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## [SHORT COMMUNICATION]

## Inhibition of a $\text{Cl}^-$ - Transporting P-type ATPase in *Aplysia* Gut

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**ABSTRACT**—Utilizing a basolateral membrane vesicle preparation containing  $\text{Cl}^-$ -ATPase from *Aplysia* foregut, it was shown that orthovanadate inhibited  $\text{Cl}^-$ -ATPase activity, ATP-dependent  $\text{Cl}^-$  transport and ATP-dependent membrane potential change. N-ethylmaleimide (NEM) and p-chloromercuribenzoate (PCMBs) also inhibited the  $\text{Cl}^-$  pump biochemical and transport characteristics. However, bafilomycin, azide, DCCD or efrapreptin had no effect on the  $\text{Cl}^-$  pump characteristics suggesting that this  $\text{Cl}^-$  pump was a P-type ATPase.

### INTRODUCTION

Two general mechanisms of intestinal transcellular  $\text{Cl}^-$  absorption have been well established (Gerencser, 1996). One of these is an electrically silent symport which drives  $\text{Cl}^-$  uphill into enterocytes by means of an inward flux of  $\text{Na}^+$  moving down a favorable electrochemical gradient as is exemplified in intestinal epithelia of prawn (Ahearn *et al.*, 1977), flounder (Field *et al.*, 1978), bullfrog (Quay and Armstrong, 1969) and human (Turnberg *et al.*, 1970). The other transcellular  $\text{Cl}^-$  absorptive mechanism is a  $\text{Cl}^-$ /anion antiport as is found in intestinal epithelia of *Amphiuma* (Gunter-Smith and White, 1979) and rabbit (Frizzell *et al.*, 1976).

However, It has been hypothesized that  $\text{Cl}^-$  absorption across the *Aplysia* gut is mediated by a primary active transport process (Gerencser and White, 1980; Gerencser, 1983) located in the basolateral membrane (BLM) [Gerencser, 1983]. Lending credence to this idea were the observations that both BLM-localized  $\text{Cl}^-$ -stimulated ATPase and ATP-dependent  $\text{Cl}^-$  transport activities were reconstituted into proteoliposomes (Gerencser, 1990) and were shown to be properties of the same transporter protein (Gerencser and Zelezna, 1993). Serosal N-ethylmaleimide (NEM) inhibited the active transepithelial  $\text{Cl}^-$  absorptive flux across the *Aplysia* gut (Gerencser, 1990) while it had been demonstrated in other tissues that NEM specifically inhibited vacuolar  $\text{H}^+$ -ATPases (V-ATPases) (Pederson and Carafoli, 1987). Therefore, the present study was undertaken to further clarify whether the nature of the *Aplysia*  $\text{Cl}^-$  pump was P, V or F (Pederson and Carafoli, 1987).

### METHODS

Seahares (*Aplysia californica*) were obtained from Marinus, Inc. (Long Beach, CA) and were maintained at 25°C in circulating filtered seawater. Adult *Aplysia* (600–1000g) were used in these experiments. Tris (hydroxymethyl) aminomethane (Tris)-ATP, phenylmethylsulfonylfluoride (PMSF) and EDTA were purchased from Sigma Chemical. (St. Louis MO). All other reagent grade purity chemicals, including sodium orthovanadate, were purchased from Fisher Scientific (Norcross, GA).  $^{36}\text{Cl}^-$ , [carboxyl- $^{14}\text{C}$ ]inulin and [methyl- $^3\text{H}$ ] triphenylphosphonium bromide ( $^3\text{H}$  TPMP $^+$ ) were purchased from New England Nuclear (Boston, MA).

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, 1990). Marker enzymes of the BLM were determined as previously described (Gerencser, 1990; Gerencser and Zelezna, 1993).

Both  $\text{Cl}^-$ -stimulated ATPase and ATP-dependent transport activities were measured as described previously (Gerencser and Lee, 1985; Gerencser, 1988). The BLMV electrical potential ( $\Delta\psi$ ) was estimated from the distribution of the lipophilic cation triphenylmethylphosphonium (TPMP $^+$ ) between the extra- and intravesicular space by ultrafiltration as described by Gerencser (1988) utilizing a double labeling method. When used, inhibitors were preincubated with the BLMV for 10 min., as was valinomycin, the  $\text{K}^+$  ionophore (Gerencser, 1988).

All data are reported as means  $\pm$  SEM. Differences between means were analyzed statistically using Student's t-test with a  $P < 0.05$  used as the statistical significant difference criterion.

### RESULTS AND DISCUSSION

The present series of experiments probed the effects of various reactants and inhibitors on ATP-dependent  $\Delta\psi$ , ATP-dependent  $\text{Cl}^-$  transport, and  $\text{Cl}^-$ -ATPase activity in the BLMV preparation. As seen in Table 1, the only inhibitors that significantly reduced ATP-driven  $\Delta\psi$ , ATP-dependent  $\text{Cl}^-$  accumu-

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lation, and  $\text{Cl}^-$ -stimulated ATPase activity in the BLMV were orthovanadate, PCMBs and NEM. The PCMBs-induced inhibition of ATP-driven  $\Delta\Psi$ , ATP-dependent  $\text{Cl}^-$  accumulation, and  $\text{Cl}^-$ -stimulated ATPase activity was reversed by the subsequent addition of dithiothreitol (DTT).

