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Sodium-Sulfate Symport by *Aplysia californica* Gut

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ABSTRACT—Sulfate transport across plasma membranes has been described in a wide variety of organisms and cell types including gastrointestinal epithelia. Sulfate transport can be coupled to proton, sodium symport or antiport processes involving chloride or bicarbonate. It had previously been observed in *Aplysia* gut that sulfate was actively absorbed. To understand the mechanism for this transport, short-circuited *Aplysia californica* gut was used. Bidirectional transepithelial fluxes of both sodium and sulfate were measured to see whether there was interaction between the fluxes. The net mucosal-to-serosal flux of Na^+ was enhanced by the presence of sulfate and it was abolished by the presence of serosal ouabain. Similarly, the net mucosal-to-serosal flux of sulfate was dependent upon the presence of Na^+ and was abolished by the presence of serosal ouabain. Theophylline, DIDS and bumetanide, added to either side, had no effect on transepithelial potential difference or short-circuit current in the *Aplysia* gut bathed in a Na_2SO_4 seawater medium. However, mucosal thiosulfate inhibited the net mucosal-to-serosal fluxes of both sulfate and Na^+ and the thiosulfate-sensitive Na^+ flux to that of sulfate was 2:1. These results suggest the presence of a Na-SO_4 symporter in the mucosal membrane of the *Aplysia californica* foregut absorptive cell.

INTRODUCTION

Gastrointestinal and renal transport of the divalent anion sulfate across epithelial apical membranes has been investigated in various vertebrate groups including mammals (Ahearn and Murer, 1984; Pritchard, 1987), teleost fish (Renfro and Pritchard, 1982, 1983) and the domestic chicken (Renfro *et al*., 1987). A number of mechanisms for brush-border carrier-mediated sulfate transport across epithelial membranes have been proposed and include sodium-sulfate cotransport (Ahearn and Murer, 1984; Lucke *et al*., 1979), anion exchange (Renfro and Pritchard, 1982; Taylor *et al*., 1987) and pH gradient-dependent transfer (Schron *et al*., 1985). These processes contribute to transepithelial regulation of sulfate levels, and may affect acid-base balance and plasma osmolarity.

However, there are very few studies of sulfate transport across epithelia of invertebrates. A proton-stimulated sulfate/chloride exchanger has recently been described in apical membranes of lobster (*Homarus americanus*) hepatopancreatic epithelial cells (Cattey *et al*, 1992), while an oxalate/sulfate antiporter has also been described in the basolateral membranes of the same cells of lobster hepatopancreas (Gerencser *et al*, 1995). Many years ago, it was shown that *Aplysia* foregut could actively absorb sulfate (Gerencser, 1979), however the mechanism for transporting sulfate was not defined. In view of this observation, the present study was undertaken to determine the nature of the sulfate transporter in *Aplysia* gut. The present study uses isolated foregut from *Aplysia californica* to characterize a sodium/sulfate symporter that is located in the mucosal membrane of the gut cells and is inhibited by the thiosulfate and ouabain. This transport mechanism may contribute, in part, in maintaining sulfate homeostasis by *Aplysia*.

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MATERIALS AND METHODS

Mollusc

Aplysia californica were obtained from Marinus (Westchester, CA) and were maintained at 25°C in circulating filtered seawater. Adult *Aplysia* (600–1000 g) were used in these experiments and in most cases only animals that had been kept in the laboratory under the above conditions for ≤1 wk were used.

Incubation media for gut tissue

The formula for the standard seawater (Ringer's) solution used was: Na_2SO_4, 231 mM; $MgSO_4 \cdot H_20$, 12.3 mM; K_2SO_4, 12.1 mM; $NaHCO_3$, 2.4 mM; Ca (Gluconate)$_2$, 11.4 mM; mannitol, 0.237 mM. A Na^+-free medium was prepared by totally replacing Na^+ with trishydroxyaminomethane$^+$ using sulfate and bicarbonate salts. A sulfate-free medium was prepared by totally replacing sulfate and mannitol with gluconate. The total osmolality of the bathing media was 1010 mOsm/Kg and their pH was 7.8 at 25°C.

