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[SHORT COMMUNICATION]

Inhibition of a Cl^- - Transporting P-type ATPase in *Aplysia* Gut

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ABSTRACT—Utilizing a basolateral membrane vesicle preparation containing Cl^--ATPase from *Aplysia* foregut, it was shown that orthovanodate inhibited Cl^--ATPase activity, ATP-dependent Cl^- transport and ATP-dependent membrane potential change. N-ethylmalemide (NEM) and p-chloromercurobenzoate (PCMBS) also inhibited the Cl^- pump biochemical and transport characteristics. However, bafilomycin, azide, DCCD or efrapeptin had no effect on the Cl^- pump characteristics suggesting that this Cl^- pump was a P-type ATPase.

INTRODUCTION

Two general mechanisms of intestinal transcellular Cl^- absorption have been well established (Gerencser, 1996). One of these is an electrically silent symport which drives Cl^- uphill into enterocytes by means of an inward flux of 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 Cl^- absorptive mechanism is a 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 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 Cl- stimulated ATPase and ATP-dependent 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-ethylmalemide (NEM) inhibited the active transepithelial Cl^- absorptive flux across the *Aplysia* gut (Gerencser, 1990) while it had been demonstrated in other tissues that NEM specifically inhibited vacuolar H^+-ATPases (V-ATPases) (Pederson and Carafoli, 1987). Therefore, the present study was undertaken to further clarify whether the nature of the *Aplysia* Cl^- pump was P, V or F (Pederson and Carafoli, 1987).

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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). $^{36}Cl^-$,[carboxyl^{14}-C]inulin and [methyl^{-3}H] triphenylphosphonium bromide ([^{3}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 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 K^+ ionophore (Gerencser, 1988).

All date are reported as means ±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 Cl^- transport, and Cl^- -ATPase activity in the BLMV preparation. As seen in Table 1, the only inhibitors that significantly reduced ATP-driven $\Delta\Psi$, ATP-dependent Cl^- accumu-

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

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 BLMV (Table 1), containing 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 Cl^- transport and Cl^--ATPase activity in the BLM (Table 1) suggesting that the 110 kDa 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 Cl^- and its associated negative charge. (Slayman and Zuckier, 1989; Pederson and Carafoli, 1987). In contrast, DCCD, an inhibitor of all known 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 Cl^- accumulation or Cl^--ATPase activity (Table 1) strongly suggesting that contaminant H^+ -ATPase (Gerencser, 1996) could not express the observed Cl^- pump activity.

As demonstrated in the present study (Table 1), the addition of PCMBS or NEM to BLM vesicles containing Cl^--ATPase evoked an inhibition of ATP-dependent $\Delta\psi$, ATP-dependent Cl^- transport and 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 isan inhibitor of V-ATPases (Pederson and Carafoli, 1987) and has been shown to inhibit Cl^- pump characteristics (Table 1) and Cl^- transport (Gerencser, 1990), it is not necessarily true that the Cl^- pump is a V-ATPase because NEM is relatively non-specific inhibitor of all types of ligands on molecules (Rothestein, 1990). The fact that the PCMBS inhibition of the 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 Cl^- pump rests with the insensitivity of the Cl^- pump characteristics to DCCD, bafilomycin and NBD- Cl^-, all of which are V-ATPase inhibitors (Pedersen and Carafoli, 1987).

Additionally, Cl^- -ATPase activity ATP-dependent $\Delta\Psi$ and ATP-dependent Cl^- transport in the *Aplysia* gut BLMV were insensitive to the F-ATPase inhibitors, efrapeptin 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 Cl^- Transport and Cl^- ATPase Activity[a]

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

[a] 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 $MgSO_4$, 25 mM choline Cl-, and 10 μM triphenylmethylphosphonium ($TPMP^+$)] plus [H3]$TPMP^+$ or ± $^{36}Cl^-$ at concentrations ranging from 10^{-7} to 5 x 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 25EC before samples were taken. Intravesicular medium matched the extravesicular medium in both composition and pH. $TPMP^+$ 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). Cl^--ATPase activity is defined as [(Mg^{2+}+Cl^-)] - $Mg2^+$-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 ±SEMof 4–6 different experiments; each experiment had triplicate determinations. Inhibition Åì was calculated by the following formula: $\frac{\text{ATPcontrol value-experiment value}}{\text{ATP control value}} \times 100$
"*" represents a significant difference from the ATP control at a P<0.05.

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

This study concurs with several others that postulate a Cl^--ATPase as a mechanism that translocates Cl^-, in a net sense, across a zoological membrane system. These include an electrogenic 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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