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SHORT COMMUNICATION

SUBCELLULAR CARBONIC ANHYDRASE PROFILE IN *APLYSIA* GUT

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The isolated gut of *Aplysia californica* (seahare), bathed in a substrate- and Na^+-free seawater bathing medium, generates a serosa-negative transepithelial potential ,difference and the concomitant short-circuit current (I_{sc}), which is inhibitable by acetazolamide, was shown to be identical to a net active absorptive flux of Cl^- (Gerencser, 1984). Additionally, Gerencser and Lee (1983, 1985) demonstrated the existence of a Cl^--stimulated ATPase activity in *Aplysia* gut absorptive cell basolateral membranes (BLM), which was also strongly inhibited by acetazolamide. Furthermore, Gerencser (1986) demonstrated acetazolamide inhibition of ATP-dependent Cl^- uptake and ATP-dependent vesicular membrane potential change in *Aplysia* inside-out absorptive cell BLM vesicles. A recent study demonstrating reconstitution of Cl^--stimulated ATPase activity into liposomes with subsequent Cl^- accumulation in the presence of ATP provided strong evidence for the existence of a primary active transport mechanism for Cl^- (Gerencser, 1990) and this, coupled with a previous observation (Gerencser, 1988) that FCCP (a protonophore) had no inhibitory effect on ATP-driven Cl^- accumulation in the BLM vesicles, strongly suggested that eukaryotic H^+-ATPases could not express Cl^- pump activity. In view of acetazolamide being a specific inhibitor of carbonic anhydrase (Maren, 1977), the present study was undertaken to determine whether carbonic anhydrase activity was an intermediate in the inhibition of the primary active Cl^- transport process in *Aplysia* gut.

Seahares (*Aplysia californica*) were obtained from Marinus Inc. (Westchester, CA) and were maintained at 25°C in circulating filtered sea water. Adult *Aplysia* (600–1200 g) were used in these experiments. The various subcellular membrane fractions were prepared from *Aplysia* gut epithelial absorptive cells by homogenization and differential and discontinuous sucrose density-gradient centrifugation techniques as described previously (Gerencser and Lee, 1985). Carbonic anhydrase activity was measured by the micromethod developed by Silverman and Gerster (1973), which based enzyme activity on enzyme-catalyzed ^{18}O exchange between bicarbonate and water. Alkaline phosphatase (brush border membrane marker), Na^+/K^+-ATPase (basolateral membrane marker), cytochrome *c* oxidase

Key words: gut, carbonic anhydrase, *Aplysia californica*.

Table 1. *Distribution of marker enzymes and carbonic anhydrase activity during preparation of cellular membranes from* Aplysia *gut absorptive cells*

Enzyme	H	BLM	BBM	M
Na^+/K^+-ATPase	0.92±0.38	7.28±1.85	ND	ND
Alkaline phosphatase	1.06±0.43	0.86±0.30	4.07±1.85	ND
Cytochrome *c* oxidase	0.72±0.20	ND	ND	4.81±1.05
Carbonic anhydrase	0.475±0.085	ND	0.634±0.112	1.175±0.386

Conditions for the enzyme assay were as described in Materials and methods. Enzyme activity is expressed as $\mu mol\,h^{-1}\,mg^{-1}$ protein for Na^+/K^+-ATPase and alkaline phosphatase; $\Delta log(ferrocytochrome\ c)\,min^{-1}\,mg^{-1}$ protein for cytochrome *c* oxidase; and $mmol\,s^{-1}$ for carbonic anhydrase.

H, homogenate; BLM, basolateral membrane; BBM, brush border membrane; M, mitochondria; ND, not detectable.

Starting gut mucosa was approximately 5.0 g.

(mitochondrial marker) and Cl^--stimulated ATPase were assayed as previously described (Gerencser and Lee, 1985), as was the ATP-dependent Cl^- transport in the BLM vesicles (Gerencser, 1986, 1988). All data are reported as means±S.E. Differences between means were analyzed statistically using Student's *t*-test with $P<0.05$ used as the statistical significant difference criterion.

As demonstrated in the present study (Table 1), carbonic anhydrase activity occurs in both the brush border membrane and the mitochondrial fractions of the *Aplysia* gut absorptive cells. It is apparent that the bulk of carbonic anhydrase activity resides in the mitochondria rather than the brush border because of the greater enrichment of this enzyme in that fraction. However, no detectable carbonic anhydrase activity resided in the basolateral membrane fraction (Table 1).

As seen in Table 2, Cl^--stimulated ATPase is significantly ($P<0.05$) more active than Mg^{2+}-stimulated ATPase activity in the BLM vesicle population. Acetazolamide ($0.1\,mmol\,l^{-1}$) inhibited this Cl^--stimulated ATPase activity. There is a significant ATP-dependent Cl^- uptake into these BLM vesicles compared with control vesicles in the absence of ATP ($P<0.05$) and this ATP-dependent Cl^- uptake is also inhibited by $0.1\,mmol\,l^{-1}$ acetazolamide (Table 2). All these values are means±S.E. for 3–5 different experiments (12–20 animals).

