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Inhibition of Cl^--stimulated ATPase activity in isolated basolateral membranes from *Aplysia* gut

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A Cl^--stimulated ATPase activity, which is sensitive to both *N*-ethylmaleimide and *p*-chloromercuribenzenesulfonate, has been localized to the basolateral membrane of *Aplysia* enterocytes. Dithiothreitol reversed the inhibition of Cl^--stimulated ATPase induced by *p*-chloromercuribenzenesulfonate. These results suggest that surface sulfhydryl ligands of the ATPase participate in the catalytic activity of the enzyme.

Anion-stimulated ATPase activity was first described in frog gastric mucosa [1] where it was thought to play a role in the simultaneous transport of H^+, HCO_3^-, and Cl^-. Thereafter, discovery of anion-stimulated ATPase was demonstrated in many mammalian tissues [2,3] and some lower vertebrates such as: *Necturus* [4], eel [5], trout [6], and goldfish [7]. With the exception of some recent studies [8,9] very little information currently exists about anion-stimulated ATPase activity in invertebrate tissue.

Transepithelial Cl^- flux studies [10,11] in *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., Cl^--stimulated ATPase) for it had been demonstrated that intracellular Cl^- activity in the villus enterocytes of *Aplysia* was at a lower electrochemical potential than in the extracellular medium [12], even in the absence of extracellular Na^+ [13]. Recently, Gerencser and Lee [14,15] and Gerencser [16] have demonstrated both the existence of a Cl^--stimulated ATPase activity and an ATP-dependent Cl^- transport in *Aplysia* enterocyte basolateral membrane vesicles strongly suggesting a linkage between ATPase activity and Cl^- transport. Therefore, the present study was undertaken to assess the effects of various sulfhydryl agents on Cl^--stimulated ATPase activity in *Aplysia* gut in order to further characterize properties of the ATPase.

Seahares (*Aplysia californica*) were obtained from Marinus Inc. (Westchester, CA) and were maintained at 25 °C in circulating filtered seawater. Adult *Aplysia* (400–1000 g) were used in these experiments. The basolateral plasma membrane vesicles were prepared from *Aplysia* enterocytes by homogenization and differential and discontinuous sucrose density-gradient centrifugation techniques as described previously [14,16]. Cl^--stimulated ATPase (EC 3.6.1.3) was measured in a reaction mixture as also previously described [14], as was the ATP-dependent Cl^- transport in the basolateral membrane vesicles [15,16]. When used, the chemical reagents *N*-ethylmaleimide (NEM), *p*-chloromercuribenzenesulfonate (PCMBS), diamide and dithiothreitol (DTT) were preincubated with the plasma membrane vesicles for 10 or 20 min at 25 °C and the time for Cl^--ATPase activity change from control was chosen at 15 min based on previous studies [14]. Differences between means were analyzed statistically using Student's *t*-test with a $P < 0.05$ used as a statistical difference criterion.

In Table I, the Cl^--stimulated ATPase activity derived from the basolateral membranes of *Aplysia* enterocytes are presented. As can be seen both 2 mM PCMBS and 2 mM NEM significantly inhibited Cl^--stimulated ATPase activities, PCMBS inhibiting the activity more than NEM. However, upon subsequent addition of 2 mM DTT to both PCMBS- and NEM-inhibited Cl^--stimulated ATPase activities, there was a complete restoration to control of Cl^--stimulated ATPase activity

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TABLE I

Effect of inhibitors on Cl^--ATPase activity

Values are means ± S.E. from six different experiments. Reactants were preincubated with enzymes in reaction mixture for 10 min at 30 °C. Conditions for enzyme assay described in Materials and Methods. The specific activities are given in μmol/15 min per mg.

