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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<sup>+</sup> 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<sup>+</sup> and was abolished by the presence of serosal ouabain. Theophylline, DIDS and bumetanide, added to either side, had no effect on transepithelial difference or short-circuit current in the *Aplysia* gut bathed in a Na<sub>2</sub>HPO<sub>4</sub> seawater medium. However, mucosal arsenate inhibited the net mucosal-to-serosal fluxes of both phosphate and Na<sup>+</sup> and the arsenate-sensitive Na<sup>+</sup> flux to that of phosphate was 2:1. These results suggest the presence of a Na-PO<sub>4</sub> 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<sub>i</sub>) transport against an electrochemical potential difference in the small intestine is dependent on the presence of sodium (Na<sup>+</sup>) (Fuchs and Peterlik, 1980). In vertebrates, this process can contribute to the transepithelial regulation of P<sub>i</sub> levels, and may affect acid-base balance and plasma osmolarity.

However, there is a dearth of studies regarding P<sub>i</sub> transport across epithelia of invertebrates. In view of this vacuum of P<sub>i</sub> transport information in invertebrates, the present study was undertaken to determine the nature of the P<sub>i</sub> transporter in the mucosa of *Aplysia* gut. The present study uses isolated foregut from *Aplysia californica* to characterize a Na<sup>+</sup>/P<sub>i</sub>

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<sub>i</sub> homeostasis by *Aplysia*.

## 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<sub>2</sub>, 30 mM; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 12.3 mM; K Gluconate, 12.1 mM; NaHCO<sub>3</sub>, 2.4 mM; Ca(Gluconate)<sub>2</sub>, 11.4 mM; mannitol, 40 mM. A Na<sup>+</sup>-free medium was prepared by totally replacing Na<sup>+</sup> with trishydroxymethane<sup>+</sup> 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.

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### 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 ( $\Psi_{MS}$ ) 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 ( $25 \pm 1^\circ C$ ).

The methods used to measure  $\Psi_{MS}$  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  $\pm$  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/or ouabain 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 $\pm$ 12.1 (9)	119.9 $\pm$ 12.8 (9)	28.3 $\pm$ 10.6 (9)	35.6 $\pm$ 8.3 (9)
NaGluconate+ $Na_2HPO_4$	185.1 $\pm$ 10.3 (6) $P < 0.05$	112.0 $\pm$ 15.8 (6) ns	73.1 $\pm$ 17.6 (6) $P < 0.05$	46.3 $\pm$ 12.8 (6) ns
NaGluconate+ $Na_2HPO_4$ +Ouabain	106.1 $\pm$ 9.3 (6) $P < 0.05$	101.2 $\pm$ 18.3 (6) ns	4.9 $\pm$ 4.1 (6) $P < 0.05$	1.7 $\pm$ 6.8 (6) $P < 0.05$

Values are expressed in nanoequivalents per square centimeter per minute (mean  $\pm$  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 $\pm$ 6.8 (9)	28.9 $\pm$ 7.3 (9)	1.2 $\pm$ 6.3 (9)	1.1 $\pm$ 4.6 (9)
NaGluconate+ $Na_2HPO_4$	44.6 $\pm$ 2.1 (4) $P < 0.05$	24.0 $\pm$ 3.2 (4) ns	20.6 $\pm$ 3.5 (4) $P < 0.05$	48.1 $\pm$ 9.3 (4) $P < 0.05$
NaGluconate + $Na_2HPO_4$ +Ouabain	31.7 $\pm$ 8.6 (5) ns	27.1 $\pm$ 1.6 (5) ns	4.6 $\pm$ 2.0 (5) ns	3.0 $\pm$ 2.1 (5) ns

Values are expressed in nanoequivalents per square centimeter per minute (mean  $\pm$  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<sup>+</sup> and Pi fluxes.

