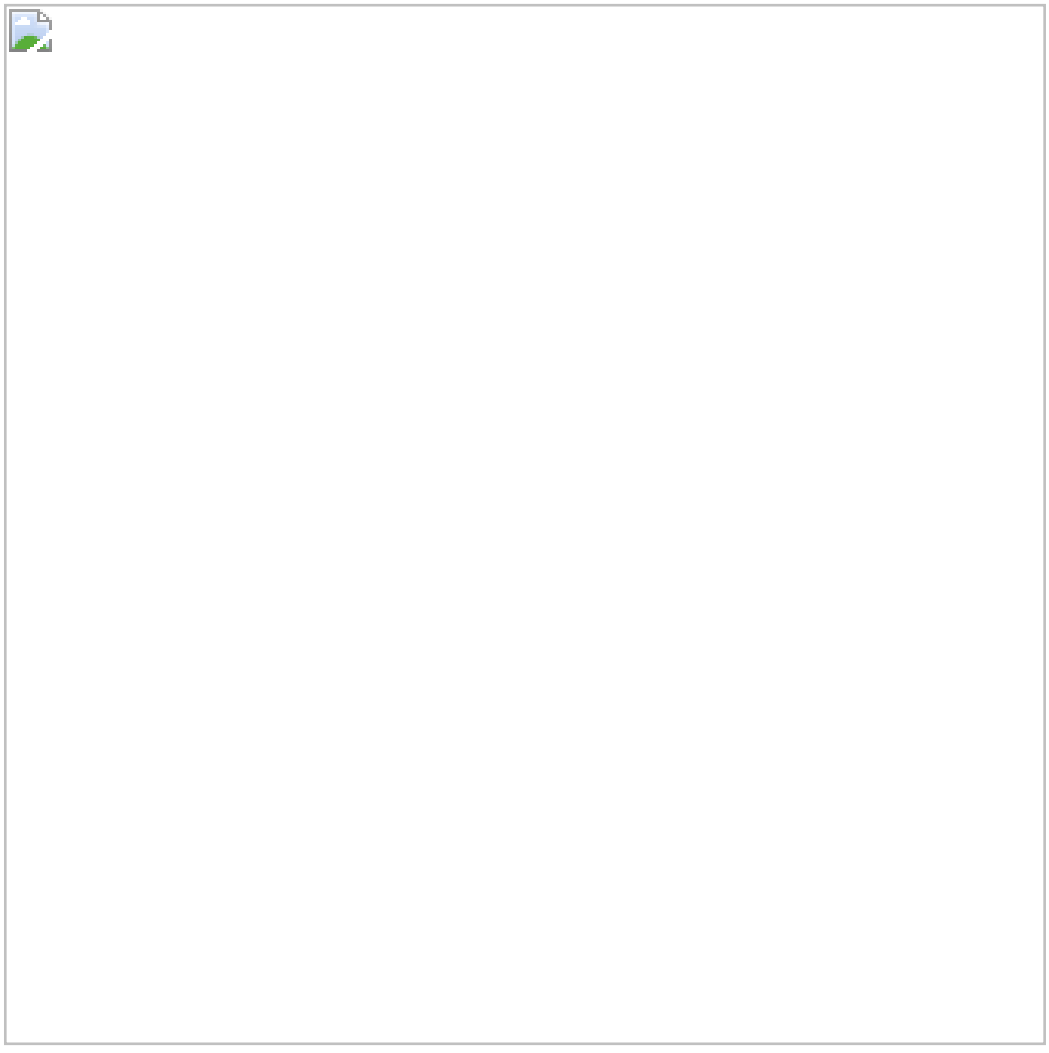

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Sodium-Phosphate Symport by *Aplysia Californica* Gut

