1996

Characterization of a Basolateral Electroneutral Na+/H+ Antiporter in Atlantic Lobster (Homarus Americanus) Hepatopancreatic Epithelial Vesicles

Jeff Duerr
George Fox University, jduerr@georgefox.edu

Gregory A. Ahearn
University of Hawaii at Manoa

Recommended Citation

Previously published in Journal of Experimental Biology, 1996, 199, pp. 643–651 http://jeb.biologists.org/content/199/3/643.full.pdf+html

CHARACTERIZATION OF A BASOLATERAL ELECTRONEUTRAL Na^+/H^+ ANTIPORTER IN ATLANTIC LOBSTER (*HOMARUS AMERICANUS*) HEPATOPANCREATIC EPITHELIAL VESICLES

JEFFREY M. DUERR AND GREGORY A. AHEARN
Department of Zoology, University of Hawaii at Manoa, Honolulu, HI 96822, USA

Accepted 24 October 1995

Summary

Purified basolateral membrane vesicles (BLMVs) were prepared from Atlantic lobster (*Homarus americanus*) hepatopancreas using a Percoll density gradient technique. Enrichments of the Na^+/K^+-ATPase and alkaline phosphatase activities of these vesicles were 15.4- and 1.2-fold, respectively. The presence of amiloride-sensitive Na^+/H^+ exchange was demonstrated. Contrary to electrogenic $2Na^+/1H^+$ exchange on apical membranes from the same tissue, kinetic studies of Na^+ transport by these basolateral membranes indicate an electroneutral antiport with a K_m of 28±1.7 mmol l^{-1} and a J_{max} of 1.74±0.13 μmol mg^{-1} min^{-1}. Amiloride interacted at a single binding site (K_i=39 μmol l^{-1}) and external Li^+ was shown to be an effective competitive inhibitor of the exchange process (K_i=493 μmol l^{-1}). The presence of a membrane-potential-sensitive, Na^+-accepting ion channel was also demonstrated. The basolateral Na^+/H^+ exchanger physiologically resembles members of the NHE family of Na^+/H^+ antiporters described in vertebrates and departs from the apical electrogenic system previously described in lobster. Whether or not the basolateral Na^+/H^+ antiporter is an NHE isoform remains to be determined.

Key words: Na^+/H^+ antiport, BLMV, basolateral membrane, vesicles, lobster, *Homarus americanus*, crustacean, epithelia, amiloride, NHE.

Introduction

Na^+/H^+ antiport has been documented and studied extensively in nearly every cell type examined, particularly in vertebrates. Both prokaryotic and eukaryotic cells generally possess a Na^+/H^+ antiporter within the plasma membrane that is responsible for the net uptake of one sodium ion in exchange for the net extrusion of one hydrogen ion (Grinstein, 1988). This 1:1 electroneutral process displays a sensitivity to amiloride and its 5-amino-substituted derivatives (Aronson, 1985), as well as an affinity for Li^+ (Dudeja *et al.* 1989). In eukaryotic cells, the Na^+/H^+ exchanger performs a variety of essential cell functions such as intracellular pH and volume regulation, transcellular Na^+ transport (Grinstein, 1988) and, in addition, is activated in response to mitogenic stimuli associated with cell growth and proliferation (Grinstein *et al.* 1989).

Studies of a number of vertebrate nonepithelial and epithelial cell types have revealed the kinetic properties of the Na^+/H^+ antiporter to be relatively similar in teleosts (Vilella *et al.* 1991), turtles (Post and Dawson, 1992), chickens (Musch *et al.* 1992) and mammals (Murer *et al.* 1976). Kinetic analyses of cultured porcine kidney cells, LLC-PK_1 (Haggerty *et al.* 1988), and cultured opossum kidney cells (Montrose and Murer, 1990) also indicate that in these polarized epithelia two pharmacologically distinct Na^+/H^+ antiporters (isoforms) co-exist within the same cell localized to either the apical or basolateral membrane (see review by Clark and Limbird, 1991). In addition to pharmacological data, immunological techniques verified the presence of two distinct Na^+/H^+ exchanger isoforms in individual epithelial cells that were restricted to either the apical or basolateral poles (Biemesderfer *et al.* 1993).

Molecular genetic methods have enabled certain laboratories to clone the gene encoding the Na^+/H^+ antiporter. The first successful clone, referred to as NHE1 (Sardet *et al.* 1989), is a ubiquitous housekeeping protein found within the basolateral membrane of nearly every polarized epithelial cell examined (Clark and Limbird, 1991). It is likely that, in polarized epithelia, the apical and basolateral Na^+/H^+ antiporters are present as isoforms on their respective membranes, with a specialized functional role fulfilled by the apical isoforms. Indeed, genes for three apically localized NHE isoforms have been cloned, corroborating the previous pharmacological and immunological evidence: NHE2 (Tse *et al.* 1991), NHE3 and NHE4 (Orlowski *et al.* 1992).

A novel class of electrogenic Na^+/H^+ antiporter was described by two independent laboratories working with invertebrate organisms (see reviews by Clark and Limbird, 1991; Towle, 1993). Amiloride-sensitive electrogenic Na^+/H^+

exchange was documented in lobster and prawn hepatopancreas (Ahearn and Clay, 1989; Ahearn *et al.* 1990), crab gill (Shetlar and Towle, 1989) and starfish pyloric cecae (Ahearn and Franco, 1990). Kinetic analysis utilizing brush-border membrane vesicles (BBMVs) isolated from Atlantic lobster (*Homarus americanus*) hepatopancreas indicated a transport stoichiometry of $2Na^+$:$1H^+$ (Ahearn and Clay, 1989). Ahearn and Franco (1990) went on to document Ca^{2+}/H^+ exchange occurring on the $2Na^+/1H^+$ exchanger in lobster antennal gland. Ca^{2+} transport *via* the electrogenic $2Na^+/1H^+$ antiporter has also been described for lobster hepatopancreas BBMVs (Ahearn and Zhuang, 1995), and in starfish pyloric cecae both Ca^{2+} and Zn^{2+} can be transported on the established electrogenic system (Zhuang *et al.* 1995). Therefore, the electrogenic $2Na^+/1H^+$ exchanger is unlike any previously described apical Na^+/H^+ antiporter and may be represented by a separate gene family.

This report utilizes a recently developed Percoll density gradient technique to isolate pure basolateral membrane vesicles (BLMVs) from Atlantic lobster hepatopancreas to investigate whether the basolateral membrane of hepatopancreatic epithelial cells possesses Na^+/H^+ exchange activity. This investigation delineates a basolateral-membrane-associated Na^+/H^+ antiporter that functionally resembles a vertebrate NHE, differing substantially from the electrogenic system proposed for both the brush-border membrane of hepatopancreatic epithelia and other membranes of crustacean and echinoderm tissues.

