCP were studied. As seen in Fig. 4A, B, and C, the ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--ATPase activity were significantly greater in those proteoliposomes whose inside and outside pH was 7.8 than in those vesicles whose intra-and extravesicular pHs were 5.8, 6.8, or 8.8.

The next series of experiments probed the effects of various reactants and inhibitors on ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--ATPase activity in the proteoliposomal preparation. As seen in Table I, the only inhibitors that significantly reduced ATP-driven $\Delta\psi$, ATP-dependent Cl^- accumulation, and Cl^--stimulated ATPase activity in the proteoliposomes were orthovanadate (10^{-7} M) and PCMBS (10^{-4} M). The PCMBS-induced inhibition of ATP-driven $\Delta\psi$, ATP-dependent Cl^- accumulation, and Cl^--stimu-

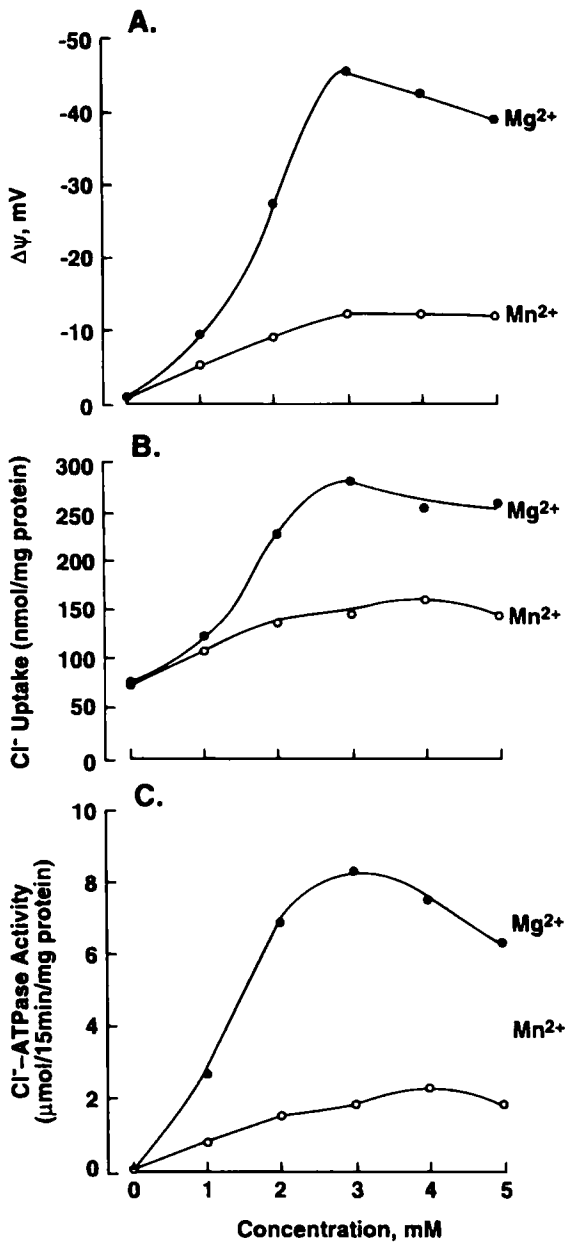

Fig. 3. Effect of divalent cation concentrations on ATP-dependent $\Delta\psi$ and Cl^- transport and Cl^--ATPase activity in proteoliposomes containing Cl^--ATPase generated from BLM of *Aplysia* gut. •, Mg^{2+} and ○, Mn^{2+}. Extra- and intravesicular medium composition was as follows: 10 mM Tris-HEPES (pH 7.8), 25 mM choline chloride, 5 mM ATP, 250 mM sucrose, and variable concentrations of either $MgSO_4$ or $MnSO_4$. Values reported are means of 5 different experiments; each experiment had triplicate determinations.