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ABSTRACT—Phosphate transport across plasma membranes has been described in a wide variety of organisms and cell types including gastrointestinal epithelia. Phosphate transport across apical membranes of vertebrate gastrointestinal epithelia requires sodium; whereas, its transport across the basolateral membrane requires antiport processes involving primarily chloride or bicarbonate. To decipher the phosphate transport mechanism in the foregut apical membrane of the mollusc, *Aplysia californica*, *in vitro* short-circuited *Aplysia californica* gut was used. Bidirectional transepithelial fluxes of both sodium and phosphate 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 phosphate and it was abolished by the presence of serosal ouabain. Similarly, the net mucosal-to-serosal flux of phosphate was dependent upon the presence of Na^+ and was abolished by the presence of serosal ouabain. Theophylline, DIDS and bumetande, added to either side, had no effect on transepithelial difference or short-circuit current in the *Aplysia* gut bathed in a Na_2HPO_4 seawater medium. However, mucosal arsenate inhibited the net mucosal-to-serosal fluxes of both phosphate and Na^+ and the arsenate-sensitive Na^+ flux to that of phosphate was 2:1. These results suggest the presence of a Na-PO_4 symporter in the mucosal membrane of the *Aplysia californica* foregut absorptive cell.

Key words: phosphate absorption, sodium phosphate symport, active transport

INTRODUCTION

Gastrointestinal and renal transport of the anion phosphate across epithelial apical membranes has been investigated in various vertebrate groups including: mammals such as rabbit (Murer *et al*., 1983) and rat (Berner *et al*., 1976); avian such as chicken (Matsumoto *et al*., 1980), and other lower vertebrates (Danisi and Murer, 1991). Studies with intact vertebrate tissue preparations have documented that transepithelial inorganic phosphate (P_i) transport against an electrochemical potential difference in the small intestine is dependent on the presence of sodium (Na^+) (Fuchs and Peterlik, 1980). In vertebrates, this process can contribute to the transepithelial regulation of P_i levels, and may affect acid-base balance and plasma osmolarity.

However, there is a dearth of studies regarding P_i transport across epithelia of invertebrates. In view of this vacuum of P_i transport information in invertebrates, the present study was undertaken to determine the nature of the P_i transporter in the mucosa of *Aplysia* gut. The present sudy uses isolated foregut from *Aplysia californica* to chracterize a Na^+/P_i symporter that is located in the mucosal membrane of the gut cells and is inhibited by arsenate and ouabain. This transport mechanism may contribute, in part, to the maintenance of P_i 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 Gluconate, 400 mM; Na_2, 30 mM; $MgSO_4 \cdot 7H_20$, 12.3 mM; K Gluconate, 12.1 mM; $NaHCO_3$, 2.4 mM; $Ca(Gluconate)_2$, 11.4 mM; mannitol, 40 mM. A Na^+-free medium was prepared by totally replacing Na^+ with trishydroxyaminomethane$^+$ using gluconate, phosphate and bicarbonate salts. A phosphate-free medium was prepared by totally replacing phosphate 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). Both the mucosal and serosal media were gassed with 100% O_2, and both aspects of the gut were independently and continuously 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 $^{32}PO_4$ (New England Nuclear), unidirectional mucosal-to-serosal (J_{MS}) and serosal-to-mucosal fluxes (J_{SM}) of Na^+ or P_i were determined on paired pieces of tissue from the same animal when their respective SCC's were comparable in magnitude (i.e. within 5% of each other). In these radioisotopic experiments the tissue was allowed to equilibrate for 30–90 min in nonradioactive seawater solution. At this electrical steady-state time (SCC changed no more than 5% of total value per hr), 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 hour 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 $^{32}PO_4$ 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 and utilizing $P > 0.05$ as the significant difference probability criterion.

RESULTS

The first group of experiments was designed to examine whether phosphate and/or ouabain had any effect on Na^+ fluxes. As can be seen in Table 1, the mean net J_{MS} of Na^+ (J_{MS}^{NET})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 both P_i and gluconate, there is a significant increase ($P<0.05$) in the J_{MS}^{NET} of Na^+. This change in Na^+ absorption is due to an increase in the unidirectional J_{MS} of Na^+. The unidirectional J_{SM} of Na^+ did not significantly change in the phosphate-based medium. Also, the mean J_{MS}^{NET} of Na^+, in the presence of phosphate, is significantly greater ($P<0.05$) than the corresponding average SCC. Serosal ouabain (10^{-4}M) abolished the phosphate-dependent J_{MS}^{NET} 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/orouabain had any effect on P_i fluxes. As can be seen in Table 2, the average net J_{MS}^{NET} of P_i is almost absent when the gut was bathed in Na^+-free bathing medium. The

