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THIOCYANATE INHIBITION OF ACTIVE CHLORIDE ABSORPTION IN *APLYSIA* INTESTINE

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This investigation was principally undertaken to examine the mechanism of active chloride absorption across the *Aplysia californica* intestine by using various inhibitors of ion transport. Isolated intestine, mounted between identical oxygenated sodium-free seawater solutions, maintained stable transmural potential differences (serosa negative) and short-circuit currents for several hours at 25°C. The metabolic inhibitors, 2,4-dinitrophenol and fluoride, reduced both transmural potential difference and short-circuit current; however, the electrical characteristics were predominantly dependent upon glycolytic energy. The addition of thiocyanate to the mucosal solution inhibited both electrical characteristics in parallel, and this inhibition could be titrated according to the thiocyanate concentration. The short-circuit current was carried wholly by a net active chloride transfer from mucosa to serosa as determined by flux measurements. These results suggest that active chloride absorption may be mediated by a primary active transport process.

Introduction

In the last several years, two general mechanisms of intestinal Cl^- transport have been reasonably well established [1,2]. The first of these is active and is thought to be effected through an electrically neutral Na^+-coupled carrier mechanism which drives Cl^- uphill into enterocytes via the inward flow of Na^+ down a favorable electrochemical potential gradient, as is exemplified in intestinal epithelia of prawn [3], flounder [4], sculpin [5], marine eel [6], bullfrog [7,8], rat [9], rabbit [10] and human [11]. The second widely accepted Cl^- transport process in enterocytes involves Cl^-/HCO_3^- countertransport, as is found in the intestinal epithelia of urodele [12], rabbit [1] and human [11].

However, there are several examples of Cl^- transport across intestinal preparations which do not conform to either of the two well-established models described above [2]. For example, White [13] described an electrogenic Cl^- uptake mechanism located in the mucosal membrane of *Amphiuma* intestine which is independent of mucosal Na^+ or HCO_3^-; Hanrahan and Phillips [14] have provided evidence for an electrogenic Cl^- transport mechanism located in the mucosal membrane of locust rectal enterocytes which is independent of Na^+ or HCO_3^-; and Gerencser [15,16] has demonstrated that the short-circuit current across *Aplysia californica* intestine is primarily a net active Cl^- absorptive flux in very low HCO_3^--containing media. 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

Abbreviation: SITS, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid stilbene.

villus enterocytes of *Aplysia* is at a lower electrochemical potential than in the extracellular medium [17], even in the absence of extracellular Na^+ [18]. Additionally, Gerencser and Lee [19,20] have demonstrated the existence of a vigorous Cl^--stimulated ATPase activity in *Aplysia* enterocyte plasma membranes, suggesting a linkage between Cl^- transport and ATPase activity.

The present study was therefore undertaken to assess the effects of various ion transport inhibitors on Na^+-independent Cl^- transport in *Aplysia* intestine and to compare the results with those found in other intestinal epithelia. The results are also discussed in reference to the knowledge of Cl^--stimulated ATPase in this preparation.

Materials and Methods

For this investigation, sea hares, *Aplysia californica*, were obtained from Pacific Bio-Marine (Venice, CA) and were maintained at 25°C in circulating filtered seawater. In most cases, only animals that had been kept in the laboratory under the above conditions for 1 week or less were used for experimental purposes. Ouabain, oligomycin, potassium thiocyanate were purchased from Sigma Chemical Co.; SITS from Pierce Chemical Co.; amiloride from Merck Sharp and Dohme Research Lab; furosemide from Hoechst Pharmaceutical Inc. All other chemicals were of reagent grade purity.