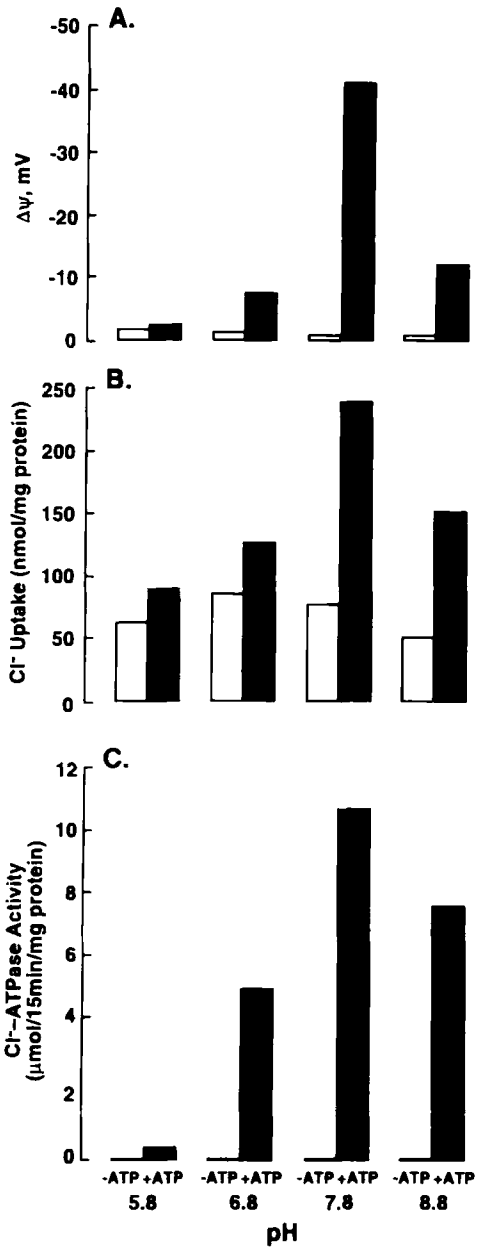

Fig. 4. Effect of bilateral iso-pH, FCCP, and ± ATP on steady-state $\Delta\psi$, Cl^- transport, and Cl^--ATPase activity in proteoliposomes containing Cl^--ATPase generated from BLM of *Aplysia* gut. FCCP (0.12 mM) was preincubated with proteoliposomal vesicles in the reaction mixture (20 μl containing 10 mM Tris-HEPES, 250 mM sucrose, 3 mM $MgSO_4$, 25 mM choline chloride, and 0 or 5 mM ATP) at the indicated pH for 10 min at 25°C. Intravesicular pH matched extravesicular pH with intravesicular medium composition as follows: 10 mM Tris-HEPES, 250 mM sucrose, 3 mM $MgSO_4$, and 25 mM choline chloride. Values reported are means of 6 different experiments; each experiment had triplicate determinations.

lated ATPase activity was reversed by the subsequent addition of DTT (10^{-4} M).

DISCUSSION

Cation-motive ATPases have been well characterized after reconstitution of their purified proteins into liposomes (Zimniak and Racker, 1978). This includes the P-type ATPases: Ca^{2+}-, Na^+/K^+- (Zimniak and Racker, 1978), H^+- (Vara and Serrano, 1982), H^+/K^+- (Schuurmans-Stekhoven and Bontig, 1981), and also F_0F_1-ATPases (Nyren and Baltscheffsky, 1983). In contrast, anion-motive ATPases such as those that are activated by Cl^- have little or no history relative to being purified and/or being reconstituted into liposomes (Pedersen and Carafoli, 1987; Gerencser and Zelezna, 1992, 1993; Slayman and Zuckier, 1989). However, a Cl^--ATPase preparation from the marine algae *Acetabularia* was reconstituted into liposomes, but the results were ambiguous because Cl^- inhibited the ATPase activity while ATP stimulated Cl^- accumulative transport (Ikeda *et al.*, 1990). Additionally, it was demonstrated that rat brain Cl^--ATPase could be reconstituted into asolectin liposomes; and the ATPase expressed its transport function through regulation by phosphoinositide turnover (Zeng *et al.*, 1994). Recently, in our laboratory, it had been demonstrated that both aspects of the primary active transporter for Cl^- (Cl^- pump), that is, Cl^--stimulated ATPase activity and ATP-dependent Cl^- transport, were reconstituted into liposomes (Gerencser, 1990). Furthermore, the reaction sequence of the Cl^--pump was delineated: Mg^{2+} stimulating phosphorylation, Cl^- stimulating dephosphorylation (Gerencser and Zelezna, 1993). The phosphorylation appeared to be of an acylphosphate nature because of its sensitivity to both hydroxylamine and to high pH (Gerencser and Zelezna, 1993; Post *et al.*, 1965), which is similar to all other P-type ATPases (Slayman and Zuckier, 1989; Post *et al.*, 1965). Combining these observations with other observations of ours (involving the proteoliposomal preparation), including (1) the isolation via polyacrylamide gel electrophoresis of a discrete, isolated, 110 kDa protein thought to be the Cl^- pump (Gerencser and Zelezna, 1993) and (2) the Mg^{2+}-driven phosphorylation reaction being inhibited by orthovanadate (Gerencser and Zelezna, 1993), strongly suggested that the Cl^- pump was a P-type ATPase (Gerencser and Zelezna, 1993) since all P-type ATPases have catalytic (sub)units whose molecu-