Materials and methods

Animals and vesicle preparation

Live intermolt Atlantic lobsters, *Homarus americanus* (H. Milne Edwards), were obtained from commercial dealers in Hawaii and maintained unfed for up to 7 days in a continuous-flow, refrigerated (10 °C) filtered seawater aquarium.

Hepatopancreatic basolateral membrane vesicles (BLMVs) were prepared from fresh organs of individual (0.5 kg) lobsters. BLMVs were generated fresh for each experiment utilizing a self-orienting Percoll gradient centrifugation technique adapted from a procedure developed for fish (Davies *et al.* 1987).

Hepatopancreatic tissue was quickly excised and placed into a hypotonic buffered sucrose medium [MSS buffer: 250 mmol l^{-1} sucrose, 20 μmol l^{-1} Tris/HCl, 300 μmol l^{-1} phenylmethylsulfonylfluoride (PMSF), pH 7.4] and homogenized with a Kinematica GmbH Polytron. An initial centrifugation step (2500 ***g***) removed large cellular debris. A crude separation of basolateral membranes was obtained from a second centrifugation at 20 400 ***g***. The crude basolateral membrane pellet was resuspended in MSS buffer and combined with a premixed dilution of Percoll and centrifuged at 47 800 ***g*** for 1 h. The resultant Percoll-gradient bands were enzymatically assayed for basolateral marker enzyme (Na^+/K^+-ATPase) and brush-border membrane marker enzyme (alkaline phosphatase) enrichment. High levels of enrichment for Na^+/K^+-ATPase (15.4-fold) and minimal alkaline phosphatase enrichment (1.2-fold) indicated a band containing a high concentration of BLM with minimal BBM contamination (Table 1). Enzyme assays were based on protocols reported by Berner and Kinne (1976) and performed at 22 °C. The basolateral membrane fraction was homogenized with preloading buffer (buffer contents varied with experiment, see Results for details) and centrifuged for 40 min at 47 800 ***g***. The resulting pellet was resuspended and washed in fresh preloading buffer and centrifuged a final time for 40 min at 47 800 ***g***.

Table 1. *Enzyme characterization of hepatopancreas basolateral membrane*

Enzyme	Activity of homogenate	Activity of the BLMVs	Purification factor
Na^+/K^+-ATPase	1.94±0.3	30±1.8	15.4±2.2
Alkaline phosphatase	13.16±0.6	15.8±3.2	1.2±0.5

Values are mean ± S.E.M., N=3.

Enzyme activities are in μmoles of product released per milligram protein per hour.

Purification factors are calculated from the mean enzyme activities of the homogenate and vesicles.

Enzyme measurements were performed on three different membrane preparations.

BLMVs, basolateral membrane vesicles.

Na^+ uptake

Isolated BLMV uptake studies were conducted at 15 °C and were started by diluting the vesicle suspension into a medium containing trace amounts of ^{22}Na and unlabeled NaCl or sodium gluconate. The compositions of the final resuspension solution and incubation media are described separately for each experiment. Na^+ uptake was terminated by placing the vesicles into 2 ml of ice-cold stop solution utilizing the Millipore filtration technique developed by Hopfer *et al.* (1973). Filters were then dissolved in liquid scintillation cocktail (Ecolume) and the radioactivity counted in a Beckman LS 8100 scintillation counter. Na^+ uptake is expressed as nmol mg^{-1} protein $filter^{-1}$ (protein levels were assayed using the BioRad assay). Na^+ uptake into BLMVs was corrected for non-specific binding by subtracting 'blank' values where vesicles and radiolabeled incubation medium were simultaneously injected into ice-cold stop medium without prior mixing. Each experiment was repeated two or three times using membranes from different animals. Each time point is presented in a figure as the mean of 3–5 replicates and the associated standard error. Statistical comparisons were accomplished using Student's *t*-tests. A value of $P<0.05$ was considered significant.

Chemicals

$^{22}NaCl$ was obtained from DuPont, Inc, USA. Amiloride,

dimethylamiloride, valinomycin, phenylmethylsulfonylfluoride (PMSF), tetramethylammonium hydroxide (TMA-OH), Percoll and other reagent grade chemicals were obtained from Sigma Chemical Co, St Louis, MO, USA.

Results

Basolateral membrane vesicles respond to changes in osmotic pressure

Freshly isolated BLMVs preloaded with 200 mmol l^{-1} mannitol and 25 mmol l^{-1} Hepes at pH 7.0 were exposed to medium containing 0, 50, 100, 300, 600 or 900 mmol l^{-1} sucrose, 25 mmol l^{-1} Hepes and 1 mmol l^{-1} ^{22}NaCl at pH 7.0 in order to ascertain whether sealed, functional vesicles were obtained. Fig. 1 illustrates the linear dependence of Na^+ uptake on the osmolality of the outside medium, thereby confirming that intravesicular volume is dependent on a transmembrane osmotic gradient. The slope statistically intersects (r^2=0.94; P<0.05) the y-axis at the origin, which indicates a negligible amount of non-specific Na^+ binding that is not accounted for by subtracting blank values. The observed osmolality of the outside medium at 0 mmol l^{-1} sucrose (60 mmol kg^{-1}) is attributable to the Hepes/Tris buffer and NaCl.

Na^+/H^+ exchange occurs in the basolateral membrane of lobster hepatopancreatic epithelium

Na^+/H^+ exchange was demonstrated in short-circuited lobster hepatopancreas BLMVs in the presence of an outwardly directed H^+ gradient as shown by a 2.5-fold ^{22}Na uptake overshoot at 1 min during a 60 min experiment (Fig. 2). This overshoot was unaffected by 10 mmol l^{-1} external Ca^{2+}, but the addition of 3 mmol l^{-1} amiloride or the absence of a pH gradient abolished the uptake overshoot, with Na^+ entry into the vesicles occurring at a greatly attenuated rate.

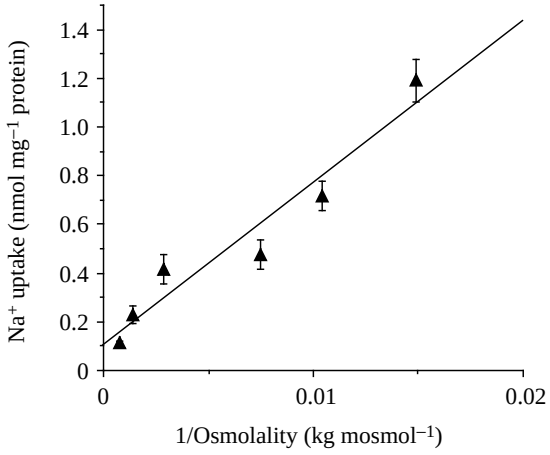

Fig. 1. Demonstration of osmotically reactive lobster hepatopancreatic BLMVs. Vesicles were preloaded 200 mmol l^{-1} mannitol and 25 mmol l^{-1} Hepes at pH 7.0 and were incubated for 90 min in medium containing 0, 50, 100, 300, 600 or 900 mmol l^{-1} sucrose, 25 mmol l^{-1} Hepes and 1 mmol l^{-1} NaCl also at pH 7.0. Values are means ± S.E.M. of five observations. The line was drawn by regression analysis (r^2=0.94, P<0.05).