The present finding that ATP, in the presence of  $\text{Cl}^-$ , can stimulate both  $\Delta\Psi$  and its associated intravesicular negativity, and  $\text{Cl}^-$  accumulation in the BLMV (Table 1), containing  $\text{Cl}^-$ -ATPase extracted from *Aplysia* gut BLM (Gerencser and Lee, 1985), strongly suggests that the mechanism responsible for this phenomenon is electrogenic. Electrogenicity is defined as generation of a potential difference and that is what was observed directly by the ATP-generated  $\Delta\Psi$  in Table 1.

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 (Pederson and Carafoli, 1987). In the present study orthovanadate inhibited the ATP-dependent  $\Delta\Psi$  and ATP-dependent  $\text{Cl}^-$  transport and  $\text{Cl}^-$ -ATPase activity in the BLM (Table 1) suggesting that the 110 kDa  $\text{Cl}^-$ -ATPase catalytic (sub)unit previously detected in the proteoliposome (Gerencser and Zelezna, 1993) was responsible for generating the negative intravesicular potential difference in the BLM preparation through transport of  $\text{Cl}^-$  and its associated negative charge. (Slayman and Zuckier, 1989; Pederson and Carafoli, 1987). In contrast, DCCD, an inhibitor of all known  $\text{H}^+$ -ATPases, whether they belong to P, V, or F groups (Pederson and Carafoli, 1987) had no effect on ATP driven  $\Delta\Psi$ , ATP-dependent  $\text{Cl}^-$  accumulation or  $\text{Cl}^-$ -ATPase activity (Table 1) strongly suggesting that contaminant  $\text{H}^+$ -

ATPase (Gerencser, 1996) could not express the observed  $\text{Cl}^-$  pump activity.

As demonstrated in the present study (Table 1), the addition of PCMBs or NEM to BLM vesicles containing  $\text{Cl}^-$ -ATPase evoked an inhibition of ATP-dependent  $\Delta\Psi$ , ATP-dependent  $\text{Cl}^-$  transport and  $\text{Cl}^-$ -ATPase activity significantly lower from that of control. Although NEM nor PCMBs are not absolutely specific for sulfhydryl ligands and have been shown to inhibit other ligands such as carboxyl, amino, phosphoryl, and tyrosyl (Rothstein, 1970) it is strongly suggested that, at least, PCMBs 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 1). Since NEM is an inhibitor of V-ATPases (Pederson and Carafoli, 1987) and has been shown to inhibit  $\text{Cl}^-$  pump characteristics (Table 1) and  $\text{Cl}^-$  transport (Gerencser, 1990), it is not necessarily true that the  $\text{Cl}^-$  pump is a V-ATPase because NEM is relatively non-specific inhibitor of all types of ligands on molecules (Rothstein, 1990). The fact that the PCMBs inhibition of the  $\text{Cl}^-$  pump is reversed by DTT and NEM inhibition is not reversed by DTT attests to the non-specificity of this molecule (Table 1). Further proofs of the non V-ATPase nature of the  $\text{Cl}^-$  pump rests with the insensitivity of the  $\text{Cl}^-$  pump characteristics to DCCD, bafilomycin and NBD- $\text{Cl}^-$ , all of which are V-ATPase inhibitors (Pedersen and Carafoli, 1987).

Additionally,  $\text{Cl}^-$ -ATPase activity ATP-dependent  $\Delta\Psi$  and ATP-dependent  $\text{Cl}^-$  transport in the *Aplysia* gut BLMV were insensitive to the F-ATPase inhibitors, efrapentin and azide (Table 1) (Pederson and Carafoli, 1987). All of these negative

**Table 1.** Effect of Inhibitors and/or Reactants on ATP-dependent  $\Delta\Psi$ , ATP-dependent  $\text{Cl}^-$  Transport and  $\text{Cl}^-$  ATPase Activity<sup>a</sup>