Table I. Effect of Inhibitors and/or Reactants on ATP-dependent $\Delta\psi$, ATP-dependent Cl^- Transport, and Cl^--ATPase Activity[a]

Inhibitors/reactants	$\Delta\psi$ (mV)	Inhibition (%)	Cl^- transport (nmole/mg protein)	Inhibition (%)	Cl^--ATPase activity (μmol/15 min/mg protein)	Inhibition (%)
None: (control, −ATP)	0.0 ± 2.5		82.5 ± 7.0			
ATP (5×10^{-3} M): (control, +ATP)	−41.6 ± 2.8		270.6 ± 16.1		7.0 ± 0.7	
Orthovanadate (10^{-7} M)	−4.5 ± 0.8	89.2	92.5 ± 10.0	94.7	0.2 ± 0.2	97.1
Efrapeptin (10^{-6} M)	−44.2 ± 3.0	—	288.6 ± 18.1	—	7.6 ± 0.8	—
Furosemide (10^{-6} M)	−39.1 ± 1.6	6.1	283.1 ± 10.0	—	6.5 ± 1.0	7.1
SITS (10^{-5} M)	−35.9 ± 4.3	13.7	275.1 ± 17.9	—	5.8 ± 1.6	17.1
Ouabain (10^{-6} M)	−42.1 ± 2.0	—	274.6 ± 16.5	—	7.1 ± 2.3	—
DCCD (10^{-5} M)	−40.3 ± 4.1	3.2	288.1 ± 19.2	—	7.0 ± 1.7	—
Ruthenium red (10^{-6} M)	−40.4 ± 3.7	2.9	281.3 ± 19.9	—	6.7 ± 1.0	4.3
Picrotoxin (10^{-6} M)	−38.1 ± 3.9	8.4	274.2 ± 16.5	—	6.8 ± 1.5	2.9
PCMBS (10^{-4} M)	−19.3 ± 3.5	53.6	155.1 ± 9.3	61.4	2.6 ± 1.6	62.9
DTT (10^{-4} M)	−40.9 ± 5.0	1.7	258.5 ± 16.3	6.4	7.3 ± 0.8	—
PCMBS (10^{-4} M) + DTT (10^{-4} M)	−41.5 ± 3.7	0.3	255.1 ± 18.1	8.2	7.2 ± 1.7	—