Experimental Procedures

The preparation and mounting of gut sheets between the two halves of a Lucite Ussing chamber that allowed measurement of transepithelial potential difference (Ψ_{MS}) and short-circuit current (SCC) across the gut have been described previously (Gerencser, 1978) (Fig. 1). Both the mucosal and serosal media were gassed with 100% O_2, and both aspects of the gut were independently and continuously

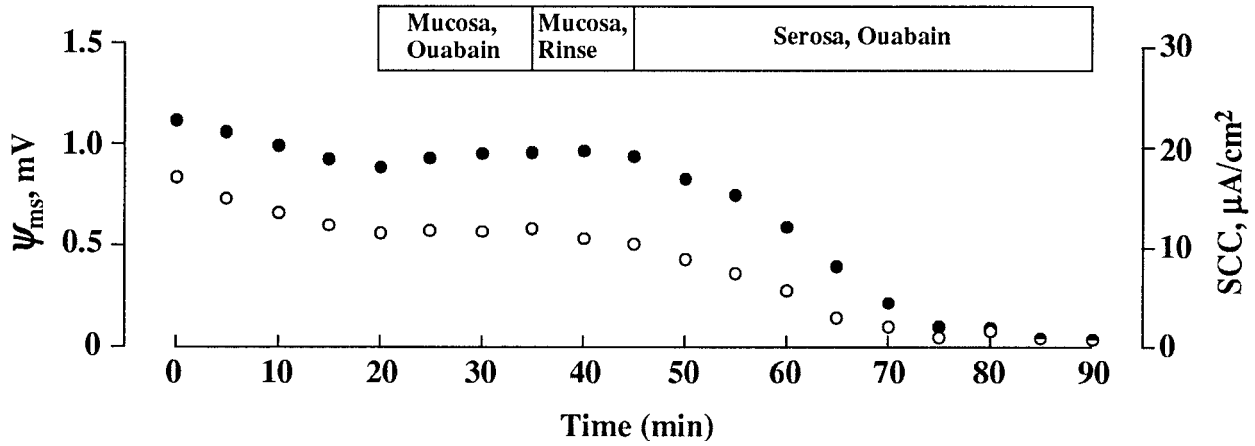

Fig. 1.

perfused by gravity with seawater medium at room temperature (25±1°C).

The methods used to measure Ψ_{MS} and SCC were essentially similar to those employed for rabbit ileum by Schultz and Zalusky (1964), except that agar bridges from calomel half-cells, instead of Ag-AgCl electrodes, were used to apply external current to the system. The electrolyte content of these bridges was identical to that of the bathing solution in each experiment to minimize diffusion currents. The agar bridges from the potential-sensing electrodes contained saturated KCl because K^+ and Cl^- have approximately equal mobility constants (Schultz and Curran, 1970). To minimize potential offset between these electrodes, the ends of these bridges were preequilibrated with the bathing medium for several hours before the experiment. Offset between the potential-sensing electrodes was measured at the beginning of the experiment and again at the end of the run following removal of the tissue and replacement of the bathing fluid. The potential drop between the potential-sensing electrodes due to the resistance of the bathing solution was compensated automatically by the voltage-clamp device as described by Rothe *et al.* (1969).

By use of ^{22}Na and $^{35}SO_4$ (New England Nuclear), unidirectional mucosal-to-serosal (J_{MS}) and serosal-to-mucosal fluxes (J_{SM}) of Na^+ or SO_4 were determined on paired pieces of tissue from the same animal when their respective SCC's 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. Thereafter, at timed intervals of approximately 20 min, 0.1 ml samples of solution were removed from the initially unlabeled half-chamber for counting. Fluxes observed during the early sampling stages, i.e., before specific activity equilibrium between tissue and bathing solution was achieved, were small. They increased to constant values by the end of the first hour following introduction of tracer. Therefore, only samples obtained following the first hr were used to estimate steady-state fluxes. Experiments were usually terminated 4–5 hr after addition of isotope. From the results obtained J_{MS} and J_{SM} of ^{22}Na and $^{35}SO_4$ were computed as described by Quay and Armstrong (1969). All data are reported as means±SEM. Differences between means were analyzed statistically using a Student's paired t-test.

