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ROLE OF THE INVERTEBRATE ELECTROGENIC $2Na^+/1H^+$ ANTIPORTER IN MONOVALENT AND DIVALENT CATION TRANSPORT

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Summary

In recent years, an electrogenic $2Na^+/1H^+$ antiporter has been identified in a variety of invertebrate epithelial brush-border membranes of gut, kidney and gill tissues. The antiporter differs significantly in its physiological properties from the electroneutral $1Na^+/1H^+$ antiporter proposed for vertebrate cells. In all invertebrate cells examined, the antiporter displayed a 2:1 transport stoichiometry, responded to an induced transmembrane potential and exhibited a high binding affinity for the divalent cation Ca^{2+}, which acted as a competitive inhibitor of Na^+ transport. A monoclonal antibody specific for the crustacean electrogenic antiporter inhibited $2Na^+/1H^+$ exchange, but was without effect on Na^+-dependent D-glucose transport. Immunoreactivity was localized at hepatopancreatic brush-border and vacuolar membranes, antennal gland coelomosac podocytes and posterior gill epithelial cells – all locations where published reports described unique cation exchange kinetics. Significant fractions of Ca^{2+} transport into invertebrate cells across brush-border membranes occurred by an electrogenic, amiloride-sensitive exchange process, probably by the $2Na^+/1H^+$ antiporter, and this transport was markedly inhibited by exogenous zinc and cadmium. A recently identified electroneutral, amiloride-sensitive, hepatopancreatic epithelial basolateral Na^+/H^+ antiporter was uninfluenced by the brush-border monoclonal antibody, exhibited an apparent 1:1 transport stoichiometry and possessed a minimal divalent cation specificity. Calcium transport at this epithelial pole occurred by the combination of a Ca^{2+}/Na^+ antiporter, an ATP-dependent Ca^{2+}-ATPase and a verapamil-sensitive calcium channel. These crustacean brush-border and basolateral transporters may play significant roles in calcification and heavy metal detoxification.

Introduction

A wide variety of epithelial and nonepithelial vertebrate cell types possess a Na^+/H^+ exchange protein in their plasma membranes that catalyzes the net uptake of extracellular sodium coupled to the net extrusion of cytoplasmic protons (Grinstein, 1988). Biological functions of this antiport mechanism include the regulation of intracellular pH and cell volume as well as the transcellular transport of sodium and bicarbonate (Murer *et al.* 1976; Knickelbein *et al.* 1983; Boron and Boulpaep, 1983; Rothstein, 1989).

Key words: Na^+/H^+ exchange, antiport, electrogenic, calcium transport, Ca^{2+}-ATPase, NHE, sodium transport, Ca^{2+}/Na^+ exchange, calcium channel, amiloride, verapamil, hepatopancreas, pyloric ceca, *Homarus americanus, Pycnopodia helianthoides.*

Distinguishing characteristics of the vertebrate electroneutral Na^+/H^+ exchanger include a 1:1 cation exchange stoichiometry for several monovalent cations, an exclusion of divalent cations from binding sites, amiloride sensitivity at the external face of the transporter, an intracellular activator site for protons, which is distinct from another proton binding site on the same membrane surface that is involved in the cation exchange process, and an external sensitivity to quinidine. Aronson and Igarashi (1986) published a model for the vertebrate electroneutral Na^+/H^+ exchanger of renal epithelial brush-border membrane that displays many properties in common with similar exchangers in a large diversity of cell types. Recently, this model has been updated by Wakabayashi *et al.* (1992), who showed that the vertebrate exchanger is an oligomeric phosphorylated glycoprotein with 815 amino acids and 12 membrane-spanning elements.

During the last 5 years, two independent laboratories, using both fluorometric and radioisotopic techniques, have identified a Na^+/H^+ antiporter in brush-border membrane vesicles (BBMV) of gill, gut and renal epithelia from crustaceans and echinoderms. The physiology of the novel antiporter differs significantly from that of the mammalian antiporter in that it exhibits an electrogenic exchange with a transport stoichiometry of $2Na^+/1H^+$ (Ahearn and Clay, 1989; Ahearn and Franco, 1990, 1991; Ahearn *et al.* 1990; Shetlar and Towle, 1989). In crustacean gut cells, two external cation binding sites with dissimilar binding properties were confirmed kinetically by external proton and amiloride inhibition of sodium transport; electrogenicity was demonstrated by the use of an imposed transmembrane electrical potential difference as the only transport driving force for cation exchange (Ahearn and Clay, 1989; Ahearn *et al.* 1990).

A monoclonal antibody has recently been developed to crustacean hepatopancreatic brush-border protein elements that may be the electrogenic $2Na^+/1H^+$ antiporter (de Couet *et al.* 1993). This antibody inhibited $2Na^+/1H^+$ exchange activity by lobster (*Homarus americanus*) hepatopancreatic BBMV, but was without effect on Na^+-dependent D-glucose transport in the same preparation. Immunoreactivity was localized at the hepatopancreatic brush border, in antennal gland coelomosac podocytes and in posterior gill epithelial cells – all locations where unique $2Na^+/1H^+$ exchange kinetics have been identified (Kimura *et al.* 1994). The antibody reacted with the digestive enzyme vacuoles of the hepatopancreatic B-cells that have a role in extracellular digestion, with the brush border of absorptive R-cells and with the calcium storage vacuoles of absorptive R-cells (Fig. 1). On Western blots of hepatopancreatic, antennal gland and gill proteins, this antibody recognized a single 185 kDa protein band with approximately twice the reported size of the mammalian Na^+/H^+ antiporter. The antigen recognized by this antibody may be widely distributed among crustacean epithelial cells, is considerably larger than the vertebrate Na^+/H^+ exchanger and appears to occur in several subcellular locations in the hepatopancreas, where it may perform a variety of different functions as a result of its unique electrogenic cation exchange properties.

This paper will present previously unpublished data concerning the role of the invertebrate electrogenic $2Na^+/1H^+$ antiporter in divalent cation transport by the crustacean hepatopancreatic epithelium and suggest ways by which this transporter may function in the processes of exoskeletal calcification and heavy metal sequestration and detoxification.

