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Ca²⁺ AND Zn²⁺ ARE TRANSPORTED BY THE ELECTROGENIC 2Na⁺/1H⁺ ANTIporter IN ECHINODERM GASTROINTESTINAL EPITHELIUM

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Summary

⁴⁵Ca²⁺ uptake by purified brush-border membrane vesicles of starfish (*Pycnopodia helianthoides*) pyloric ceca was stimulated by an outwardly directed H⁺ gradient and this stimulation was enhanced by the simultaneous presence of an induced membrane potential (inside negative; K⁺/valinomycin). External amiloride (competitive inhibitor; K_i=660 μmol l⁻¹) and a monoclonal antibody raised against proteins associated with the lobster (*Homarus americanus*) electrogenic 2Na⁺/1H⁺ antiporter both inhibited approximately half of the proton-gradient-stimulated ⁴⁵Ca²⁺ uptake. These results suggested that Ca²⁺ might be transported by the electrogenic antiporter and that the crustacean antibody was inhibitory to the exchange function in echinoderms, as was recently shown in crustacean epithelial brush-border membrane vesicles. Carrier-mediated ⁴⁵Ca²⁺ influx by amiloride-sensitive and amiloride-insensitive systems displayed the following kinetic constants: (amiloride-sensitive) K_t=66±2 μmol l⁻¹; J_{max}=0.173±0.002 pmol μg⁻¹ protein 8 s⁻¹; (amiloride-

insensitive) K_t=18±0.3 μmol l⁻¹; J_{max}=0.100±0.001 pmol μg⁻¹ protein 8 s⁻¹. Zn²⁺ was a mixed inhibitor of ⁴⁵Ca²⁺ influx by carrier-mediated transport, displaying a K_i of 920 μmol l⁻¹. Mn²⁺, Cu²⁺, Fe²⁺ and Mg²⁺ also inhibited ⁴⁵Ca²⁺ uptake, but the mechanism(s) of inhibition by these other cations was not disclosed. An equilibrium shift experiment showed that both Na⁺ and Zn²⁺ were able to exchange with equilibrated ⁴⁵Ca²⁺ in these vesicles, suggesting that both monovalent and divalent cations were able to enter pyloric cecal cells through a common carrier-mediated transport system. In addition, the echinoderm electrogenic system appeared to exhibit a molecular component recognized by the crustacean antibody that may imply a similar epitope in the two animals.

Key words: brush-border membrane, calcium, zinc, antiport, electrogenic Ca²⁺/H⁺ exchange, channel, 2Na⁺/1H⁺ exchange, heavy metal, starfish, pyloric ceca, Echinodermata, *Pycnopodia helianthoides*, amiloride.

Introduction

Recent studies with brush-border membrane vesicles (BBMVs) of crustacean hepatopancreatic and antennal gland epithelia have characterized the monovalent cation exchange properties of a Na⁺/H⁺ antiporter protein which differs significantly from analogous transporters of vertebrate cells (Ahearn and Clay, 1989; Ahearn and Franco, 1990; Ahearn *et al.* 1990; Aronson, 1985; Aronson and Igarashi, 1986). These studies suggested that the invertebrate protein was electrogenic and exhibited a transport stoichiometry of 2Na⁺/1H⁺ as a result of possessing two external cation and amiloride binding sites and only a single cytoplasmic binding site. The driving force for electrogenic exchange was the combination of the transapical Na⁺ gradient and the brush-border membrane potential which was sufficient to acidify the hepatopancreatic lumen to observed physiological levels (Gibson and Barker, 1979).

In an effort to determine the phylogenetic distribution of this apparently unique monovalent cation antiporter, Na⁺/H⁺ exchange by pyloric cecal BBMVs of the starfish *Pycnopodia*

helianthoides was characterized (Ahearn and Franco, 1991). The results of this study showed that gastrointestinal epithelia from both protostomes (arthropods) and deuterostomes (echinoderms) exhibited apical membrane electrogenic 2Na⁺/1H⁺ antiporters, strongly supporting the view that this protein may be widely distributed among the invertebrates.

