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ELECTROGENIC $2Na^+/H^+$ ANTIPORT IN ECHINODERM GASTROINTESTINAL EPITHELIUM

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Summary

Purified brush-border membrane vesicles (BBMV) of starfish (*Pycnopodia helianthoides*) pyloric caecal epithelium were prepared by a magnesium precipitation technique in order to compare the properties of Na^+/H^+ exchange in this invertebrate tissue with those of an apparently unique recently described crustacean electrogenic antiporter. In starfish BBMV ^{22}Na uptake was markedly enhanced by an outwardly directed pH gradient and membrane potential (inside negative) compared to control short-circuited vesicles. External amiloride abolished the stimulatory capacity of the proton gradient and membrane potential as driving forces for sodium transport. Sodium influx, in the presence of an outwardly directed proton gradient, was a sigmoidal function of $[Na^+]_o$ and yielded a Hill coefficient of 2.6, suggesting that more than one sodium ion was exchanged with each internal proton during the exchange event. Two additional findings were used to establish the number of external Na^+ binding sites and the transport stoichiometry of the starfish antiporter. First, amiloride acted as a competitive inhibitor of Na^+ binding to two external sites with markedly dissimilar apparent amiloride affinities ($K_{i1}=28\,\mu mol\,l^{-1}$; $K_{i2}=1650\,\mu mol\,l^{-1}$). Second, a static head flux ratio analysis resulted in a $2Na^+/H^+$ exchange stoichiometry where a balance of driving forces (e.g. no net Na^+ flux) was attained with a combination of a 10:1 Na^+ gradient and a 100:1 H^+ gradient. Results suggest that the electrogenic $2Na^+/H^+$ exchanger previously characterized for crustacean epithelia also occurs in echinoderm cells and may be a widely distributed invertebrate antiporter.

Introduction

In vertebrate cells Na^+/H^+ exchange occurs *via* an electroneutral plasma membrane carrier protein exhibiting a 1:1 flux stoichiometry (Grinstein, 1988). This mechanism is responsible for the net uptake of extracellular sodium ions and the net extrusion of cytoplasmic protons, employing the transmembrane chemical gradients of the respective cations to determine the direction and magnitude of the exchange event (Aronson, 1985; Aronson and Igarashi, 1986). Because of the

Key words: Na^+/H^+ exchange, antiport, electrogenic, epithelium, brush-border membrane vesicles, stoichiometry, pyloric caeca, echinoderm, starfish, *Pycnopodia helianthoides*.

virtually universal occurrence of the electroneutral Na^+/H^+ antiporter among vertebrate cells where it has been studied, this mechanism has become a paradigm for cellular sodium and hydrogen regulation for all animals.

Recently, radioisotopic and fluorometric methods used in two independent laboratories with three different crustacean epithelia disclosed that the cellular Na^+/H^+ antiport processes of these animals were electrogenic and exhibited a transport stoichiometry of $2Na^+/H^+$ (Ahearn and Clay, 1989; Ahearn and Franco, 1990; Ahearn *et al.* 1990; Shetlar and Towle, 1989). In these arthropod cells the occurrence of two external cation binding sites with dissimilar binding properties was confirmed kinetically with external proton and amiloride inhibition of Na^+ transfer, while electrogenicity was demonstrated by the use of an imposed transmembrane electrical potential difference as the only driving force for cation exchange (Ahearn and Clay, 1989; Ahearn and Franco, 1990; Ahearn *et al.* 1990). Although the results of these studies provided strong support for the occurrence of a plasma membrane Na^+/H^+ antiport mechanism in crustacean gut, renal and gill epithelial cells, differing markedly in physiological properties from the paradigm established for all vertebrate cells, there is no evidence to suggest that this unusual transport protein occurs in animals other than arthropods.

The present investigation is a characterization of Na^+/H^+ exchange in pyloric caecal epithelial cells of the starfish gastrointestinal tract using the same methods previously applied to the analysis of this transporter in crustacean cells. Results indicate a remarkable similarity between the antiporters of both invertebrate phyla, suggesting a potentially broad distribution of this electrogenic mechanism among non-vertebrate animals.

