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# ATP-dependent chloride transport in plasma membrane vesicles from *Aplysia* intestine

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**A $Cl^-$-stimulated ATPase activity, which is sensitive to both thiocyanate and vanadate, has been localized to the plasma membrane of *Aplysia* enterocytes. Utilizing plasma membrane vesicles from *Aplysia* enterocytes, ATP stimulated $Cl^-$ uptake to approximately 2.5-times that of control in a $Na^+$, $K^+$ and $HCO_3^-$-free medium. This ATP-dependent $Cl^-$ uptake was sensitive to both thiocyanate and vanadate. These results are consistent with the hypothesis that the active $Cl^-$ absorptive process in *Aplysia* intestine could be a $Cl^-$-stimulated ATPase found in the enterocyte plasma membrane.**

In the last several years two mechanisms of intestinal $Cl^-$ transport have been well established [1,2]. The first of these is an electrically-neutral $Na^+$ symport process which drives $Cl^-$ uphill into enterocytes via the inward flow of $Na^+$ down a favorable electrochemical potential gradient as is exemplified in gut epithelia of: prawn [3], flounder [4], sculpin [5], marine eel [6], bullfrog [7], rat [8], rabbit [9] and human [10]. The second $Cl^-$ transport process in enterocytes involves $Cl^-/HCO_3^-$ or $Cl^-/OH^-$ antiport as is found in the intestinal epithelia of: urodele [11], rabbit [1], and human [10].

However, there are several examples of $Cl^-$ absorption across intestinal preparations which do not conform to either of the two models described above [2]. For example, White [12] described an electrogenic $Cl^-$ uptake mechanism located in the mucosal membrane of *Amphiuma* intestine which is independent of mucosal $Na^+$ or $HCO_3^-$; and Gerencser [13] has demonstrated that the short-circuit current (SCC) across *Aplysia californica* intestine, bathed in a $Na^+$-free seawater saline, is identical to a net active $Cl^-$ absorptive flux. It was hypothesized that $Cl^-$ absorption across the *Aplysia* intestine 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 [14], even in the absence of extracellular $Na^+$ [15]. Therefore it has been concluded that the active transport process for $Cl^-$ in the *Aplysia* enterocyte is located at the basolateral border [13–15]. Recently, Gerencser and Lee [16] have demonstrated the existence of a $Cl^-$-stimulated ATPase activity in *Aplysia* enterocyte plasma membranes suggesting a linkage between ATPase activity and $Cl^-$ transport. Therefore the present study was undertaken to assess $Cl^-$ transport, and its possible dependence on ATP, in plasma membrane vesicles of *Aplysia* enterocytes. The results are discussed in reference to the knowledge of

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$Cl^-$-stimulated ATPase activity in this plasma membrane preparation.

Seahares (*Aplysia californica*) were obtained from Marinus Inc. (Westchester, CA) and were maintained at 25°C in circulating filtered seawater. Adult *Aplysia* (600–1000 g) were used in these experiments. The plasma membrane vesicles were prepared from *Aplysia* intestinal enterocytes by homogenization and differential and discontinuous sucrose density-gradient centrifugation techniques as described previously [16]. Plasma membrane vesicles (20–30 $\mu$g of protein in 10 $\mu$l) were incubated at 25°C in 50 $\mu$l of reaction mixture containing 10 mM Tris-Hepes (pH 7.8), 250 mM sucrose, 3 mM $MgSO_4$ and 25 mM choline chloride, the concentration of $Cl^-$ at which $V_{max}$ for the $Cl^-$-stimulated ATPase activity was reached [16]. The reaction mixture also contained approx. $1.4 \cdot 10^6$ cpm of $^{36}Cl^-$. Both membrane vesicles and reaction mixture were preincubated at 25°C for 5 min, and $Cl^-$ uptake was initiated by the addition of the membrane vesicles to the reaction mixture. Therefore, at this stage of the methodology, the vesicles were under isoosmotic equilibrium tracer exchange (initial *cis*, zero *trans* $^{36}Cl^-$) conditions. Tris-ATP (5 mM), titrated to pH 7.8 by Tris base, was routinely part of the reaction mixture in one of the two membrane vesicle aliquots. When used, the inhibitors thiocyanate (10 mM) and vanadate (1.0 mM) were preincubated with the plasma membrane vesicles for 10 min at 25°C and the time interval for inhibition of $Cl^-$ uptake in the membrane vesicles was arbitrarily chosen at 15 s (see Fig. 1). At indicated time intervals (5, 10, 15, 30 s, 1.0, 2.5, 5.0, 10.0, 30.0 min), samples were removed and immediately diluted in 2.0 ml of chilled stop solution containing 10 mM Tris-Hepes (pH 7.8), 250 mM sucrose and 10 $\mu$M 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS). The suspensions were rapidly filtered on a membrane filter (Millipore, 0.45 $\mu$m) and washed with 2.0 ml of the same buffer. The radioactivity retained on the filters was assayed in Aquasol in a liquid scintillation counter (Packard Prias).