Experiments using crustacean hepatopancreatic and antennal gland BBMVs disclosed that Ca²⁺ acted as a competitive inhibitor of Na⁺ uptake by the amiloride-sensitive 2Na⁺/1H⁺ exchanger in these tissues (Ahearn and Franco, 1990). Additional studies with these crustacean membrane preparations suggested that the electrogenic antiporter represented a significant pathway for cellular uptake of Ca²⁺ (Ahearn and Franco, 1993; Zhuang and Ahearn, 1995). Because the starfish pyloric cecal brush-border membrane was shown to possess an electrogenic 2Na⁺/1H⁺ antiporter with physiological properties similar to those described for gut and renal tissues of crustaceans, the possibility that this transporter may also have a role in divalent cation transport in

echinoderms was evaluated. The findings indicate that Ca^{2+} is transported by both amiloride-sensitive and amiloride-insensitive transporters in starfish epithelia. In addition, Zn^{2+} , and possibly other heavy metal ions, is transferred by the amiloride-sensitive process in these echinoderm cells and, as a result, this protein may have a significant role in both Ca^{2+} and heavy metal ion metabolism in invertebrates. Preliminary results of this work have been recently published in abstract form (Zhuang and Ahearn, 1994).

Materials and methods

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

The pooled pyloric cecal sample was homogenized in hypotonic buffer and mixed with 10mmol l^{-1} MgCl_2 for selective precipitation of most cellular membranes except the brush border. This was followed with purification by a series of centrifugations at either $3000g$ or $27000g$ and homogenization in additional hypotonic buffer using a glass tissue grinder. The resulting purified sample of brush-border membranes was resuspended in a small volume of appropriate internal medium by 10–15 passages through a syringe fitted with a 22 gauge needle. This final vesicle suspension exhibited a total protein content of $10\text{--}15\ \mu\text{g}\ \mu\text{l}^{-1}$ (Bio-Rad protein assay). Using this preparative method, previous studies with gastrointestinal and renal organs from such diverse organisms as crustaceans, fish and mammals have produced final purified vesicle suspensions that exhibited significant enrichments of brush-border marker enzymes, such as alkaline phosphatase (Ahearn *et al.* 1985), leucine aminopeptidase (Reshkin and Ahearn, 1987) and sucrase (Hopfer *et al.* 1973), while concurrently displaying reduced occurrence of enzymes associated with other cell membranes, such as Na^+/K^+ -ATPase (basolateral membranes) and cytochrome *c* oxidase (mitochondrial membranes). Table 1 shows the results of enzyme assays performed on final vesicle pellets of starfish pyloric ceca and indicates that the brush-border marker alkaline phosphatase was enriched almost 10-fold, while the basolateral enzyme Na^+/K^+ -ATPase displayed only a 0.63-fold purification with these methods. These results suggest that a relatively pure brush-border preparation with only minimal basolateral contamination had been generated using the above procedures.

Transport studies using these pyloric cecal brush-border

Table 1. *Enzymatic characterization of sea star (Pycnopodia helianthoides) pyloric cecal brush-border membrane vesicles*

Enzyme	Activity		Purification factor
	Homogenate	Final vesicles	
Na^+/K^+ -ATPase	9.67 ± 0.28	6.07 ± 0.67	0.63 ± 0.02
Alkaline phosphatase	4.00 ± 0.16	38.85 ± 0.40	9.73 ± 0.37

Values are mean \pm S.E.M. (three animal preparations per mean value).

Enzyme activities are in μmol product released mg^{-1} protein h^{-1} .