Phosphate Fluxes				
Seawater Media	J <sub>MS</sub>	J <sub>SM</sub>	J <sub>MS</sub> <sup>NET</sup>	SCC
NaGluconate+ Na <sub>2</sub> HPO <sub>4</sub>	41.2±7.3 (4)	22.6±5.9 (4)	18.6±4.3 (4)	37.1±2.8 (4)
NaGluconate+ Na <sub>2</sub> HPO <sub>4</sub> +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 <sub>MS</sub>	J <sub>SM</sub>	J <sub>MS</sub> <sup>NET</sup>	SCC
NaGluconate+ Na <sub>2</sub> HPO <sub>4</sub>	216.1±15.0 (5)	114.6±10.1 (5)	101.5±14.7(5)	40.6±7.8 (5)
NaGluconate+ Na <sub>2</sub> HPO <sub>4</sub> +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<sub>ms</sub>, mucosal-to-serosal flux; J<sub>sm</sub>, serosal-to-mucosal flux; SCC, short-circuit current, ns, not significant

corresponding average SCC is also close to zero. However, when the Na<sup>+</sup>-free P<sub>i</sub> bathing medium was replaced with a Na<sup>+</sup>-containing P<sub>i</sub> medium, the average J<sub>MS</sub><sup>NET</sup> of P<sub>i</sub> increased significantly (P<0.05) over control. This increase in the J<sub>MS</sub><sup>NET</sup> of P<sub>i</sub> was entirely attributable to an increase in the unidirectional J<sub>MS</sub> of P<sub>i</sub> because there was no significant change in the unidirectional J<sub>SM</sub> of P<sub>i</sub> in the presence of Na<sup>+</sup>. The average SCC, in the presence of Na<sup>+</sup>, was significantly greater than zero (P<0.05) and it was also greater than the J<sub>MS</sub><sup>NET</sup> of P<sub>i</sub>. Serosal ouabain (10<sup>-4</sup>M) inhibited both the J<sub>MS</sub><sup>NET</sup> of P<sub>i</sub> and the SCC. The unidirectional J<sub>MS</sub> of P<sub>i</sub> was the only flux of P<sub>i</sub> that was affected by serosal ouabain.

The next series of experiments were designed to examine the effects of arsenate on Na<sup>+</sup> and P<sub>i</sub> fluxes in *Aplysia* gut. The addition of arsenate (10<sup>-2</sup>M) to the mucosal compartment of a Na Gluconate + Na<sub>2</sub>HPO<sub>4</sub> bathing medium inhibited the unidirectional J<sub>MS</sub> of P<sub>i</sub>, but not the J<sub>SM</sub> of P<sub>i</sub>, resulting in the complete depression of J<sub>MS</sub><sup>NET</sup> of P<sub>i</sub> (Table 3). In contrast, the serosal addition of 10<sup>-2</sup>M arsenate to the serosal bathing solution had no effect on either the unidirectional J<sub>MS</sub> or J<sub>SM</sub> of P<sub>i</sub> [data not shown (n=3)]. The addition of 10<sup>-2</sup>M arsenate to the mucosal bathing solution also inhibited the unidirectional J<sub>MS</sub> of Na<sup>+</sup> without affecting the unidirectional J<sub>SM</sub> of Na<sup>+</sup>. The ratio of the arsenate -sensitive Na<sup>+</sup> and P<sub>i</sub> fluxes was 2:1 in both J<sub>MS</sub> and J<sub>MS</sub><sup>NET</sup>. On the other hand, arsenate had no significant effect on SCC across the *Aplysia* gut.

Theophylline (10<sup>-6</sup>M), bumetanide (10<sup>-5</sup>M) nor 10<sup>-5</sup>M 4,4'-diisothiocyano-2,2'-disulfonic stilbene (DIDS) added to either the mucosal or serosal bathing medium had no effect on J<sub>MS</sub> of Na<sup>+</sup> or P<sub>i</sub> 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<sup>+</sup>- P<sub>i</sub> 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<sup>+</sup>- P<sub>i</sub> cotransport, P<sub>i</sub>-anion exchange mechanisms and proton-dependent P<sub>i</sub> 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<sub>i</sub>; Na<sup>+</sup>- P<sub>i</sub> cotransport and P<sub>i</sub>-HCO<sub>3</sub><sup>-</sup> exchange (Matsumoto *et al.*, 1980; Gmaj and Murer, 1986). However, there is a paucity of studies on lower vertebrates and invertebrates relative to P<sub>i</sub> transport. Therefore, one of the reasons for studying the *Aplysia* gut was to provide evidence for the existence of a P<sub>i</sub>-transporter and, also to identify the nature of this transporter.