RESULTS

The first group of experiments was designed to examine whether sulfate and/or ouabain had any effect on Na^+ fluxes. As can be seen in Table 1, the mean net J_{MS} of Na^+ (J^{NET}_{MS}) is approximately equal to the average SCC with gluconate being the major anion in the bathing medium. However, upon replacing both the mucosal and serosal bathing media with a media containing sulfate as its major anion, there is a significant increase ($P<0.05$) in the J^{NET}_{MS} of Na^+. This change in Na^+ absorption is due to an increase in unidirectional J_{MS} of Na^+. The unidirectional J_{SM} of Na^+ did not significantly change in the sulfate-based medium. Also, the mean J^{NET}_{MS} of Na^+, in the presence of sulfate, is significantly greater ($P<0.05$) than the corresponding average SCC. Serosal ouabain (10^{-4}M) abolished both the basal and sulfate-dependent J^{NET}_{MS} of Na^+ by inhibiting solely the unidirectional J_{MS} of Na^+. Ouabain also abolished the SCC.

The next group of experiments was designed to examine if Na^+ and/or ouabain had any effect on sulfate fluxes. As can be seen in Table 2, the average net J^{NET}_{MS} of sulfate is almost absent when the gut was bathed in Na^+-free bathing media. The corresponding average SCC is also close to zero. However, when the Na^+-free sulfate bathing medium was replaced with a Na^+-containing sulfate medium, the average J^{NET}_{MS} of sulfate increased significantly ($P<0.05$). This increase in the J^{NET}_{MS} of sulfate was entirely attributable to the increase in the unidi-

Table 1. Na^+ fluxes in various seawater media

Seawater Media	J_{MS}	J_{SM}	J^{NET}_{MS}	SCC
Na Gluconate	148.2±12.1 (6)	119.9±12.8 (6)	28.3±10.6 (6)	35.6±8.3 (6)
Na_2SO_4	205.5±16.3 (6)	125.1±18.3 (6)	80.4±15.1 (6)	42.1±8.9 (6)
Na_2SO_4+Ouabain	125.4±15.1 (6)	116.4±15.3 (6)	19.0±11.9 (6)	2.3±9.1 (6)

Values are means±SEM in neq/cm².min. No of experiments shown in parentheses.

Table 2. Sulfate fluxes in various seawater media.

Seawater Media	J_{MS}	J_{SM}	J^{NET}_{MS}	*SCC*
$Tris_2SO_4$	30.1±6.8 (5)	28.9±7.3 (5)	1.2±6.3 (5)	1.1±4.6 (5)
Na_2SO_4	48.6±5.3 (5)	26.7±8.1 (5)	21.9±5.9 (5)	38.6±8.1(5)
Na_2SO_4+Ouabain	33.6±7.1 (5)	29.6±6.35 (5)	4.0±5.9 (5)	4.2±4.3 (5)

Values are means±SEM in neq/cm^2.min. No of experiments shown in parentheses.

Table 3. Effect of thiosulfate on Na^+ and sulfate fluxes.

Sulfate Fluxes				
Seawater Media	J_{MS}	J_{SM}	J^{NET}_{MS}	*SCC*
Na_2SO_4	45.2±8.1 (4)	25.3±6.3 (4)	19.9±6.8 (4)	30.3±3.2 (4)
Na_2SO_4+thiosulfate	28.4±6.1 (4)	27.3±4.9 (4)	1.1±5.6 (4)	25.6±4.3 (4)
Significance	P<0.05	N.S.	P<0.05	N.S.
Sodium Fluxes				
Seawater Media	J_{MS}	J_{SM}	J^{NET}_{MS}	*SCC*
Na_2SO_4	193.1±12.2 (4)	116.9±16.3 (4)	76.2±11.1(4)	35.3±9.1 (4)
Na_2SO_4+thiosulfate	150.6±13.1(4)	120.3±10.6 (4)	30.3±9.8 (4)	28.1±5.6 (4)
Significance	P<0.05	N.S.	P<0.05	N.S.