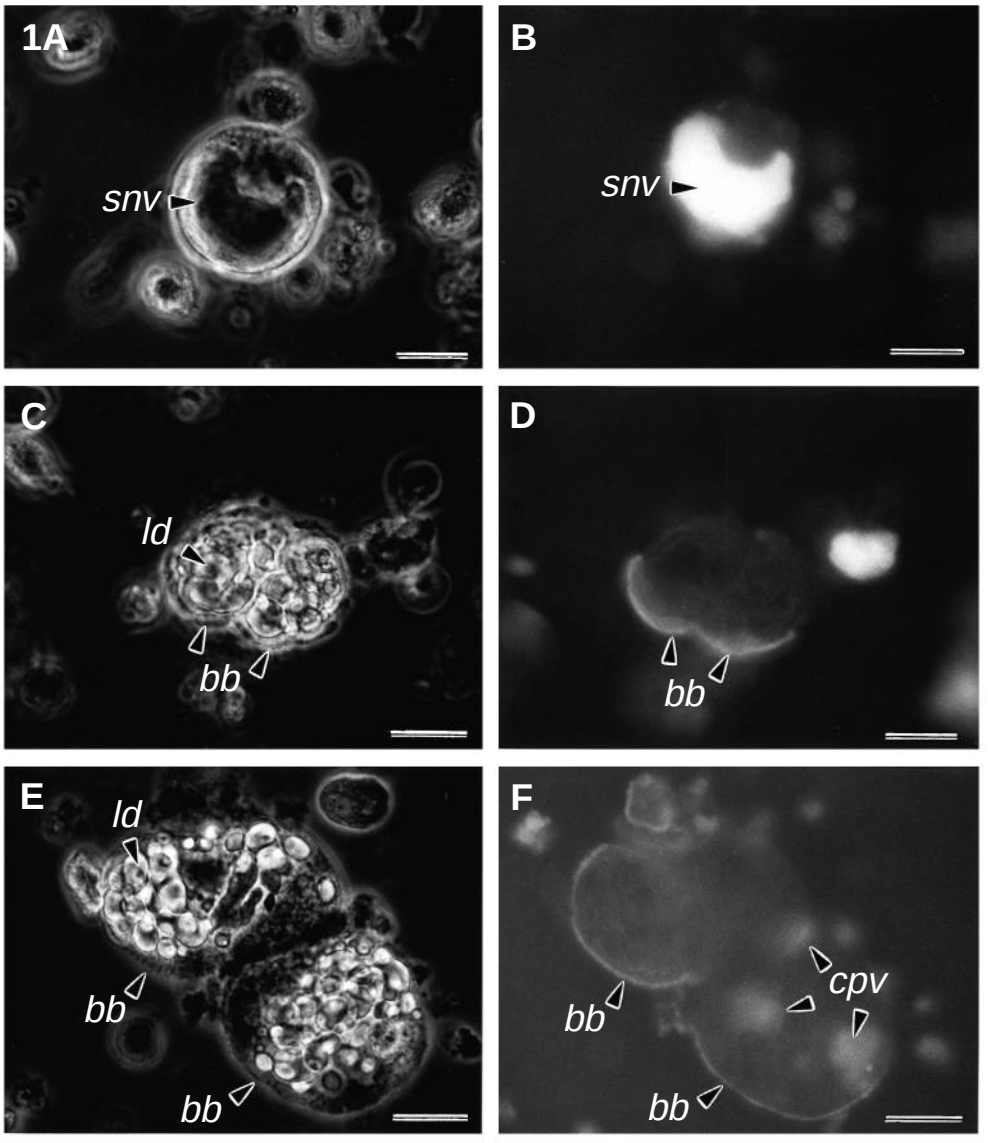

Fig. 1. Immunocytological localization of mouse monoclonal antibody to lobster (*Homarus americanus*) hepatopancreatic epithelial cells in suspension after dissociation from intact tissue. The secondary antibody was a FITC-labelled goat anti-mouse IgG. Control antibody was produced against mammal keratin and was non-reactive to all labelled structures in this figure. (A) Hepatopancreatic B-cell (digestive enzyme producing cell) with a large supranuclear vacuole (*snv*) in phase contrast (A) that is immunogenically reactive in epifluorescence (B). (C) Phase-contrast images of R-cells (absorptive cells) with abundant cytoplasmic lipid droplets (*ld*). (D) An epifluorescent image of the same cells showing immunoreactivity to the antibody localized to the brush border (*bb*). (E) A phase contrast and (F) an epifluorescent picture of two R-cells showing fluorescent labelling of the brush-border membranes and of intracellular vacuoles containing putative calcium phosphate concretions (*cpv*). Scale bars, 10 μm (from Kimura *et al.* 1994).

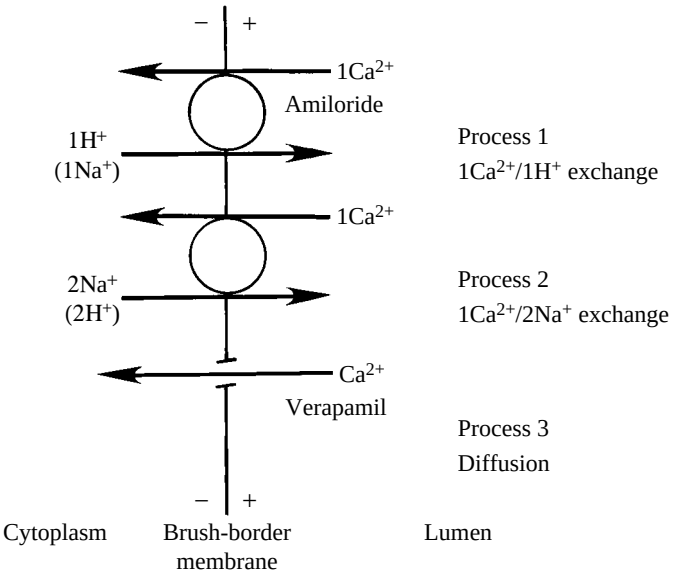

Fig. 2. Proposed Ca^{2+} uptake pathways of lobster (*Homarus americanus*) hepatopancreatic brush-border membrane. Process 1 is an electrogenic $1Ca^{2+}/1H^{+}$ exchanger which is amiloride-sensitive and probably corresponds to the previously described electrogenic $2Na^{+}/1H^{+}$ antiporter in these crustacean epithelia. Process 2 is an electroneutral $1Ca^{2+}/2Na^{+}$ exchanger which is amiloride-insensitive. Process 3 is a verapamil-inhibited, membrane-potential-sensitive, diffusional process that could be a transmembrane Ca^{2+} channel (data from Z. Zhuang and G. A. Ahearn, in preparation).

Role of the electrogenic $2Na^{+}/1H^{+}$ antiporter in brush-border Ca^{2+} transport

The crustacean hepatopancreas is a diverticulum of the gut which consists of a single-layer epithelium that functions in digestive, absorptive and secretory processes. We have isolated, purified and characterized, in membrane vesicles from hepatopancreatic epithelial brush-border and basolateral membranes, transport proteins for a variety of organic and inorganic substances (Ahearn, 1988; Ahearn and Clay, 1988, 1989; Ahearn *et al.* 1990, 1992). The hepatopancreatic brush-border membrane of the Atlantic lobster *Homarus americanus* possesses three transport processes for the uptake of luminal Ca^{2+}: (1) an amiloride-sensitive porter; (2) an amiloride-insensitive porter; and (3) a verapamil-inhibited, non-saturable, diffusion-like process responding to an induced transmembrane electrical potential (Fig. 2; Z. Zhuang and G. A. Ahearn, in preparation). Both porter processes were mediated by antiporters that were capable of exchanging external Ca^{2+} for either intravesicular Na^{+} or H^{+}. The amiloride-sensitive system was electrogenic and exhibited the following Michaelis–Menten kinetic values: (1) K_t=0.58±0.02 mmol l^{-1} (mean ± S.E.M., N=3); (2) J_{max}=1.67±0.04 pmol μg^{-1} protein $3\,s^{-1}$. The amiloride-insensitive system was electroneutral and possessed the following values: (1) K_t=0.52±0.02 mmol l^{-1}; (2) J_{max}=0.55±0.01 pmol μg^{-1} protein $3\,s^{-1}$ (N=3). External amiloride was a competitive inhibitor of $^{45}Ca^{2+}$ influx, inhibiting entry of the divalent cation at a single binding site with a K_i of 920 μmol l^{-1}. A significant fraction of Ca^{2+}

entry into hepatopancreatic epithelial cells was postulated to occur by a previously reported electrogenic $2Na^+/1H^+$ antiporter (Ahearn and Clay, 1989; Ahearn *et al.* 1990), by an electroneutral $2Na^+/1Ca^{2+}$ antiporter (Ahearn and Franco, 1990, 1993) and by a verapamil-sensitive putative Ca^{2+} channel. This array of hepatopancreatic brush-border Ca^{2+} transport processes qualitatively resembled that previously reported for the luminal membrane of lobster antennal glands (Ahearn and Franco, 1993) and suggested that crustacean epithelial cells from different organs may handle this divalent cation by remarkably similar physiological means.