Materials and methods

Starfish [*Pycnopodia helianthoides* (Brandt)] were collected between June and August from waters near the Friday Harbor Marine Laboratory of the University of Washington in the San Juan Islands and maintained unfed in flowing sea water until needed. Two to three arms were removed from an individual initially possessing 15–20 arms and the dissected diverticula were pooled for further treatment. The procedure for producing purified brush-border vesicles from this pooled sample was generally the same as the magnesium precipitation technique previously used to make similar membrane preparations from mammalian (Hopfer *et al.* 1973; Kessler *et al.* 1978), fish (Reshkin and Ahearn, 1987) and crustacean (Ahearn *et al.* 1985; Behnke *et al.* 1990) epithelia.

The pooled pyloric caecal sample was homogenized in hypotonic buffer and mixed with $10\,mmol\,l^{-1}$ $MgCl_2$ for selective precipitation of most cellular membranes except the brush border. This was followed with purification by centrifugation at $3000\,g$ and $27\,000\,g$ and homogenization in additional hypotonic buffer using a glass homogenizer. A further sequence of precipitation, purification and homogenization in the proper internal buffer was then carried out, followed by centrifugation at $27\,000\,g$. The resulting purified sample of brush-border mem

brane was resuspended in a small volume of internal medium by passage 10–15 times through a syringe fitted with a 22-gauge needle. This final vesicle suspension exhibited a total protein content of approximately 10 mg ml^{-1} (Bio-Rad protein assay). Using this preparative method, previous studies with gastrointestinal and renal organs from such diverse organisms as crustaceans, fish and mammals produced final purified vesicle suspensions that exhibited significant enrichments of brush-border enzyme markers such as alkaline phosphatase (specific activities: homogenate, 1.28±0.37 μmol mg^{-1} h^{-1}; vesicles, 10.32±2.26 μmol mg^{-1} h^{-1}; purification factor, 15.26±4.65; Ahearn *et al.* 1985), leucine aminopeptidase (specific activities: homogenate, 18.8±1.6 μmol mg^{-1} h^{-1}; vesicles, 168.7± 3.9 μmol mg^{-1} h^{-1}; purification factor, 9.1±0.8; Reshkin and Ahearn, 1987) and sucrase (specific activities: homogenate, 0.08 μmol mg^{-1} h^{-1}; vesicles, 2.63 μmol mg^{-1} min^{-1}; purification factor, 31.1; Hopfer *et al.* 1973), while concurrently displaying reduced occurrence of enzymes associated with other cell membranes such as Na^+/K^+-ATPase (basolateral membranes) and cytochrome *c* oxidase (mitochondrial membranes).

Transport studies using these pyloric caecal brush-border membrane vesicles (BBMV) were conducted at 18°C with freshly prepared membrane preparations using the Millipore filtration technique of Hopfer *et al.* (1973). At the beginning of a transport experiment, a volume (e.g. 20 μl) of membrane vesicles was added to a volume of radiolabelled medium (e.g. 160 μl) containing ^{22}Na (New England Nuclear, Corp.). Following incubation times varying from a few seconds to 180 min (equilibrium of isotope across vesicle membranes), a known volume of this reaction mixture (e.g. 20 μl) was withdrawn and plunged into 2 ml of ice-cold stop solution (composition varying with experiment; see figure legends). The resulting suspensions were rapidly filtered through Millipore filters (0.45 μm pore diameter) to retain the vesicles and washed with another 5 ml of stop solution. Filters were then added to ICN Ecolume scintillation cocktail and counted for radioactivity in a Beckman LS-9000 scintillation counter. Sodium uptake was expressed (using specific activity of ^{22}Na in the medium) as $nmol^{-1}$ mg^{-1} protein $filter^{-1}$. Each experiment was repeated two or three times using membranes prepared from different animals, yielding qualitatively and quantitatively similar results from each experiment. Within a given experiment each point was analyzed using 3–5 replicates and values are presented in the figures as means±S.E.

Results

Effects of a transmembrane H^+ gradient and membrane potential on the time course of ^{22}Na uptake

To establish the presence of a Na^+/H^+ exchanger in BBMV of starfish pyloric caeca, ^{22}Na uptake was examined in vesicles possessing an outwardly directed proton gradient, while the possible electrogenicity of the system was assessed by observing the effect of an imposed transmembrane electrical potential on the exchange process. Vesicles were loaded with 102.5 mmol l^{-1} tetramethylammo-