Values are means±SEM in neq/cm^2.min. No of experiments shown in parentheses. Level of significant difference is at P<0.05; N.S., not significant.

rectional J_{MS} of sulfate because there was no significant change in the undirectional J_{SM} of sulfate in the presence of Na^+. The average SCC, in the presence of Na^+, was significantly greater than zero (P<0.05) and it was also greater than the J^{NET}_{MS} of sulfate. Serosal ouabain (10^{-4}M) inhibited both the J^{NET}_{MS} of sulfate and the SCC. The unidirectional J_{MS} of sulfate was the only flux of sulfate that was affected by serosal ouabain.

The next series of experiments were designed to examine the effects of thiosulfate on Na^+ and sulfate fluxes in *Aplysia* gut. The addition of thiosulfate (10^{-2}M) to the mucosal compartment of a Na_2SO_4 bathing medium inhibited the unidirectional J_{MS} of sulfate, but not the J_{SM} of sulfate, resulting in the complete depression of J^{NET}_{MS} of sulfate (Table 3). In contrast, the serosal addition of 10-2M thiosulfate to the serosal bathing solution had no effect on either the unidirectional J_{MS} or JSM of sulfate [data not shown (n=3)]. The addition of 10^{-2}M thiosulfate to the mucosal bathing solution also inhibited the unidirectional J_{MS} of Na^+ without affecting the unidirectional J_{SM} of Na^+. The ratio of the thiosulfate-sensitive Na^+ and sulfate fluxes was 2:1 in both J_{MS} and J^{NET}_{MS}. On the other hand, thiosulfate had no significant effect on SCC across the *Aplysia* gut.

Theophylline (10^{-6}M), bumetanide (10^{-5}M) nor 10^{-5}M 4,4'-diisothiocyano-2,2'-disulfonic stilbene (DIDS) added to either the mucosal or serosal bathing medium had no effect on J_{MS} or SCC in the *Aplysia* gut preparation. Each of these chemical agents were used in three experiments.

DISCUSSION

In the current investigation we presented suggestive evidence for the existence of a carrier-mediated Na^+-sulfate symport located in the apical membrane of *Aplysia californica* foregut epithelium. Sulfate carriers have been described in the apical membranes of several vertebrate epithelial tissues (Cattey *et al*., 1994). Both sodium-sulfate cotransport and sulfate-hydroxyl exchange mechanisms have been demonstrated in rabbit ileal brush border (Schron *et al*., 1985; Schneider *et al*., 1984). In avian renal apical membranes multiple pathways were shown to transport sulfate; sodium-sulfate cotransport, sulfate-bicarbonate exchange and proton-dependent sulfate transport (Renfro *et al*., 1987). Marine teleost renal tubule apical membranes have been shown to contain a sulfate-anion exchange mechanism which is most effective with bicarbonate (Renfro and Pritchard, 1983). In lobster hepatopancreatic apical membranes sulfate uptake was not stimulated by inwardly directed cation gradients of either Na^+ or K^+ (Cattey *et al*., 1992). However, intravesicular Cl^- stimulated the influx of radiolabeled sulfate which was interpreted as there being a SO_4/Cl antiporter in the apical membrane.