In 1991, the electrogenic $2Na^+/1H^+$ antiporter that had been originally identified only in epithelia of protostomes (e.g. crustaceans) was reported in cells of deuterostomes [e.g. asteriod echinoderm (*Pycnopodia helianthoides*) pyloric ceca epithelia] as well (Ahearn and Franco, 1991), suggesting that this invertebrate exchanger may be widely distributed. As in crustacean cells, $^{22}Na^+/H^+$ exchange by BBMV of the echinoderm epithelium was electrogenic and was inhibited by external amiloride at two external monovalent cation binding sites with markedly dissimilar apparent amiloride affinities (K_i=28 μmol l^{-1} and 1650 μmol l^{-1}). A static head flux ratio analysis revealed a $2Na^+/1H^+$ exchange stoichiometry in which a balance of driving forces (i.e. no net Na^+ flux) was attained with a combination of a 10:1 Na^+ gradient and a 100:1 H^+ gradient. $^{45}Ca^{2+}$ uptake by purified pyloric cecal BBMV of the starfish occurred by electrogenic, amiloride-sensitive and electroneutral, amiloride-insensitive transporters, as found in crustacean epithelia (Z. Zhuang and G. A. Ahearn, in preparation). The amiloride-sensitive antiporter displayed the following apparent kinetic values: K_t=0.31±0.04 mmol l^{-1}; J_{max}= 0.16±0.01 pmol μg^{-1} protein 3 s^{-1} (N=3), whereas values of the amiloride-insensitive system were: K_t=1.11±0.03 mmol l^{-1}; J_{max}=0.88 pmol μg^{-1} 3 s^{-1} (N=3).

Calcium and heavy metal interactions at invertebrate epithelial brush-border membranes

$^{45}Ca^{2+}$ uptake in the presence of an outwardly directed H^+ gradient and induced potential difference ($\Delta\Psi$) (inside negative) by pyloric cecal brush-border membrane vesicles of the starfish *Pycnopodia helianthoides* was significantly reduced by the addition of Zn^{2+} to the external medium (Fig. 3; Z. Zhuang and G. A. Ahearn, in preparation). Similarly, exogenous Fe^{2+} and Cd^{2+} were also found to inhibit Ca^{2+}/H^+ exchange by this membrane preparation. As shown in Fig. 3, exogenous Zn^{2+} acted as a competitive inhibitor of Ca^{2+} uptake, exhibiting a K_i of 6.3 mmol l^{-1}. As discussed above, the apparent Ca^{2+} binding affinity (K_t) of the starfish amiloride-sensitive transporter in pyloric cecal epithelium was 0.31±0.04 mmol l^{-1} (N=3), whereas the competitive inhibitor constant (K_i) for Zn^{2+} was 6.3 mmol l^{-1} (Fig. 3). Because the shared carrier exhibited a 20-fold higher apparent binding affinity for Ca^{2+} than for Zn^{2+}, and because environmental concentrations of Ca^{2+} far exceed those of Zn^{2+} (Forstner and Wittmann, 1979), even in the most highly polluted waters, inhibition of Ca^{2+} uptake by Zn^{2+} from sea water may be minimal. However, starfish are carnivorous animals, and the food they consume, in comparison with sea water, may contain much higher concentrations of Zn^{2+} or other potential heavy metal competitive inhibitors of Ca^{2+} transport as a result of

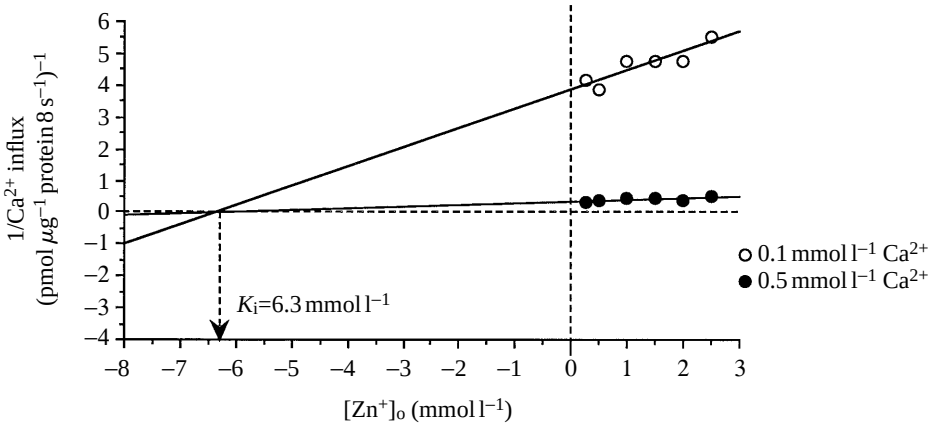

Fig. 3. Dixon plot of an experiment to demonstrate the effects of variable external Zn^{2+} concentrations on $^{45}Ca^{2+}/H^{+}$ exchange in short-circuited starfish (*Pycnopodia helianthoides*) pyloric cecal BBMV. Vesicles were loaded with 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 (25 mmol l^{-1} Mes/Tris) and were incubated for 8 s in medium at pH 8.5 (25 mmol l^{-1} Hepes/Tris) containing either 0.1 or 0.5 mmol l^{-1} $^{45}Ca^{2+}$ gluconate, 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate and one of the following concentrations of $ZnCl_2$: 0.25, 0.5, 1.0, 1.5, 2.0 or 2.5 mmol l^{-1}. Lines were drawn by linear regression analysis (from Z. Zhuang and G. A. Ahearn, in preparation).

bioaccumulation and sequestration mechanisms in the cells of the prey. It is possible that gastrointestinal heavy metal concentrations during prey digestion could be much higher than those found in the surrounding the sea water or sediments and, therefore, could pose a potentially far more serious physiological problem.

A similar experiment was conducted with lobster hepatopancreatic BBMV to ascertain whether heavy metal inhibition of Ca^{2+} uptake by gastrointestinal epithelial cells is a common phenomenon among invertebrates. In this instance, $^{45}Ca^{2+}$ influx (0.1 and 0.5 mmol l^{-1} Ca^{2+}) was observed in proton-loaded vesicles in the presence and absence of external Cd^{2+} (0.01–5 mmol l^{-1} Cd^{2+}). Cadmium significantly reduced the uptake of Ca^{2+} at both concentrations, the metal acting as a competitive inhibitor of $^{45}Ca^{2+}$ influx into BBMV with a K_i of 66.5 μmol l^{-1} (Fig. 4). This value was considerably lower than the K_i determined for Zn^{2+} inhibition of calcium uptake by starfish pyloric ceca and suggests that cadmium may be a more effective binding agent at the calcium transport site than is zinc. These data also suggest that cadmium may exert a significant inhibitory effect on calcium uptake at far lower environmental concentrations than would zinc. Future experiments with these alkaline metal ions will determine whether all agents are capable of moving across the hepatopancreatic brush-border membrane and into the cytoplasm.