Purification factors are means of individual vesicle activities/individual homogenate activities.

membrane vesicles (BBMVs) were conducted at $20\pm 2^\circ\text{C}$ with freshly produced membrane preparations using the Millipore filtration technique of Hopfer *et al.* (1973). At the beginning of a transport experiment, a volume (e.g. $20\ \mu\text{l}$) of membrane vesicles was added to a volume of radiolabelled medium (e.g. $160\ \mu\text{l}$) containing $^{45}\text{Ca}^{2+}$ (ICN Radiochemicals). Following incubation times ranging from 15 s to 60 min (to equilibrate the isotope across the vesicle membranes), a known volume of this reaction mixture (e.g. $20\ \mu\text{l}$) was withdrawn and plunged into 2 ml of ice-cold stop solution (composition differing with different experiments; see figure legends). The resulting suspensions were rapidly filtered through Millipore filters ($0.65\ \mu\text{m}$ pore diameter) to retain the vesicles and washed with another 5 ml of stop solution. Filters were then added to ICN Ecolume scintillation cocktail and counted for radioactivity in a Beckman LS-9000 scintillation counter. Ca^{2+} uptake was expressed, using the specific activity of $^{45}\text{Ca}^{2+}$ in the medium, as $\text{pmol}\ \mu\text{g}^{-1}$ protein filter $^{-1}$. Within a given experiment, each time point was analyzed using 3–5 replicates and values are presented in the figures as means \pm S.E.M.

Monoclonal antibodies used in this study were prepared against purified protein elements (molecular mass 185 kDa) associated with the electrogenic $2\text{Na}^+/\text{1H}^+$ antiporter of lobster (*Homarus americanus*) hepatopancreatic epithelial brush-border membrane, as previously described (de Couet *et al.* 1993). These antibodies inhibited both electrogenic $2\text{Na}^+/\text{1H}^+$ exchange and $1\text{Ca}^{2+}/\text{1H}^+$ exchange by lobster hepatopancreatic brush-border membrane vesicles, but were without effect on Na^+ -dependent D-glucose transport by the same membranes. Their possible inhibitory effect on amiloride-sensitive $^{45}\text{Ca}^{2+}/\text{H}^+$ exchange by starfish pyloric cecal brush-border membrane vesicles was assessed in the present study using the antibody concentration (1:100 dilution; antibody protein content $20\ \text{mg ml}^{-1}$) that had proved effective in reducing cation exchange in lobster vesicles.

Results

Osmotic reactivity and calcium binding properties of starfish pyloric cecal BBMVs

To confirm the closure of pyloric cecal BBMVs and that Ca^{2+} transported by these vesicles was moving into an

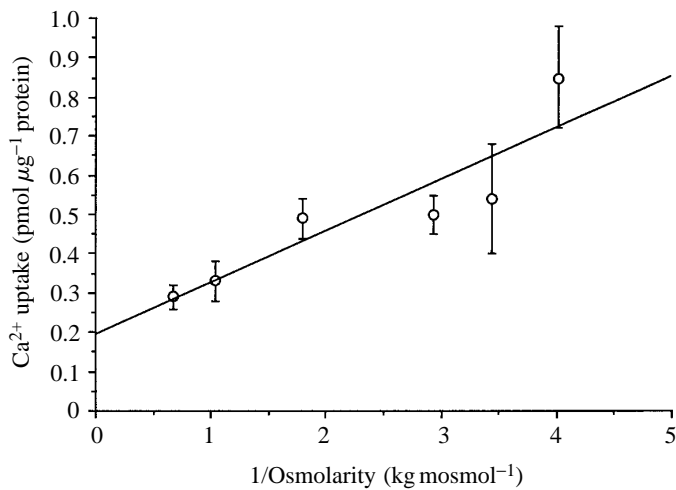


Fig. 1. Effect of transmembrane osmotic gradients on equilibrium uptake of $^{45}\text{Ca}^{2+}$ by brush-border membrane vesicles (BBMVs) of starfish pyloric ceca. Vesicles were loaded with 200 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes/Tris at pH 7.0 and were incubated for 90 min in an identical medium containing 0.1 mmol l^{-1} $^{45}\text{Ca}^{2+}$ gluconate and one of the following concentrations of sucrose (in mmol l^{-1}): 0, 50, 100, 300, 600 or 900. In this and later figures, the line drawn on the figure was computed using linear regression analysis, while symbols are means \pm S.E.M., $N=3-5$.

osmotically reactive space rather than simply binding to the membrane surface, 90 min equilibrium uptake of 0.1 mmol l^{-1} $^{45}\text{Ca}^{2+}$ by starfish vesicles 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 incubated in identical external media containing the labelled Ca^{2+} and 0–900 mmol l^{-1} sucrose.