When the *Aplysia* foregut was bathed in a sulfate-free (Table 1) or chloride-free (Gerencser, 1981; Gerencser, 1985) Na^+-containing seawater media, the net active absorptive flux of Na^+ was equivalent to the SCC. This observation is interpreted as Na^+ being the only ion actively translocated, in a net sense, across the gut tissue. However, when sulfate replaced gluconate [a non-transportable anion (Cattey *et al*., 1992)] in the bathing media, the net active absorptive flux of Na^+ increased solely due to the increase in the unidirectional J_{MS} of Na^+. This suggests that sulfate stimulates the absorptive flux of Na^+. However, the J^{NET}_{MS} of Na^+ is significantly greater than the corresponding SCC (Table 1). This disparity in J^{NET}_{MS} of Na^+ and SCC could be accounted for by a net active absorptive flux of an anion such as sulfate. Serosally-applied ouabain inhibited both J^{NET}_{MS} of Na^+ and the SCC, accompanied an inhibition of the unidirectional J_{MS} of Na^+ (Table 1). These observations suggest that Na^+ transport and SCC are depen-

dent on the activity of the Na^+/K^+-ATPase (Gerencser and Lee, 1985; Skou, 1965).

In a Na^+-free seawater bathing medium there is no net transport of sulfate nor a SCC across the *Aplysia* gut (Table 2). However, upon replacing the Na^+-free seawater medium with a medium containing Na^+, there is a finite J_{MS}^{NET} of sulfate under short-circuited conditions. These observations suggest that active sulfate absorption is dependent upon the presence of Na^+ and that there is coupling between these two ions in their transit from the mucosal to the serosal bathing solutions. This is because, in the presence of Na^+, there is a finite SCC, part of which can be accounted for by the J_{MS}^{NET} of sulfate while the remainder of the SCC can be accounted for by a net mucosal-to-serosal movement of Na^+ (Tables 1,2,3). The substantiation of Na+ as the co-transported ion species with that of sulfate is shown with the inhibition of both the unidirectional J_{MS} of sulfate and the SCC by serosally-applied ouabain (Table 2). As previously stated ouabain specifically inhibits active Na^+ transport (Skou, 1965; Schultz and Zalusky, 1964). Therefore, its inhibition of active sulfate absorption implies a degree of coupling between the two undirectional fluxes (J_{MS}'s) of both Na^+ and sulfate.

Thiosulfate is a known inhibitor of sulfate transport (Schneider *et al*., 1984; Turner, 1984). In the present study, mucosally-applied thiosulfate inhibited the J_{MS} of sulfate such that the active component of sulfate absorption was abolished (Table 3). In addition mucosally-applied thiosulfate also inhibited the unidirectional J_{MS} of Na^+ (Table 3). Together, these results strongly suggest a coupling between Na^+ and sulfate transport, in their co-movement from mucosa to sersosa. The result that serosally-applied thiosulfate had no effect on either Na^+ or sulfate transport suggests that the transporter for both ions resides in the apical membrane of the *Aplysia* foregut absorptive cell and not in the basolateral membrane. Since thiosulfate significantly inhibited both unidirectional J_{MS}'s of Na^+ and sulfate, but did not significantly inhibit the corresponding SCC (Table 3), the decrease in coupled Na^+-sulfate flux, from mucosa-to-serosa, must be electrically silent. In addition, as seen in Table 1, sulfate stimulated the J_{MS} of Na^+ without an increase in SCC. The SCC's under these different experimental conditions did not change. This suggested that the coupled Na^+/sulfate cotransport, from mucosa-to-serosa was electrically neutral. Since Na^+ is a univalent cation and sulfate is a divalent anion, the stoichiometry of coupled Na^+/sulfate transport in the *Aplysia* gut could be two Na^+ per one sulfate per cycle of transport, or some mathematical equivalent of 2 Na^+ per 1 sulfate in order for electroneutrality to be maintained. In fact, the ratio of the thiosulfate-sensitive Na^+ to sulfate fluxes was 2:1.

In summary, we have presented suggestive evidence for the existence of a Na-SO_4 symporter located in the apical membrane of the *Aplysia californica* foregut absorptive cell that could be responsible for the net absorption of sulfate by this animal. This event could be beneficial for cellular viability of cellular metabolic reactions such as: 1) sulfur conjugation (Gerencser, 1996; Turner, 1984; and/or Pritchard, 1987) complexing with heavy metals such as what happens in lobster hepatopancreas (Gerencser *et al*., 1995). Sulfate homeostasis in the *Aplysia* is, at least, partly maintained by this luminal Na/SO_4 symport transport mechanism.

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