Sodium and calcium transport across invertebrate epithelial basolateral membranes

A Percoll gradient centrifugation method originally developed for purifying epithelial basolateral membranes of mammalian cells (Davies *et al.* 1987) was adapted to lobster

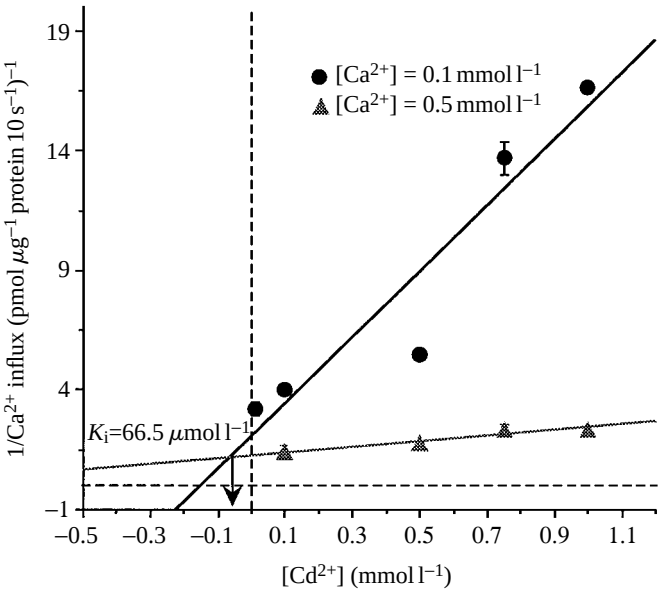

Fig. 4. Dixon plot of the effects of variable external Cd^{2+} concentration on $^{45}Ca^{2+}/H^+$ exchange in short-circuited lobster (*Homarus americanus*) hepatopancreatic BBMV. Vesicles were loaded with 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.85 (25 mmol l^{-1} Hepes/Tris) and were incubated for 10 s in medium at pH 7.85 (25 mmol l^{-1} Hepes/Tris) containing either 0.1 or 0.5 mmol l^{-1} $^{45}Ca^{2+}$ gluconate, 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate and one of the following concentrations of $CdCl_2$: 0.01, 0.1, 0.5, 0.75, 1.0 or 5.0 mmol l^{-1}. Lines were drawn by linear regression analysis. Values are mean ± S.E.M., N=3.

hepatopancreatic tissue. A vesicle band removed from near the top of this gradient was highly enriched in basolateral membrane marker enzymes (Na^+/K^+-ATPase purification factor=15.4-fold) with minimal contamination by brush-border membranes (alkaline phosphatase purification factor=1.2-fold) (Table 1). These putative basolateral membranes were used in flux studies to assess the nature of their cation transport processes.

The presence and characteristics of Na^+/H^+ exchange in purified lobster hepatopancreatic basolateral membrane vesicles (BLMV) were determined by preloading the preparation with buffer at pH 5.5 and incubating it in a medium at pH 8.5 containing 1 mmol l^{-1} $^{22}Na^+$. Some of the vesicles preloaded in this manner were short-circuited (valinomycin; potassium gluconate both sides) or exhibited a transient inside-negative membrane potential (valinomycin; potassium gluconate inside, tetramethylammonium gluconate outside). In some cases, vesicles were exposed to incubation media containing 3 mmol l^{-1} amiloride, 10 mmol l^{-1} calcium or an inhibitory monoclonal antibody (de Couet *et al.* 1993).

A transmembrane pH gradient plus a transient potential difference ($\Delta\Psi$) caused a rapid uptake of $^{22}Na^+$ in exchange for protons (Fig. 5A). The fourfold overshoot phenomenon was maximal at approximately 1 min of incubation and gradually returned to equilibrium by 20 min. A transmembrane pH gradient alone stimulated the uptake of $^{22}Na^+$ about

Table 1. *Enzyme characterization of hepatopancreas basolateral membrane*

Enzyme	Activity of homogenate (μmol mg^{-1} protein h^{-1})	Activity of the BLMV (μmol mg^{-1} protein h^{-1})	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±0.50

Values are mean ± S.E.M.
Enzyme activities are in μmol product released per mg protein per hour.
Purification factors were calculated from the mean enzyme activities of the homogenate and vesicles.
Enzyme measurements were performed on three different membrane preparations.

twofold over its equilibrium value. The presence of 3 mmol l^{-1} amiloride completely blocked uptake of the isotope (Fig. 5A). Calcium was a partial inhibitor of the $^{22}Na^+$ uptake overshoot process, presumably interacting with Na^+ on the Ca^{2+}/Na^+ antiporter described below (Fig. 5B). In contrast, the presence of a monoclonal antibody against protein elements of the brush-border electrogenic $2Na^+/1H^+$ antiporter, which has previously been shown to inhibit the exchange process in BBMV (de Couet *et al.* 1993), was without effect on Na^+/H^+ exchange in BLMV.

In order to assess the nature of $^{22}Na^+$ influx by BLMV, vesicles were loaded at pH 5.5 with an induced inside-negative $\Delta\Psi$ and were incubated for 2.5 s in medium containing $^{22}Na^+$ concentrations varying from 5 to 100 mmol l^{-1}. After correction for non-specific $^{22}Na^+$ binding to membranes and filters, the entry rate of sodium was a hyperbolic function of external sodium concentration (Fig. 6A). Apparent kinetic constants for basolateral sodium entry are displayed on the figure. In contrast with the hyperbolic kinetics of influx data for basolateral Na^+/H^+ exchange in lobster hepatopancreas, influx data for monovalent cation antiport in BBMV preparations from hepatopancreas from the marine lobster, the freshwater prawn (*Macrobrachium rosenbergii*) and the seawater starfish (*Pycnopodia helianthoides*) were all sigmoidal functions of exogenous sodium concentration (Fig. 6B; Ahearn *et al.* 1990; Ahearn and Franco, 1991). These comparisons between the Na^+/H^+ exchange kinetics of invertebrate brush-border and basolateral membranes suggest that markedly dissimilar transporters are present in the two locations and that this difference may be related to the apparent number of cation binding sites on each protein.

Further evidence for a difference in the apparent number of sodium binding sites on brush-border and basolateral Na^+/H^+ antiporters in lobster hepatopancreatic epithelium was obtained by examining the inhibitory effect of external amiloride on the exchange process in BLMV. Hepatopancreatic BLMV were loaded at pH 5.5 (inside-negative $\Delta\Psi$) and were exposed for 5 s to external medium containing $^{22}Na^+$ at either 1 or 5 mmol l^{-1} and concentrations of amiloride ranging from 0.05 to 1 mmol l^{-1}. Amiloride inhibited Na^+/H^+ exchange in BLMV by acting as a competitive inhibitor at a single binding site (single slope at each $[Na^+]$) on the antiporter (Fig. 7, Dixon plot). The single inhibitor constant (K_i=108.6 μmol l^{-1}) fell near the range for the effect of this drug on Na^+/H^+ exchange in vertebrate cells (i.e. 10–100 μmol l^{-1}). In contrast with the results from