Fig. 1 indicates that, for all membrane preparations, a significant ($P<0.01$) linear relationship existed between vesicular $^{45}\text{Ca}^{2+}$ content at equilibrium and the reciprocal of the incubation medium osmolarity. Extrapolation of the curve to the vertical axis provided an index of nonspecific surface binding of $^{45}\text{Ca}^{2+}$ to vesicles at equilibrium and amounted to approximately 23.5% of total $^{45}\text{Ca}^{2+}$ uptake under control osmotic conditions (0 mmol l^{-1} sucrose). These results suggest that pyloric cecal vesicles were sealed, were osmotically reactive and displayed a significant binding component which had to be considered during subsequent influx assessments. In order to reduce the contribution to total $^{45}\text{Ca}^{2+}$ uptake values of non-specific binding, vesicle blanks were made during uptake experiments by exposing membranes and isotope simultaneously to ice-cold stop solution. The resulting bound activity was subtracted from total uptake at selected exposure intervals to provide estimates of transport activity alone.

Effects of a transmembrane H^+ gradient and membrane potential on the time course of $^{45}\text{Ca}^{2+}$ uptake

Our previous studies with starfish pyloric cecal BBMVs suggested that an outwardly directed proton gradient provided an adequate driving force for vesicular accumulation of

exogenous $^{22}\text{Na}^+$ by electrogenic $2\text{Na}^+/\text{H}^+$ antiport (Ahearn and Franco, 1991). Other studies with crustacean hepatopancreatic epithelial BBMVs indicated that Ca^{2+} shared this amiloride-sensitive antiporter with Na^+ (Ahearn and Franco, 1990). In order to evaluate the possible extent to which Ca^{2+} was able to use the amiloride-sensitive electrogenic $2\text{Na}^+/\text{H}^+$ antiporter in starfish pyloric cecal BBMVs, vesicles were loaded with an internal medium at pH 5.5 and incubated with radiolabelled $^{45}\text{Ca}^{2+}$ at pH 8.5. In some treatments, the external medium also contained 2 mmol l^{-1} amiloride or 50 mmol l^{-1} Na^+ .

The results in Fig. 2 suggest that greater $^{45}\text{Ca}^{2+}$ uptake occurred in vesicles with an outwardly directed proton gradient ($\text{pH}_i=5.5$; $\text{pH}_e=8.5$) than when equal pH values were present on both vesicle surfaces ($\text{pH}_i=\text{pH}_e=5.5$). In addition, when a transmembrane proton gradient was used as a driving force for the vesicular accumulation of $^{45}\text{Ca}^{2+}$, external amiloride (2 mmol l^{-1}) or Na^+ (50 mmol l^{-1}) acted as potent inhibitors of the exchange process. There remained a significant uptake of Ca^{2+} in the presence of either inhibitor, suggesting the presence of a Ca^{2+} transport process that was refractory to inhibition by these substances. These results suggest that Ca^{2+} uptake by starfish pyloric cecal BBMVs occurs by antiport with protons at an amiloride-sensitive exchanger which is probably the electrogenic $2\text{Na}^+/\text{H}^+$ transporter previously characterized for this membrane (Ahearn and Franco, 1991).

Fig. 3 describes the effects of an imposed transmembrane potential (inside negative; K^+ /valinomycin) on proton-gradient-dependent $^{45}\text{Ca}^{2+}$ uptake in starfish pyloric cecal BBMVs. This figure indicates that an outwardly directed