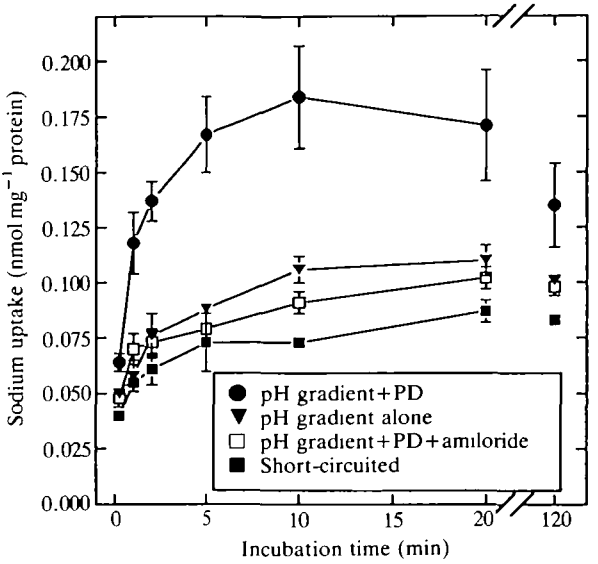

Fig. 1. Effect of transmembrane H^+ gradient and membrane potential on the time course of ^{22}Na uptake by brush-border membrane vesicles (BBMV) of *Pycnopodia helianthoides*. Vesicles were loaded with 102.5 mmol l^{-1} TMA^+ gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at either pH 6.0 or 8.0 and were then incubated in a medium at pH 8.0 containing 2.5 mmol l^{-1} [^{22}Na] gluconate, 100 mmol l^{-1} TMA^+ gluconate and 50 mmol l^{-1} potassium gluconate or in a medium at pH 8.0 containing 2.5 mmol l^{-1} [^{22}Na] gluconate and 150 mmol l^{-1} TMA^+ gluconate. In addition, one of the external media contained 2.0 mmol l^{-1} amiloride, while the other lacked the drug. Stop solution: 102.5 mmol l^{-1} TMA^+ gluconate, 50 mmol l^{-1} potassium gluconate and 25 mmol l^{-1} Hepes–Tris, pH 8.0. PD, potential difference. Values are presented as mean±S.E. (N=3–5) in the figures.

nium (TMA^+) gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin (K^+ ionophore) at either pH 6.0 (25 mmol l^{-1} Mes–Tris) or pH 8 (25 mmol l^{-1} Hepes–Tris). Preloaded vesicles were then incubated for varying times in a medium at pH 8.0 containing 2.5 mmol l^{-1} [^{22}Na] gluconate, 100 mmol l^{-1} TMA^+ gluconate and 50 mmol l^{-1} potassium gluconate, or in a medium at pH 8.0 containing 2.5 mmol l^{-1} [^{22}Na] gluconate and 150 mmol l^{-1} TMA^+ gluconate. One batch of the second incubation medium contained 2.0 mmol l^{-1} amiloride, while another lacked the drug.

Fig. 1 shows the time course of ^{22}Na uptake by starfish pyloric caecal BBMV under four conditions: short-circuited (control), pH gradient alone, pH gradient plus membrane potential difference and pH gradient plus membrane potential difference plus amiloride. A small, but significant (P<0.05), stimulation of sodium uptake occurred in vesicles possessing an outwardly directed proton gradient compared to short-circuited vesicles lacking any driving forces. Addition of a transmembrane electrical potential difference with K^+ and the ionophore valinomycin markedly enhanced the stimulatory capacity of the outwardly directe

proton gradient and led to a transient concentrative ^{22}Na uptake in which internal sodium concentration temporarily exceeded that at equilibrium by a factor of two. By 180 min of incubation vesicles of all experimental treatments did not differ significantly ($P<0.05$) in ^{22}Na content (0.095 nmol mg^{-1} protein; data not shown). External amiloride abolished the sodium uptake overshoot in the presence of a proton gradient and membrane potential. These results suggest that starfish pyloric caeca BBMV possess an amiloride-sensitive Na^+/H^+ exchanger that is responsive to membrane potential.

Kinetics of Na^+/H^+ exchange

Preliminary time course experiments of ^{22}Na uptake with starfish pyloric caeca BBMV indicated that an incubation period of 10 s was short enough to ensure unidirectional influx of isotope and long enough to accumulate sufficient labelled substrate for detection. Based on the results of these preliminary studies, a 10 s incubation of vesicles with [^{22}Na] gluconate was used in all kinetic experiments reported here.