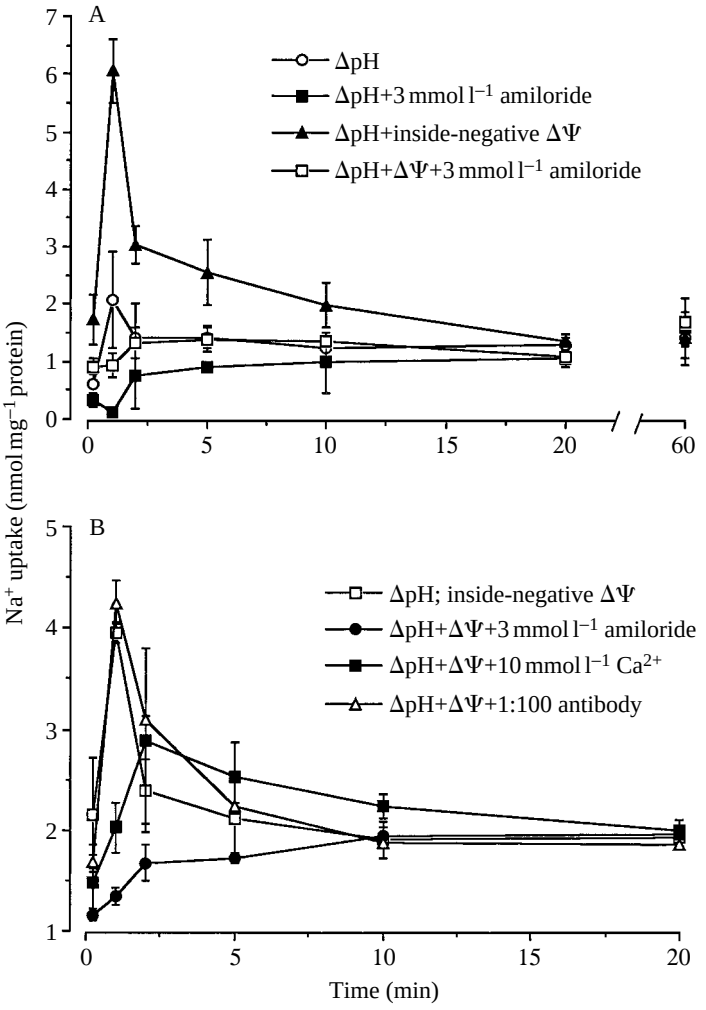

Fig. 5. (A) Time course of $^{22}Na^+/H^+$ exchange by lobster hepatopancreatic BLMV: effects of $\Delta\Psi$ and amiloride. Vesicles were loaded with 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 (50 mmol l^{-1} Mes/Tris) and were incubated in medium at pH 8.5 (50 mmol l^{-1} Hepes/Tris) containing 1 mmol l^{-1} $^{22}Na^+$ gluconate, 100 mmol l^{-1} mannitol and either 100 mmol l^{-1} potassium gluconate (short-circuited) or 100 mmol l^{-1} tetramethylammonium (TMA^+) gluconate (inside-negative $\Delta\Psi$). In some cases, 3 mmol l^{-1} amiloride was added to the external medium. (B) Time course of $^{22}Na^+/H^+$ exchange by lobster hepatopancreatic BLMV: effects of external Ca^{2+} and brush-border monoclonal antibody. Vesicles were prepared as described above (inside negative $\Delta\Psi$) but, in this instance, one group of membranes was incubated in medium containing 10 mmol l^{-1} calcium gluconate, while another was exposed to a 1:100 dilution of an inhibitory brush-border antibody. Values are mean ± S.E.M., N=3.

BLMV, amiloride was a competitive inhibitor of $2Na^+/H^+$ exchange by invertebrate brush-border membranes at two external sites with markedly dissimilar apparent binding affinities (Ahearn and Clay, 1989; Ahearn *et al.* 1990; Ahearn and Franco, 1991).

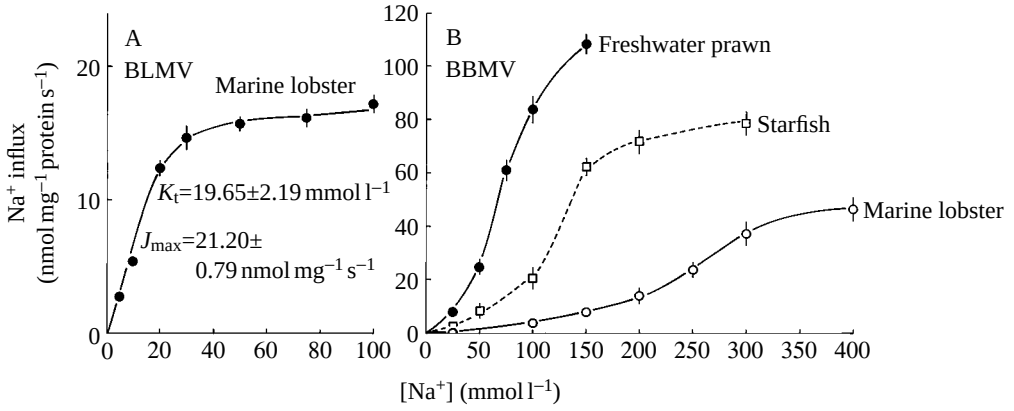

Fig. 6. (A) $^{22}Na^+/H^+$ exchange kinetics of lobster hepatopancreatic epithelium: BLMV properties. Vesicles were loaded at pH 5.5 with an inside-negative $\Delta\Psi$ as described in Fig. 5 and were incubated for 2.5 s in medium at pH 8.0 containing variable concentrations of $^{22}Na^+$ gluconate from 5 to 100 mmol l^{-1}. The hyperbolic line and apparent influx kinetics were obtained from an iterative computer curve-fitting program. (B) $^{22}Na^+/H^+$ exchange kinetics of lobster hepatopancreatic epithelium: BBMV properties. Vesicles were loaded as above and in Fig. 5 and were incubated for 2.5 s in medium at pH 8.5 containing variable concentrations of $^{22}Na^+$ gluconate from 5 to 400 mmol l^{-1}. Sigmoidal lines were obtained from an iterative computer curve-fitting program. Brush-border exchange kinetics from the freshwater prawn (*Macrobrachium rosenbergii*) and the starfish (*Pycnopodia helianthoides*) are also displayed for comparative purposes (data from Ahearn *et al.* 1990; Ahearn and Franco, 1991). Values are mean ± S.E.M., N=3.

These results with hepatopancreatic BLMV suggest that Na^+/H^+ exchange at the basolateral surface of the epithelium is significantly different from the analogous process at the brush border. Whereas influx of Na^+ in BBMV occurs by an electrogenic exchanger with two external cation binding sites, entry of the cation at the basolateral border takes place by a transporter with a single sodium/amiloride binding site and, therefore, presumably displays an electroneutral $1Na^+/1H^+$ exchange stoichiometry. Electrogenicity demonstrated in Fig. 5A under these conditions would result from the simultaneous operation of a membrane-potential-sensitive ion channel accommodating a diffusional flow of Na^+ in response to an imposed membrane potential.

A conductive Na^+ channel in the hepatopancreatic basolateral membrane is suggested by the results in Fig. 8. In this experiment, ΔpH was zero and $\Delta\Psi$ was the only driving force affecting the movements of $^{22}Na^+$. The uptake of isotope by BLMV with an inside-negative $\Delta\Psi$ was faster than that of vesicles possessing an electrically positive $\Delta\Psi$ (Fig. 8). Exposure of vesicles under both conditions to a low concentration of amiloride (10 μmol l^{-1}) significantly reduced (P<0.05) their respective apparent initial uptake rates between 15 s and 1 min compared with that of vesicles lacking the drug. These results suggest that an amiloride-sensitive conductive ion channel is present in the basolateral membrane and support identification of the electroneutral Na^+/H^+ antiport discussed above.

