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Ca^{2+} TRANSPORT PROCESSES OF LOBSTER HEPATOPANCREATIC BRUSH-BORDER MEMBRANE VESICLES

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Summary

$^{45}Ca^{2+}$ uptake by hepatopancreatic brush-border membrane vesicles of Atlantic lobster (*Homarus americanus*) occurred by a combination of three independent processes: (1) an amiloride-sensitive carrier-mediated transport system; (2) an amiloride-insensitive carrier-mediated transport system; and (3) a verapamil-inhibited channel process responsive to transmembrane potential. Both carrier-mediated processes were antiporters and capable of exchanging external Ca^{2+} with intravesicular Na^+ or H^+. The kinetic parameters of both carrier-mediated processes have been reported previously. External amiloride and Zn^{2+} were both competitive inhibitors of $^{45}Ca^{2+}$ influx, reducing entry of the divalent cation at a single binding site with K_i values of 370 $\mu mol\,l^{-1}$ for amiloride and 940 $\mu mol\,l^{-1}$ for Zn^{2+}. It is concluded that the mechanisms controlling Ca^{2+} entry into hepatopancreatic epithelial cells include a previously reported electrogenic $2Na^+/1H^+$ antiporter, an electroneutral $2Na^+/1Ca^{2+}$ antiporter and a verapamil-sensitive Ca^{2+} channel, which might also be used for the entry of Zn^{2+} and possibly other heavy metals. Evidence from an equilibrium-shift experiment, based on the thermodynamics of a coupled transport process, suggested that both monovalent (Na^+) and divalent (Ca^{2+} and Zn^{2+}) cations may enter hepatopancreatic epithelial cells through a common carrier-mediated transport protein. This suite of hepatopancreatic brush-border Ca^{2+} transport processes qualitatively resembles that previously reported for the luminal membrane of lobster antennal glands and suggests that crustacean epithelial cells from different organs may handle this divalent cation by similar means.

Key words: Na^+/Ca^+ exchange, Na^+/H^+ exchange, Ca^{2+} channel, antiporter, electrogenic, hepatopancreas, lobster, *Homarus americanus.*

Introduction

Transcellular Ca^{2+} transport in vertebrate intestinal or renal epithelia consists of passive uptake across the brush-border membrane into the cytoplasm down a concentration gradient and active transport up a concentration gradient into the blood across the basolateral membrane (Bronner, 1989; Carafoli, 1984; Van Os, 1987). The carrier-mediated brush-border entry mechanism in these animals has been characterized as a uniport process responsive to the transapical membrane potential and stimulated by the presence of vitamin D (Liang *et al.* 1986; Rasmussen *et al.* 1982; Schachter and Kowarski, 1982).

The crustacean hepatopancreas has been characterized as an organ with digestive, absorptive and secretory functions (Ahearn *et al.* 1992; Gibson and Barker, 1979; Lozzi, 1971; Yonge, 1924), based historically on morphological and histochemical evidence and, more recently, on the results from experiments using purified hepatopancreatic epithelial brush-border and basolateral membrane vesicles. Previous studies on ecdysis in crustaceans have also suggested that this organ may play a critical role in the storage of Ca^{2+} during the molt cycle, sequestering the ion in calcium phosphate or calcium sulfate granules within absorptive epithelial cells during intermolt and premolt, and releasing it to the hemolymph for incorporation into the new exoskeleton during postmolt (Johnson, 1980). Recent studies characterizing the ion transport mechanisms of the crustacean hepatopancreas, using purified epithelial brush-border membrane vesicles, have disclosed the presence of an apparently unique electrogenic $2Na^+/1H^+$ antiporter that differs significantly in its physiological properties from the more thoroughly investigated electroneutral $1Na^+/1H^+$ exchanger of mammals (Ahearn and Clay, 1989; Ahearn and Franco, 1990; Aronson, 1985). Additional experiments using brush-border vesicles of lobster hepatopancreas have also shown an electrogenic anion antiporter which catalyzes the exchange of 1 SO_4^{2-} for 1 Cl^- and presumably leads to the secretion of the divalent ion (Cattey *et al.* 1992). At present, there are few detailed studies describing the transport properties of the crustacean hepatopancreatic luminal membrane for Ca^{2+} even though this ion is of major significance during molting.

Vesicle studies with lobster antennal gland epithelium have shown that a portion of the total $^{45}Ca^{2+}$ flux across the luminal membrane of this organ occurs by way of the electrogenic

$2Na^+/1H^+$ antiporter that is also present in this membrane, with Ca^{2+} and Na^+ competing for binding to the external face of the transport system (Ahearn and Franco, 1990, 1993). Because the same electrogenic $2Na^+/1H^+$ antiporter occurs in both the kidney and the hepatopancreas of the lobster, the role of this antiporter in Ca^{2+} uptake from dietary constituents is of interest. The present investigation employed brush-border membrane vesicles of lobster hepatopancreatic epithelium to characterize and compare quantitatively the mechanisms for Ca^{2+} uptake into this organ. Results suggest that influx of the divalent cation occurred by three distinct processes: (1) an amiloride-sensitive carrier mechanism, which is probably the electrogenic $2Na^+/1H^+$ antiporter; (2) an amiloride-insensitive carrier mechanism, which appears to be an electroneutral $1Ca^{2+}/2Na^+$ exchanger; and (3) a verapamil-sensitive ion channel, which may also play a role in the entry of Zn^{2+} into the hepatopancreatic epithelial cells.

Materials and methods

Live Atlantic lobster (*Homarus americanus* H. Milne Edwards; 0.5 kg each) were purchased from commercial dealers in Hawaii and maintained unfed at 10 °C for up to 1 week in filtered sea water. All animals were in either intermolt or early premolt as assessed by the molt stage classification scheme introduced by Aiken (1973).

Hepatopancreatic brush-border membrane vesicles (BBMVs) were prepared from fresh tissue removed from individual lobsters. Each membrane batch was produced from one or two organs using a method of combined osmotic disruption, differential centrifugation and magnesium precipitation described previously (Ahearn *et al.* 1985). Marker enzyme assays confirmed that vesicles prepared by these methods were highly enriched in brush-border membranes of all four cell types, with minimal contamination from basolateral or organelle membranes.

Transport experiments were conducted at 20 °C using the rapid filtration technique developed by Hopfer *et al.* (1973). For time-course experiments, a volume of vesicles (e.g. 20 μl; approximately 0.2 mg total protein) was added to a volume of incubation medium (e.g. 160 μl) containing $^{45}Ca^{2+}$. At various incubation times, a known volume (20 μl) of the reaction mixture was pipetted out and plunged into 2 ml of ice-cold stop solution (stop solution composition varied in different experiments and generally consisted of incubation medium without any calcium) to stop the Ca^{2+} uptake process. The vesicle suspension was then rapidly filtered through 0.65 μm Millipore filters (presoaked in distilled water overnight) and washed with another 6 ml of ice-cold stop solution. Filters were transferred to scintillation vials, which were filled with Beckman Ready Solv HP scintillation cocktail and measured for radioactivity in a Beckman LS-8100 scintillation counter.

Transport experiments involving incubations shorter than 10 s were conducted using a rapid-exposure uptake apparatus (Inovativ Labor, Adliswil, Switzerland). Uptake was initiated by mixing 3 μl of vesicles with 24 μl of radiolabeled incubation medium (the same ratio as above), and filters were washed and counted for radioactivity as above. For all experiments, a 'blank uptake' was performed for each condition by mixing vesicles and radiolabeled incubation medium and then almost simultaneously (within less than 1 s after the mixing) adding the ice-cold stop solution. The resulting value was subtracted from corresponding experimental results before the final uptake was determined. Incubation and intravesicular media varied for different experiments and compositions are indicated in the figure legends. Ca^{2+} uptake values were expressed as picomoles per microgram of protein (Bio-Rad protein assay) per filter using the specific activity of Ca^{2+} in the incubation media.

To confirm the closure of hepatopancreatic brush-border membrane vesicles (BBMVs) and to substantiate that Ca^{2+} transport by these vesicles was into an osmotically reactive space rather than just binding to the membrane surface, 90 min equilibrium uptake of 0.05 mmol l^{-1} $^{45}Ca^{2+}$ by BBMVs was assessed at a series of transmembrane osmotic gradients. Vesicles were loaded with 200 mmol l^{-1} mannitol and 25 mmol l^{-1} Hepes/Tris at pH 7.0 and were incubated in identical external media containing labeled Ca^{2+} and 0–900 mmol l^{-1} sucrose.

For equilibrium shift experiments, short-circuited vesicles were loaded with 150 mmol l^{-1} KCl, 5 mmol l^{-1} Hepes and 50 μmol l^{-1} valinomycin at pH 6.0 and were pre-incubated in an identical medium containing 0.05 mmol l^{-1} $^{45}Ca^{2+}$ gluconate at pH 8.0. At the end of this pre-incubation period, triplicate samples of the reaction mixture were taken to estimate vesicular isotope content. Subsequently, a small volume of a highly concentrated solution of mannitol, Na^+, Ca^{2+} or Zn^{2+} (final concentrations in reaction mixture were 10 mmol l^{-1} for mannitol and Na^+, 1 mmol l^{-1} for Ca^{2+} and Zn^{2+}) was added to the remaining reaction mixtures to initiate the countertransport process. Triplicate samples of these reaction mixtures were subsequently taken at 0.5, 1.5, 3, 5 and 60 min, plunged into ice-cold stop solution, filtered, and counted for radioactivity.

Unless otherwise indicated, valinomycin (50 μmol l^{-1}) and bilaterally equal K^+ concentrations across the vesicular wall were present to short-circuit the membranes. Each experiment was generally repeated three times using membranes prepared from different animals. Within a given experiment, each point was determined from 3–5 replicate samples. Data are presented as mean ± S.E.M. of a single representative experiment (N=3–5 for each mean value in a figure). Similar qualitative findings were obtained in the repetition of an experiment. Data were analyzed using the computer program SigmaPlot (Jandel), which provides an iterative best fit to experimental values.