Vesicles were loaded at pH 5.5 (Mes–Tris) with 400 mmol l^{-1} TMA^+ gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin, and were incubated for 10 s in media at pH 8.5 (Hepes–Tris) containing variable [^{22}Na] gluconate and TMA^+ gluconate concentrations. In the presence of a fixed outwardly directed proton gradient, Na^+ influx (J_{Na}) was a sigmoidal function of $[Na^+]_o$ and followed the Hill equation for multisite binding cooperativity (Fig. 2):

$$J_{Na} = \frac{J_{max}[Na^+]_o}{K_{Na}^n + [Na^+]_o}, \quad (1)$$

where J_{max} is maximal Na^+ influx, K_{Na}^n is an apparent affinity constant, modified to accommodate multisite interactions (interaction coefficient), and the Hill coefficient, n, is an estimate of the number of reactive Na^+ binding sites. Quantitative values for these transport constants were estimated using a Marquardt non-linear iterative computer program providing the best-fitting curve through the experimental points in Fig. 2. From these estimates, values of sodium influx kinetic constants were: $K_{Na}^n = 120.2 \pm 23.0$ mmol l^{-1}; $J_{max} = 98.7 \pm 17.4$ nmol mg^{-1} protein s^{-1}; and $n = 2.6 \pm 1.0$. The sigmoidal nature of the influx curve and the occurrence of a Hill coefficient of approximately 2.0, indicates that Na^+/H^+ exchange by these membrane preparations probably occurs by a carrier process with at least two external cation sites and exhibiting binding cooperativity.

Effect of external amiloride concentration on Na^+/H^+ exchange

Fig. 1 indicated that 2.0 mmol l^{-1} external amiloride abolished Na^+/H^+ exchange in starfish BBMV. To characterize the nature of the inhibitory action of this drug on the antiport process, an experiment was conducted in which vesicles were loaded with 100 mmol l^{-1} TMA^+ gluconate, 50 mmol l^{-1} potassium gluconate, 50 μmol l^{-1} valinomycin and 25 mmol l^{-1} Mes–Tris (pH 5.5), and were incubated for 10 s in media containing 5 or 25 mmol l^{-1} [^{22}Na] gluconate,

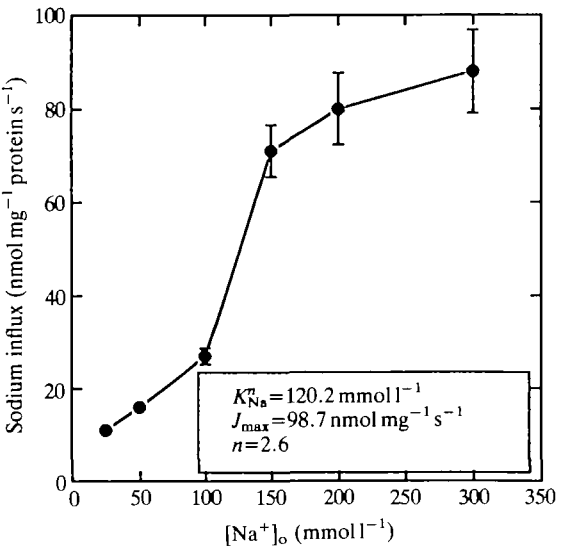

Fig. 2. Effect of external sodium concentration $[Na^+]_o$ on the kinetics of Na^+/H^+ exchange in BBMV of the starfish *Pycnopodia helianthoides*. Vesicles were loaded at pH 5.5 with 400 mmol l^{-1} TMA^+ gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin, and were incubated for 10 s in media at pH 8.5 containing variable [^{22}Na] gluconate and TMA^+ gluconate concentrations. Stop solution: 450 mmol l^{-1} TMA^+ gluconate and 25 mmol l^{-1} Hepes–Tris, pH 8.5.

150 mmol l^{-1} TMA^+ gluconate, 25 mmol l^{-1} Hepes–Tris (pH 8.5) and one of the following concentrations of amiloride (in μmol l^{-1}): 0, 10, 50, 100, 500, 1000 or 10 000.

A control experiment was first conducted using the same internal and external media as described above to establish whether variable concentrations of amiloride had differential effects on ^{22}Na binding to vesicles. In this instance, however, rather than allowing the radiolabelled Na^+ to enter the vesicles over a 10-s period, vesicles and labelled external media were injected directly into ice-cold stop solution simultaneously, followed by filtering and washing. The nonspecific binding at 25 mmol l^{-1} Na^+ was approximately five times that at 5 mmol l^{-1} Na^+, and there was no significant difference ($P>0.05$), at each Na^+ concentration, between the various amiloride concentrations on the respective radioactive binding values (data not shown).