In three different BLMV experiments, hepatopancreatic Ca^{2+} transport by basolateral

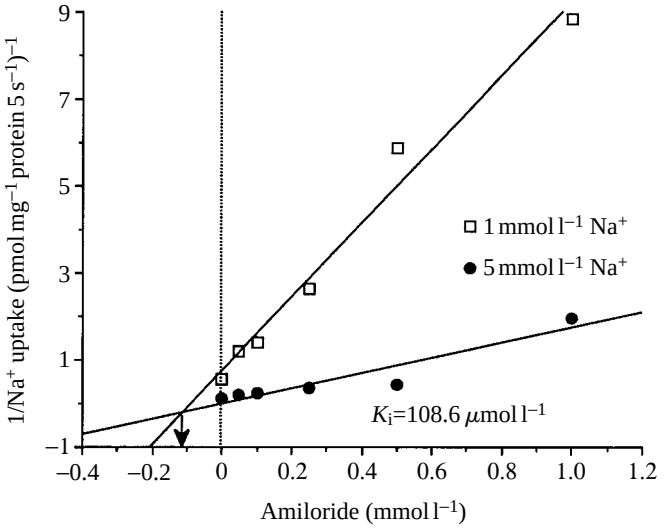

Fig. 7. Dixon plot of an experiment to show the effects of variable external amiloride concentrations on $^{22}Na^+/H^+$ exchange in lobster hepatopancreatic BLMV loaded at pH 5.5 (inside-negative $\Delta\Psi$) and exposed for 5 s to external medium at pH 8.0 containing either 1 or 5 mmol l^{-1} $^{22}Na^+$ gluconate and concentrations of amiloride ranging from 0.05 to 1 mmol l^{-1}. Lines were drawn by linear regression analysis.

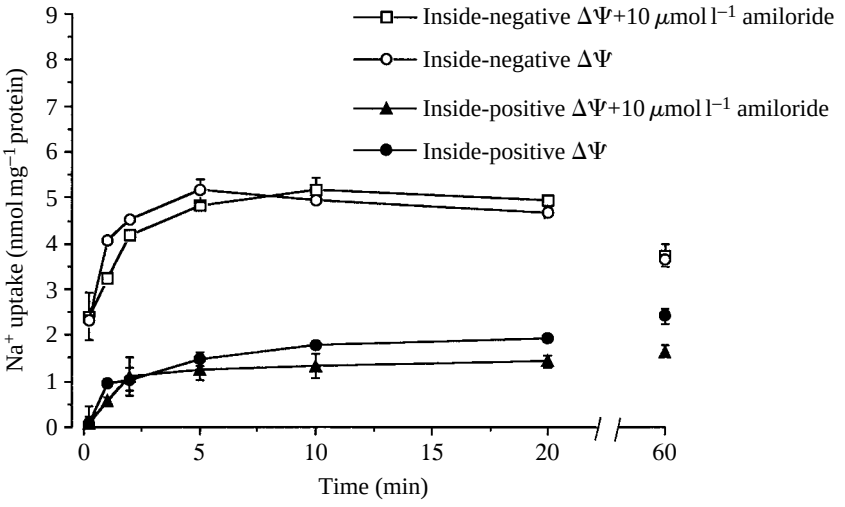

Fig. 8. Effect of a transmembrane $\Delta\Psi$ as the sole driving force for $^{22}Na^+$ uptake by lobster hepatopancreatic BLMV. Vesicles were loaded with either 100 mmol l^{-1} TMA^+ gluconate (induced inside-positive $\Delta\Psi$) or 100 mmol l^{-1} potassium gluconate (induced inside-negative $\Delta\Psi$) and 50 mmol l^{-1} valinomycin at pH 7.0 (25 mmol l^{-1} Hepes/Tris) and were incubated in external medium at the same pH containing 1 mmol l^{-1} $^{22}Na^+$ gluconate and either 100 mmol l^{-1} potassium gluconate or 100 mmol l^{-1} TMA^+ gluconate. In some cases, 10 μmol l^{-1} amiloride was added to the external medium. Values are mean ± S.E.M., N=3.

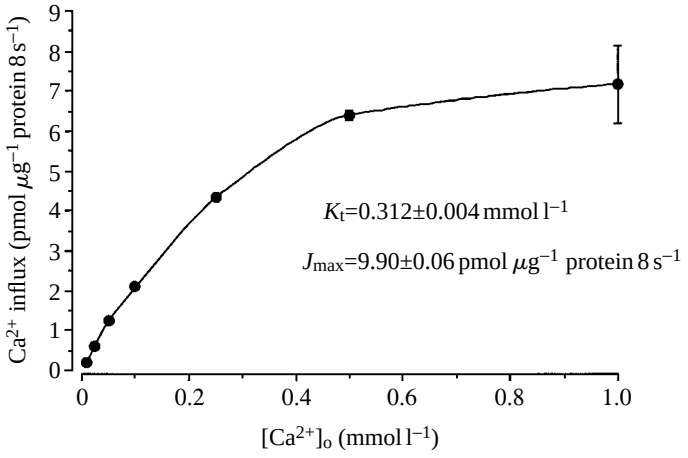

Fig. 9. Effect of external Ca^{2+} concentration on the kinetics of $^{45}Ca^{2+}/Na^+$ exchange in lobster hepatopancreatic BLMV. Vesicles were loaded with 50 mmol l^{-1} sodium gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 7.5 (25 Hepes/Tris) and were incubated for 8 s in medium of the same pH containing 50 mmol l^{-1} TMA^+ gluconate, 50 mmol l^{-1} potassium gluconate and varying external calcium gluconate concentrations from 0.01 to 1.0 mmol l^{-1}. Kinetic constants were obtained from an iterative computer curve-fitting program. Values are mean ± S.E.M., N=3.

membranes was compared with divalent cation uptake by the brush-border membrane. The first experiment was designed to assess whether a Ca^{2+}/Na^+ exchanger is present on the basolateral membrane. Short-circuited vesicles were loaded with 50 mmol l^{-1} sodium at pH 7.5 and were incubated for 8 s in medium of the same pH containing $^{45}Ca^{2+}$ at Ca^{2+} concentrations from 0.01 to 1 mmol l^{-1}. There was a hyperbolic relationship between $^{45}Ca^{2+}$ influx, by Ca^{2+}/Na^+ exchange, and external calcium concentration (Fig. 9). These data provide evidence for an antiporter with the following apparent kinetic constants for Ca^{2+} influx: K_t=0.312±0.004 mmol l^{-1}; J_{max}=9.90±0.06 pmol μg^{-1} protein $8\,s^{-1}$ (N=3). Detailed characterization of this antiporter and comparison of it with the brush-border Ca^{2+}/H^+ antiporter are planned.

The presence of a Ca^{2+}-ATPase in lobster hepatopancreatic BLMV was confirmed in a second series of experiments with vesicles prepared without any transmembrane ion gradients or $\Delta\Psi$ as potential driving forces. In these experiments $^{45}Ca^{2+}$ uptake by BLMV was measured in the presence of 5 mmol l^{-1} exogenous ATP and in the presence and absence of the ATPase inhibitor vanadate. ATP significantly increased the rate of $^{45}Ca^{2+}$ accumulation and vanadate abolished it (Fig. 10). These results suggest the presence of a Ca^{2+}-ATPase in this membrane and imply that intracellular calcium regulation in lobsters may involve the integration of secondary Ca^{2+}/Na^+ antiport and primary Ca^{2+}-ATPase transport.