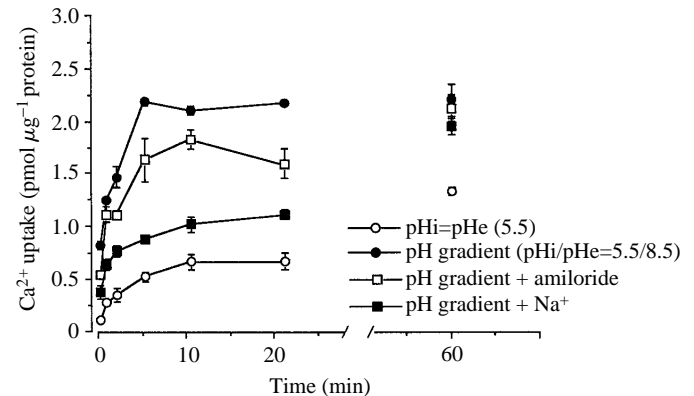


Fig. 2. Effects of an outwardly directed proton gradient and external amiloride or Na^+ on $^{45}\text{Ca}^{2+}$ uptake by pyloric cecal BBMVs. Vesicles were loaded with 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate and 50 mmol l^{-1} valinomycin at pH 5.5 (25 mmol l^{-1} Mes/Tris) and were incubated in media at either pH 5.5 or pH 8.5 (25 mmol l^{-1} Hepes/Tris) containing 0.1 mmol l^{-1} $^{45}\text{Ca}^{2+}$ gluconate, 100 mmol l^{-1} potassium gluconate and an appropriate mannitol concentration to maintain osmolarity. One series of vesicles exhibiting an outwardly directed proton gradient was exposed to 2 mmol l^{-1} amiloride and another series was exposed to 50 mmol l^{-1} sodium gluconate. After 20 min, the Ca^{2+} ionophore A23187 ($50\text{ }\mu\text{mol l}^{-1}$ in 90% ethanol) was added to all vesicles.

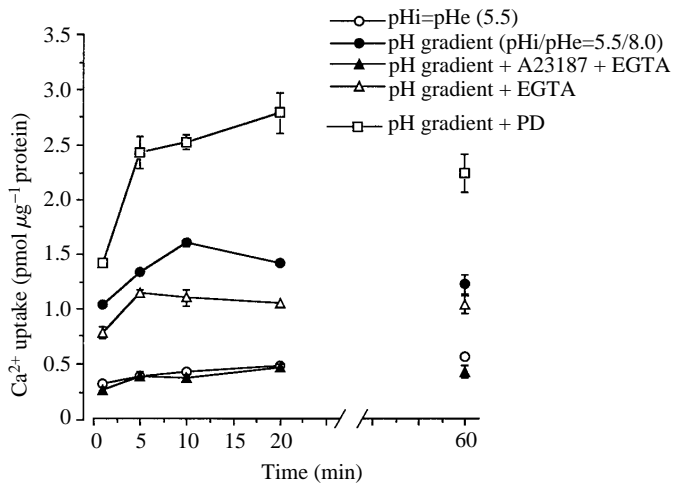


Fig. 3. Effects of an outwardly directed proton gradient and inside negative transmembrane electrical potential difference (PD) on $^{45}\text{Ca}^{2+}$ uptake by pyloric cecal BBMVs. Vesicles were loaded with the same inside medium as in Fig. 2 at pH 5.5 and were incubated in media at pH 8.5 containing 0.1 mmol l^{-1} $^{45}\text{Ca}^{2+}$ gluconate, 100 mmol l^{-1} mannitol and 100 mmol l^{-1} trimethylammonium gluconate or 100 mmol l^{-1} potassium gluconate, producing short-circuit conditions or imposed membrane potential conditions. One series of vesicles exhibiting an outwardly directed proton gradient after each incubation period was exposed for 3 min to ice-cold stop solution containing 5 mmol l^{-1} EGTA before assessing its radioactivity, while a second series was incubated for 3 min in ice-cold stop solution containing 5 mmol l^{-1} EGTA plus $20 \mu\text{mol l}^{-1}$ A23187 before counting.