Fig. 3 shows that increasing external amiloride concentration had a marked inhibitory effect on 10-s uptake of ^{22}Na at both 5 and 25 mmol l^{-1}. Significant reductions in Na^+ entry were observed at every amiloride concentration used, with maximal inhibition occurring at 10 000 μmol l^{-1}, where Na^+ uptake was not significantly different ($P<0.05$) from binding values measured using vesicles injected into ice-cold stop solution simultaneously with radiolabelled incubation medium.

Fig. 4 gives Dixon plots of the effects of external amiloride concentration o

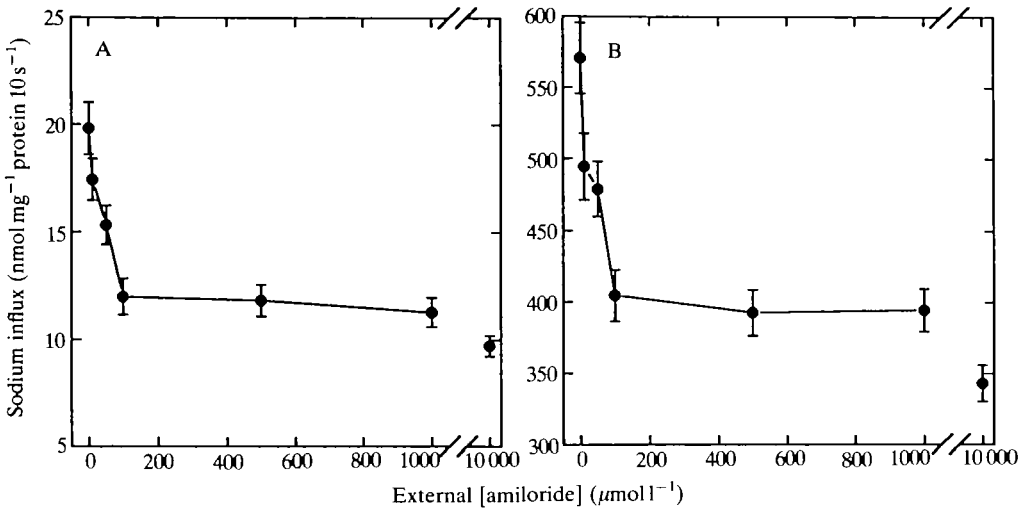

Fig. 3. Effect of external amiloride concentration on Na^+/H^+ exchange in BBMV of the starfish *Pycnopodia helianthoides*. Vesicles were loaded with 100 mmol l^{-1} TMA^+ gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 and were incubated for 10 s in media at pH 8.5 containing either (A) 5 or (B) 25 mmol l^{-1} [^{22}Na] gluconate, 150 mmol l^{-1} TMA^+ gluconate and one of the following concentrations of amiloride: 0, 10, 50, 100, 500, 1000 or 10 000 μmol l^{-1}. Stop solution: 175 mmol l^{-1} TMA^+ gluconate and 25 mmol l^{-1} Hepes–Tris at pH 8.5.

10-s uptake of 5 and 25 mmol l^{-1} Na^+. Data for these analyses were derived from values in Fig. 3 after subtraction of the ^{22}Na binding values obtained at 10 000 μmol l^{-1} amiloride from each of the remaining uptake values. Both Dixon plots exhibited two slopes, suggesting the interaction of the drug at two independent binding sites with markedly different apparent binding affinities. Inhibitor constants (K_i) for amiloride competing with Na^+ at both high- and low-affinity sites were estimated using computer-fitted linear regression analysis of the data in Fig. 4 by extrapolating each curve to the left of the vertical axis. A vertical line, drawn from the respective intersections of each pair of lines to the x-axis, provided values for these constants. Using these methods, the inhibitor constants for amiloride inhibition of Na^+ influx at the high- and low-affinity sites were 28 μmol l^{-1} and 1650 μmol l^{-1}, respectively, suggesting that the two external cation sites exhibited a 58-fold difference in apparent binding affinity for this drug.