When the *Aplysia* foregut was bathed in a P<sub>i</sub>-free (Table 1) or Cl<sup>-</sup>-free (Gerencser, 1981; Gerencser, 1985) Na<sup>+</sup>-containing seawater media, the net active absorptive flux of Na<sup>+</sup> was equivalent to the SCC. This observation is interpreted as Na<sup>+</sup> being the only ion actively translocated, in a net sense, across the gut tissue. However, when P<sub>i</sub> partially replaced gluconate [a non-transportable anion (Cattley *et al.*, 1992)] in the bathing media, the net active absorptive flux of Na<sup>+</sup> increased solely due to the increase in the unidirectional J<sub>MS</sub> of Na<sup>+</sup>. This suggests that P<sub>i</sub> stimulated the absorptive flux of Na<sup>+</sup>. However, the J<sub>MS</sub><sup>NET</sup> of Na<sup>+</sup> is significantly greater than the corresponding SCC (Table 1). This disparity in J<sub>MS</sub><sup>NET</sup> of Na<sup>+</sup> and SCC could be accounted for by a net active absorptive flux of an anion such as P<sub>i</sub>. Serosally-applied ouabain inhibited both J<sub>MS</sub><sup>NET</sup> of Na<sup>+</sup> and the SCC, accompanied by an inhibition of the unidirectional J<sub>MS</sub> of Na<sup>+</sup> (Table 1). These observations suggest that Na<sup>+</sup> transport and SCC are dependent on the activity of the Na<sup>+</sup>/K<sup>+</sup>-ATPase (Gerencser and Lee, 1985; Skou, 1965).

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

Arsenate is a known inhibitor of  $\text{P}_i$  transport (Murer and Hildmann, 1981). In the present study, mucosally-applied arsenate inhibited the  $J_{\text{MS}}$  of  $\text{P}_i$  such that the active component of  $\text{P}_i$  absorption was abolished (Table 3). In addition mucosally-applied arsenate also inhibited the unidirectional  $J_{\text{MS}}$  of  $\text{Na}^+$  (Table 3). Together, these results strongly suggest a coupling between  $\text{Na}^+$  and  $\text{P}_i$  transport, in their co-movement from mucosa to serosa. The result that serosally-applied arsenate had no effect on either  $\text{Na}^+$  or  $\text{P}_i$  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  $J_{\text{MS}}\text{'s}$  of  $\text{Na}^+$  and  $\text{P}_i$ , but did not significantly inhibit the corresponding SCC (Table 3), the decrease in coupled  $\text{Na}^+$ -  $\text{P}_i$  flux, from mucosa-to-serosa, must be electrically neutral. In addition, as seen in Table 1, phosphate stimulated the  $J_{\text{MS}}$  of  $\text{Na}^+$  without an increase in SCC. The SCC's under these different experimental conditions did not change. This suggested that the coupled  $\text{Na}^+$ /  $\text{P}_i$  cotransport, from mucosa-to-serosa was electrically neutral at a pH=7.8. Since  $\text{Na}^+$  is a univalent cation and phosphate is a divalent anion at a pH=7.8, the stoichiometry of coupled  $\text{Na}^+$ /  $\text{P}_i$  transport in the *Aplysia* gut could be two  $\text{Na}^+$  per one  $\text{P}_i$  per cycle of transport, or some mathematical equivalent of 2  $\text{Na}^+$  per 1  $\text{P}_i$  in order for electroneutrality to be maintained. In fact, the ratio of the arsenate-sensitive  $\text{Na}^+$  to  $\text{P}_i$  fluxes was 2:1.

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

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