In Fig. 1 the uptake of $Cl^-$ by isolated *Aplysia* intestinal enterocyte plasma membrane vesicles in both the presence and absence of ATP as a function of time is presented. $Na^+$, $K^+$ and $HCO_3^-$

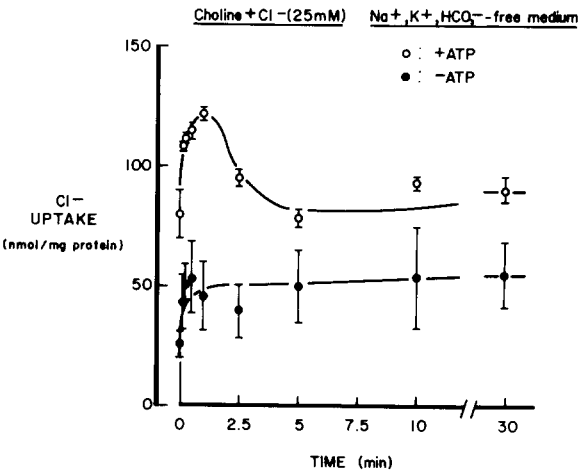


Fig. 1. Time-course of $Cl^-$ uptake. The reaction mixture, assay of transport and incubation times (abscissa) were as described in the text. Values are means ± S.E. from four different experiments (12–14 animals). ATP (5 mM): ○; ATP-free: ●.

were omitted from both the ATP-containing and the ATP-free reaction mixtures. Also, no pH gradient existed between the extra- and intravesicular space in both the presence and absence of ATP. As can be seen in Fig. 1, external 5 mM ATP increased the initial rate and the extent of $Cl^-$ uptake by the membrane vesicles approx. 2.5-fold compared with the ATP-free control. A significant difference in $Cl^-$ uptake in the two populations of vesicles was maintained for, at least, 30 min.

The effects of the inhibitors, vanadate and thiocyanate on ATP-dependent $Cl^-$ uptake was also studied. Thiocyanate (10 mM) reduced the ATP-dependent $Cl^-$ uptake by an average of 23.7 ± 5.9% (S.E.) (four experiments, 12 animals) while 1 mM vanadate reduced the ATP-dependent $Cl^-$ uptake by an average of 24.4 ± 6.4% (S.E.) (four experiments, 12 animals).

To assign a direct role of $Cl^-$ transport to an ATPase, the enzyme should be shown to be an integral component of the plasma membrane. Thus, in principle, the energy for active $Cl^-$ transport can be obtained from the hydrolysis of ATP. The inside-out plasma membrane vesicles from *Aplysia* enterocytes used in the present study were obtained via homogenization and centrifugation techniques that yielded a purified plasma membrane fraction that had little or no intracellular

membrane contamination [16]. Additionally, these plasma membrane vesicles contained $Cl^-$-stimulated ATPase activity which was sensitive to both thiocyanate and vanadate, but not sensitive to ouabain, oligomycin or efrapeptin [16].

As demonstrated in the present study (Fig. 1), the addition of ATP, in the presence of magnesium, to plasma membrane vesicles of *Aplysia* enterocytes, evoked a rapid $Cl^-$ uptake significantly above that of control. The ATP-driven $Cl^-$ uptake was obtained in the absence of $Na^+$, $K^+$, $HCO_3^-$, or a pH gradient between the intra- and extravesicular space which is strong suggestive evidence that the $(Na^+ + K^+)$-ATPase enzyme [17], $Na^+/Cl^-$ symport [1,2], $K^+/Cl^-$ symport [18], $Na^+/K^+/Cl^-$ symport [18], $Cl^-/HCO_3^-$ or $Cl^-/OH^-$ antiport [2] or $K^+/H^+$ antiport [17] are not mechanisms that are involved in the accumulation of $Cl^-$ within the vesicles in the presence of ATP.

The present finding that thiocyanate inhibited the ATP-dependent $Cl^-$ uptake in *Aplysia* enterocyte plasma membrane vesicles is consistent with the following previous findings: (I) thiocyanate inhibition of active $Cl^-$ absorption and short circuit current in in vitro *Aplysia* intestine [13], and (II) thiocyanate inhibition of $Cl^-$-stimulated ATPase activity in *Aplysia* enterocyte plasma membranes [16]. The parallels between thiocyanate sensitivity to $Cl^-$-stimulated ATPase activity and net active $Cl^-$ transport in both tissue and plasma membrane vesicle preparations justifies the speculation that the ATP-dependent $Cl^-$ transport mechanism could be driven by a $Cl^-$-stimulated ATPase found in the enterocyte plasma membrane. Additional supporting data for this hypothesis are: (I) the present finding that vanadate inhibited the ATP-dependent $Cl^-$ transport in *Aplysia* enterocyte plasma membrane vesicles and (II) the previous finding that vanadate inhibited $Cl^-$-stimulated ATPase activity in *Aplysia* enterocyte plasma membrane vesicles [16].

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