Inhibitors/reactants	$\Delta\Psi$ (mV)	% Inhibition	$\text{Cl}^-$ transport (nmol/mg protein)	% Inhibition	$\text{Cl}^-$ -ATPase activity (umol/15min/mg protein)	% Inhibition
None: (control, -ATP)	0.0 ± 0.2		47.8 ± 4.3			
ATP(5x10 <sup>-3</sup> M): (control, +ATP)	-33.6 ± 2.8		99.8 ± 8.3		2.3 ± 0.8	
Orthovanadate (10 <sup>-7</sup> M)	*-3.9 ± 0.6	88.3	*55.6 ± 4.8	85.0	*0.3 ± 0.1	86.9
DTT (10 <sup>-4</sup> M)	-33.8 ± 4.3	0.0	99.6 ± 6.3	00.0	2.1 ± 0.9	8.6
NEM (10 <sup>-5</sup> M)	*-4.3 ± 0.9	87.2	*52.1 ± 5.6	91.8	*0.2 ± 0.2	91.3
NEM(10 <sup>-5</sup> M)+DTT(10 <sup>-4</sup> M)	*-4.6 ± 1.5	86.3	*59.1 ± 8.9	79.3	*0.5 ± 0.1	78.2
PCMBs (10 <sup>-4</sup> M)	*-6.1 ± 2.0	81.8	*58.1 ± 7.3	80.2	*0.3 ± 0.1	86.9
PCMBs (10 <sup>-4</sup> M)+DTT(10 <sup>-4</sup> M)	-35.8 ± 1.6	-6.5	106.5 ± 10.9	-6.6	2.3 ± 1.2	0.0
DCCD (10 <sup>-5</sup> M)	-36.1 ± 0.5	-7.4	99.7 ± 5.8	0.0	2.6 ± 1.1	-13.0
Bafilomycin (10 <sup>-5</sup> M)	-32.0 ± 5.3	4.8	96.1 ± 9.3	7.1	2.4 ± 1.4	3.1
NBD-Cl (10 <sup>-5</sup> M)	-33.6 ± 1.4	0.0	95.3 ± 10.1	8.6	2.1 ± 0.5	8.6
Azide (10 <sup>-5</sup> M)	-34.1 ± 2.8	-1.5	106.1 ± 15.3	-7.1	2.4 ± 0.5	-4.0
Efrapentin (10 <sup>-6</sup> M)	-32.9 ± 1.5	2.0	99.7 ± 14.6	0.0	2.2 ± 1.0	4.3

<sup>a</sup> Inhibitors and/or reactants were preincubated with BLM in the reaction mixture [50 µl containing 10 mM imidazole-HCl (pH 7.8), 250 mM sucrose, 3 mM MgSO<sub>4</sub>, 25 mM choline Cl<sup>-</sup>, and 10 µM triphenylmethylphosphonium (TPMP<sup>+</sup>) plus [H<sup>3</sup>]TPMP<sup>+</sup> or <sup>36</sup>Cl<sup>-</sup> at concentrations ranging from 10<sup>-7</sup> to 5 x 10<sup>-3</sup>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<sup>-4</sup>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<sup>+</sup> nonspecifically bound to BLM was accounted for in final computation of vesicular electrical potential ( $\Delta\Psi$ ). Negative sign in  $\Delta\Psi$  denotes intravesicular polarity relative to extracellular bathing medium (reaction mixture).  $\text{Cl}^-$ -ATPase activity is defined as [(Mg<sup>2+</sup>+Cl<sup>-</sup>)] - Mg<sup>2+</sup>-ATPase]. Mg<sup>2+</sup>-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  $\text{Cl}^-$  transport in the absence of ATP or on Mg<sup>2+</sup>-ATPase activity. Values reported are means ± SEM of 4–6 different experiments; each experiment had triplicate determinations. Inhibition % was calculated by the following formula:  $\frac{\text{ATP control value} - \text{experiment value}}{\text{ATP control value}} \times 100$

\*\*\* represents a significant difference from the ATP control at a P<0.05.

results suggest that neither proton pump, nor F-ATPase, nor V-ATPase activity is involved in ATP-dependent  $\Delta\Psi$ , ATP-dependent  $\text{Cl}^-$  transport or  $\text{Cl}^-$ -ATPase activity. The present results warrant strong speculation that the active  $\text{Cl}^-$  absorptive mechanism in the *Aplysia* gut BLM is electrogenic and is driven by a  $\text{Cl}^-$  stimulated ATPase which is a P-type ATPase. Since all F- and V-ATPases are proton pumps (Pederson and Carafoli, 1987), the finding that the  $\text{Cl}^-$ -ATPase is a P-type ATPase attests to a biological efficiency of this unimolecular mechanism (Gerencser and Zelezna, 1993). Otherwise, a primary proton pump (i.e. a V-ATPase) would need a second transporter in order to translocate  $\text{Cl}^-$  up an electrochemical potential gradient (i.e. a  $\text{H}^+$ - $\text{Cl}^-$  symporter). This event would be after the proton pump had set up a favorable  $\text{H}^+$  gradient that would drive  $\text{Cl}^-$  energetically uphill by the symporter as has been described by Wieczorek (1992) in his "proton recycling process" hypothesis.

This study concurs with several others that postulate a  $\text{Cl}^-$ -ATPase as a mechanism that translocates  $\text{Cl}^-$  in a net sense, across a zoological membrane system. These include an electrogenic  $\text{Cl}^-$  transporter found in locust rectal epithelium (Hanrahan and Phillips, 1983) rat brain (Shiroya *et al.*, 1989), blue crab gill (Lee, 1982) and human placenta (Boyd and Chipperfield, 1982).

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