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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 bumetanide, added to either side, had no effect on transepithelial difference or short-circuit current in the *Aplysia* gut bathed in a Na₂HPO₄ 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-PO₄ 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 (Pᵢ) 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 Pᵢ levels, and may affect acid-base balance and plasma osmolarity.

However, there is a dearth of studies regarding Pᵢ transport across epithelia of invertebrates. In view of this vacuum of Pᵢ transport information in invertebrates, the present study was undertaken to determine the nature of the Pᵢ transporter in the mucosa of *Aplysia* gut. The present study uses isolated foregut from *Aplysia californica* to characterize a Na⁺/Pᵢ 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 Pᵢ 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; Na₂, 30 mM; MgSO₄·7H₂O, 12.3 mM; K Gluconate, 12.1 mM; NaHCO₃, 2.4 mM; Ca(Gluconate)₂, 11.4 mM; mannitol, 40 mM. A Na⁺-free medium was prepared by totally replacing Na⁺ with tris(hydroxymethyl)aminomethane⁺ 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 (ΨMS) and short-circuit current (SCC) across the gut have been described previously (Gerencser, 1978). Both the mucosal and serosal media were gassed with 100% O2, 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 ΨMS 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 offset between the potential-sensing electrodes, the ends of these bridges were compensated automatically by the voltage-clamp device as described by Rothe et al. (1969).

By use of 22Na and 32P04 (New England Nuclear), unidirectional mucosal-to-serosal (JMS) and serosal-to-mucosal fluxes (JSM) of Na+ or P 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 unlabelled 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 JMS and JSM of 22Na and 32P04 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 JMS of Na+ (JNET) 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 and gluconate, there is a significant increase (P<0.05) in the JNET of Na+. This change in Na+ absorption is due to an increase in the unidirectional JMS of Na+. The unidirectional JSM of Na+ did not significantly change in the phosphate-based medium. Also, the mean JNET of Na+, in the presence of phosphate, is significantly greater (P<0.05) than the corresponding average SCC. Serosal ouabain (10−4M) abolished the phosphate-dependent JNET of Na+ by inhibiting solely the unidirectional JMS 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 fluxes. As can be seen in Table 2, the average net JNET of P is almost absent when the gut was bathed in Na+-free bathing medium. The

| Table 1. Na+ fluxes in various seawater media |
| Seawater Media | JMS | JSM | JNET | SCC |
| Na Gluconate | 148.2±12.1 (9) | 119.9±12.8 (9) | 28.3±10.6 (9) | 35.6±8.3 (9) |
| NaGluconate+N2HPO4 | 185.1±10.3 (6) | 112.0±15.8 (6) | 73.1±17.6 (6) | 46.3±12.8 (6) |
| NaGluconate+N2HPO4+Ouabain | 106.1±9.3 (6) | 101.2±18.3 (6) | 4.9±4.1 (6) | 1.7±1.6 (6) |

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

| Table 2. Phosphate fluxes in various seawater media |
| Seawater Media | JMS | JSM | JNET | SCC |
| Tris Gluconate +Tris2HPO4 | 30.1±6.8 (9) | 28.9±7.3 (9) | 1.2±6.3 (9) | 1.1±4.6 (9) |
| NaGluconate+N2HPO4 | 44.6±2.1 (4) | 24.0±3.2 (4) | 20.6±3.5 (4) | 48.1±9.3 (4) |
| NaGluconate +Na2HPO4+Ouabain | 31.7±8.6 (5) | 27.1±1.6 (5) | 4.6±2.0 (5) | 3.0±2.1 (5) |

Values are expressed in nanoequivalents per square centimeter per minute (mean±SEM). Numbers in parentheses show the number of experiments; Jms, mucosal-to-serosal flux; Jsm, serosal-to-mucosal flux; SCC, short-circuit current, ns, not significant.
corresponding average SCC is also close to zero. However, when the Na+-free Pi bathing medium was replaced with a Na+-containing Pi medium, the average JMS of Pi increased significantly (P<0.05) over control. This increase in the JMS of Pi was entirely attributable to an increase in the unidirectional JMS of Pi because there was no significant change in the unidirectional JSM of Pi 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 JMS of Pi. Serosal ouabain (10–4M) inhibited both the JMS of Pi and the SCC. The unidirectional JMS of Pi was the only flux of Pi that was affected by serosal ouabain.