$^{45}Ca^{2+}$ was obtained from New England Nuclear Corp., Boston, USA; reagent-grade chemicals, valinomycin, amiloride, verapamil, tetramethylammonium hydroxide (TMA-OH) and D-gluconic acid lactone, were purchased from Sigma Chemical Co., St Louis, USA.

Results

Osmotic reactivity and $^{45}Ca^{2+}$ binding properties of lobster hepatopancreatic BBMVs

Fig. 1 indicates that a significant (r^2=0.94, P=0.01) linear relationship existed between vesicular $^{45}Ca^{2+}$ content at equilibrium for all membrane preparations and the reciprocal of the incubation medium osmolarity. Extrapolation of the line to the vertical axis provided an index of non-specific surface $^{45}Ca^{2+}$ binding to vesicles at equilibrium and amounted to approximately 40 % of total $^{45}Ca^{2+}$ uptake under control osmotic conditions (0 mmol l^{-1} sucrose) after 90 min of incubation. These results suggest that hepatopancreatic brush-border vesicles were sealed, osmotically reactive and displayed a significant binding component that had to be considered during subsequent influx assessments. In order to reduce the contribution of non-specific binding to total $^{45}Ca^{2+}$ uptake values, blanks were made during all uptake experiments by exposing membranes and isotope simultaneously to ice-cold stop solution, and then filtering immediately to collect vesicles for the counting of $^{45}Ca^{2+}$. The resulting bound activity was subtracted from total uptake at selected exposure intervals to provide estimates of transport activity alone.

Components of apparent $^{45}Ca^{2+}$ uptake

Our previous studies with lobster hepatopancreatic BBMVs suggested that an outwardly directed proton gradient provided an adequate driving force for vesicular accumulation of exogenous $^{45}Ca^{2+}$ by electrogenic $2Na^+/1H^+$ antiport and that this accumulation was enhanced in the presence of an induced membrane potential (K^+, valinomycin, negative inside) (Ahearn and Zhuang, 1996). Four components may contribute to this apparent $^{45}Ca^{2+}$ accumulation: (1) non-specific binding to filters; (2) external binding to membranes; (3) internal binding to membranes; and (4) accumulation in an intravesicular soluble Ca^{2+} pool. To evaluate the contribution of each of these components to the total $^{45}Ca^{2+}$ uptake, vesicles were loaded with an internal medium at pH 5.5 and incubated with $^{45}Ca^{2+}$ at pH 8.5. The results shown in Fig. 2 indicate that after taking samples at 20 min, the addition of the Ca^{2+} ionophore A23187 (20 μmol l^{-1}) and EGTA (5 mmol l^{-1}) to the incubation mixture produced a fall in the $^{45}Ca^{2+}$ accumulation. Addition of 5 mmol l^{-1} EGTA alone caused a similar decrease but not as great as the one produced in the presence of A23187 and EGTA, while addition of A23187 alone led to no such decrease (Brunette and Leclerc, 1992). Incubation of the reaction mixture for 2 min in ice-cold stop solution with the cation chelator EGTA or with EGTA plus the

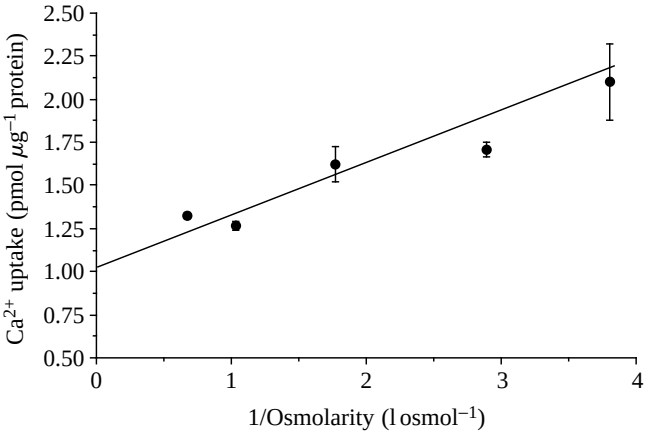

Fig. 1. The effect of a transmembrane osmotic gradient on equilibrium uptake of $^{45}Ca^{2+}$ by brush-border membrane vesicles (BBMVs) from lobster hepatopancreas. Vesicles were loaded with 200 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes/Tris at pH 7.0 and were incubated for 90 min in an identical medium containing 0.05 mmol l^{-1} $^{45}Ca^{2+}$ gluconate and one of the following concentrations of sucrose in (mmol l^{-1}): 900, 600, 300, 100, 50 or 0. Osmolarity was measured using a Wescor 5500 vapor pressure osmometer. The line drawn on the figure was computed using linear regression analysis, while symbols are means ± S.E.M. (N=5 for each mean value, N=5 for sample size of regression analysis, r^2=0.94, P=0.01).

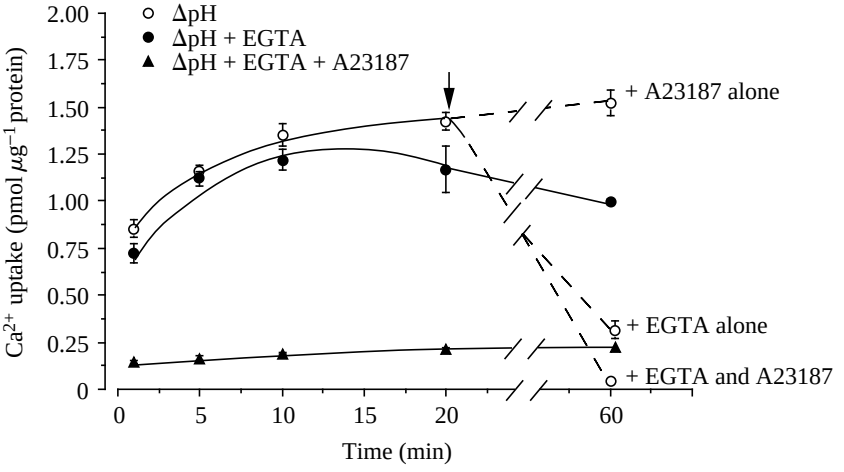

Fig. 2. The effect of an outwardly directed proton gradient (ΔpH) on $^{45}Ca^{2+}$ uptake by hepatopancreatic BBMVs. Vesicles were loaded with 100 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 (25 mmol l^{-1} Mes/Tris) and were incubated in media at pH 8.5 (25 mmol l^{-1} Hepes/Tris) containing 0.05 mmol l^{-1} $^{45}Ca^{2+}$ gluconate, 50 mmol l^{-1} potassium gluconate and an appropriate quantity of mannitol to maintain osmolarity. One sample of vesicles exhibiting an outwardly directed proton gradient was exposed for 2 min, after each incubation period, to ice-cold stop solution containing 5 mmol l^{-1} EGTA before assessing its radioactivity. A second sample was incubated for 2 min in ice-cold stop solution containing 5 mmol l^{-1} EGTA plus 20 μmol l^{-1} A23187 before measuring its radioactivity. The third sample was stopped in a general ice-cold stop solution without incubation as a control and, after 20 min (as shown by the arrow), the whole reaction mixture was divided into three equal parts. To one of them A23187 was added, to another one EGTA, and to the last one A23187 plus EGTA (all having the same final concentrations as above). Bars represent ± S.E.M., N=3 for each mean value.

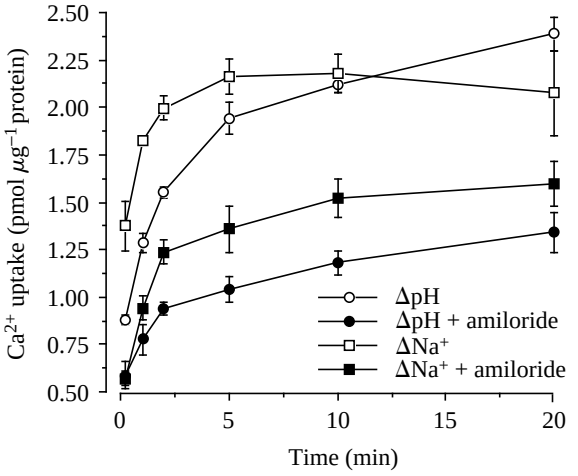

Fig. 3. The effects of 4 mmol l^{-1} amiloride on the exchange of internal H^+ or Na^+ with external $^{45}Ca^{2+}$. Vesicles were loaded with either 50 mmol l^{-1} TMA gluconate, 25 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 or 50 mmol l^{-1} sodium gluconate, 25 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 7.5, and were incubated in media at either pH 8.5 or pH 7.5 containing 0.05 mmol l^{-1} calcium gluconate, as well as appropriate TMA gluconate and potassium gluconate concentrations. Two other groups of vesicles exhibiting either an outwardly directed proton gradient or a Na^+ gradient (ΔNa^+) were exposed to 4 mmol l^{-1} amiloride. Bars represent ± S.E.M., N=3 for each mean value.

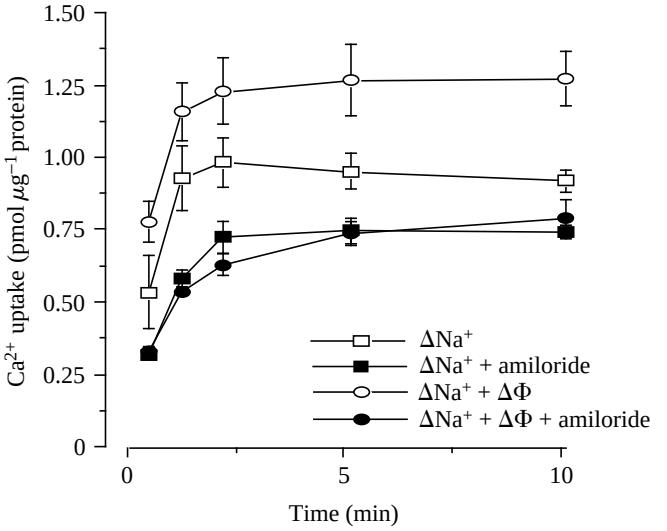

Fig. 4. The effects of 4 mmol l^{-1} amiloride on the exchange of internal Na^+ with external $^{45}Ca^{2+}$ in the presence or absence of a potential difference (ΔΦ) across the vesicle membrane. Vesicles were loaded with 50 mmol l^{-1} sodium gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 8.0 and were then incubated in media at pH 8.0 containing 0.05 mmol l^{-1} calcium gluconate and either 50 mmol l^{-1} TMA gluconate and 50 mmol l^{-1} potassium gluconate or 100 mmol l^{-1} TMA gluconate only. One sample of vesicles possessing an outwardly directed Na^+ gradient (ΔNa^+) either with or without an induced potential difference was exposed to 4 mmol l^{-1} amiloride. Bars represent ± S.E.M., N=3 for each mean value.