[a] Inhibitors and/or reactants were preincubated with proteoliposomes in reaction mixture [50 μl containing 10 mM imidazole-HCl (pH 7.8), 250 mM sucrose, 3 mM $MgSO_4$, 25 mM choline Cl^-, and 10 μM triphenylmethylphosphonium ($TPMP^+$)] plus [H^3] $TPMP^+$ or $\pm$ $^{36}Cl^-$ at concentrations ranging from 10^{-7} to 5×10^{-3} M for 10 min at 25°C. ATP (5 mM) was added to reaction mixture to initiate 15-S incubation period, which was done at 25°C. When DTT (10^{-4} M) was added to ATP + PCMBS, an additional 10 min incubation period was done at 25°C before samples were taken. Intravesicular medium matched the extravesicular medium in both composition and pH. $TPMP^+$ nonspecifically bound to proteoliposomes was accounted for in final computation of transproteoliposomal electrical potential ($\Delta\psi$). Negative sign in $\Delta\psi$ denotes intravesicular polarity relative to extracellular bathing medium (reaction mixture). Cl^--ATPase activity is defined as $[(Mg^{2+} + Cl^-)] - [Mg^{2+}$-ATPase]. Mg^{2+}-ATPase activity was experimentally determined to be 2.6 μmol/15 min/mg protein. All inhibitors/reactants had no significant effect on $\Delta\psi$ or Cl^- transport in the absence of ATP or on Mg^{2+}-ATPase activity. Values reported are means of 4–6 different experiments; each experiment had triplicate determinations.

lar weights approximate 100 kDa and are inhibited, universally, by orthovanadate, the competitive inhibitor of transition-state phosphate binding (Pedersen and Carafoli, 1987; Slayman and Zuckier, 1989). Further, it was demonstrated by thermodynamic means that the Cl^- pump was a uniporter which transported 1 Cl^- for each ATP hydrolyzed per reaction cycle (Gerencser, 1993a). This finding strongly suggested that the pump was electrogenic in nature. If this was true, the transport component of the Cl^- pump could be further separated into two subcomponents: (1) the transport of Cl^- and (2) the transport of net negative charge. Therefore, we thought that it was reasonable to use the Cl^--ATPase-containing proteoliposomal preparation to analyze and compare both transport facets or subcomponents of the Cl^- pump with its catalytic (ATPase) component. This work is one of the few kinetic examinations of any aspect of an anion pump and the only study of an anion pump that has been done at the level of reconstituted protein (Pedersen and Carafoli, 1987; Gerencser and Zelezna, 1992; Slayman and Zuckier, 1989).

The present finding that ATP, in the presence of Cl^-, can stimulate both $\Delta\psi$ and its associated intravesicular negativity, and Cl^- accumulation in the proteoliposomal vesicle (Fig. 1A and B), containing Cl^--ATPase (Gerencser and Zelezna, 1993) extracted from *Aplysia* gut BLM (Gerencser and Lee, 1985a), strongly suggests that the mechanism responsible for this phenomenon is electrogenic. This conclusion is supported by the present findings with the ionophore valinomycin (Fig. 1A and B). Both ATP-driven $\Delta\psi$ and Cl^- accumulation across the proteoliposomal membrane, in the presence of valinomycin and an external to internal K^+ gradient, was always greater than in their absence. The results with

valinomycin plus K^+ suggest that the positive K^+ diffusion potential, induced from the extra- to intravesicular space by addition of the ionophore, stimulated both ATP-dependent $\Delta\psi$ and ATP-dependent Cl^- accumulation by lowering the extra- to intravesicular electrochemical potential for Cl^-. Conversely, both ATP-driven $\Delta\psi$ and ATP-dependent Cl^- accumulation across the proteoliposomal membrane, in the presence of valinomycin and an internal to external K^+ gradient, was always less than in their absence (Fig. 1A and B). These results with valinomycin plus K^+ suggest that the positive K^+ diffusion potential, induced from the intra- to extravesicular space by addition of the ionophore, inhibits both ATP-driven $\Delta\psi$ and Cl^- accumulation by raising the extra- to intravesicular electrochemical potential for Cl^- by increasing intravesicular negativity. If the Cl^- transporter was nonelectrogenic, then the ionophore-induced electrical potential changes would not affect ATP-dependent Cl^-transport and its associated $\Delta\psi$ (Gerencser, 1988). Therefore, the electrogenicity of the Cl^--pump is defined by the inferential valinomycin/K^+ experiments and also, directly, by the ATP-generated $\Delta\psi$.