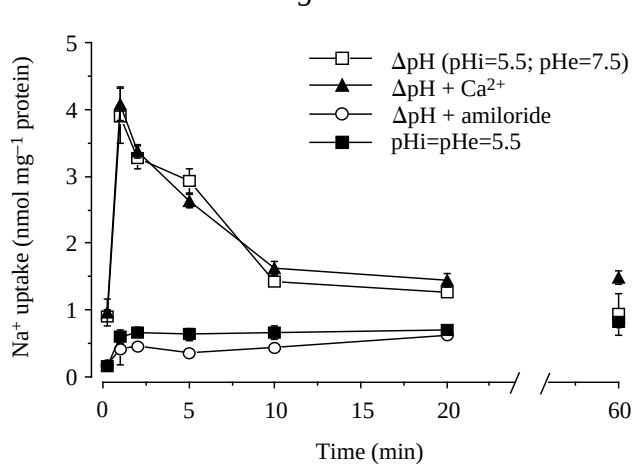

Fig. 2. Time course of $^{22}Na^+/H^+$ exchange by lobster hepatopancreatic BLMVs: effects of external Ca^{2+} and amiloride. Vesicles were loaded with 100 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 (50 mmol l^{-1} Mes/Tris) and were incubated in medium at either pH 5.5 (no pH gradient) or pH 7.5 (50 mmol l^{-1} Hepes/Tris), containing 1 mmol l^{-1} radiolabeled sodium gluconate, 100 mmol l^{-1} mannitol and 50 mmol l^{-1} potassium gluconate. In some instances, either 10 mmol l^{-1} Ca^{2+} or 3 mmol l^{-1} amiloride was added to the external medium. pHi, pH inside vesicles; pHe, pH of medium; ΔpH, pH gradient. Values are means ± S.E.M., N=5.

As previously mentioned, the apical membrane of lobster hepatopancreatic epithelium features an electrogenic $2Na^+/1H^+$ antiporter. The following experiment was designed to examine the possibility that the BLM porter may be a similar protein. Na^+/H^+ exchange is enhanced in the presence of a membrane potential compared with short-circuited conditions (Fig. 3). Amiloride does not completely inhibit Na^+/H^+ exchange relative to conditions in which there is no pH gradient. This is in contrast to the effect of amiloride on Na^+/H^+ exchange under electroneutral experimental conditions, suggesting an amiloride-insensitive, electrogenic component to Na^+ uptake in BLMVs. External Ca^{2+} (10 mmol l^{-1}) only partially inhibited Na^+ uptake, but did not eliminate the attenuated overshoot at 5 min.

In order to clarify the sensitivity of voltage-dependent Na^+ uptake to external Ca^{2+} and amiloride, an experiment was conducted in which Na^+ uptake into BLMVs was a function of a transmembrane potential only (inside negative). Relative to control uptake values, 3 mmol l^{-1} amiloride had no inhibitory activity, yet 10 mmol l^{-1} Ca^{2+} did reduce Na^+ uptake into BLMVs during the first 20 min (Fig. 4). Taken together, the time course data clearly indicate the presence of a basolateral Na^+/H^+ exchanger that may operate as an electroneutral ($1Na^+$:$1H^{+)}$ antiporter, unlike that described for apical membranes from the same tissue.

Kinetics of the basolateral Na^+/H^+ exchanger

A time course of $^{22}Na^+$ uptake using three different Na^+

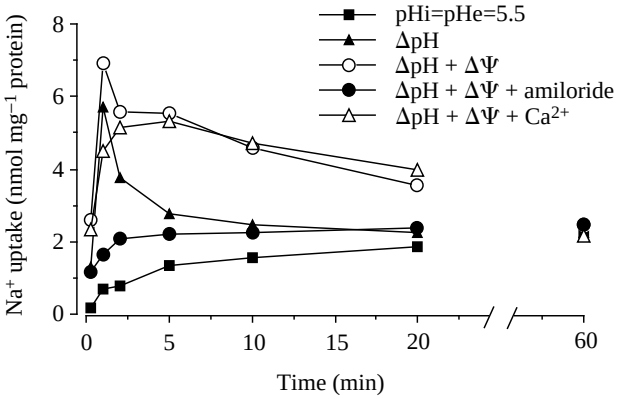

Fig. 3. Time course of $^{22}Na^+/H^+$ exchange by lobster hepatopancreatic BLMVs: effects of membrane potential, external Ca^{2+} and external amiloride. Vesicles were preloaded with 100 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 (25 mmol l^{-1} Mes/Tris) and were incubated in medium at either pH 5.5 (no pH gradient) or pH 7.5 (25 mmol l^{-1} Hepes/Tris), containing 1 mmol l^{-1} radiolabeled sodium gluconate, 100 mmol l^{-1} mannitol and either 50 mmol l^{-1} potassium gluconate (voltage-clamped) or 50 mmol l^{-1} tetramethylammonium gluconate (inside-negative membrane potential). In some instances, either 10 mmol l^{-1} Ca^{2+} or 3 mmol l^{-1} amiloride was added to the external medium. Values are means ± S.E.M., N=5. $\Delta\Psi$, transmembrane potential difference.

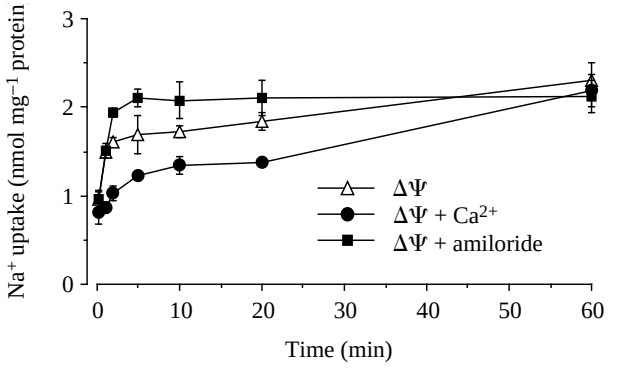

Fig. 4. Time course of ^{22}Na uptake by lobster hepatopancreatic BLMVs: effect of Ca^{2+} and amiloride on voltage-dependent Na^+ uptake. Vesicles were loaded with 100 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin (pH 8.0, 25 mmol l^{-1} Hepes/Tris) and were incubated in medium at pH 8.0 (25 mmol l^{-1} Hepes/Tris) containing 1 mmol l^{-1} radiolabeled sodium chloride, 100 mmol l^{-1} mannitol and 50 mmol l^{-1} tetramethylammonium gluconate. In some instances, 10 mmol l^{-1} Ca^{2+} or 3 mmol l^{-1} amiloride was added to the external medium. Values are means ± S.E.M., N=5.

concentrations (5, 50 and 100 mmol l^{-1}) for periods of 1, 2, 3, 4, 6, 8, 10 and 12 s was examined in order to determine a range over which Na^+ entry remained linear for each Na^+ concentration (not shown). The maximum time point at which all three cation concentrations illustrated a linear uptake was 2.5 s. Therefore, Na^+ uptake recorded at 2.5 s would yield accurate initial rates of Na^+ entry.