Adult *A. californica* were used in these experiments. The preparation and mounting of intestinal sheets between the two sides of a divided Lucite Ussing chamber have been described elsewhere [21]. The mucosal and serosal media were gassed and circulated continuously though Krogh-type airlifts with 100% oxygen by the method described by Ussing and Zerahn [22]. The experiments were run at 25°C. The formula for the Na^+-free seawater medium used was (mM): Tris-HCl, 462; $MgSO_4 \cdot 7H_2O$, 2.4; KCl, 9.7, $KHCO_3$, 2.4; $MgCl_2 \cdot 6H_2O$, 9.8; $CaCl_2$, 11.4. The total osmolality of the bathing medium was 1000 mosmol/l and its pH was 7.8 at 25°C. An automatic voltage clamp device was employed to record transmural potential difference and short-circuit current [23]. This device monitors transmural potential difference and short-circuit current with uncertainties of 0.2 mV and 0.2 μA, respectively, has a 90% response time of 0.1 s, and has provision for automatic compensation of the voltage drop due to the bathing solution.

The experiments with the Tris-HCl seawater medium were performed as follows. After the tissue was excised and mounted, short-circuit current and transmural potential difference were recorded every 5 min until an electrical steady state was reached. This usually required from 15 to 50 min. When sufficient measurements had been made to establish the steady-state short-circuit current and transmural potential difference, the inhibitor to be tested was added, either by removing the control seawater medium from the chamber and replacing it with one containing the inhibitor at the concentration desired, or by directly adding a small volume (usually 10–15 μl) of a stock solution of the substance to one or both chamber compartments. Following this above procedure, measurement of short-circuit current and transmural potential difference were continued as before.

Using ^{36}Cl (New England Nuclear), undirectional mucosal-to-serosal fluxes (J_{ms}) and serosal-to-mucosal fluxes (J_{sm}) of Cl^- were determined on paired pieces of tissue from the same animal when their respective short-circuit currents were comparable in magnitude. In these radioisotopic experiments the tissue was allowed to equilibrate for 30–90 min in nonradioactive seawater solution. At this electrical steady-state time, a trace amount of isotope was directly added to the chamber and allowed to reach an isotopic steady-state transport across the intestine. Thereafter, at timed intervals of approx. 20 min, 0.1 ml samples of solution were removed from the initially unlabeled half-chamber for counting. The samples were counted in a three-channel Beckman LS-330 liquid scintillation counter. From the results obtained, J_{ms} ^{36}Cl and J_{sm} ^{36}Cl were computed as described by Quay and Armstrong [8]. All data are reported as means $\pm$ S.E. Differences between means were analyzed statistically using Student's t-test with $P < 0.05$ used as the statistical significant difference criterion.

Results

It was observed in a Tris-HCl seawater medium that the spontaneous transmural potential dif-

ference generated by the isolated *Aplysia* intestine was oriented with the serosa being negative relative to the mucosa. After an initial transient period, the steady-state transmural potential difference and concomitant short-circuit current remained stable for 3–4 h ($n = 4$).

As seen in Fig. 1, the addition of 1 mM 2,4-dinitrophenol to both the mucosal and serosal bathing solutions elicited a small inhibition of transmural potential difference and short-circuit current. The remaining transmural potential difference and short-curcuit current was markedly reduced or abolished by the addition of 1 mM fluoride (F^-) to the mucosal solution ($N = 5$) or to the mucosal and serosal solutions ($N = 3$). The application of iodoacetate (1 mM) to the mucosal solution ($N = 2$) after 2,4-dinitrophenol application abolished the electrical characteristics. Rinsing and replacing the mucosal and serosal solutions with freshly oxygenated, inhibitor-free Tris-HCl seawater partially restored the electrical characteristics, transmural potential difference and short-circuit current ($n = 8$).

A dose vs. response relationship between three concentrations of thiocyanate (SCN^-) administered to the mucosal solution and inhibition of short-circuit current was studied. As was observed, short-circuit current decreased curvilinearly with increasing concentrations of mucosally applied SCN^- until total inhibition occurred at 10 mM SCN^-. The average inhibition (percentage) relative to control short-circuit current at 0.1 mM SCN^- was $32 \pm 6\%$, while 1 mM SCN^- gave a $85 \pm 11\%$ inhibitory response relative to control. The addition of 0.1, 1.0 or 10.0 mM SCN^- to the serosal solution had little or no effect on either transmural potential difference or short-circuit current ($n = 4$).