proton gradient and membrane potential were additive in their stimulatory effects on Ca^{2+} uptake by these membrane preparations compared with uptake occurring under short-circuit conditions. Fig. 3 also shows the effects of incubating $^{45}\text{Ca}^{2+}$ -labelled vesicles for 3 min in ice-cold stop solution with the cation chelator EGTA, or with EGTA plus the Ca^{2+} ionophore A23187, following isotope exposure in buffer with an imposed transmembrane pH gradient. The results of these latter two treatments suggest that EGTA alone is able to remove a fraction of $^{45}\text{Ca}^{2+}$ activity, which probably

corresponds to non-specific external binding, while addition of the ionophore and the chelator together also leads to loss of isotope from the osmotically reactive compartment within the vesicles. The time course of uptake under short-circuit conditions with bilaterally equal pH conditions (i.e. no driving forces for Ca^{2+} uptake) therefore appears largely to represent isotopic binding to vesicles and is approximately equivalent to that fraction removed by the addition of EGTA. The high $^{45}\text{Ca}^{2+}$ content at 60 min resulting from combined pH gradients and potential differences was probably the result of enhanced internal isotope binding under these conditions which did not allow the vesicles to reach isotopic equilibration.

Effect of Ca^{2+} channel blockers on $^{45}\text{Ca}^{2+}$ uptake

In order to determine whether $^{45}\text{Ca}^{2+}$ entered BBMVs of starfish pyloric ceca through a membrane-potential-sensitive Ca^{2+} channel, two known Ca^{2+} channel blockers, verapamil and nifedipine, were used as possible inhibitors of Ca^{2+} uptake in response to an imposed membrane potential (K^+ /valinomycin) and bilaterally equal pH values (pH 8.0 or pH 6.5). Under these experimental conditions, the only driving force for $^{45}\text{Ca}^{2+}$ uptake was the membrane potential. As shown in Fig. 4, Ca^{2+} uptake was greater in vesicles with an imposed membrane potential (inside negative) than in its absence or in the presence of an inside-positive potential (pH 8.0). While addition of $100 \mu\text{mol l}^{-1}$ verapamil to these vesicles did not abolish the membrane potential enhancement of $^{45}\text{Ca}^{2+}$ uptake, the addition of $10 \mu\text{mol l}^{-1}$ nifedipine did significantly lower isotope uptake. $^{45}\text{Ca}^{2+}$ uptake at pH 6.5 (both vesicle surfaces) under short-circuit conditions was approximately the same as that at pH 8.0 was used. However, addition of a membrane potential (inside negative) to pH 6.5 vesicles did not result in enhanced uptake of Ca^{2+} , as was displayed at pH 8.0, suggesting that acidification inhibited the transfer of Ca^{2+} through the membrane-potential-sensitive pathway.

These results suggest that a nifedipine- and proton-sensitive Ca^{2+} channel, which responds to an imposed membrane potential, may be present in starfish pyloric cecal BBMVs and that diffusional transport of the divalent cation through such an aqueous pathway seems likely.

Fig. 4. Effect of membrane potential only on 0.1 mmol l^{-1} $^{45}\text{Ca}^{2+}$ uptake by pyloric cecal BBMVs. Vesicles were loaded with either 100 mmol l^{-1} potassium gluconate or 100 mmol l^{-1} trimethylammonium gluconate, 25 mmol l^{-1} Mes/Tris or 25 mmol l^{-1} Hepes/Tris, 100 mmol l^{-1} mannitol and $50 \mu\text{mol l}^{-1}$ valinomycin at either pH 6.5 or at pH 8.0. Both groups of vesicles were then incubated in media of the same respective pH containing 0.1 mmol l^{-1} $^{45}\text{Ca}^{2+}$ gluconate, 100 mmol l^{-1} potassium gluconate or 100 mmol l^{-1} trimethylammonium gluconate to produce either short-circuit conditions or imposed membrane potential conditions (PD). One series of vesicles with an induced membrane potential was preincubated with $100 \mu\text{mol l}^{-1}$ external verapamil, while another was preincubated with $10 \mu\text{mol l}^{-1}$ nifedipine.