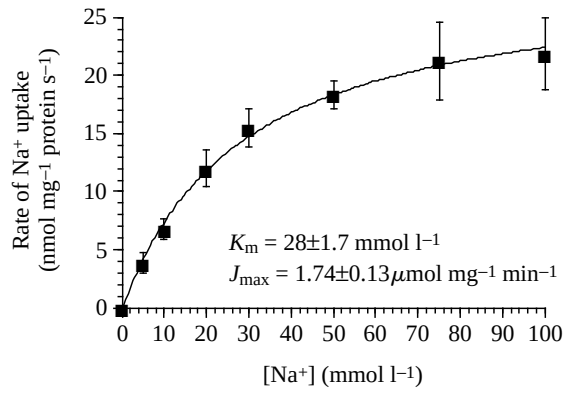

Fig. 5. $^{22}Na^+/H^+$ exchange kinetics of lobster hepatopancreatic BLMVs. Vesicles were preloaded at pH 5.5 as described in Results and were incubated for 2.5 s in medium at pH 7.5 containing concentrations of radiolabeled sodium gluconate ranging from 0 to 100 mmol l^{-1}. Data plotted represent means ± S.E.M. of five observations, and the hyperbolic curve and kinetic constants were calculated using iterative curve-fitting software (Deltagraph Professional 2.0.3).

The kinetics of Na^+ uptake at 2.5 s as a function of external Na^+ concentration was examined for Na^+ concentrations ranging from 0 to 100 mmol l^{-1} in BLMVs. BLMVs were preloaded with 300 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate, 25 mmol l^{-1} Mes/Tris and 50 μmol l^{-1} valinomycin at pH 5.5, and incubated in medium containing 50 mmol l^{-1} potassium gluconate, 25 mmol l^{-1} Hepes/Tris and variable sodium gluconate and tetramethylammonium gluconate concentrations at pH 7.5. Na^+ uptake into BLMVs was terminated at 2.5 s and corrected for non-specific binding by subtracting 'blank' values. The final net uptake values for each Na^+ concentration are shown in Fig. 5.

Na^+ influx could be described as a function of a distinct saturable carrier exhibiting Michaelis–Menten kinetic characteristics. The system could be quantitatively described by the following equation:

$$J_{Na} = J_{max} \times [Na^+]/\{K_m + [Na^+]\}\,, \qquad (1)$$

where J_{Na} is the inward Na^+ flux in nmol mg^{-1} s^{-1}, J_{max} is the maximal carrier-mediated Na^+ flux in μmol mg^{-1} min^{-1}, K_m is the concentration of Na^+ resulting in 0.5J_{max} and $[Na^+]$ is the concentration of Na^+ (in mmol l^{-1}). The hyperbolic curve in Fig. 5 represents the carrier-mediated Na^+ uptake process and suggests a 1:1 stoichiometry for Na^+/H^+ exchange. The kinetic constants were calculated using a Michaelis–Menten function, giving a K_m of 28±1.7 mmol l^{-1} and a J_{max} of 1.74±0.13 μmol mg^{-1} min^{-1}.

Effect of external amiloride concentration on Na^+ influx

Fig. 2 indicated that 3 mmol l^{-1} effectively abolished Na^+ influx into hepatopancreatic BLMVs. In order to define more accurately the inhibitory kinetics of amiloride on the BLMV Na^+/H^+ exchange mechanism, an experiment was conducted in which vesicles were preloaded with 100 mmol l^{-1} mannitol,

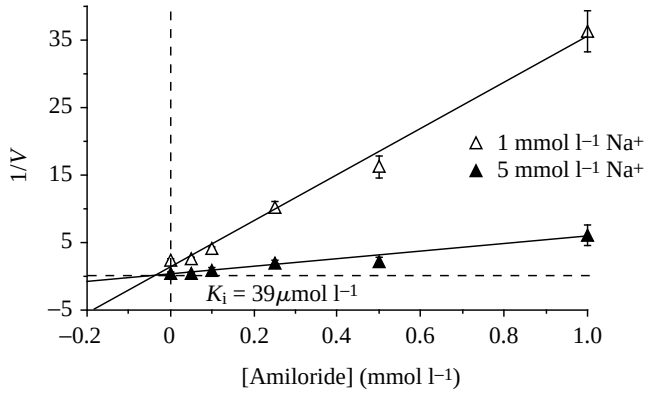

Fig. 6. Dixon plot of the effects of external amiloride concentration on $^{22}Na^+/H^+$ exchange in lobster hepatopancreatic BLMVs at pH 5.5 (valinomycin/K^+ voltage-clamp) and exposed for 5 s to external medium at pH 7.5 and containing either 1 mmol l^{-1} or 5 mmol l^{-1} radiolabeled sodium gluconate and concentrations of amiloride ranging from 0.05 to 1.0 mmol l^{-1}. Lines were drawn by regression analysis (r^2=0.99 for 1 mmol l^{-1} sodium gluconate; r^2=0.95 for 5 mmol l^{-1} sodium gluconate; P<0.05 for both slopes). Values are means ± S.E.M., N=5. V, rate of Na^+ uptake (nmol mg^{-1} protein $5\,s^{-1}$).

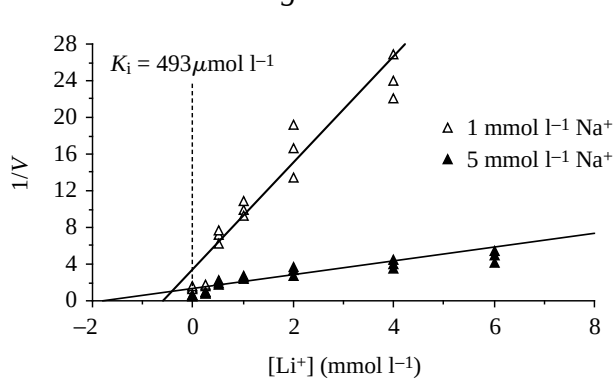

Fig. 7. Dixon plot of the effects of external Li^+ concentration on $^{22}Na^+/H^+$ exchange in lobster hepatopancreatic BLMVs. Short-circuited vesicles were prepared as previously described (see Fig. 6) with the external medium containing variable concentrations of Li^+ ranging from 0 to 6 mmol l^{-1}. Lines were drawn by linear regression analysis (r^2=0.87 for 1 mmol l^{-1} sodium gluconate; r^2=0.88 for 5 mmol l^{-1} sodium gluconate; P<0.05 for both slopes). Values are means ± S.E.M., N=5. V, rate of Na^+ uptake (nmol mg^{-1} protein $5\,s^{-1}$).