Static head demonstration of Na^+/H^+ exchange stoichiometry

Fig. 2 indicated that Na^+ influx as a function of external sodium concentration followed sigmoidal kinetics, resulting in a Hill coefficient of approximately 2.0, suggesting the possible involvement of more than one Na^+ binding site during Na^+/H^+ exchange. Fig. 4 shows that external amiloride acted as a competitive inhibitor of Na^+ binding to the antiport process at two distinct sites with markedly different apparent binding affinities for the drug. Both figures, therefore, suggest

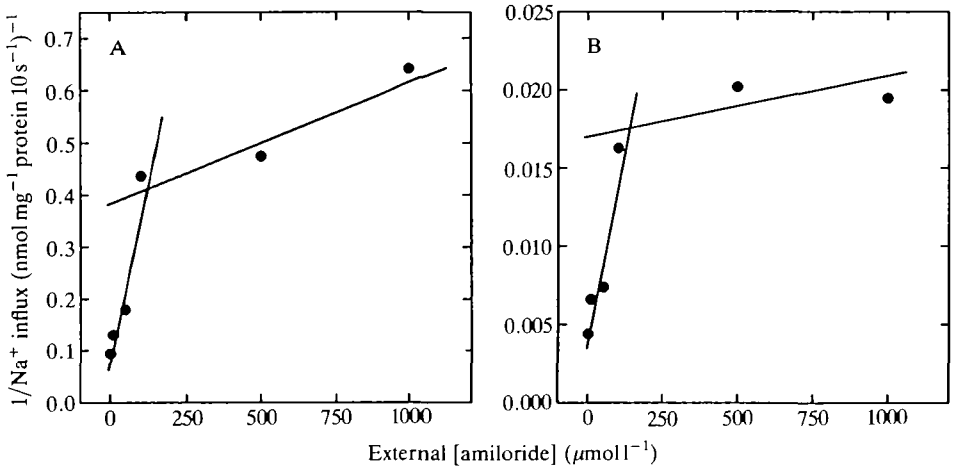

Fig. 4. Dixon plots of the effects of external amiloride concentration on Na^+/H^+ exchange in BBMV of the starfish *Pycnopodia helianthoides*. Data were taken from Fig. 3 after subtraction of ^{22}Na binding values obtained at 10000 μmol l^{-1} amiloride from each of the remaining uptake values. Stop solution: same as that described for Fig. 3.

that the transport stoichiometry of Na^+/H^+ exchange in starfish pyloric caecal BBMV may depart significantly from the established electroneutral Na^+/H^+ antiport system characterized for vertebrate cells (Aronson, 1985).

To clarify the stoichiometric relationship between the simultaneous fluxes of sodium ions and protons in this vesicle preparation, the static head method of transport analysis, proposed by Turner and Moran (1982) for mammalian cells and recently applied to Na^+/H^+ exchange in BBMV of crustacean epithelia (Ahearn *et al.* 1990; Ahearn and Franco, 1990), was used with these echinoderm membranes. In this procedure, a pH gradient was established across the vesicle wall (pHi=5.0; pHo=7.0). This served as a fixed driving force for $^{22}Na/H^+$ exchange, and various Na^+ concentration gradients were tested as opposing driving forces to balance this proton concentration gradient. When the driving forces from proton and Na^+ concentration gradients were balanced, no net flux of ^{22}Na across the membrane in exchange for protons was observed. The thermodynamic equation that describes the condition of no net flux of either H^+ or Na^+ by way of an Na^+/H^+ antiporter when driving forces for both cations are balanced in short-circuited (K^+/valinomycin) BBMV is:

$$\ln([H^+]_i/[H^+]_o) = N\ln([Na^+]_i/[Na^+]_o), \quad (2)$$

where N is the number of sodium ions simultaneously transported for each proton (transport stoichiometry).

Two groups of BBMV were preloaded for 30 min with 100 mmol l^{-1} [^{22}Na] gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.0 (25 mmol l^{-1} Mes–Tris). One group of preloaded vesicles was then incubated fo