Ca^{2+} ionophore, following isotope exposure for the time indicated in buffer with an imposed transmembrane pH gradient, provided further evidence that 5 mmol l^{-1} EGTA alone removed a fraction of $^{45}Ca^{2+}$ activity. This fraction probably corresponded to non-specific external binding (approximately 10 %). The presence of both the ionophore and the chelator led to a loss in the intravesicular load of $^{45}Ca^{2+}$, which included the osmotically reactive component, of about 80 %. The remaining radioactivity that could not be removed was probably internally bound. Because of these different uptake components, isotope equilibration between the internal soluble pool and the external medium might not be readily apparent in time-course experiments.

The effect of external amiloride on Na^+- or H^+-gradient-stimulated $^{45}Ca^{2+}$ uptake in short-circuited vesicles is shown in Fig. 3. $^{45}Ca^{2+}$ uptake by these vesicles was greater with an outwardly directed Na^+ gradient than when an outwardly directed H^+ gradient was employed, but in both instances the presence of 4 mmol l^{-1} amiloride strongly reduced the effect of the monovalent cation gradients on the uptake of the divalent cation, suggesting that a significant portion of $^{45}Ca^{2+}$ uptake by these vesicles occurred by way of an amiloride-sensitive transport system. The control condition, short-circuited vesicles in the absence of any exchangeable cations, has been described previously (Ahearn and Zhuang, 1996) and is also shown in Figs 5 and 6.

Stimulation of $^{45}Ca^{2+}$ uptake in the presence of a transmembrane potential (Ahearn and Zhuang, 1996) suggests that an electrogenic process is partially responsible for the exchange of $^{45}Ca^{2+}$ with either Na^+ or H^+ in the absence of amiloride. In contrast, the results displayed in Fig. 4 suggest that the cation exchange that continued in the presence of amiloride was probably an electroneutral process, since in the presence of amiloride, there was no difference in $^{45}Ca^{2+}$ uptake by vesicles with or without a potential difference across the vesicle membrane. The results shown in Figs 5 and 6 indicate that incubation of the vesicles with 100 μmol l^{-1} verapamil abolished the stimulatory effect of membrane potential on the cation exchange, suggesting the occurrence of a Ca^{2+} channel in the brush-border membrane. Such an inhibitory effect was also found in vesicles with only an outwardly directed Na^+ gradient, but not in vesicles with a H^+ gradient, suggesting that part of the Ca^{2+}/Na^+ exchange mechanism was verapamil-sensitive and that this might be an electroneutral, amiloride-insensitive Ca^{2+}/nNa^+ carrier-mediated system.

Initial rate of $^{45}Ca^{2+}/H^+$ exchange

To establish an exposure interval that approximated to the initial $^{45}Ca^{2+}$ uptake rate, the time course of Ca^{2+} transfer by BBMVs with an induced transmembrane electrical potential and an outwardly directed proton gradient was examined at two different external Ca^{2+} concentrations ($[Ca^{2+}]_e$, 0.025 and 0.5 mmol l^{-1}) for very short time intervals (1–15 s), using a rapid uptake apparatus that automatically controlled incubation time to 1 s. Fig. 7 shows that, after subtraction of non-specific

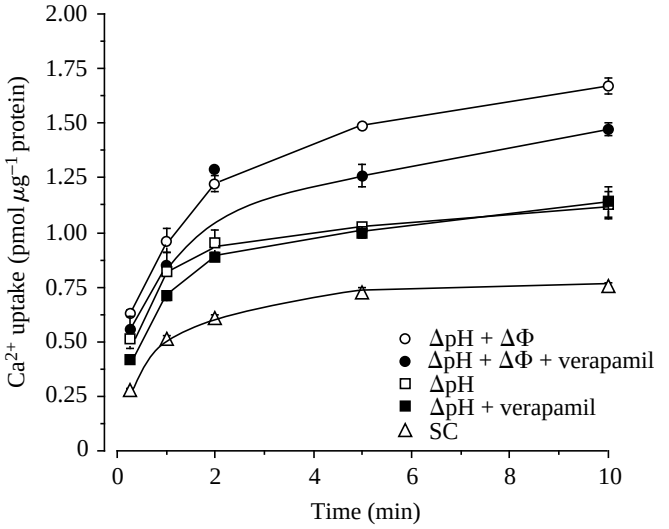

Fig. 5. Effects of an outwardly directed proton gradient (ΔpH), with an inside-negative transmembrane potential difference (ΔΦ) and verapamil on $^{45}Ca^{2+}$ uptake by hepatopancreatic BBMVs. Vesicles were loaded with 100 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 (25 mmol l^{-1} Mes/Tris) and were incubated in media at pH 8.5 (25 mmol l^{-1} Hepes/Tris) containing 0.05 mmol l^{-1} $^{45}Ca^{2+}$ gluconate and 100 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate or 200 mmol l^{-1} mannitol to produce short-circuited (SC) conditions or imposed membrane potential conditions. One sample of vesicles possessing an outwardly directed proton gradient and a potential difference was preincubated with 100 μmol l^{-1} verapamil for 30 min. A second sample of vesicles with the proton gradient alone was also preincubated with 100 μmol l^{-1} verapamil for 30 min. Bars represent ± S.E.M., N=3 for each mean value.

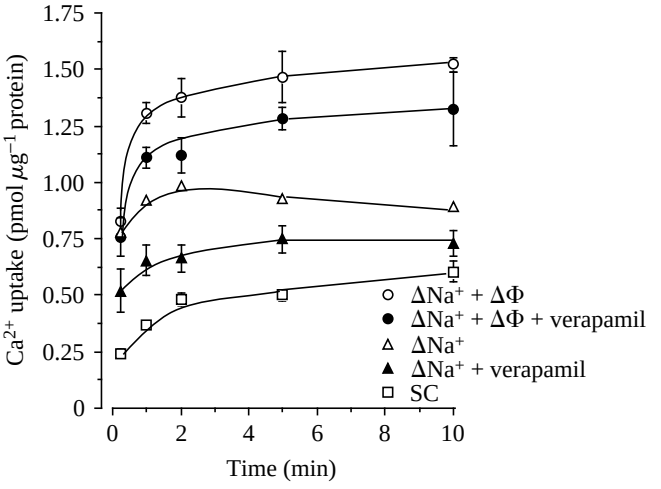

Fig. 6. The effects of an outwardly directed Na^+ gradient (ΔNa^+) with an inside-negative transmembrane potential difference (ΔΦ) and verapamil on $^{45}Ca^{2+}$ uptake by hepatopancreatic BBMVs. Vesicles were loaded with 50 mmol l^{-1} sodium gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 8.0 (25 mmol l^{-1} Hepes/Tris) and were incubated in media at pH 8.5 (25 mmol l^{-1} Hepes/Tris) containing 0.05 mmol l^{-1} $^{45}Ca^{2+}$ gluconate, and 50 mmol l^{-1} sodium gluconate, 50 mmol l^{-1} potassium gluconate or 200 mmol l^{-1} mannitol, producing short-circuited (SC) conditions or imposed membrane potential conditions. One sample of vesicles possessing an outwardly directed Na^+ gradient and a potential difference was preincubated with 100 μmol l^{-1} verapamil for 30 min. A second sample of vesicles with only a Na^+ gradient was also preincubated with 100 μmol l^{-1} verapamil for 30 min. Bars represent ± S.E.M., N=3 for each mean value.

binding, uptake of $^{45}Ca^{2+}$ was a linear function of time from 1 to 6 s for 0.025 mmol l^{-1} $[Ca^{2+}]_e$ and from 1 to 4 s for 0.5 mmol l^{-1} $[Ca^{2+}]_e$. Slopes of linear regression lines through the data provided estimates of unidirectional $^{45}Ca^{2+}$ influx at each $[Ca^{2+}]_e$. In the absence of amiloride, $^{45}Ca^{2+}$ influx at 0.025 mmol l^{-1} $[Ca^{2+}]_e$ was 0.02 pmol μg^{-1} protein s^{-1}, while at 0.5 mmol l^{-1} $[Ca^{2+}]_e$ it was 0.91 pmol μg^{-1} protein s^{-1}. When 2 mmol l^{-1} amiloride was added to the incubation medium, these influx rates were decreased to 0.015 and 0.21 pmol μg^{-1} protein s^{-1}, respectively. The occurrence of a significant accumulation of $^{45}Ca^{2+}$ in the presence of 2 mmol l^{-1} amiloride may indicate either that insufficient amiloride was used to block $^{45}Ca^{2+}$ uptake completely by the amiloride-sensitive transport pathway or that amiloride-insensitive processes for $^{45}Ca^{2+}$ transfer, which are stimulated by outwardly directed proton gradients and membrane potential, may also occur in this membrane.

Nature of amiloride inhibition of $^{45}Ca^{2+}/H^+$ exchange

Figs 3 and 4 indicate that 4 mmol l^{-1} amiloride significantly reduced $^{45}Ca^{2+}/H^+$ or $^{45}Ca^{2+}/Na^+$ exchange in lobster hepatopancreatic BBMVs. In order to characterize the nature of the inhibitory action of this drug on the antiport process, an experiment was conducted in which short-circuited vesicles possessing an outwardly directed proton gradient (pHi 5.5; pHe 8.5) were incubated for 3 s in media containing either 0.05 or 0.25 mmol l^{-1} calcium gluconate and one of the following concentrations of amiloride: 0, 0.1, 0.25, 0.5, 1, 2, 4 or 8 mmol l^{-1}. Fig. 8A shows that increasing external amiloride concentration had a marked inhibitory effect on uptake of $^{45}Ca^{2+}$ over 3 s at both 0.05 and 0.25 mmol l^{-1} Ca^{2+}. Significant reductions in $^{45}Ca^{2+}$ entry were observed at every amiloride concentration used (P=0.05), and no significant differences were seen among the concentrations of 2, 4 and 8 mmol l^{-1}. Fig. 8 also indicates that some $^{45}Ca^{2+}$ influx still occurred at maximal concentrations of amiloride, suggesting that an amiloride-insensitive transport process is still operative at these extreme inhibitor concentrations.