50 mmol l^{-1} potassium gluconate and 25 mmol l^{-1} Mes at pH 5.5. Vesicles were incubated in medium consisting of 100 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 25 mmol l^{-1} Hepes at pH 7.5 and variable amiloride concentrations (0–3 mmol l^{-1}) with either 1 or 5 mmol l^{-1} NaCl present. Fig. 6 shows a Dixon plot of the effects of increasing amiloride concentrations on uptake over a 5 s period (time increased to accommodate binding of amiloride) at both 1 mmol l^{-1} and 5 mmol l^{-1} Na^+. Single slopes for each Na^+ concentration suggest a single binding site for amiloride on the Na^+/H^+ antiporter. The intersections of the plots at 1 mmol l^{-1} and 5 mmol l^{-1} Na^+ are above the horizontal axis, which may suggest a competitive inhibitory effect of amiloride, and yield an apparent K_i of 39 μmol l^{-1}. However, the intersection is not significantly (P>0.05) above the horizontal axis and therefore does not permit a definitive statement about the nature of the inhibition to be made.

The kinetics of uptake of Na^+ into BLMVs as a function of Na^+ or amiloride concentration is consistent with the vertebrate paradigm of an electroneutral Na^+/H^+ exchange and departs from the electrogenic model described for the apical membrane of the same cell. To investigate further the similarity of BLMV Na^+/H^+ exchange to the process described in vertebrate cells, the effect of externally applied Li^+ was assessed. Fig. 7 is a Dixon plot of the inhibitory effects of variable Li^+ concentrations on ^{22}Na influx. Vesicles were preloaded with 100 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 25 mmol l^{-1} Mes at pH 5.5 and were incubated for 5 s in 100 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate, 25 mmol l^{-1} Hepes at pH 7.5 with either 1 or 5 mmol l^{-1} NaCl and different concentrations of LiCl (0–6 mmol l^{-1}). One slope for each Na^+ concentration suggests a single binding site for Li^+, and the intersection of these two slopes above the horizontal axis suggests a competitive inhibition with an apparent K_i of 493 μmol l^{-1}.

Static head conditions predict a 1Na^+:1H^+ stoichiometry

In order to confirm the stoichiometric relationship between Na^+ and H^+ as 1:1, as suggested by hyperbolic influx kinetics, the static head method of Turner and Moran (Turner, 1983) was employed. In this procedure, a pH gradient was established across the vesicular membrane (pHi=5.5; pHe=6.5) which served as a fixed driving force for Na^+ uptake. Various gradients of Na^+ were tested as opposing driving forces to balance the H^+ gradient, until an appropriate concentration of Na^+ was employed which resulted in no net flux of Na^+ across the membrane. The thermodynamic equation that describes the condition of no net flux of either Na^+ or H^+ is:

$$\ln[H^+]_i/[H^+]_e = n(\ln[Na^+]_i/[Na^+]_e + F\Delta\Psi/\boldsymbol{R}T) \ , \qquad (2)$$

where $\Delta\Psi$ is the transmembrane potential, n is the number of sodium ions simultaneously transported for each proton, and F, $\boldsymbol{R}$ and T have their usual meanings. Since our experiments were performed under valinomycin/K^+ voltage-clamped conditions, the above equation reduces to:

$$\ln[H^+]_i/[H^+]_e = n(\ln[Na^+]i/[Na^+]_e) \ . \qquad (3)$$

Two groups of vesicles were preloaded for 30 min at room temperature (24 °C) with 5 mmol l^{-1} radiolabeled sodium gluconate, 50 mmol l^{-1} potassium gluconate, 100 mmol l^{-1} mannitol and 50 μmol l^{-1} valinomycin at pH 5.5 (25 mmol l^{-1} Mes/Tris). One group was incubated for 2.5 s in external medium at pH 6.5 (25 mmol l^{-1} Mes/Tris), containing 0.25, 0.5 or 5.0 mmol l^{-1} radiolabeled sodium gluconate (same specific activity as internal medium), 50 mmol l^{-1} potassium gluconate, and mannitol for osmotic balance. The other group was exposed to an identical set of external media with the addition of 3 mmol l^{-1} amiloride. Samples of preloaded

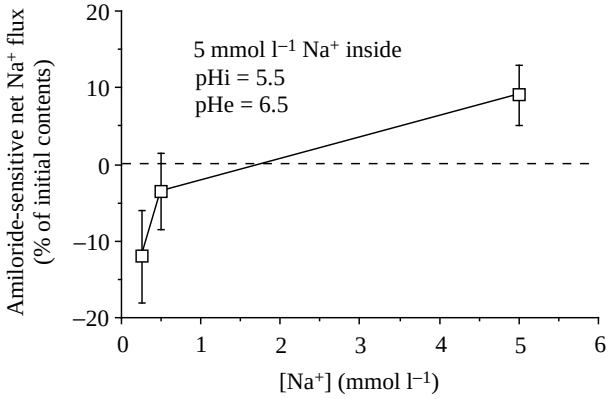

Fig. 8. Static head analysis of Na^+/H^+ exchange. Two groups of vesicles were preloaded for 30 min at 24 °C with 5 mmol l^{-1} radiolabeled sodium gluconate, 50 mmol l^{-1} potassium gluconate, 100 mmol l^{-1} mannitol and 50 μmol l^{-1} valinomycin at pH 5.5 (25 mmol l^{-1} Mes/Tris) and incubated for 2.5 s in external medium at pH 6.5 (25 mmol l^{-1} Mes/Tris) containing 0.25, 0.5 or 5 mmol l^{-1} radiolabeled sodium gluconate, 50 mmol l^{-1} potassium gluconate and a variable concentration of mannitol for osmotic balance. One group of vesicles was exposed to the external medium with the addition of 3 mmol l^{-1} amiloride. Values displayed represent the percentage change in vesicular ^{22}Na content over a 2.5 s incubation resulting from amiloride-sensitive net influx or efflux of labeled Na^+ in response to variable driving forces. Values are means ± S.E.M., N=4.