Table 1. Na^+ fluxes in various seawater media

Seawater Media	J_{MS}	J_{SM}	J_{MS}^{NET}	SCC
Na Gluconate	148.2±12.1 (9)	119.9±12.8 (9)	28.3±10.6 (9)	35.6±8.3 (9)
NaGluconate+Na_2HPO_4	185.1±10.3 (6) P<0.05	112.0±15.8 (6) ns	73.1±17.6 (6) P<0.05	46.3±12.8 (6) ns
NaGluconate+Na_2HPO_4+Ouabain	106.1±9.3 (6) P<0.05	101.2±18.3 (6) ns	4.9±4.1 (6) P<0.05	1.7±6.8 (6) P<0.05

Values are expressed in nanoequivalents per square centimeter per minute (mean±SEM). Numbers in parentheses show the number of experiments; J_{ms}, mucosal-to-serosal flux; J_{sm}, serosal-to-mucosal flux; SCC, short-circuit current; ns, not significant.

Table 2. Phosphate fluxes in various seawater media.

Seawater Media	J_{MS}	J_{SM}	J_{MS}^{NET}	SCC
Tris Gluconate +$Tris_2HPO_4$	30.1±6.8 (9)	28.9±7.3 (9)	1.2±6.3 (9)	1.1±4.6 (9)
NaGluconate+Na_2HPO_4	44.6±2.1 (4) P<0.05	24.0±3.2 (4) ns	20.6±3.5 (4) P<0.05	48.1±9.3 (4) P<0.05
NaGluconate +Na_2HPO_4+Ouabain	31.7±8.6 (5) ns	27.1±1.6 (5) ns	4.6±2.0 (5) ns	3.0±2.1 (5) ns

Values are expressed in nanoequivalents per square centimeter per minute (mean±SEM). Numbers in parentheses show the number of experiments; J_{ms}, mucosal-to-serosal flux; J_{sm}, serosal-to-mucosal flux; SCC, short-circuit current, ns, not significant

Table 3. Effect of arsenate on Na^+ and Pi fluxes.

	Phosphate Fluxes			
Seawater Media	J_{MS}	J_{SM}	J_{MS}^{NET}	SCC
NaGluconate+ Na_2HPO_4	41.2±7.3 (4)	22.6±5.9 (4)	18.6±4.3 (4)	37.1±2.8 (4)
NaGluconate+ Na_2HPO_4+Arsenate	23.1±6.1 (4)	25.2±3.6 (4)	–2.2±4.1 (4)	29.3±5.1 (4)
Significance	P<0.05	ns	P<0.05	ns
	Sodium Fluxes			
Seawater Media	J_{MS}	J_{SM}	J_{MS}^{NET}	SCC
NaGluconate+ Na_2HPO_4	216.1±15.0 (5)	114.6±10.1 (5)	101.5±14.7(5)	40.6±7.8 (5)
NaGluconate+ Na_2HPO_4+Arsenate	170.1± 9.3 (5)	110.1±12.6 (5)	60.0±10.9(5)	32.6±5.8 (5)
Significance	P<0.05	ns	P<0.05	ns

Values are expressed in nanoequivalents per square centimeter per minute (mean±SEM). Numbers in parentheses show the number of experiments; J_{ms}, musosal-to-serosal flux; J_{sm}, serosal-to-mucsal flux; SCC, short-circuit current, ns, not significant

corresponding average SCC is also close to zero. However, when the Na^+-free P_i bathing medium was replaced with a Na^+-containing P_i medium, the average J_{MS}^{NET} of P_i increased significantly (P<0.05) over control. This increase in the J_{MS}^{NET} of P_i was entirely attributable to an increase in the unidirectional J_{MS} of P_i because there was no significant change in the undirectional J_{SM} of P_i 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_{MS}^{NET} of P_i. Serosal ouabain (10^{-4}M) inhibited both the J_{MS}^{NET} of P_i and the SCC. The unidirectional J_{MS} of P_i was the only flux of P_i that was affected by serosal ouabain.