Fig. 8B is a Dixon plot of the $^{45}Ca^{2+}$ influx over 3 s by short-circuited hepatopancreatic BBMVs. Both $[Ca^{2+}]_e$ values exhibited Dixon plots with single slopes over the range of external amiloride concentrations used, suggesting that the drug inhibited $^{45}Ca^{2+}$ transport at a single binding site. The amiloride K_i value derived from this graphical evaluation of the data was 370 μmol l^{-1}, very close to one of the apparent amiloride binding affinities reported for the kidney of the same animal (Ahearn and Franco, 1990).

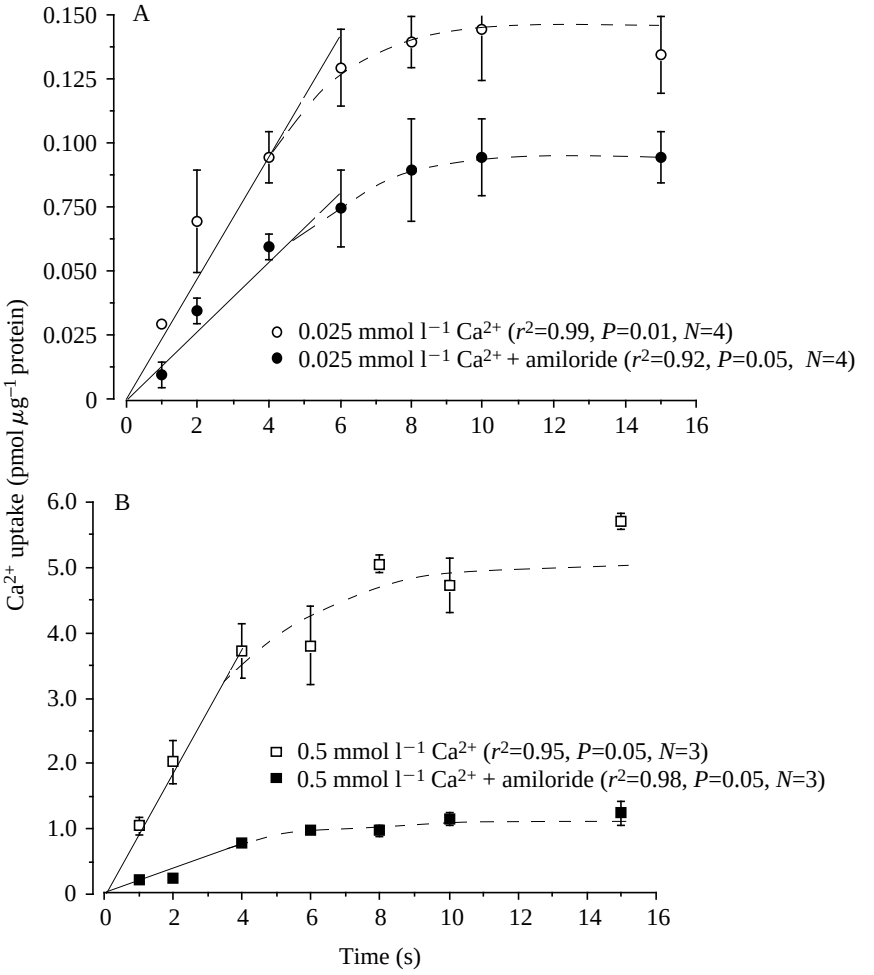

Fig. 7. The initial rate of $^{45}Ca^{2+}/H^+$ exchange in short-circuited hepatopancreatic BBMVs. Vesicles were loaded with 50 mmol l^{-1} TMA gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 (Mes/Tris) and were then incubated in media at pH 8.5 (Hepes/Tris) containing either 0.025 mmol l^{-1} (A) or 0.5 mmol l^{-1} (B) $^{45}Ca^{2+}$ gluconate and 100 mmol l^{-1} TMA gluconate for osmotic balance. Bars represent ± S.E.M. Amiloride was added at 2 mmol l^{-1}.

Nature of Zn^{2+} inhibition of $^{45}Ca^{2+}/H^+$ exchange

The same experimental approach as above was used to characterize the nature of the inhibitory action of Zn^{2+} on Ca^{2+} uptake, as shown in Fig. 9A. Short-circuited vesicles with an outwardly directed proton gradient (pHi 5.5; pHe 8.5) were incubated for 3 s in media containing either 0.05 or 0.25 mmol l^{-1} calcium gluconate and one of the following concentrations of $ZnCl_2$: 0, 0.25, 0.5, 1, 3, 5 or 10 mmol l^{-1}. Fig. 9A shows that increasing external Zn^{2+} concentration had a marked inhibitory effect on uptake of $^{45}Ca^{2+}$ over 3 s at both Ca^{2+} concentrations. Significant reductions in $^{45}Ca^{2+}$ entry were observed at every Zn^{2+} concentration used (P=0.05) and maximal inhibition occurred at 5 and 10 mmol l^{-1} Zn^{2+}.

Fig. 9B is a Dixon plot of 3 s $^{45}Ca^{2+}$ influx data by short-circuited hepatopancreatic BBMVs. Both levels of $[Ca^{2+}]_e$ exhibited Dixon plots with single slopes over the range of external Zn^{2+} concentrations used, suggesting that Zn^{2+} inhibited $^{45}Ca^{2+}$ transport at a single binding site. The Zn^{2+} K_i value of 940 μmol l^{-1} is about the same as the apparent K_i reported for the pyloric ceca of starfish (*Pycnopodia helianthoides*) (Zhuang *et al.* 1995).

Effect of internal H^+ concentration on $^{45}Ca^{2+}/H^+$ exchange

The influence of an outwardly directed proton gradient on the initial rate of 0.05 mmol l^{-1} $^{45}Ca^{2+}$ uptake (3 s incubations) by short-circuited hepatopancreatic BBMVs was investigated in the presence of 4 mmol l^{-1} amiloride. All uptake values were corrected for non-specific binding as discussed previously. Fig. 10A indicates that $^{45}Ca^{2+}$ influx was a hyperbolic function of internal proton concentration both in the presence and in the absence of amiloride, suggesting that $^{45}Ca^{2+}/H^+$ exchange by these membrane preparations occurred by a combination of both amiloride-sensitive and amiloride-insensitive carrier-mediated transfer processes, each possessing a rate that can be described by the Michaelis–Menten equation:

$$J = (J_{max} \times [H^+]_i)/(K_H + [H^+]_i), \quad (1)$$

where J is total $^{45}Ca^{2+}$ influx in pmol μg^{-1} protein $3\,s^{-1}$, J_{max} is the apparent maximal carrier-mediated influx, K_H is the apparent proton concentration (μmol l^{-1}) resulting in half-maximal uptake and $[H^+]_i$ is the intravesicular proton concentration.

The amiloride-sensitive component of total $^{45}Ca^{2+}$ influx at each $[H^+]_i$ (Fig. 10B) was obtained by subtracting each individual Ca^{2+} uptake value in the presence of the drug from the corresponding mean uptake value in its absence (Fig. 10A) to yield an estimate of the two components of $^{45}Ca^{2+}$ entry (Fig. 10B,C). A nonlinear, iterative best-fit computer program

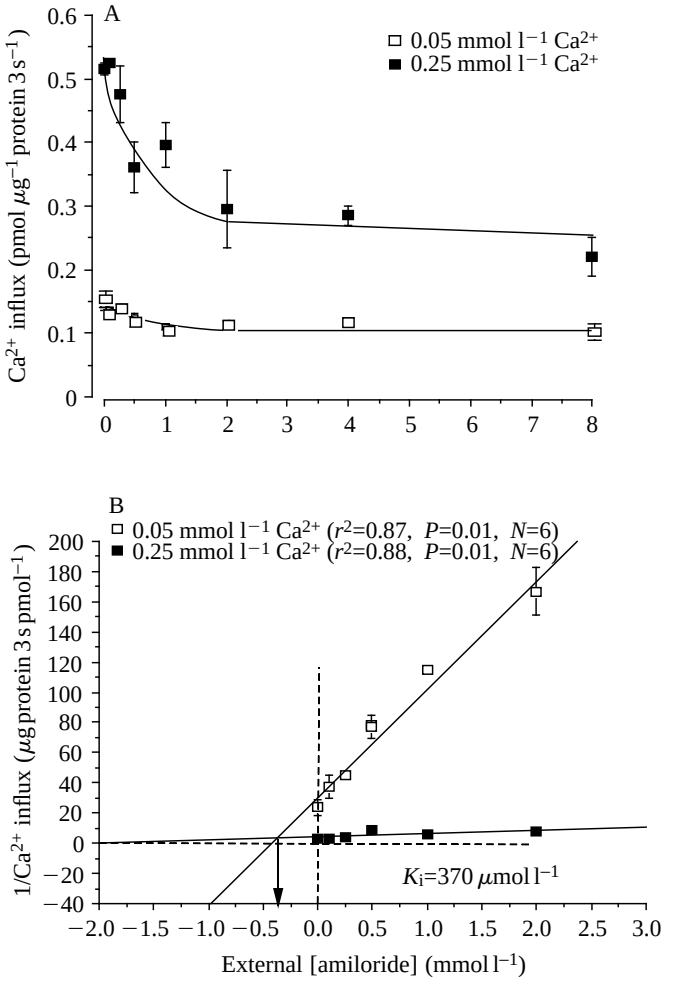

Fig. 8. (A) Effects of external variable amiloride concentrations on $^{45}Ca^{2+}/H^+$ exchange in short-circuited hepatopancreatic BBMVs. Vesicles were loaded with 50 mmol l^{-1} TMA gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 (Mes/Tris), and were then incubated for 3 s in media containing either 0.05 or 0.25 mmol l^{-1} $^{45}Ca^{2+}$ gluconate, 50 mmol l^{-1} TMA gluconate, 50 mmol l^{-1} potassium gluconate at pH 8.5 (25 mmol l^{-1} Hepes/Tris) and one of the following concentrations of amiloride: 0, 0.1, 0.25, 0.5, 1, 2, 4 or 8 mmol l^{-1}. (B) Dixon plot of data from A. Lines are drawn by linear regression analysis. Arrow shows the K_i value. Bars represent ± S.E.M., *N* indicates the sample size of the regression analysis.