The possible presence of a calcium channel in the hepatopancreatic basolateral membrane was investigated in the third group of experiments by eliminating all possible driving forces for $^{45}Ca^{2+}$ uptake by BLMV except for an imposed transmembrane $\Delta\Psi$. In this instance, K^+ and valinomycin were used to establish a transient inside-negative or

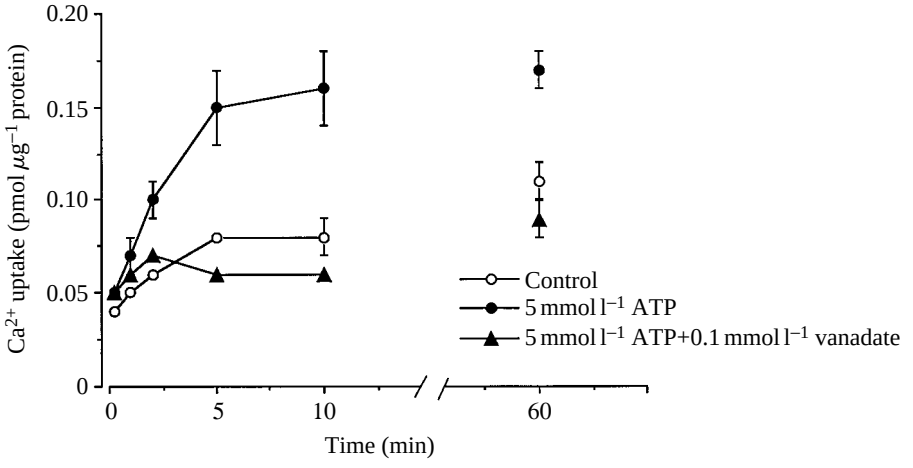

Fig. 10. Evidence for an ATP-dependent Ca^{2+}-ATPase in lobster hepatopancreatic BLMV. Vesicles were loaded with 100 mmol l^{-1} KCl and 5 mmol l^{-1} $MgCl_2$ at pH 7.4 (25 mmol l^{-1} Hepes/Tris) and were incubated in medium of the same pH containing 5 mol l^{-1} $^{45}Ca^{2+}$ gluconate, 5 mmol l^{-1} Tris–ATP, 100 mmol l^{-1} KCl and 5 mmol l^{-1} $MgCl_2$. In one instance, 0.1 mmol l^{-1} vanadate was added to the external medium as an ATPase inhibitor. Values are mean ± S.E.M., N=3.

inside-positive $\Delta\Psi$ in BLMV and the Ca^{2+} channel blocker verapamil was added to the external medium to block diffusional flow of the divalent cation across the membrane in response to the established $\Delta\Psi$. In the presence of an inside-negative $\Delta\Psi$, $^{45}Ca^{2+}$ uptake was enhanced compared with that in the condition involving the application of an inside-positive $\Delta\Psi$ (Fig. 11). In addition, external 100 μmol l^{-1} verapamil significantly reduced $\Delta\Psi$-driven $^{45}Ca^{2+}$ flux. These data suggest that a verapamil-sensitive calcium channel may occur in the hepatopancreatic basolateral membrane and may play a significant role in calcium regulation.

Potential roles of cation transporters in calcification and heavy metal detoxification

Invertebrate gastrointestinal diverticula, such as the crustacean hepatopancreas or echinoderm pyloric ceca, perform a wide range of physiological activities that include digestion of food and absorption of amino acids, sugars, lipids and other organic solutes (Ahearn *et al.* 1992). In addition, these tissues secrete enyzmes, acids and other ionic substances such as the divalent anions (Cattey *et al.* 1994). These organs also appear to have a significant, but less studied, role in divalent cation regulation. The crustacean hepatopancreatic epithelium is known to be a cyclical storage site for exoskeletal calcium during the premolt phase of ecdysis. As an animal approaches molting, much of the calcium that is contained in the exoskeleton is transferred to the blood and either lost to the environment, across the gills and other permeable sites, or stored as calcium phosphate granules in hepatopancreatic epithelial cells and elsewhere (Greenaway, 1985). Following the loss of the old skeleton, hepatopancreatic calcium is mobilized and again transferred to the blood, where integumentary uptake mechanisms rapidly

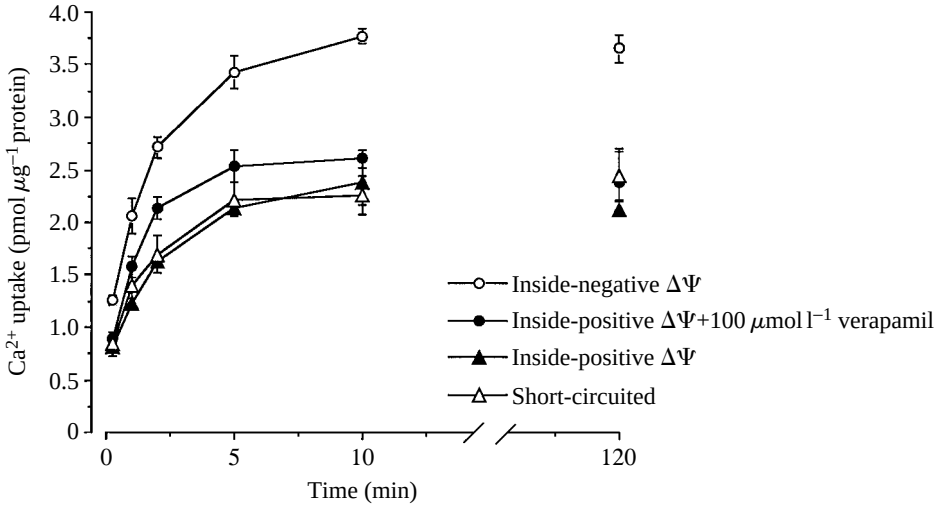

Fig. 11. Effect of a transmembrane $\Delta\Psi$ as the sole driving force for $^{45}Ca^{2+}$ uptake by lobster hepatopancreatic BLMV. Vesicles were either loaded with 100 mmol l^{-1} TMA^+ gluconate (induced inside-positive $\Delta\Psi$) or 100 mmol l^{-1} potassium gluconate (induced inside-negative $\Delta\Psi$ and short-circuited condition) and 50 μmol l^{-1} valinomycin at pH 7.0 (25 mmol l^{-1} Hepes/Tris) and were incubated in external medium at the same pH containing 0.1 mmol l^{-1} $^{45}Ca^{2+}$ gluconate and either 100 mmol l^{-1} potassium gluconate or 100 mmol l^{-1} TMA^+ gluconate where appropriate. In one case, 100 mol l^{-1} verapamil was added to the external medium. Values are mean ± S.E.M., N=3.

incorporate the divalent cation into the newly formed shell. Little is known about uptake or loss mechanisms for calcium by either hepatopancreatic brush-border or basolateral membranes during different phases of the crustacean molt cycle and even less is understood about the processes responsible for sequestration of the divalent cation in cytoplasmic calcium phosphate vacuoles.