The maximal ATP-driven $\Delta\psi$, ATP-driven Cl^- accumulation, and Cl^--ATPase activity (Fig. 1A, B, and C) all occurred at approximately 25 mM Cl^-. Also, the apparent K_m's for Cl^- in stimulating $\Delta\psi$, Cl^- accumulation and Cl^--ATPase activity, in the presence of ATP, were 9.7, 9.6, and 9.6 mM, respectively, which are similar to the 10.3 mM which had been previously reported for Cl^--ATPase activity in BLM's of *Aplysia* gut (Gerencser and Lee, 1985a). The findings that neither NO_3^- nor SO_4^{-2} could generate $\Delta\psi$ or anion stimulation of Mg^{2+}-ATPase activity, in the presence of ATP (Fig. 1), suggests a relative specificity for the ATPase; among these three anions, Cl^- was absolutely necessary for the generation and maintenance of the negative intraproteoliposomal potential difference and anion-induced ATPase activity. These results concur with previous *Aplysia* BLM ATPase studies which revealed that Cl^- could stimulate ATPase activity whereas NO_3^- nor SO_4^{-2} could not (Gerencser and Lee, 1985a; Gerencser, 1988; Gerencser and Zelezna, 1992).

ATP, and its subsequent hydrolysis, was found to be necessary for generating a negative intraproteoliposomal potential difference (Fig. 2A), Cl^- accumulation (Fig. 2B), and Cl^--ATPase activity (Fig. 2C) because neither CTP, ADP, nor the non hydrolyzable ATP analog, AdoP[NH]P, could generate $\Delta\psi$, Cl^- transport, or Cl^--ATPase activity significantly above that of control. $\Delta\psi$ (intravesicular negativity), Cl^- accumulation and Cl^--ATPase activity were ATP concentration dependent in a curvilinear fashion with apparent K_m's for ATP of 2.0, 1.9, and 1.6 mM, respectively. These values are very similar to the apparent K_m for ATP found for Cl^--ATPase (2.6 mM) in the BLM of *Aplysia* (Gerencser and Lee, 1985a) and for ATP-induced phosphorylation of Cl^--ATPase in the same proteoliposomal preparation (Gerencser, 1993b) of *Aplysia*.

The findings that ATP-stimulated $\Delta\psi$ (Fig. 3A), ATP-stimulated Cl^- transport (Fig. 3B), and Cl^--stimulated ATPase activity (Fig. 3C) increased with increasing concentrations of Mg^{2+} suggest that Mg^{2+} is necessary for these transport and catalytic phenomena to occur because in the absence of Mg^{2+} and in the presence of ATP, $\Delta\psi$, Cl^- transport, and Cl^--ATPase activity were not significantly different from control values. This conforms to the idea that all ion-motive ATPases, including Cl^--ATPase, are Mg^{2+}-dependent mechanisms (Pedersen and Carafoli, 1987; Slayman and Zuckier, 1989). The Mg^{2+} concentration optimum for ATP-dependent $\Delta\psi$, ATP-dependent Cl^- accumulation, and Cl^--ATPase activity are identical (3 mM) and is the same optimum determined for (1) Mg^{2+}-stimulating ATPase activity in the BLMs of this same preparation (Gerencser and Lee, 1985a) and (2) Mg^{2+}-stimulating phosphoenzyme formation (phosphorylation) in the proteoliposomes of this same preparation (Gerencser and Zelezna, 1993). These experiments, past and present, demonstrate the correspondence between overall ATPase activity (Gerencser and Lee, 1985a), Cl^--ATPase phosphorylation (Gerencser and Zelezna, 1993; Gerencser, 1993b), ATP-dependent Cl^- transport, ATP-dependent $\Delta\psi$, and Cl^--ATPase activity (Figs. 1, 2, and 3), which is similar to those characteristics detected in cation-activated and cation-motive ATPases (Pedersen and Carafoli, 1987; Slayman and Zuckier, 1989).