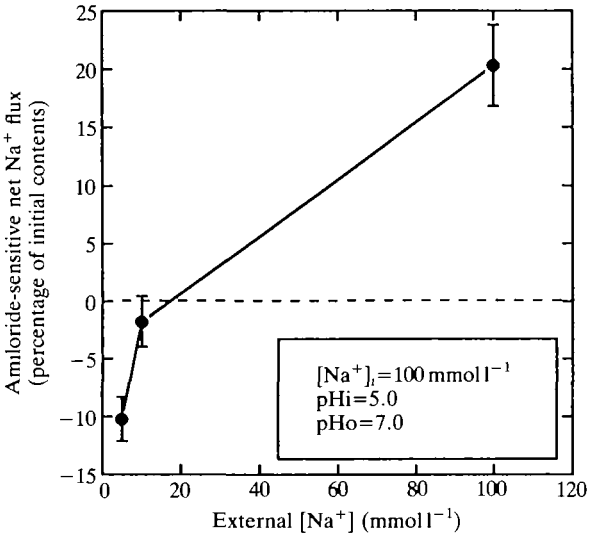

Fig. 5. Static head analysis of Na^+/H^+ flux ratio. Two groups of vesicles were preloaded for 30 min with 100 mmol l^{-1} [^{22}Na] gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.0. One of these groups was then incubated for 5 s in external media at pH 7.0 containing 5, 10 or 100 mmol l^{-1} [^{22}Na] gluconate, 50 mmol l^{-1} potassium gluconate and mannitol for osmotic balance. The second group was exposed to the same external media, but in this case they also contained 2.0 mmol l^{-1} amiloride. Amiloride-sensitive net Na^+ flux for each external medium was the difference between the sodium uptake values of these two vesicle groups. Stop solution: 100 mmol l^{-1} TMA^+ gluconate, 50 mmol l^{-1} potassium gluconate and 25 mmol l^{-1} Hepes–Tris at pH 7.0.

5 s in external media at pH 7.0 (25 mmol l^{-1} Hepes–Tris) containing 5, 10 or 100 mmol l^{-1} [^{22}Na] gluconate (same specific activity as in the internal medium), 50 mmol l^{-1} potassium gluconate and mannitol for osmotic balance. The second group was exposed to identical external media except that 2.0 mmol l^{-1} amiloride was added to each medium. Preloaded vesicle samples were taken and analyzed for ^{22}Na content at the end of the preloading period, for comparison with vesicle isotopic content following exposure to each external medium. Amiloride-sensitive net Na^+ flux for each external medium represents the difference between the 5-s ^{22}Na uptake in the presence and absence of the drug.

Fig. 5 shows the relationship between amiloride-sensitive net Na^+ flux (expressed as a percentage of the preloaded vesicle isotope contents) and external sodium concentration. Static head conditions were attained at 100 mmol l^{-1} internal sodium and 10 mmol l^{-1} external sodium. At either 5 or 100 mmol l^{-1} external sodium, significant net flow of ^{22}Na across the vesicle wall was observed, directionality being determined by the predominant cation driving force. The 10-fold transmembrane gradient of Na^+ balancing a 100-fold transmembrane gradient of protons, producing static head conditions, suggests that the transport stoichiometry of Na^+/H^+ exchange in starfish pyloric caeca BBMV is approximately 2.0.

Discussion

The results of the present investigation suggest that brush-border membranes of starfish pyloric caecal epithelial cells possess an amiloride-sensitive, electrogenic $2Na^+/H^+$ antiporter for the regulation of intracellular sodium and hydrogen concentrations. It has kinetic properties remarkably similar to those recently described for such an exchanger in crustacean hepatopancreatic and renal cells (Ahearn and Clay, 1989; Ahearn *et al.* 1990; Ahearn and Franco, 1990). In epithelial cells of both invertebrate groups the exchange events were electrogenic and responsive to alterations in membrane potential (Fig. 1), suggesting that the combination of transmembrane chemical gradients of the respective cations and the membrane potential itself were capable of acting synergistically as driving forces for the transporter. In mammalian cells, directionality and magnitude of Na^+/H^+ exchange is determined solely by the relative transmembrane chemical concentrations of the two cations as a result of the electroneutral nature of the process (Aronson, 1985; Aronson and Igarashi, 1986; Grinstein and Rothstein, 1986; Sacktor and Kinsella, 1986).

Electrogenicity in the invertebrate systems is a result of the occurrence of two external cation binding sites, both of which interact with the drug amiloride in a competitive fashion (Figs 2–4), and a single internal cation binding site, which exhibits a considerably higher apparent binding affinity for protons than for sodium ions (Ahearn *et al.* 1990). In crustacean cells, there is no indication of an intracellular proton activator site for the regulation of Na^+/H^+ exchange, as has been described for the electroneutral process in mammalian cells (Aronson *et al.* 1982). Because of the differences in number of external cation binding sites between invertebrate and vertebrate Na^+/H^+ exchangers, Dixon analyses of sodium influx inhibition by amiloride in vesicles made from cells of the two groups of animals yield dissimilar results. Whereas single-slope Dixon plots reported for mammalian preparations suggest competitive inhibition between the cation and the drug at single external sites (Aronson *et al.* 1983; Aronson and Igarashi, 1986; Moran, 1987; Kleinman *et al.* 1988), the two-slope Dixon plots of crustacean and echinoderm BBMV indicate that the drug is competing with sodium entry at two distinct binding sites with very different apparent affinities (Ahearn *et al.* 1990; Ahearn and Franco, 1990; Fig. 4).