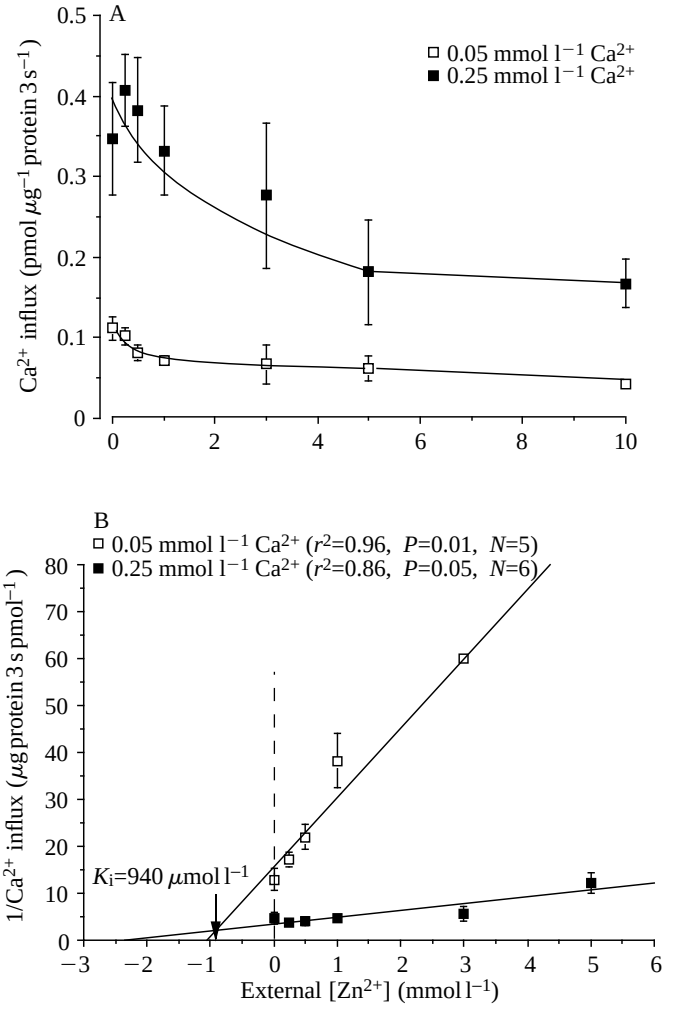

Fig. 9. (A) The effects of external variable Zn^{2+} concentrations on $^{45}Ca^{2+}/H^+$ exchange in short-circuited hepatopancreatic BBMVs. Vesicles were loaded with 50 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 (Mes/Tris), and were then incubated for 3 s in media containing either 0.05 or 0.25 mmol l^{-1} $^{45}Ca^{2+}$ gluconate, 50 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate at pH 8.5 (25 mmol l^{-1} Hepes/Tris) and one of the following concentrations of $ZnCl_2$: 0, 0.25, 0.5, 1, 3, 5 or 10 mmol l^{-1}. (B) Dixon plot of data from A. Lines are drawn by linear regression analysis. Arrow shows the K_i value. Bars represent ± S.E.M., *N* represents the sample size of the regression analysis.

was utilized to analyze the data in Fig. 10B,C using equation 1. The apparent kinetic constants for the two carrier processes calculated in this manner were, for the amiloride-sensitive system, apparent K_H=0.29±0.02 μmol l^{-1} and apparent J_{max}=0.19±0.002 pmol μg^{-1} protein 3 s^{-1} and for the amiloride-insensitive system, apparent K_H=0.32±0.04 μmol l^{-1} and apparent J_{max}=0.032±0.001 pmol μg^{-1} protein 3 s^{-1}.

Effects of internal Na^+ concentrations on $^{45}Ca^{2+}/Na^+$ exchange

The effects of intravesicular Na^+ concentration ($[Na^+]_i$) on 3 s influx of 0.05 mmol l^{-1} $^{45}Ca^{2+}$ in short-circuited BBMVs are displayed in Fig. 11A–C. Vesicles were preloaded for 30 min at room temperature (22 °C) with 0, 5, 10, 25, 60, 80 or 100 mmol l^{-1} sodium gluconate at pH 8.5 and were incubated in media containing 4 mmol l^{-1} amiloride at pH 8.5. After subtracting uptake in the presence of the drug from the total uptake shown in Fig. 11A, the results displayed in Fig. 11B,C indicate that $^{45}Ca^{2+}$ influx was a hyperbolic function of $[Na^+]_i$ in the absence of amiloride with an apparent K_{Na} of 82.5±6.1 mmol l^{-1} and apparent J_{max} of 5.80±0.04 pmol μg^{-1} protein 3 s^{-1}, and that $^{45}Ca^{2+}$ influx was a linear function of $[Na^+]_i$ in the presence of amiloride, suggesting a lower-affinity carrier-mediated process.

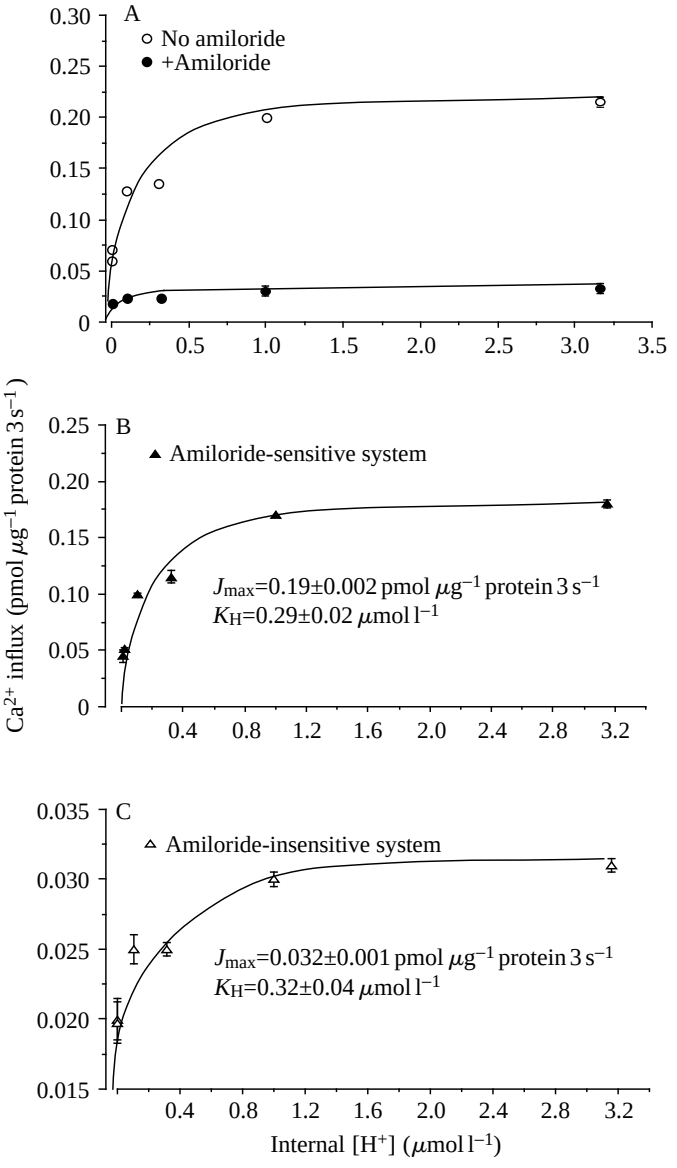

Fig. 10. (A) The effects of varying the internal H^+ concentration on 0.05 mmol l^{-1} $^{45}Ca^{2+}$ influx in short-circuited hepatopancreatic BBMVs. Vesicles were loaded with 50 mmol l^{-1} TMA gluconate, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 8.0 to pH 5.5 (25 mmol l^{-1} Mes/Tris or Hepes/Tris provide $[H^+]$ from 0.01 to 3.16 μmol l^{-1}) and were then incubated in media containing 50 mmol l^{-1} TMA gluconate, 50 mmol l^{-1} potassium gluconate at pH 8.5 (25 mmol l^{-1} Hepes/Tris). One sample of the vesicles was exposed to 4 mmol l^{-1} amiloride. (B) Intravesicular H^+ binding properties of amiloride-sensitive, carrier-mediated Ca^{2+} influx. Data are mean values from A after subtraction of the amiloride-insensitive carrier influx from the total carrier influx. Kinetic constants displayed on the figure were obtained using a computer curve-fitting program and the Michaelis–Menten equation, as described in the text. (C) The intravesicular H^+ binding properties of amiloride-insensitive, carrier-mediated Ca^{2+} influx. Kinetic constants displayed on the figure were obtained using a computer curve-fitting program and the Michaelis–Menten equation, as described in the text. Values are means ± S.E.M., N=5.