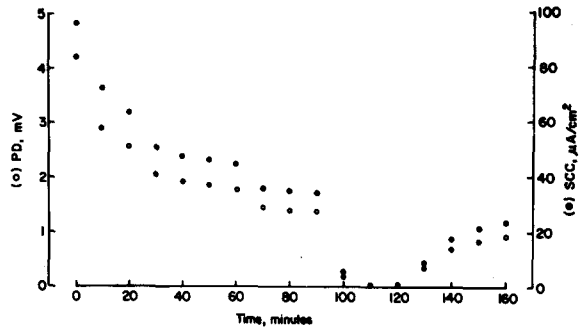

Fig. 1. Typical experiment showing inhibition of transmural potential difference (○) and short-circuit current (●) by addition of 1 mM 2,4-dinitrophenol and 1 mM fluoride to both the mucosal and serosal bathing solutions. Rinsing both mucosal and serosal compartments with inhibitor-free media partially restored transmural potential difference and short-circuit current. Transmural potential difference is oriented with the mucosa being positive relative to the serosa.

Ouabain (1.5 mM) added to either the mucosal ($n = 2$) or serosal ($n = 5$) bathing solutions had virtually no effect on potential difference or short-circuit current. Oligomycin (2 μM) added to either the mucosal ($n = 4$) or serosal ($n = 2$) bathing solutions also had no effect on the electrical characteristics. Albumin-bound oligomycin ($7 \cdot 10^{-5}$ M) added to either the mucosal ($n = 3$) or serosal ($n = 2$) bathing solutions had no effect on transmural potential difference or short-circuit current. When 1 mM furosemide or 1 mM amiloride was added to the mucosal ($n = 4$) or the serosal ($n = 2$) bathing solutions no change in transmural potential difference or short-circuit current was noted. SITS (0.1 mM) had no effect on transmural potential difference or short-circuit current when added to the mucosal ($n = 3$) or serosal ($n = 2$) bathing solutions. However, when 1 mM acetazolamide was added to both the mucosal

TABLE I

CHLORIDE FLUXES IN TRIS-HCl SEAWATER MEDIA

Values are means ± S.E. in nequiv.·cm^{-2}·min^{-1}. Number of experiments shown in parentheses.

	J_{ms}	J_{sm}	J_{ms}^{NET}	Short-circuit current
Before thiocyanate addition	216.7 ± 14.2(6)	180.1 ± 13.2(6)	36.6 ± 6.8	30.3 ± 7.9(6)
After thiocyanate addition	175.7 ± 8.1(6)	173.9 ± 8.6(6)	1.8 ± 1.3	0.8 ± 0.7(6)

and serosal bathing solutions ($n = 5$), an average inhibition of $31 \pm 4\%$ from control was observed for transmural potential difference and short-circuit current.

In order to determine the ionic nature of the measured electrical characteristics, in the absence and presence of SCN^-, measurements of the J_{ms} and J_{sm} of Cl^- were made under short-circuited conditions. As shown in Table I, the mean J_{ms} of Cl^- was significantly greater ($P < 0.05$) than its paired mean J_{sm} of Cl^-. The average net J_{ms} of Cl^- was not significantly different from the average short-circuit current under these control conditions. The addition of 10 mM SCN^- to the mucosal bathing solution significantly reduced, in parallel, both the mean J_{ms} of Cl^- and the average short-circuit current, but had no significant effect on the mean J_{sm} of Cl^-.

Discussion

Several studies have demonstrated active transport of anions in a broad range of tissues including intestine [13,24], stomach [25], kidney [26], gill [27,28] and even nerve [29]. Chloride and bicarbonate have been the most frequently studied anions [2,30]. Cl^-/HCO_3^--stimulated ATPase activity has also been found in various membrane fractions of cell homogenates in these tissues [2,31] fuelling speculation of a cause-and-effect relationship between the ATPase and active anion transport, respectively [28,32,33].