The asymmetrical arrangement of cation binding sites on the internal and external faces of the invertebrate Na^+/H^+ exchanger leads to very different transmembrane flux ratios for the cations from those described for virtually all analogous mammalian antiporters. Among mammalian cells, and those of other vertebrate species as well, the Na^+/H^+ flux ratio is universally approximately 1.0 (Aronson *et al.* 1983; Knickelbein *et al.* 1983; Kleinman *et al.* 1988; Moran, 1987). In the four invertebrate gastrointestinal and renal epithelia where Na^+/H^+ exchange has now been examined in this regard, static head analysis indicates a flux ratio not significantly different from 2.0 (Fig. 5; Ahearn and Clay, 1989; Ahearn *et al.* 1990; Ahearn and Franco, 1990). In all cases the presence of the second sodium binding site and the resulting electrogenicity of the exchange eve

confer upon the transporter the thermodynamic capability of moving protons out of the epithelium against a 10000- to 100000-fold concentration gradient (cell pH=7.0; lumen pH=2.0). In the present study the flux ratio determined by static head analysis (Fig. 5) was slightly, but not significantly ($P>0.05$), smaller than the Hill coefficient determined in Fig. 2. While both techniques appear to provide a coupling coefficient of $2Na^+/H^+$, the slightly higher value obtained by Hill analysis may suggest that more than two sodium ions are involved in activating the transporter, but that only two are actually translocated across the membrane. The differences between solute activation and energization of carrier processes in other invertebrate cells have been discussed previously (Gerencser and Stevens, 1989).

In crustaceans, the contents of the stomach lumen, receiving secretions from the hepatopancreatic epithelium by way of the hepatopancreatic ducts, have been measured at pH 4.0–5.0 during digestion (Gibson and Barker, 1979), and the suggestion was recently proposed that a major physiological function of this electrogenic antiporter may be to provide the necessary protons to aid in food breakdown (Ahearn *et al.* 1990; Ahearn and Franco, 1990). While extracellular digestion, involving pH reduction of an epithelial secretion in crustaceans, may be one operation that is assisted by the presence of this transporter, it is clear that the function of the carrier mechanism cannot be restricted to this activity alone. Both the crustacean kidney (Ahearn and Franco, 1990) and starfish pyloric caecum (present study) possess this electrogenic mechanism and neither of these organ systems produces a particularly acidic secretion. The pH of crab urine, measured by cannula insertion through the nephropore, is approximately 8.0 (Cameron and Batterton, 1978), while starfish evert their stomachs and digest prey outside their own bodies at a pH near that of sea water (pH 8.2). In both of these cases, as well as in the crustacean hepatopancreas, the potential thermodynamic capabilities of proton secretion by this electrogenic process appear to be under-utilized if production of a strongly acidic secretion is the sole function of this carrier. It is likely that significant quantities of basic buffering agents are being simultaneously secreted with protons by these three epithelia, using a variety of possible mechanisms that may be directly or indirectly coupled to electrogenic cation exchange and which elevate the pH of the respective secretions to values markedly above the physiological potential of the $2Na^+/H^+$ antiporter. At present the nature of these possible coupled reactions, and the specific chemical species that may be transported by them, is unclear.

The Arthropoda and Echinodermata are invertebrate phyla representing the two major divisions of the Animal Kingdom based on embryological development. The Protostomia, embracing flatworms, annelids, mollusks and arthropods, exhibit spiral determinate cleavage and the embryonic blastopore gives rise to both mouth and anus. In contrast, the Deuterostomia are composed of the echinoderms, hemichordates and chordates and exhibit radial indeterminate cleavage, where the mouth does not develop from the embryonic blastopore, but is formed independently. Other characteristics also differentiate these two main lines of animal development. While echinoderms are therefore closely related to

chordates embryologically, they display Na^+/H^+ exchange characteristics more similar to those of the arthropods, which are phylogenetically distant relatives. Because representative invertebrate organisms from the two major animal divisions possess Na^+/H^+ exchangers with very similar properties, the electrogenic $2Na^+/H^+$ antiporter found in crustaceans and echinoderms may be widely distributed among the lower animals. If this proves to be the case, the thoroughly investigated electroneutral Na^+/H^+ exchanger of virtually all mammalian cells may represent an evolutionary departure from the animal norm, as represented by the electrogenic system of invertebrates.

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