Effects of external $[Na^+]$ on $^{45}Ca^{2+}/H^+$ exchange

The joint use of an electrogenic amiloride-sensitive $2Na^+/1H^+$ antiporter by both external Na^+ and Ca^{2+} has been reported for antennal gland BBMVs of the lobster (Ahearn and Franco, 1990, 1993). In order to examine the possible use of the hepatopancreatic $2Na^+/1H^+$ exchanger by both Na^+ and Ca^{2+}, an experiment was conducted measuring $^{45}Ca^{2+}$ uptake by short-circuited hepatopancreatic BBMVs at two values of $[Ca^{2+}]_e$ and variable values of $[Na^+]_e$ in the presence and absence of 2.0 mmol l^{-1} amiloride. In this experiment, vesicles were loaded at pH 5.5 and were incubated for 3 s in media at pH 8.5 containing either 0.025 or 0.25 mmol l^{-1} calcium gluconate and sodium gluconate concentrations from 0 to 200 mmol l^{-1}. All $^{45}Ca^{2+}$ uptake values were corrected for non-specific binding as discussed above.

Fig. 12 shows that $^{45}Ca^{2+}$ influx at both external concentrations of the Ca^{2+} was strongly stimulated by increasing $[Na^+]_e$ from 0 to 15 mmol l^{-1}, but further increases of external Na^+ concentration resulted in a significant inhibition of $^{45}Ca^{2+}$ influx both in the presence and in the absence of amiloride. These results provide additional support for the presence of both amiloride-sensitive and amiloride-insensitive $^{45}Ca^{2+}/H^+$ exchange processes in the membranes. Furthermore, these data suggest that both $^{45}Ca^{2+}/H^+$ exchange mechanisms were affected by the external Na^+ concentration, showing a stimulatory response at lower $[Na^+]_e$ and an inhibitory effect at the higher $[Na^+]_e$.

Effects of verapamil on $^{45}Ca^{2+}$ uptake in the absence of a pH gradient

The possible presence of a Ca^{2+} channel in hepatopancreatic BBMVs was investigated in an experiment in which the drug verapamil was used to block membrane-potential-stimulated diffusion of Ca^{2+} across vesicle membranes. In this experiment, vesicles were loaded with 50 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 8.5 (Hepes/Tris), and were preincubated with either 50 or 100 μmol l^{-1} verapamil for 30 min prior to exposure to media at the same pH containing 50 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate or TMA gluconate plus 0.05 mmol l^{-1} calcium gluconate. Under these conditions, vesicles were either short-circuited, with no driving forces affecting $^{45}Ca^{2+}$ accumulation, or had an induced membrane potential (inside negative) stimulating diffusional entry of the labeled cation.

Fig. 13A shows that $^{45}Ca^{2+}$ uptake was significantly (P<0.01, Student's t-test) greater in vesicles possessing a transmembrane electrical potential driving force than in short-circuited vesicles. Furthermore, these data show that the addition of either 50 or 100 μmol l^{-1} verapamil to vesicles with an induced membrane potential led to significant reductions in $^{45}Ca^{2+}$ uptake compared with the control condition. No difference was observed in the inhibitory effect produced by the two drug concentrations, suggesting that a maximal influence occurred at the lower concentration. A

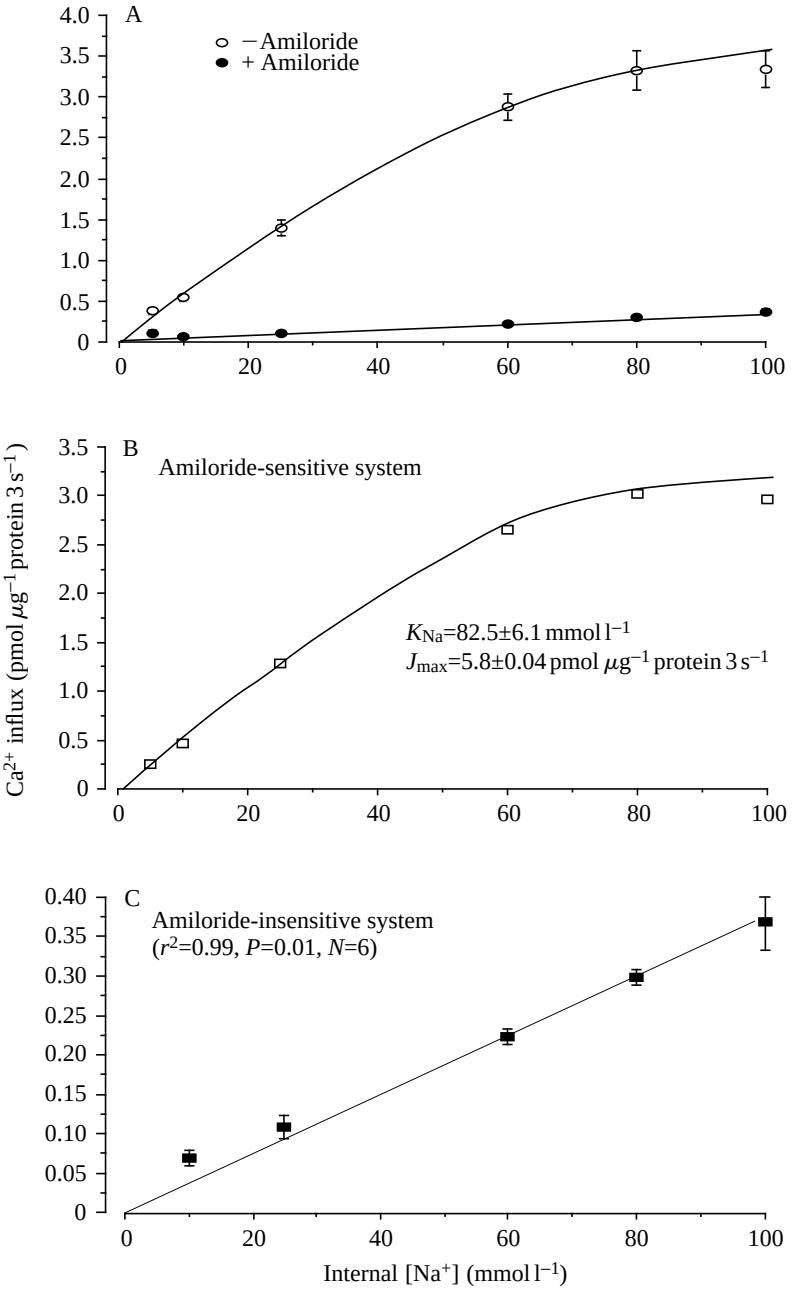

Fig. 11. (A) Effects of variable internal Na^+ concentration on 0.05 mmol l^{-1} $^{45}Ca^{2+}$ influx in short-circuited hepatopancreatic BBMVs. Vesicles were loaded with 50 mmol l^{-1} TMA gluconate, 50 mmol l^{-1} potassium gluconate, 50 μmol l^{-1} valinomycin and varying sodium gluconate concentrations (0, 5, 10, 25, 60, 80 or 100 mmol l^{-1}) and appropriate concentrations of TMA gluconate at pH 8.5 (25 mmol l^{-1} Mes/Tris) and were then incubated in media either with or without 4 mmol l^{-1} amiloride, containing 50 mmol l^{-1} TMA gluconate, 50 mmol l^{-1} potassium gluconate at pH 8.5 (25 mmol l^{-1} Hepes/Tris). (B) The intracellular Na^+ binding properties of amiloride-sensitive, carrier-mediated Ca^{2+} influx. Data were derived from A after subtraction of the amiloride-insensitive carrier influx from the total carrier influx. Kinetic constants and lines displayed on this figure were obtained as described in Fig. 10. (C) The intravesicular Na^+ binding properties of amiloride-insensitive, carrier-mediated Ca^{2+} influx. Values are means ± S.E.M.

small, but significant (P<0.05), membrane-potential-stimulated $^{45}Ca^{2+}/H^+$ exchange occurred in vesicles exposed to the drug compared with values observed in short-circuited vesicles. These results suggest that a verapamil-sensitive Ca^{2+} channel is present in hepatopancreatic BBMVs in addition to amiloride-sensitive and amiloride-insensitive carrier processes for the cation. Meanwhile, the significant amount of $^{45}Ca^{2+}$ accumulation in the presence of 100 μmol l^{-1} verapamil suggested that a potential-difference-dependent, verapamil-insensitive $^{45}Ca^{2+}$ diffusional component might also occur in these membrane preparations.

Effects of verapamil on $^{65}Zn^{2+}$ uptake

The possible role of a Ca^{2+} channel in Zn^{2+} transport was investigated in an experiment in which the drug verapamil was used to block membrane-potential-stimulated diffusion of the divalent cation across vesicle membranes. In this experiment, vesicles were loaded with 50 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 8.5 (Hepes/Tris), and were preincubated with 100 μmol l^{-1} verapamil for 30 min prior to exposure to media at the same pH containing mannitol, potassium gluconate, or TMA gluconate plus 0.5 mmol l^{-1} $^{65}Zn^{2+}$. Under these conditions, vesicles were either short-circuited with no driving forces affecting $^{65}Zn^{2+}$ accumulation or had an induced membrane potential (inside negative) which stimulated diffusional entry of the labeled cation.