The next series of experiments were designed to examine the effects of arsenate on Na^+ and P_i fluxes in *Aplysia* gut. The addition of arsenate (10^{-2}M) to the mucosal compartment of a Na Gluconate + Na_2HPO_4 bathing medium inhibited the unidirectional J_{MS} of P_i, but not the J_{SM} of P_i, resulting in the complete depression of J_{MS}^{NET} of P_i (Table 3). In contrast, the serosal addition of 10^{-2}M arsenate to the serosal bathing solution had no effect on either the unidirectional J_{MS} or J_{SM} of P_i [data not shown (n=3)]. The addition of 10^{-2}M arsenate 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 arsenate -sensitive Na^+ and P_i fluxes was 2:1 in both J_{MS} and J_{MS}^{NET}. On the other hand, arsenate 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} of Na^+ or P_i or SCC in the *Aplysia* gut preparation. Each of these chemical agents were used in three experiments described above.

DISCUSSION

In the current investigation we presented suggestive evidence for the existence of a carrier-mediated Na^+- P_i symport located in the apical membrane of *Aplysia californica* foregut epithelium. Phosphate carriers have been described in the apical membranes of several vertebrate epithelial tissues (Danisi and Murer, 1991). Na^+ - P_i cotransport, P_i -anion exchange mechanisms and proton-dependent P_i transport have been demonstrated in mammalian ileal brush border and basolateral membranes (Murer *et al*., 1983; Sactor and Cheng, 1981). In avian renal membranes multiple pathways were shown to transport P_i; Na^+ - P_i cotransport and P_i -HCO_3^- exchange (Matsumoto *et al*., 1980; Gmaj and Murer, 1986). However, there is a paucity of studies on lower vertebrates and invertebrates relative to P_i transport. Therefore, one of the reasons for studying the *Aplysia* gut was to provide evidence for the existence of a P_i -transporter and, also to identify the nature of this transporter.

When the *Aplysia* foregut was bathed in a P_i -free (Table 1) or Cl^--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 P_i partially 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 P_i stimulated the absorptive flux of Na^+. However, the J_{MS}^{NET} of Na^+ is significantly greater than the corresponding SCC (Table 1). This disparity in J_{MS}^{NET} of Na^+ and SCC could be accounted for by a net active absorptive flux of an anion such as P_i. Serosally-applied ouabain inhibited both J_{MS}^{NET} of Na^+ and the SCC, accompanied by an inhibition of the unidirectional J_{MS} of Na^+ (Table 1). These observations suggest that Na^+ transport and SCC are dependent 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 P_i 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 P_i under short-circuited conditions. These observations suggest that active P_i 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 P_i 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 P_i is shown with the inhibition of both the unidirectional J_{MS} of P_i 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 P_i absorption implies a degree of coupling between the two undirectional fluxes (J_{MS}'s) of both Na^+ and P_i.

Arsenate is a known inhibitor of P_i transport (Murer and Hildmann, 1981). In the present study, mucosally-applied arsenate inhibited the J_{MS} of P_i such that the active component of P_i absorption was abolished (Table 3). In addition mucosally-applied arsenate also inhibited the unidirectional J_{MS} of Na^+ (Table 3). Together, these results strongly suggest a coupling between Na^+ and P_i transport, in their comovement from mucosa to sersosa. The result that serosally-applied arsenate had no effect on either Na^+ or P_i 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 arsenate significantly inhibited both unidirectional J_{MS}'s of Na^+ and P_i, but did not significantly inhibit the corresponding SCC (Table 3), the decrease in coupled Na^+- P_i flux, from mucosa-to-serosa, must be electrically neutral. In addition, as seen in Table 1, phosphate 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^+/ P_i cotransport, from mucosa-to-serosa was electrically neutral at a pH=7.8. Since Na^+ is a univalent cation and phosphate is a divalent anion at a pH=7.8, the stoichiometry of coupled Na^+/ P_i transport in the *Aplysia* gut could be two Na^+ per one P_i per cycle of transport, or some mathematical equivalent of 2 Na^+ per 1 P_i in order for electroneutrality to be maintained. In fact, the ratio of the arsenate-sensitive Na^+ to P_i fluxes was 2:1.

In summary, we have presented suggestive evidence for the existence of a Na^+/ P_i symporter located in the apical membrane of the *Aplysia californica* foregut absorptive cell that could be responsible for the net absorption of P_i by this animal. This event could be beneficial for the viability of cellular metabolic reactions such as phosphorylation. P_i homeostasis in the *Aplysia* is, at least, partly maintained by this luminal Na/ P_i symport transport mechanism.

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