It is known that hepatopancreatic calcium phosphate ‘concretions’ contain a variety of divalent and trivalent cationic heavy metals that are accumulated from the environment or diet and are concentrated in vacuoles by the epithelium as complexes with sulfate and phosphate (Viarengo, 1989; Viarengo and Nott, 1993). Concentrations of metals in vacuolar concretions can be several 100-fold higher than environmental concentrations of the individual metal. Heavy metal concretion formation is a detoxification mechanism used by numerous invertebrates to remove and isolate potentially lethal substances from the blood. Such concretions are eventually eliminated from the storage cells by exocytosis into the gastrointestinal lumen, where they are voided from the animal in the feces. The mechanisms by which divalent and trivalent heavy metal cations enter invertebrate epithelial cells, and the processes responsible for their sequestration, and therefore their detoxification, within epithelial vacuolar concretions are unknown. This study has begun to shed some light on the physiological mechanisms responsible for divalent cation transport across specific invertebrate gastrointestinal epithelial cell membranes that are closely involved in the processes of calcification and heavy metal detoxification.

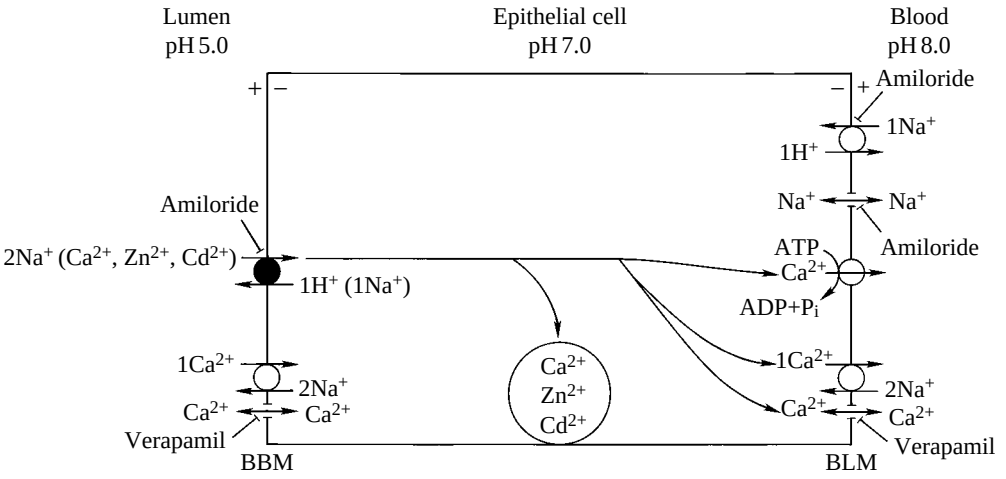

Fig. 12. Working model of monovalent and divalent cation transport mechanisms of crustacean hepatopancreatic epithelial cells. The brush-border membrane (BBM) possesses the three transport processes described in Fig. 2, but this model also accounts for heavy metal (e.g. Zn^{2+}, Cd^{2+}, etc.) inhibition of Ca^{2+} influx by way of a shared amiloride-sensitive transporter which is probably the electrogenic $2Na^+/1H^+$ antiporter described previously. On the basolateral side of the cell (BBM), two apparent transport processes for Na^+ and three systems for Ca^{2+} may be located. In contrast to the electrogenic brush-border mechanism, basolateral Na^+/H^+ exchange probably occurs by an amiloride-sensitive, electroneutral system, which may be linked to a conductive Na^+ channel that responds to an induced $\Delta\Psi$. An ATP-dependent Na^+/K^+-ATPase, of undefined kinetic properties, is also probably present on this membrane (Table 1). Basolateral Ca^{2+} transfer may take place by an ATP-dependent Ca^{2+}-ATPase, a Ca^{2+}/Na^+ exchanger and a verapamil-sensitive ion channel. During the processes of calcification and detoxification, Ca^{2+} and heavy metals may also be accumulated within epithelial vacuoles as 'concretion' mixtures of cations and either sulfate or phosphate. This accumulation process may involve one or more of the plasma membrane transport proteins or channels described above.

Fig. 12 is a working model of monovalent and divalent cation transport mechanisms of the crustacean epithelial hepatopancreatic brush-border and basolateral membranes. Echinoderm pyloric cecal epithelial cells exhibit brush-border transporters similar to those described in this model, but the kinetic details of each mechanism are not currently as well clarified as those for crustaceans. Calcium and the heavy metal ions Zn^{2+} and Cd^{2+} are able to bind competitively to the previously described electrogenic $2Na^+/H^+$ antiporters of hepatopancreatic and pyloric cecal brush-border membranes. The relative apparent affinities of the shared carrier for the cations were: $Cd^{2+} > Ca^{2+} > Zn^{2+}$. Whether the bound metals eventually enter the epithelial cytoplasm and undergo subsequent intracellular physiological sequestration is unknown. Fig. 1 shows the subcellular localization of the antigen for the monoclonal antibody produced against protein elements of the electrogenic brush-border cation exchanger to hepatopancreatic calcium phosphate concretion vacuolar membranes and suggests that the same exchanger may be present in this location as occurs on the luminal membrane and that it may regulate Ca^{2+} and metal sequestration events at these vacuoles as well as cellular cation uptake across the brush

border. Potential roles of the other brush-border cation transport mechanisms in heavy metal transport remain to be discovered, particularly in view of a recent study (Verbost *et al.* 1989) suggesting that cadmium may enter fish branchial epithelial cells through calcium channels.

Na^+/H^+ exchange at the hepatopancreatic basolateral membrane appears to use an electroneutral antiporter in combination with a membrane-potential-sensitive Na^+ channel, rather than the electrogenic $2Na^+/H^+$ antiporter (Fig. 12). The apparent electroneutral basolateral antiporter of hepatopancreas may be an isoform of the NHE gene family which has been thoroughly characterized in vertebrate cells (Tse *et al.* 1993) and which may also occur in the nematode *Caenorhabditis elegans* (Marra *et al.* 1993).

Three apparently distinct Ca^{2+} transport processes have been identified on the hepatopancreatic basolateral membrane: (1) a Ca^{2+}/Na^+ antiporter; (2) an ATP-dependent calcium ATPase, and (3) a verapamil-sensitive Ca^{2+} channel (Fig. 12). All of these proteins may be involved in intracellular Ca^{2+} regulation during intermolt, when an expected net absorption of Ca^{2+} from the luminal contents to the blood might occur in order to provide sufficient levels of the cation in the blood to supply organismic requirements. At premolt, when a large flux of Ca^{2+} into the blood from the old exoskeleton occurs, significant participation of the suggested basolateral verapamil-sensitive Ca^{2+} channel in uptake from the blood might occur prior to intracellular vacuolar sequestration.

Numerous invertebrate representatives of the Arthropoda, Mollusca, Echinodermata, Annelida and other phyla possess epithelial concretion vacuoles, which are apparently involved in detoxifying heavy metals. The mechanism(s) of metal sequestration and detoxification by these vacuoles has not been identified in any of these animals. Considerable work remains to substantiate the details of the model shown in Fig. 12 for crustacean hepatopancreatic epithelium and to link each transport mechanism to biological activities, such as calcification and heavy metal detoxification. In addition, separate studies of calcium phosphate concretion vacuolar membranes in crustaceans have yet to be undertaken to identify the processes responsible for metal and complexing anion accumulation. Characterization of the transport events associated with Ca^{2+} and heavy metal metabolism in this group of animals may provide an insight into universal cellular processes for regulating divalent cation levels in many invertebrate phyla.

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