Reactant	$(Mg^{2+} + Cl^-)$-ATPase	Cl^--ATPase	% Inhibition	Significance from control
Control	7.30 ± 0.70	2.15 ± 0.29	–	–
PCMBS	5.24 ± 0.61	0.09 ± 0.06	96	$P < 0.05$
PCMBS + DTT	8.48 ± 0.91	3.33 ± 0.65	–	–
NEM	6.18 ± 0.75	1.03 ± 0.15	48	$P < 0.05$
NEM + DTT	6.02 ± 0.74	0.87 ± 0.18	40	$P < 0.05$
Diamide	7.65 ± 0.55	2.50 ± 0.40	–	–

in the PCMBS-treated membranes while DTT had no effect on the NEM-inhibited Cl^--stimulated ATPase activity. Diamide (2 mM) had no significant effect on Cl^--stimulated ATPase activity (Table I). Additionally, PCMBS, NEM, DTT, diamide, PCMBS + DTT, or NEM + DTT had no significant effect on Mg^{2+}-ATPase activity at concentrations of 2 mM.

The effects of serially added PCMBS and DTT on ATP-dependent Cl^- uptake into isolated basolateral membrane vesicles was also studied. PCMBS (2 mM) reduced the ATP-dependent Cl^- uptake (21 ± 4 (4) nmol · mg protein^{-1}) by an average of 76.4% from control (89 ± 9 (4) nmol · mg protein^{-1}) while subsequent addition of 2 mM DTT partially and significantly ($P < 0.05$) reversed the PCMBS inhibition to 15.8% from control (75 ± 10 (4) nmol · mg protein^{-1}). PCMBS and/or DTT had no significant effect on the ATP-independent component of Cl^- transport, which was 55 ± 6 (4) nmol · mg protein^{-1}.

As demonstrated in the present study (Table I) the addition of either NEM or PCMBS to plasma membrane vesicles of *Aplysia* enterocytes evoked an inhibition of Cl^--stimulated ATPase activity significantly below that of control. Although NEM and PCMBS are not absolutely for sulfhydryl ligands and have been shown to inhibit other ligands such as carboxyl, amino, phosphoryl, and tyrosyl [17], it is strongly suggestive that their inhibition was through sulfhydryl ligand binding since DTT, a specific thiol reducing agent [18], completely reversed the inhibition by PMCBS (Table I). 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 [17]. Again, the reversibility of Cl^--stimulated ATPase inhibition by DTT (Table I) suggests sulfhydryl ligand participation in the activity of the enzyme. NEM interaction with sulfhydryl ligands is through alkylation and these covalent bonds are not easily reversed through chemical means [17]. Therefore, possibly, the reason why DTT had no effect on the NEM-induced inhibition of Cl^--stimulated ATPase activity (Table I). Of course, this does not rule out NEM effects on other ligands which could, directly or indirectly, inhibit the Cl^--stimulated ATPase activity. Paralleling these observations was the result where DTT reversed the PCMBS inhibition of ATP-dependent Cl^- uptake into the basolateral membrane vesicles (vide supra) similar to previous observations where thiocyanate, acetazolamide and vanadate inhibited both Cl^--stimulated ATPase activity [14] and ATP-dependent Cl^- transport [16].

PCMBS is thought to interact with surface sulfhydryl groups because of its negatively charged sulfonic group which reduces its lipid solubility [17]. In contrast, NEM is very lipid soluble and binds to both surface and intramembranous sulfhydryl ligands [17]. Since Cl^--stimulated ATPase inhibition was almost complete with PCMBS and was also greater than that induced by equal concentration of NEM, it is highly suggestive that surface sulfhydryl ligands not intramembranous sulfhydryl groups, are at least in part, responsible, either directly or indirectly, for the Cl^--stimulated ATPase activity in *Aplysia* gut. Since diamide had no significant effect on Cl^--stimulated ATPase activity (Table I), it is not unreasonable to assume that the oxidized disulfide group plays no role relative to the catalytic activity of the ATPase since diamide is a known oxidizing agent of sulfhydryl ligands [18].

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