The previous findings, combined with the present findings that acetazolamide inhibits (i) active Cl^- absorption and I_{sc} in *Aplysia* gut *in vitro* (Gerencser, 1984); (ii) Cl^--stimulated ATPase activity in *Aplysia* gut absorptive cell BLM (Gerencser and Lee, 1985, and Table 2); and (iii) the ATP-dependent Cl^- uptake (Table 2) and ATP-dependent intravesicular negative potential change in *Aplysia* gut absorptive cell BLM vesicles (Gerencser, 1986, 1988; Gerencser *et al.* 1988), are consistent with the possibility that carbonic anhydrase activity is an intermediate, directly or indirectly, in active Cl^- transport in the *Aplysia* gut because acetazolamide, at low concentrations, has been shown to be a specific inhibitor of

Table 2. *Inhibition of Cl^- catalytic and transport activities*

Mg^{2+}-ATPase	$(Mg^{2+}+Cl^-)$-ATPase	$(Mg^{2+}+Cl^-)$-ATPase acetazolamide
BLM vesicle ATPase activity		
5.40±1.21	10.41±1.43	7.50±1.25
−ATP	+ATP	+ATP+acetazolamide
Cl^- uptake into BLM vesicles		
68.0±8.7	144.6±12.9	97.1±10.3

Enzyme activity is expressed as μmol 15 min^{-1} mg^{-1} protein for Mg^{2+}- and $(Mg^{2+}+Cl^-)$-ATPase. Conditions for the enzyme assay are described in Materials and methods. The time period of assay for V_i had been determined previously (Gerencser and Lee, 1985).

Acetazolamide (0.1 mmol l^{-1}) had no significant effect on Mg^{2+}-ATPase activity. Acetazolamide (0.1 mmol l^{-1}) was either preincubated with the BLM vesicles in the reaction mixture (50 μl containing 10 mmol l^{-1} imidazole–HCl, 250 mmol l^{-1} sucrose, 3 mmol l^{-1} $MgSO_4$ and 25 mmol l^{-1} choline chloride) at pH 7.8 for 10 min at 25 °C or 5 mmol l^{-1} ATP was added to the reaction mixture to initiate the incubation for the transport experiments. The incubation for the uptake of ^{36}Cl was measured for 10 min at 25 °C. The time for steady-state values for both ATP-independent and ATP-dependent ^{36}Cl uptakes was based on previous observations (Gerencser, 1986, 1988). ^{36}Cl uptake is expressed as nmol mg^{-1} protein.

+ Represents a compound's presence in the reaction mixture; − represents its absence.

carbonic anhydrase (Maren, 1977). However, since no carbonic anhydrase activity was detectable in the BLM of *Aplysia* gut (Table 1) and acetazolamide inhibited both Cl^--stimulated ATPase activity and ATP-dependent Cl^- transport in the BLM (Table 2), this hypothesis is not tenable. Lee (1982) has demonstrated acetazolamide inhibition of blue crab gill HCO_3^--ATPase, while White (1980) has shown acetazolamide to be a Cl^- transport inhibitor, supporting a precedent for acetazolamide inhibition of both anion-ATPase activity and anion transport. Thus, the present data further strengthen the idea that the BLM-localized Cl^- pump, which is inhibitable by acetazolamide, is independent of carbonic anhydrase activity.

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References

GERENCSER, G. A. (1984). Thiocyanate inhibition of active chloride absorption in *Aplysia* intestine. *Biochim. biophys. Acta* **775**, 389–394.

GERENCSER, G. A. (1986). Inhibition of a chloride pump by acetazolamide in the gut of *Aplysia californica*. *J. exp. Biol.* **125**, 391–393.

GERENCSER, G. A. (1988). Electrogenic ATP-dependent Cl^- transport by plasma membrane vesicles from *Aplysia* intestine. *Am. J. Physiol.* **254**, R127–R133.

GERENCSER, G. A. (1990). Reconstitution of a chloride-translocating ATPase from *Aplysia californica* gut. *Biochim. biophys. Acta* **1030**, 301–303.

GERENCSER, G. A. AND LEE, S. H. (1983). Cl^--stimulated adenosine triphosphatase: existence, location and function. *J. exp. Biol.* **106**, 142–161.

GERENCSER, G. A. AND LEE, S. H. (1985). Cl^--HCO_3^--stimulated ATPase in intestinal mucosa of *Aplysia*. *Am. J. Physiol.* **348**, R241–R248.

GERENCSER, G. A., WHITE, J. F., GRADMANN, D. AND BONTING, S. L. (1988). Is there a Cl^- pump? *Am. J. Physiol.* **255**, R677–R692.

LEE, S. H. (1982). Salinity adaptation of HCO_3^--dependent ATPase activity in the gills of the blue crab (*Callinectes sapidus*). *Biochim. biophys. Acta* **689**, 143–154.

MAREN, T. H. (1977). Use of inhibitors in physiological studies of carbonic anhydrase. *Am. J. Physiol.* **232**, F291–F297.

SILVERMAN, D. N. AND GERSTER, R. (1973). The detection and localization of carbonic anhydrase in the rabbit cornea. *Expl Eye Res.* **17**, 129–136.

WHITE, J. F. (1980). Bicarbonate-dependent chloride absorption in small intestine: ion fluxes and intracellular chloride activities. *J. Membr. Biol.* **53**, 95–107.