The findings that the ATP-driven $\Delta\psi$, ATP-dependent Cl^- accumulation, and Cl^--ATPase activity were much reduced in inside-out proteoliposomal preparations in which intra- and extravesicular pH was 5.8, 6.8, or 8.8, rather than 7.8, in the presence of FCCP, suggest that pH itself played a major role in determining ATP-driven $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--ATPase activity (Fig. 4A, B, and C). Supporting this contention was the finding that there was a greater ATP-dependent $\Delta\psi$ and ATP-dependent Cl^- accumulation in FCCP-loaded proteoliposomes whose intra- and extravesicular pH was 7.8 rather than 5.8, 6.8, or 8.8

(Fig. 4A and B). Similarly, there was greater Cl^--ATPase activity in FCCP-loaded proteoliposomes whose intra- and extravesicular pH was 7.8 rather than 5.8, 6.8, or 8.8 (Fig. 4C). The Cl^--ATPase (Gerencser and Lee, 1985a) and ATP-dependent Cl^- transport (Gerencser, 1988) activity found in the *Aplysia* BLM preparation have shown identical pH profiles with a pH optimum of 7.8. Since both $\Delta\psi$ and Cl^- transport are ATP-driven transport expressions of the Cl^--ATPase (Gerencser and Lee, 1985a; Gerencser, 1988; Gerencser *et al.*, 1988), it would be expected that the pH optima of Cl^--ATPase activity, ATP-dependent Cl^- transport, and ATP-dependent $\Delta\psi$ would correspond with each other as has been demonstrated in the present study (Fig. 4).

Orthovanadate is a specific competitive inhibitor of phosphate-binding on P-type ATPase catalytic (sub)units, and all P-type ATPases have a catalytic (sub)unit whose molecular weight approximates 100 kDa (Pedersen and Carafoli, 1987; Slayman and Zuckier, 1989). In the present study orthovanadate inhibited the (ATP + Cl^-)-dependent $\Delta\psi$ and ATP-dependent Cl^- transport and Cl^--ATPase activity (Table I), suggesting that the 110 kDa Cl^--ATPase catalytic (sub)unit detected in the proteoliposome (Gerencser and Zelezna, 1993) was responsible for generating the negative intravesicular potential difference in that same proteoliposomal preparation through transport of Cl^- and its associated negative charge. Previously, orthovanadate had been shown to inhibit Cl^--ATPase activity (Gerencser and Lee, 1985a) and ATP-dependent Cl^- transport (Gerencser, 1988) in BLM's of *Aplysia* gut and Mg^{2+}-induced Cl^--ATPase phosphorylation in the proteoliposomal preparation (Gerencser and Zelezna, 1993). All of these observations, past and present, suggest that Cl^--ATPase is effecting electrogenicity through transport of Cl^- because it was demonstrated that there is only one ATPase present in the proteoliposome (Gerencser and Zelezna, 1993) and this ATPase has a molecular size and is of the type (Pedersen and Carafoli, 1987; Slayman and Zuckier, 1989) that could specifically bind orthovanadate (Pedersen and Carafoli, 1987; Gerencser and Zelezna, 1993). In contrast, DCCD, an inhibitor of all known H^+-ATPases, whether they belong to P, V, or F groups (Gerencser, 1993b), had no effect on ATP-driven $\Delta\psi$, ATP-dependent Cl^- accumulation, or Cl^--ATPase activity (Table I) strongly suggesting that contaminant H^+-ATPase (Gerencser *et al.*, 1988; Gerencser and Zelezna, 1992) could not express the observed Cl^- pump activity.

As demonstrated in the present study (Table I), the addition of PCMBS to proteoliposomal vesicles containing Cl^--ATPase (Gerencser and Zelezna, 1993) evoked an inhibition of ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--ATPase activity significantly lower from that of control. Although PCMBS is not absolutely specific for sulfhydryl ligands and has been shown to inhibit other ligands such as carboxyl, amino, phosphoryl, and tyrosyl (Rothstein, 1970), it is strongly suggested that its inhibition was, in a major part if not totally, through sulfhydryl ligand binding, since DTT, a specific thiol-reducing agent (Rothstein, 1970), almost totally reversed the inhibition by PCMBS (Table I). Also buttressing this argument is the fact that PCMBS binding to a sulfhydryl ligand forms a mercaptide complex, which is an easily reversible complex in the presence of thiol-reducing agents (Rothstein, 1970). Again, the almost total reversibility of ATP-dependent Cl^- transport, ATP-dependent $\Delta\psi$, and Cl^--ATPase activity by DTT, in the presence of PCMBS (Table I), suggests sulfhydryl-ligand participation in these ATP-dependent transport and catalytic activities.