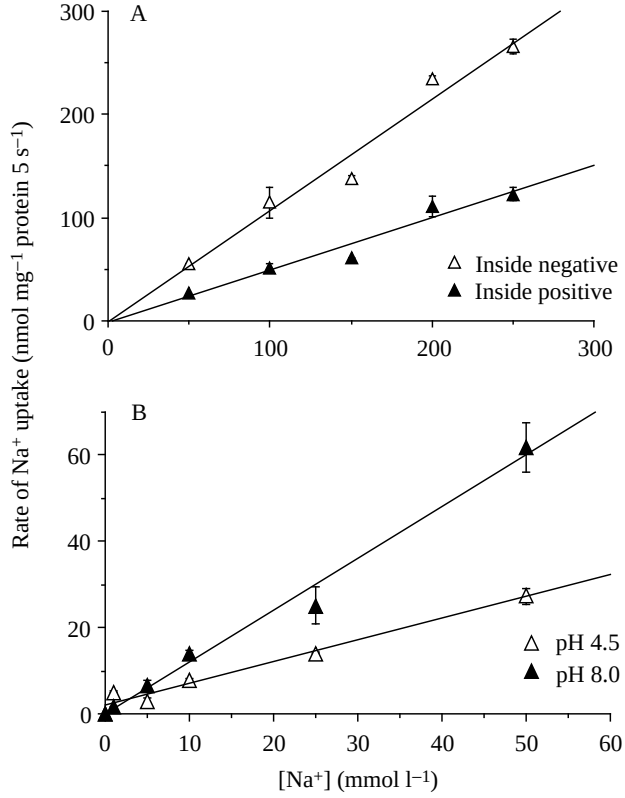

Fig. 9. (A) Effect of transmembrane potential difference as the sole driving force for Na^+ uptake by lobster hepatopancreatic BLMVs. Vesicles were loaded with 100 mmol l^{-1} mannitol and either 100 mmol l^{-1} tetramethylammonium gluconate (induced inside-positive condition, open triangles) or 100 mmol l^{-1} potassium gluconate (induced inside-negative condition, filled triangles) and 50 μmol l^{-1} valinomycin at pH 8.0 (25 mmol l^{-1} Hepes/Tris) and were incubated in external media at the same pH containing concentrations of radiolabeled sodium gluconate ranging from 50 to 250 mmol l^{-1}. (B) Effect of transmembrane potential difference as the sole driving force for Na^+ uptake by lobster hepatopancreatic BLMVs. Vesicles were prepared as indicated in A with an inside-negative potential at either pH 4.5 (25 mmol l^{-1} Mes/Tris HCl) or pH 8.0 (25 mmol l^{-1} Hepes) and incubated in external medium at the same pH containing concentrations of radiolabeled sodium gluconate ranging from 0 to 50 mmol l^{-1}. Values are means ± S.E.M., N=5.

vesicles were analyzed for ^{22}Na content at the end of the preincubation period and this was compared with the total isotopic content of vesicles after exposure to each medium. Amiloride-sensitive net Na^+ flux for each external medium represents the difference between the ^{22}Na uptake during a 2.5 s period in the presence and absence of the drug.

Fig. 8 illustrates the relationship between amiloride-sensitive Na^+ uptake and the external Na^+ concentration as a percentage of the preloaded vesicle isotopic contents. Static head conditions (no net flux) were met at an external Na^+ concentration of 0.5 mmol l^{-1}. Equation 3 predicts a value of n approximately equal to 1.0, and it may be subsequently predicted that the transport stoichiometry of Na^+/H^+ exchange is likely to be approximately 1.0.

Presence of a conductive pathway for Na^+ uptake

To rule out the possibility of a basolateral membrane-potential-sensitive carrier, an experiment was conducted in which vesicles were preloaded with 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate and 25 mmol l^{-1} Hepes at pH 8.0. The vesicles were then incubated in 100 mmol l^{-1} mannitol, 100 mmol l^{-1} tetramethylammonium gluconate and 25 mmol l^{-1} Hepes at pH 8.0 in order to produce an inside-negative potential (inside-positive potentials were generated by reversing the preloading and incubation media). External Na^+ concentration was varied from 50 to 250 mmol l^{-1} using sodium gluconate and was osmotically balanced by increasing the mannitol concentration in the preloading medium. Fig. 9A illustrates a direct linear relationship (r^2=0.95, inside negative; r^2=0.97, inside positive; P<0.05 for both lines) between Na^+ concentration and influx rate, supporting the possible presence of a functional channel. This linear uptake is significantly reduced (P<0.05) when the inside of the vesicle is positive relative to the external medium, supporting the electrogenic component of Na^+ influx.

In order to strengthen the argument that a non-carrier mechanism is operating over the same concentration range that the electroneutral Na^+/H^+ antiporter is functional, BLMVs were preloaded as described above such that they possessed an inside-negative membrane potential. Na^+ concentration was varied externally from 0 to 50 mmol l^{-1} with sodium gluconate and osmotically balanced with appropriate amounts of mannitol present in the preloading medium. Na^+ influx as a

function of external Na^+ concentration was assessed at pH 8.0 and 4.5. Fig. 9B illustrates a linear function (r^2=0.94, pH=4.5; r^2=0.96, pH=8,0; P<0.05 for both lines) for BLMV Na^+ influx that is significantly reduced (P<0.05) at pH 4.5, suggesting that a non-saturatable, pH-sensitive process is accommodating Na^+ entry into BLMVs.

Discussion

The present study strongly supports the existence of an electroneutral Na^+/H^+ antiporter on the basolateral membrane of lobster hepatopancreatic epithelial cells which physiologically resembles members of the NHE family of Na^+/H^+ antiporters (Fig. 10). The lobster BLM antiporter differs from the previously documented apical electrogenic Na^+/H^+ exchanger reported in lobster and prawn hepatopancreas (Ahearn and Clay, 1989; Ahearn *et al.* 1990), in starfish pyloric ceca (Ahearn and Franco, 1990) and in blue crab gill (Shetlar and Towle, 1989).

Na^+ uptake by the basolateral Na^+/H^+ antiporter is a hyperbolic function of Na^+ concentration, as illustrated in Fig. 5, with a calculated K_m of 28±1.7 mmol l^{-1} and a J_{max} of 1.74±0.13 μmol mg^{-1} min^{-1}, values that fall within the range reported for the vertebrate NHE isoforms (Clark and Limbird, 1991).

In order to verify the 1:1 stoichiometric relationship between the simultaneous flux of Na^+ and H^+ predicted by the Michealis–Menten kinetic model, the static head method of Turner and Moran (Turner, 1983) was applied to Na^+/H^+ antiport in BLMVs. Because a 10-fold transmembrane gradient of Na^+ balanced a 10-fold gradient of H^+ and led to static head conditions, the transport stoichiometry of Na^+/H^+ exchange was predicted to be 1.0.

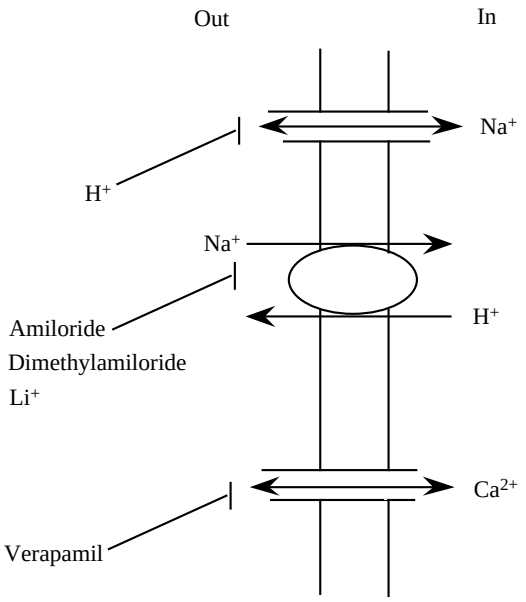

Fig. 10. Model of the basolateral membrane as determined using isolated BLMVs. Na^+ transport occurs *via* an electroneutral Na^+/H^+ antiporter and through a pH-sensitive Na^+-accepting ion channel. Ca^{2+} entry is *via* a verapamil-sensitive Ca^{2+} channel. The Na^+/H^+ antiporter is inhibited by Li^+ as well as amiloride and dimethylamiloride.