The next series of experiments were designed to examine the effects of arsenate on Na+ and Pi fluxes in Aplysia gut. The addition of arsenate (10–5M) to the mucosal compartment of a Na Gluconate + Na2HPO4 bathing medium inhibited the unidirectional JMS of Pi, but not the JSM of Pi, resulting in the complete depression of JNET of Pi (Table 3). In contrast, the serosal addition of 10–2M arsenate to the serosal bathing solution had no effect on either the unidirectional JMS or JSM of Pi (data not shown (n=3)). The addition of 10–2M arsenate to the mucosal bathing solution also inhibited the unidirectional JMS of Na+ without affecting the unidirectional JSM of Na+. The ratio of the arsenate-sensitive Na+ and Pi fluxes was 2:1 in both JMS and JNET. On the other hand, arsenate had no significant effect on SCC across the Aplysia gut.

Theophylline (10–6M), bumetanide (10–5M) nor 10–5M 4,4'-diisothiocyano-2,2'-disulfonic stilbene (DIDS) added to either the mucosal or serosal bathing medium had no effect on JMS of Na+ or Pi 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+-Pi 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+-Pi cotransport, Pi-anion exchange mechanisms and proton-dependent Pi 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 Pi; Na+ - Pi cotransport and Pi–HCO3– exchange (Matsumoto et al., 1980; Gmaj and Murer, 1986). However, there is a paucity of studies on lower vertebrates and invertebrates relative to Pi transport. Therefore, one of the reasons for studying the Aplysia gut was to provide evidence for the existence of a Pi-transporter and, also to identify the nature of this transporter.

When the Aplysia foregut was bathed in a Pi-, 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 Pi, 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 JMS of Na+. This suggests that Pi, stimulated the absorptive flux of Na+. However, the JNET of Na+ is significantly greater than the corresponding SCC (Table 1). This disparity in JNET of Na+ and SCC could be accounted for by a net active absorptive flux of an anion such as Pi. Serosally-applied ouabain inhibited both JNET of Na+ and the SCC, accompanied by an inhibition of the unidirectional JMS 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 Pi, 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 JNET of Pi, under short-circuited conditions. These observations suggest that active Pi 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

| Table 3. Effect of arsenate on Na+ and Pi fluxes. |
|---------------------------------|----------------|------------------|------------------|-----------|
| **Phosphate Fluxes**            |                |                  |                  |          |
| **Seawater Media**              | JMS            | JSM              | JNET             | SCC       |
| NaGluconate+ Na2HPO4            | 41.2±7.3 (4)   | 22.6±5.9 (4)     | 18.6±4.3 (4)     | 37.1±2.8 (4) |
| NaGluconate+ Na2HPO4+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**              | JMS            | JSM              | JNET             | SCC       |
| NaGluconate+ Na2HPO4            | 216.1±17.0 (5) | 114.6±10.1 (5)   | 101.5±14.7(5)    | 40.6±7.8 (5) |
| NaGluconate+ Na2HPO4+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               | ns               | ns        |

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

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solutions. This is because, in the presence of Na\(^+\), there is a finite SCC, part of which can be accounted for by the J\(_{\text{MS}}\) of Pi, 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 Pi is shown with the inhibition of both the unidirectional J\(_{\text{MS}}\) of Pi 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 Pi absorption implies a degree of coupling between the two unidirectional fluxes (J\(_{\text{MS}}\)'s) of both Na\(^+\) and Pi.

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

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

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