The bar chart shown in Fig. 13B indicates that $^{65}Zn^{2+}$ uptake

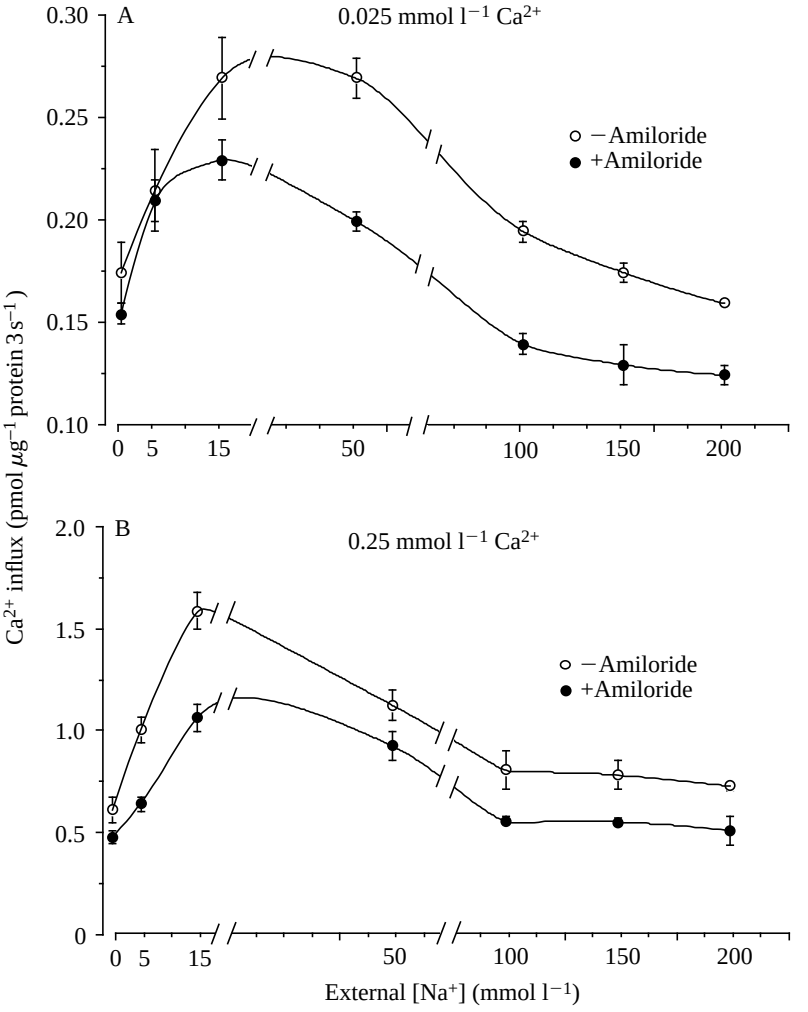

Fig. 12. The effects of external [Na^+] on 0.025 mmol l^{-1} (A) and 0.25 mmol l^{-1} (B) $^{45}Ca^{2+}/H^+$ exchange in hepatopancreatic BBMVs. Vesicles were loaded with 200 mmol l^{-1} TMA gluconate, 25 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 5.5 and were then incubated in media containing 25 mmol l^{-1} potassium gluconate, sodium gluconate at concentrations from 0 mmol l^{-1} to 200 mmol l^{-1} and appropriate TMA gluconate concentrations for osmotic balance at pH 8.5. One sample of the vesicles was exposed to 2 mmol l^{-1} amiloride. Bars represent ± S.E.M., N=5 for each mean value.

was significantly (P<0.05) greater in vesicles possessing a transmembrane electrical potential driving force than in short-circuited vesicles. Furthermore, these data show that addition of 100 μmol l^{-1} verapamil to vesicles with an induced membrane potential led to significant reductions in $^{65}Zn^{2+}$ uptake compared with the control ($\Delta\Phi$ only) condition (P<0.05). These results suggest that the heavy metal zinc might enter the vesicle through a verapamil-sensitive Ca^{2+} channel as well.

An equilibrium shift experiment providing further evidence of cation exchange

Our previously reported results showing the effects of external cations on $^{45}Ca^{2+}/H^+$ exchange in lobster hepatopancreatic BBMVs (Ahearn *et al.* 1994) suggested that a variety of substances were able to inhibit the uptake of $^{45}Ca^{2+}$. Even though we saw a reduced $^{45}Ca^{2+}$ entry into these membrane preparations, none of the results confirmed that the inhibiting agent was transported across the membrane in the place of $^{45}Ca^{2+}$ and in exchange for internal protons. In order to assess whether the $^{45}Ca^{2+}$ exchange processes characterized in this study were capable of transporting either monovalent or divalent cations in place of $^{45}Ca^{2+}$, a series of equilibrium shift experiments, based on the thermodynamics of coupled transport processes, were conducted. In these experiments, $^{45}Ca^{2+}$ was first equilibrated across short-circuited vesicle membranes and then a small volume (4–5 μl) of a solution containing a relatively high concentration of an unlabeled potential exchange substrate was introduced into the equilibrated vesicles without disruption of the osmolarity. If radiolabeled Ca^{2+} and the external substrate to be tested were capable of antiport through a common mechanism, the addition of the test solute to equilibrated vesicles would drive $^{45}Ca^{2+}$ out of the vesicles against a concentration gradient by exchange and thereby reduce the level of radioactivity associated with the membranes. This type of experiment could demonstrate two phenomena. First, a countertransport of labeled Ca^{2+} by the test solute would suggest that both substances were able to use a common carrier protein for exchange and, second, this result would imply that *in vivo* the test solute could gain access to the intracellular compartment of hepatopancreatic cells by way of this membrane protein.

Fig. 14 shows that 10 mmol l^{-1} mannitol had no effect on the amount of radiolabeled Ca^{2+} remaining in vesicles during a 60 min exchange period. In contrast, 10 mmol l^{-1} Na^+, 1 mmol l^{-1} Ca^{2+} and 1 mmol l^{-1} Zn^{2+} all stimulated the efflux

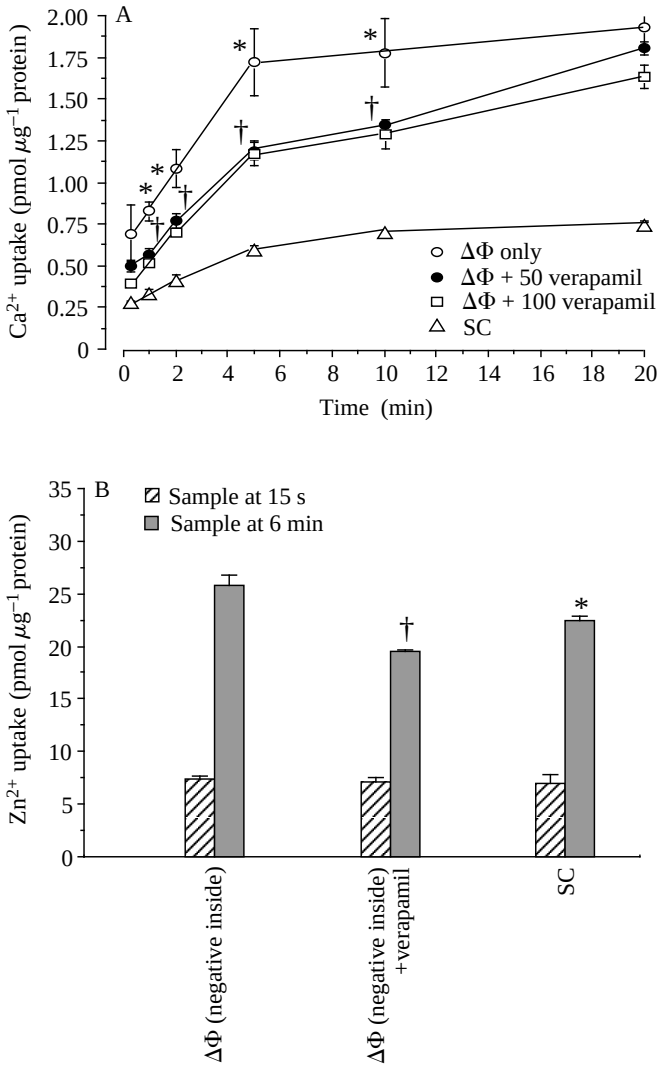

Fig. 13. The effects of external verapamil on 0.05 mmol l^{-1} $^{45}Ca^{2+}$ (A) or 0.5 mmol l^{-1} $^{65}Zn^{2+}$ (B) uptake in hepatopancreatic BBMVs. Vesicles were loaded with 50 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin at pH 8.5 and were then incubated in media containing 0.05 mmol l^{-1} $^{45}Ca^{2+}$ or 0.5 mmol l^{-1} $^{65}Zn^{2+}$, 50 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate or TMA gluconate concentrations to produce short-circuited conditions (SC) and imposed membrane potential conditions (ΔΦ). Vesicles with a membrane potential were also preincubated with either 50 or 100 μmol l^{-1} verapamil (A) or 100 μmol l^{-1} verapamil (B). A sample size of 10 was taken for each treatment and Student's *t*-test was used to determine significance. * indicates a significant difference (P<0.05) between the SC and ΔΦ conditions; † indicates a significant difference (P<0.02) between the ΔΦ condition and the ΔΦ+100 μmol l^{-1} verapamil condition. Values are means ± S.E.M.

of $^{45}Ca^{2+}$ from the pre-loaded vesicles, with unlabeled Ca^{2+} being the best *trans*-stimulator. Measurable countertransport of internal Ca^{2+} for external Na^+ and Zn^{2+} strongly suggests that both monovalent and divalent cations were able to employ a common carrier mechanism for exchange and to gain access to the intracellular compartment of hepatopancreatic cells.

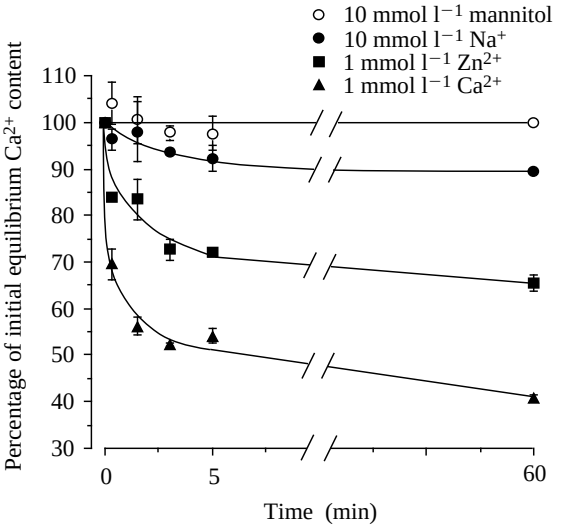

Fig. 14. Equilibrium shift experiment demonstrating *trans*-stimulation of 0.05 mmol l^{-1} $^{45}Ca^{2+}$ efflux from equilibrated hepatopancreatic BBMVs by external Na^+, Ca^{2+} or Zn^{2+} (mannitol serving as a control). Vesicles were preincubated and then exposed to various external substrates as described in the text. Values are means ± S.E.M., N=5.

Discussion

The crustacean hepatopancreas, a large multilobate diverticulum of the pyloric stomach, is an important organ for digestion and absorption of organic nutrients (Ahearn *et al.* 1992) and is known to have a significant role in organismic ion balance. Despite the important Ca^{2+} storage function that hepatopancreatic cells exhibit during the molt cycle, little is known about how this cation enters hepatopancreatic epithelial cells across the brush-border membrane from the gastrointestinal contents or of the physiological details of the processes regulating Ca^{2+} incorporation into storage vacuoles (Becker *et al.* 1974).