In contrast, kinetic analysis of Na^+ influx on the BBM from the same tissue revealed a sigmoidal curve reflecting the cooperative binding of, probably, 2 Na^+ externally and 1 H^+ internally, resulting in an electrogenic antiport process subsequently corroborated by static head analysis (Ahearn and Clay, 1989). It is thus clear that the apical and basolateral membranes of hepatopancreas epithelium possess functionally different Na^+/H^+ antiporters. Furthermore, Ca^{2+}/H^+ exchange in BLMVs is undetectable (Z. Zhuang, unpublished data), whereas in BBMVs Ca^{2+} has been shown to be exchanged in lieu of Na^+ by the apical $2Na^+/1H^+$ antiporter (Ahearn and Franco, 1990).

Over a broad range of external Na^+ concentrations, the presence of an inside-negative potential alone produced a linear influx over the entire range examined (Fig. 9A). This figure also indicates the inhibitory effect of an inside-positive potential on solute influx. To eliminate the possibility that, at lower Na^+ concentrations, an electrogenic carrier may be contributing to the electrogenic influx pathway, Na^+ uptake by BLMVs was monitored while external Na^+ concentrations ranged from 0 to 50 mmol l^{-1}. Diffusion across the membrane was observed; it was significantly reduced at pH 4.5 relative to pH 8.0. Ion channels are indeed sensitive to pH (Palmer and Frindt, 1987). According to Hille (1968), at pH 4.5, Na^+ channel conductivity diminished by approximately 50 % compared with that measured at physiological pH, consistent with the effect observed for Na^+ influx into lobster BLMVs. The precise nature of this putative basolateral membrane ion channel is unknown.

Na^+ conductance in epithelial basolateral membranes is not a novel observation. Post and Dawson (1992), in their description of a turtle colon basolateral Na^+/H^+ antiporter, described an amiloride-sensitive electroneutral exchanger that was also capable of an uncoupled Na^+-selective conductance. Garty *et al.* (1987) described an amiloride-sensitive Na^+ conductance in the basolateral membrane of toad bladder as an L-type Na^+ channel (K_i=13 μmol l^{-1}) and did not consider the possibility that the conductance was a property of the Na^+/H^+ antiporter. The dual nature of the antiporter proposed by Post and Dawson (1992) was supported in part by the observation that both Na^+/H^+ exchange and Na^+ conductance were blocked by similar concentrations of amiloride. In our studies, Na^+ conductance was unaffected by externally applied 3 mmol l^{-1} amiloride or 500 μmol l^{-1} dimethylamiloride (data not shown), suggesting that the Na^+/H^+ antiporter was not responsible for the observed basolateral Na^+ conductance. However, Palmer and Frindt (1987) reported that at pH 6.5 a marked decrease occurs in Na^+ channel activity in rat cortical collecting tubules, whereas both Post and Dawson's (1992) and our own data (not shown) do not reveal diminished Na^+ conductance at pH 6.5.

In a wide range of mammalian cells, amiloride appears to

interact at a single site that blocks transport function in the Na^+/H^+ exchanger with K_i values ranging from 7 to 99 μmol l^{-1} (Aronson, 1985). Lobster BLMV Na^+/H^+ exchange is also inhibited by amiloride with a K_i of 39 μmol l^{-1}. In general, amiloride has been shown to be a competitive inhibitor of Na^+ entry (Aronson, 1985), interacting with at least two amino acids, one of which also binds Na^+ (Counillon and Pouyssegur, 1993). Our data suggest competitive inhibition by amiloride on lobster BLMV Na^+/H^+ exchange; however, the intercept is not significantly above the horizontal axis and therefore inhibition may be non-competitive.

In addition to amiloride, Li^+ (K_i=493 μmol l^{-1}) also acts as a competitive inhibitor of Na^+/H^+ exchange in lobster BLMVs, although with less inhibitory efficacy. Li^+ is typically transported as an alternative substrate in Na^+/H^+ exchange (Aronson, 1985). Mahnensmith and Aronson (1985) present evidence that Li^+ and amiloride compete for a single site in renal epithelial vesicles, which is consistent with the effects of both amiloride and Li^+ on Na^+/H^+ exchange in lobster BLMVs. An inhibitory effect of Li^+ is in contrast to the physiology of the apical $2Na^+/1H^+$ antiporter, which is not affected by Li^+ (Z. Zhuang, unpublished observation).

This report is the first physiological characterization of an electrically silent Na^+/H^+ antiporter in crustacean epithelia. The antiporter resembles the NHE family of Na^+/H^+ antiporters described for all vertebrate species studied and is dissimilar to the electrogenic model of crab and lobster brush-border epithelia. It is unknown at present whether this novel electroneutral Na^+/H^+ exchanger is a unique or a homologous NHE isoform.

Recent attempts (Towle and Wu, 1994) to clone the gene encoding the electrogenic $2Na^+/1H^+$ antiporter in crab, using degenerate primers based on NHE sequences in order to screen crab gill cDNA, have produced a gene sequence which shows extensive homology with vertebrate NHE sequences. Hydropathy analysis yielded a pattern of hydrophobic and hydrophilic domains which closely resembled the pattern of the NHE1 isoform. NHE1 is the ubiquitous 'housekeeping' antiporter and, in polarized vertebrate epithelia, is generally localized to the basolateral membrane (Biemesderfer *et al.* 1993). Unfortunately, neither the tissue specificity nor the cellular localization of the cloned crab sequence is known and functional expression of cDNA has not been reported.

It is plausible that the basolateral electroneutral Na^+/H^+ antiporter described for the lobster hepatopancreatic epithelium plays a housekeeping role in maintaining intracellular pH and cell volume akin to the role of NHE1. The electrogenic $2Na^+/1H^+$ antiporter on the brush-border membrane may reflect an adaptive response of Na^+/H^+ antiport to specific aspects of Na^+ and/or Ca^{2+} transport (Ahearn *et al.* 1994) and may be representative of a novel gene family. Future investigations into the regulatory events and structural components of the lobster BLMV Na^+/H^+ antiporter should clarify its precise physiological role and homology to the NHE gene family.

The authors thank Z. Zhuang, M. Klein, D. Tupper and V. Pennington for technical support and helpful discussions. This study was supported by grants from the National Science Foundation (DCB89-0365 and IBN-9317230).

References

Ahearn, G. A. and Clay, L. P. (1989). Kinetic analysis of 2 Na^+–1 H^+ antiport in crustacean hepatopancreas. *Am. J. Physiol.* **257**, R484–R493.