Additionally, ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, and Cl^--ATPase activity in the *Aplysia* gut proteoliposomes were insensitive to the Na^+/K^+-ATPase inhibitor, ouabain; F_1-ATPase inhibitor, efrapeptin; Na^+/Cl^- symport inhibitor, bumetamide; Cl^-/HCO_3^- antiport inhibitor, SITS; Ca^{2+}-ATPase inhibitor, ruthenium red; and the Cl^- channel inhibitor, picrotoxin (Table I). These negative results suggest that neither Na^+ pump, proton pump, Na^+-coupled symport, anion-coupled antiport, Ca^{2+} pump, nor GABA-receptor Cl^- channel complex is involved in ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport, or Cl^--ATPase activity (Pedersen and Carafoli, 1987; Slayman and Zuckier, 1989; Shiroya *et al.*, 1989).

The present kinetics are uniquely significant not only because they are the first and only results obtained with a reconstituted anion transporter ATPase (Gerencser and Zelezna, 1993) but because they demonstrate the interrelationship and interchangability between both transport and catalysis of this primary active transporter for Cl^-. These results are strongly consistent with the hypothesis that the Cl^--ATPase in *Aplysia* foregut is an electrogenic ATP-driven Cl^- pump (Gerencser, 1993a). A working model of the Cl^- pump is depicted in Fig. 5.

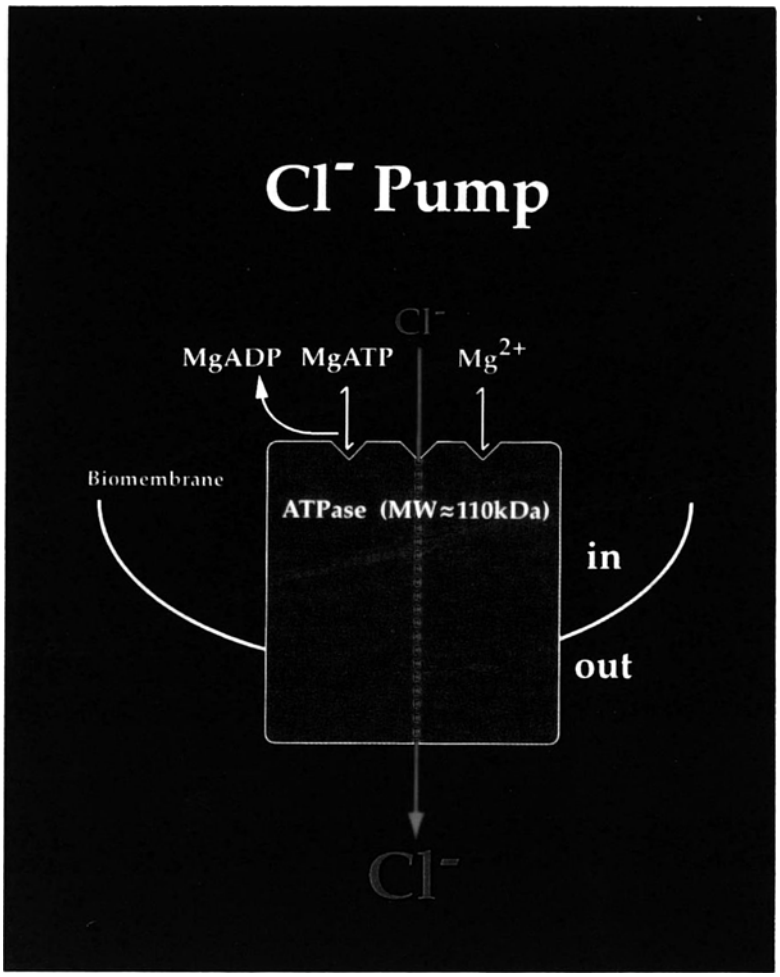

Fig. 5. Working model of the Cl^--stimulated, Cl^--translocating ATPase, or Cl^- pump.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of F. Robbins and C. Burgin. This investigation was supported by the Eppley Foundation for Research, Inc.

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