Sea water contains approximately 10 mmol l^{-1} Ca^{2+}, and it has been reported that lobsters (*Homarus americanus*) drink a considerable volume of sea water during the process of molting (Mykles, 1980). Therefore, a significant amount of Ca^{2+} is available to crustacean hepatopancreatic epithelial cells during ecdysis, and this ion is also likely to be present in the stomach and the hepatopancreatic ducts during normal feeding activities during intermolt. Our previously reported results (Ahearn and Zhuang, 1996) together with the data from the present investigation suggest that Ca^{2+} transport across lobster hepatopancreatic brush-border membrane vesicles occurs by a combination of three transport processes: (1) an amiloride-sensitive carrier system; (2) an amiloride-insensitive carrier system, and (3) a verapamil-sensitive ion channel (Table 1). In addition, a verapamil-insensitive, potential-difference-dependent diffusional component might be present in the brush-border membrane preparation, but further investigation is needed to characterize this component. Similar findings were recently reported for Ca^{2+} transport by apical membrane vesicles of the kidney in the same animal, suggesting a

Table 1. *Kinetic constants and properties of the brush-border Ca^{2+} transport processes*

Transport processes	Proposed transport stoichiometry	Kinetic constants	Other properties
Amiloride-sensitive system			
Ca^{2+}/H^+	1:1	K_H=0.29±0.02 μmol l^{-1}	Electrogenic, may also be involved in Zn^{2+} transport
Ca^{2+}/Na^+	1:1	K_{Na}=82.5±6.1 mmol l^{-1}	
Amiloride-insensitive system			
Ca^{2+}/H^+	1:2	K_H=0.32±0.04 μmol l^{-1}	Electroneutral, may also be involved in Zn^{2+} transport
Ca^{2+}/Na^+	1:2		Probably the component that is sensitive to verapamil
Verapamil-sensitive ion channel			Sensitive to a transmembrane potential difference and probably involved in Ca^{2+} and Zn^{2+} transport
Verapamil-insensitive ion channel			Probably involved in Ca^{2+} and Zn^{2+} transport

common physiological theme for regulating the transmembrane flow of this divalent cation by marine crustacean epithelia (Ahearn and Franco, 1993). It is interesting to note that very similar mechanisms for Ca^{2+} transport have been found in the pyloric ceca brush-border membranes of the starfish *Pycnopodia helianthoides*, except that the stimulation of Ca^{2+} uptake by membrane potential alone was sensitive to nifedipine rather than to verapamil (Zhuang *et al.* 1995).

We previously reported that zinc and cadmium inhibited $^{45}Ca^{2+}/H^+$ exchange by starfish pyloric ceca and lobster hepatopancreas (Ahearn *et al.* 1994). The present study verifies that zinc is a competitive inhibitor of carrier-mediated $^{45}Ca^{2+}$ influx, with a K_i of 940 μmol l^{-1} (see Fig. 9), suggesting that both divalent cations are able to use a common exchanger for uptake into the apical cells. Equilibrium shift experiments confirmed the capability of Ca^{2+}, Na^+ and Zn^{2+} to exchange with each other by way of a common transport protein, thereby strengthening the argument that each has the potential to gain access to the hepatopancreatic intracellular compartment *in vivo* (Fig. 14). In addition, Zn^{2+} may also enter the hepatopancreatic apical cells by way of the verapamil-inhibited Ca^{2+} channel (Fig. 13). Because Zn^{2+} and Ca^{2+} act as competing substrates at the same carrier binding site, the cation with the higher affinity will be more likely to associate with the transporter (at equal environmental concentrations) and enter the cell. Sea water has a Ca^{2+} concentration near 10 mmol l^{-1}, while Zn^{2+} concentrations are generally many times lower than this (Forstner and Wittmann, 1979). Our previous investigation (Ahearn and Zhuang, 1996) reported the apparent Ca^{2+} binding affinities (K_t) of lobster amiloride-sensitive transporters in hepatopancreatic epithelium to be 58±2 μmol l^{-1} and of amiloride-insensitive transporters to be 52±2 μmol l^{-1} (means ± S.E.M.; N=7), while the inhibitor constant (K_i) for Zn^{2+} was 940 μmol l^{-1} (Fig. 9). Because the shared cation carrier exhibits a considerably higher apparent binding affinity for Ca^{2+} than for Zn^{2+}, and because environmental concentrations of Ca^{2+} far exceed those of Zn^{2+}, even in the most highly polluted water areas, inhibition of Ca^{2+} uptake by Zn^{2+} from sea water may be minimal. However, despite their reputation as scavengers and omnivores, lobsters appear to be somewhat selective in the food they eat, and small crustaceans, molluscs and echinoderms are the main constituents of the diet of *H. americanus* (Phillips *et al.* 1980). These prey organisms are likely to contain much higher concentrations of Zn^{2+} in their cells due to bioaccumulation and sequestration mechanisms than those that occur in sea water or sediments and thus they pose a potentially far more serious physiological problem than direct uptake from the environment. It is important therefore that specific detoxification mechanisms occur in the cells of carnivores such as *H. americanus* to limit the bioavailability of these metals in their hemolymph. The transport mechanisms described in this report for Zn^{2+} might be part of an overall cellular detoxification response to potentially toxic heavy metals (Al-Mohanna and Nott, 1985).

A model for $^{45}Ca^{2+}$ transport by lobster antennal gland epithelial brush-border membrane vesicles was recently proposed (Ahearn and Franco, 1993) that also adequately describes the transfer processes for this cation across hepatopancreatic epithelial apical membrane. This model suggests that two cation exchangers occur on the epithelial brush-border membrane of both organs and that these can exchange either cytoplasmic H^+ or Na^+ for extracellular Ca^{2+}. One is inhibited by amiloride while the other appears refractory to the drug. A verapamil-sensitive $^{45}Ca^{2+}$ channel also occurs at this cell pole and accounts for diffusional flow of the divalent cation across the apical membranes in response to an induced membrane potential. The amiloride-sensitive Ca^{2+} transport process is probably the electrogenic $2Na^+/1H^+$ antiporter that has been previously described for both tissues (Ahearn and Clay, 1989; Ahearn and Franco, 1990; Ahearn *et al.* 1990). The use of a monovalent cation exchanger by a divalent cation distinguishes this invertebrate transport protein from the analogous electroneutral exchanger of vertebrate membranes, which does not accommodate Ca^{2+} (Aronson, 1985). The amiloride-insensitive Ca^{2+} transport process of lobster antennal glands and hepatopancreas is largely a $^{45}Ca^{2+}/Na^+$ exchanger, and it appears to be electroneutral, probably

exchanging 2 Na^+ for 1 Ca^{2+} in both organs (Ahearn and Franco, 1993).

The occurrence of amiloride-sensitive $^{45}Ca^{2+}/H^+$ exchangers in both antennal glands and the hepatopancreas of lobster, which probably represent the transport activities of previously characterized electrogenic $2Na^+/1H^+$ antiporters, is in agreement with the reported tissue distribution of this protein visualized by antibody reactivity. In a recent study, a monoclonal antibody, raised in mice against proteins of hepatopancreatic brush-border membranes, abolished $^{22}Na^+/H^+$ and $^{45}Ca^{2+}/H^+$ exchange by hepatopancreatic brush-border membrane vesicles, but was without effect on Na^+-dependent D-glucose transport in the same preparation (De Couet *et al*. 1993). The antigen responding to this antibody was found in the hepatopancreas, antennal gland and gills of the lobster and had a molecular mass of 185 kDa. Thus, both physiological $^{45}Ca^{2+}$ influx measurements and antigen tissue distribution patterns suggest that the electrogenic $2Na^+/1H^+$ antiporter is located in both the hepatopancreas and antennal glands and that it is responsible for a significant fraction of epithelial Ca^{2+} uptake by brush-border membranes of both organs.

Although brush-border Ca^{2+} transport mechanisms of the lobster kidney and hepatopancreas are similar and may represent identical transport proteins in the two locations, the array of transfer processes in these crustacean tissues differs considerably from those proposed for apical membrane Ca^{2+} transport in vertebrate epithelia. Ca^{2+} influxes across the luminal membranes of mammalian and fish intestines are the result of a combination of saturable and non-saturable processes operating simultaneously (Klaren *et al.* 1993; Van Os, 1987; Wilson *et al.* 1989). Whereas the mammalian duodenal carrier processes exhibit relatively low binding affinities for Ca^{2+} (approximately 1 mmol l^{-1}; Schachter and Kowarski, 1982), that for the teleost *Oreochromis mossambicus* has a considerably higher Ca^{2+} affinity (5.8 μmol l^{-1}; Klaren *et al.* 1993). The nature of these carrier processes was not elucidated in detail, but studies with mammalian intestine showed that $^{45}Ca^{2+}$ uptake by brush-border membrane vesicles preloaded with strontium was increased compared with the uptake by similar vesicles lacking strontium (Van Os, 1987). This suggests a possible antiport capability of the transporter similar to that reported for Ca^{2+} uptake in lobster hepatopancreatic brush-border membrane vesicles in the present study. Presumably in mammalian cells, when strontium was absent, other cytoplasmic cations such as Na^+ or H^+ might be able to exchange with external Ca^{2+}. A similar cation antiporter could equally well account for carrier-mediated Ca^{2+} influx in teleost intestine (Klaren *et al.* 1993). In vertebrates, Na^+/H^+ exchange occurs by an electroneutral $1Na^+/1H^+$ antiporter which has no divalent cation specificity (Aronson, 1985). However, the electroneutral $2Na^+(2H^+)/1Ca^{2+}$ antiporter described for the luminal hepatopancreatic and antennal gland membranes of lobsters (Ahearn and Franco, 1993; present investigation) could also account for the brush-border carrier-mediated Ca^{2+} influx in intestines of both mammals and teleosts, but appropriate experiments are needed to support this hypothesis.

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