Ahearn, G. A. and Franco, P. (1990). Sodium and calcium share the electrogenic 2 Na^+–1 H^+ antiporter in crustacean antennal glands. *Am. J. Physiol.* **259**, F758–F767.

Ahearn, G. A., Franco, P. and Clay, L. P. (1990). Electrogenic 2 Na^+/1 H^+ exchange in crustaceans. *J. Membr. Physiol.* **116**, 215–226.

Ahearn, G. A. and Zhuang, Z. (1995). Cellular mechanisms of calcium transport in crustaceans. *Physiol. Zool.* (in press).

Ahearn, G. A., Zhuang, Z., Duerr, J. and Pennington, V. (1994). Role of the invertebrate electrogenic 2 Na^+–1 H^+ antiporter in monovalent and divalent cation transport. *J. exp. Biol.* **196**, 319–335.

Aronson, P. S. (1985). Kinetic properties of the plasma membrane Na^+–H^+ exchanger. *A. Rev. Physiol.* **47**, 545–560.

Berner, W. and Kinne, R. (1976). Transport of *p*-aminohippuric acid by plasma membrane vesicles isolated from rat kidney cortex. *Pflügers Arch.* **361**, 269–277.

Biemesderfer, D., Pizzonia, J., Abu-Alfa, A., Exner, M., Reilly, R., Igarashi, P. and Aronson, P. (1993). NHE3: a Na^+–H^+ exchanger isoform of renal brush border. *Am. J. Physiol.* **265**, F736–F742.

Clark, J. D. and Limbird, L. E. (1991). Na^+–H^+ exchanger subtypes: a predictive review. *Am. J. Physiol.* **261**, C945–C953.

Counillon, L. and Pouyssegur, J. (1993). Molecular biology and hormonal regulation of vertebrate Na^+–H^+ exchanger isoforms. In *Molecular Biology and Function of Carrier Proteins* (ed. L. Ruis, J. M. Russel and M. L. Jennings), pp. 169–185. New York: Rockefeller University Press.

Davies, S., Maenz, D. D. and Cheeseman, C. I. (1987). A novel imino-acid carrier in the enterocyte basolateral membrane. *Biochim. biophys. Acta* **896**, 247–255.

Dudeja, P. K., Foster, E. S. and Brasitus, T. A. (1989). Na^+/H^+ antiporter of rat colonic basolateral membrane vesicles. *Am. J. Physiol.* **257**, G624–G632.

Garty, H., Warncke, J. and Lindemann, B. (1987). An amiloride-sensitive Na^+ conductance in the basolateral membrane of toad urinary bladder. *J. Membr. Biol.* **95**, 91–103.

Grinstein, S. (1988). *Na^+/H^+ Exchange*. Boca Raton, FL: CRC Press.

Grinstein, S., Rotin, S. D. and Mason, M. J. (1989). Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. *Biochemistry, N.Y.* **988**, 73–97.

Haggerty, J. G., Agarwal, N., Reilly, R. F., Adelberg, E. A. and Slayman, C. W. (1988). Pharmacologically different Na/H antiporters on the apical and basolateral surfaces of cultured porcine kidney cells (LLC-PK_1). *Proc. natn. Acad. Sci. U.S.A.* **85**, 6797–6801.

Hille, B. (1968). Charge and potentials at the nerve surface: divalent ions and pH. *J. gen. Physiol.* **51**, 221–236.

Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, J. (1973).

Glucose transport in isolated brush border membrane from rat intestine. *J. biol. Chem.* **248**, 25–32.

MAHNENSMITH, R. L. AND ARONSON, P. (1985). The plasma membrane sodium–hydrogen exchanger and its role in physiological and pathophysiological processes. *Circulation Res.* **57**, 773–788.

MONTROSE, M. H. AND MURER, H. (1990). Polarity and kinetics of Na^+/H^+ exchange in cultured opossum kidney cells. *Am. J. Physiol.* **259**, C121–C133.

MURER, H., HOPFER, U. AND KINNE, R. (1976). Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. *Biochem. J.* **154**, 597–560.

MUSCH, M. W., BOOKSTEIN, C., ARVANS, D. L., CRAGOE, E. J., JR, ROE, M. C. AND CHANG, E. B. (1992). Characterization of chicken intestinal brush border membrane Na/H exchange. *Comp. Biochem. Physiol.* **103**A, 439–444.

ORLOWSKI, J., KANDASAMY, R. AND SHULL, G. E. (1992). Molecular cloning of putative members of the Na^+/H^+ exchanger gene family. *J. biol. Chem.* **267**, 9331–9339.

PALMER, L. G. AND FRINDT, G. (1987). Effects of cell Ca and pH on Na channels from rat cortical collecting tubule. *Am. J. Physiol.* **253**, F333–F339.

POST, M. A. AND DAWSON, D. C. (1992). Basolateral Na–H antiporter: uncoupled Na transport produces an amiloride-sensitive conductance. *Am. J. Physiol.* **262**, C1089–C1094.

SARDET, C., FRANCHI, A. AND POUYSSEGUR, J. (1989). Molecular cloning, primary structure and expression of the human growth factor-activatable Na^+/H^+ antiporter. *Cell* **56**, 271–280.

SHETLAR, R. E. AND TOWLE, D. W. (1989). Electrogenic sodium–proton exchange in membrane vesicles from crab (*Carcinus meanas*) gill. *Am. J. Physiol.* **257**, R924–R931.

TOWLE, D. W. (1993). Ion transport systems in membrane vesicles isolated from crustacean tissues. *J. exp. Zool.* **265**, 387–396.

TOWLE, D. W. AND WU, W. S. (1994). Isolation and sequencing of the electrogenic sodium/proton antiporter cDNA from crustacean gill epithelium. *FASEB J.* **8**, A68.

TSE, C. M., MA, A. I., YANG, V. W., WATSON, A., LEVINE, S., MONTROSE, M., POTTER, J., SARDET, C., POUYSSEGUR, J. AND DONOWITZ, M. (1991). Molecular cloning of a cDNA encoding the rabbit ileal villus cell basolateral membrane Na^+/H^+ exchanger. *EMBO J.* **10**, 1957–1967.

TURNER, R. J. (1983). Quantitative studies of cotransport systems: models and vesicles. *J. Membr. Biol.* **76**, 1–15.

VILELLA, S., ZONNO, V., CASSANO, G., MAFFIA, M. AND STORELLI, C. (1991). Na^+/H^+ exchange in the kidneys of eels (*Anguilla anguilla*) adapted to seawater or to freshwater environments: studies with brush border membrane vesicles. *Comp. Biochem. Physiol.* **100**A, 455–460.

ZHUANG, Z., DUERR, J. M. AND AHEARN, G. A. (1995). Ca^{2+} and Zn^{2+} transported by the electrogenic 2 Na^+/1 H^+ antiporter in echinoderm gastrointestinal epithelium. *J. exp. Biol.* **198**, 1207–1217.