The previous finding that chloride exists in an electrochemical well inside the *Aplysia* enterocyte, even in the absence of extracellular sodium, necessitated the invokation of a serosally located active transport system for that ion, in the absorptive direction, that was different from that involved in the activation of the chloride-independent sodium pump in this tissue [18]. The present results confirm previous observations [18] on the serosa-negative electrical orientation and on the temporal stability of the tissue in the absence of extracellular sodium. The previous demonstration of total electrical recovery of both transmural and transmucosal potential difference when Tris replaced sodium and, conversely, when sodium replaced Tris in the bathing medium [18] attests to the stability afforded the transport properties of the *Aplysia* intestinal epithelium. The results indicate that the origin of the electrical characteristics in isolated *Aplysia* intestine stem from an active chloride transport mechanism which transfers chloride in a net sense from the mucosal to the serosal compartment. This is directly demonstrated by the chloride flux experiments of Table I where the mean J_{ms} of chloride is significantly greater than its paired mean J_{sm} of chloride. Also, the average net mucosal to serosal chloride flux is identical to the corresponding mean short-circuit current. This result compares favorably with those found in locust rectum [14] and *Amphiuma* intestine [12] under similar experimental conditions.

Since active transport processes are coupled (directly or indirectly) to metabolism, the findings that the metabolic inhibitors DNP and F^- inhibited transmural potential difference and short-circuit current (Fig. 1) further support the hypothesis of an active chloride transport process generating and maintaining the measured electrical characteristics. The finding that F^- or iodoacetate caused the major portion of inhibition to transmural potential difference and short-circuit current suggests that glycolysis, and not aerobic metabolism, is the primary source of energy powering chloride absorption because F^- and iodoacetate are known glycolytic inhibitors [7,16].

Thiocyanate has been shown to inhibit anion transport across a variety of epithelial systems [34,35]. The observation that mucosally-applied SCN^- inhibited the active chloride flux (Table I) and short-circuit current suggests that the SCN^- action is either on the highly conductive mucosal membrane of the *Aplysia* enterocyte [36,37], where chloride moves energetically downhill into the cell, or on the intracellular aspect of the active extrusion mechanism for chloride located in the basolateral membrane [18]. The finding that serosally applied SCN^- had little or no effect on chloride transport across the intestine suggests that the SCN^- is directly blocking the transport of chloride, whether its site of action is the mucosal or basolateral membrane or both, since the active component of chloride transport in this preparation is mucosa to serosa (Ref. 18, and Table I).

The finding that furosemide, amiloride or ouabain had no effect on transmural potential difference or short-circuit current further substan-

tiates the independence of the chloride transport mechanism from the presence or movement of sodium, since these drugs are known inhibitors of sodium transport [1,2]. The observation that SITS had no effect on potential difference or short-circuit current suggests the independence of the chloride transport mechanism from the simultaneous counter transport of another anion, since this compound is a known anion-anion exchange inhibitor [38].

The results with these inhibitors suggest a possible participation by a primary active chloride transport mechanism, for previous observations [19,20] have shown that a purified plasma membrane fraction from *Aplysia* enterocytes contains a vigorous Cl^--stimulated ATPase activity. This enzyme has been shown to be insensitive to ouabain, oligomycin, SITS, amiloride and furosemide, but sensitive to SCN^- and acetazolamide. These findings have also been demonstrated in a number of tissues known to carry out active anion transport and to contain anion-stimulated ATPase activity [33,39,40]. The parallelism of ouabain and oligomycin insensitivity and SCN^- sensitivity to net active chloride absorption and Cl^--stimulated ATPase activity justifies the speculation that the active chloride absorptive mechanism could be a Cl^--stimulated ATPase found in the enterocyte plasma membrane.

The previous finding that acetazolamide inhibited Cl^--stimulated ATPase activity has also been made in blue crab gill [41]. The present observation that acetazolamide inhibited chloride absorption has been also demonstrated in *Amphiuma* intestine [13]. Thus, the data further strengthen the notion that the Cl^--stimulated ATPase, which is inhibited by acetazolamide, may be involved in active chloride absorption across the *Aplysia* intestine.

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