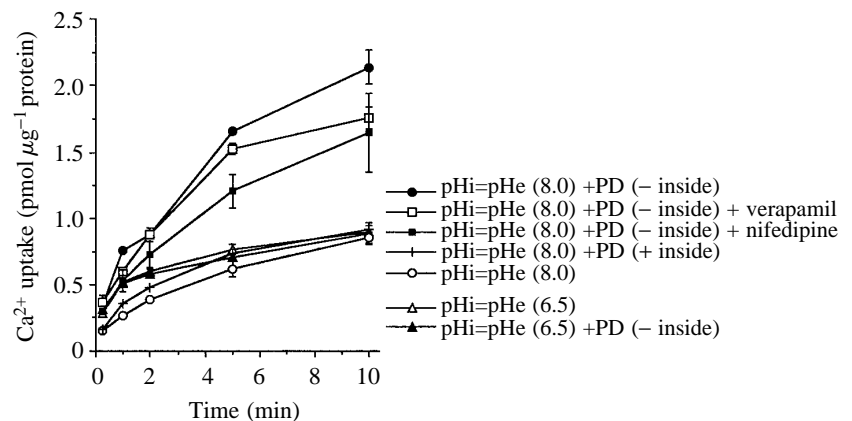
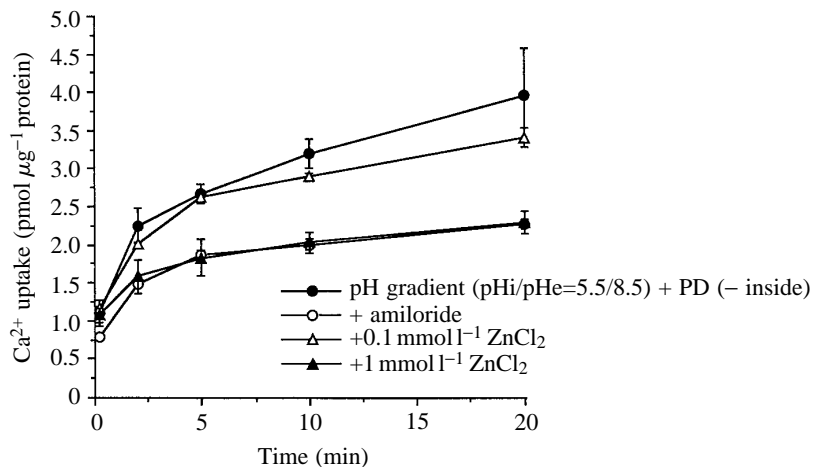


Fig. 5. Effects of two external Zn^{2+} concentrations (0.1 and 1.0 $mmol\ l^{-1}$) on $^{45}Ca^{2+}/H^+$ exchange in vesicles possessing an outwardly directed proton gradient and induced transmembrane potential difference. Vesicles were loaded with the same internal medium as in Fig. 2 at pH 5.5 and were incubated in media at pH 8.5 containing 0.1 $mmol\ l^{-1}$ $^{45}Ca^{2+}$ gluconate, 100 $mmol\ l^{-1}$ mannitol and 100 $mmol\ l^{-1}$ trimethylammonium gluconate. One series of vesicles was exposed to 3 $mmol\ l^{-1}$ amiloride, while the other two were exposed to one or other of the Zn^{2+} concentrations (as $ZnCl_2$).



Effects of Zn^{2+} on $^{45}Ca^{2+}$ uptake

To clarify the possible role of the electrogenic $2Na^+/1H^+$ antiporter in the detoxification of heavy metals, the time course of 0.1 $mmol\ l^{-1}$ $^{45}Ca^{2+}$ uptake by pyloric cecal BBMVs with an outwardly directed H^+ gradient and transmembrane potential difference (inside negative) was examined at two external Zn^{2+} concentrations (0.1 and 1 $mmol\ l^{-1}$). One sample of vesicles was exposed to 3 $mmol\ l^{-1}$ external amiloride and was used as a control.

Fig. 5 shows the effects of Zn^{2+} on the time course of $^{45}Ca^{2+}$ uptake in the presence of an H^+ gradient and a membrane potential. Vesicles which were not exposed to either amiloride or Zn^{2+} exhibited the greatest accumulation of $^{45}Ca^{2+}$ over the time intervals selected. Addition of 3 $mmol\ l^{-1}$ external amiloride, or 1 $mmol\ l^{-1}$ Zn^{2+} , significantly ($P < 0.01$) reduced the uptake of $^{45}Ca^{2+}$, but Zn^{2+} at only 0.1 $mmol\ l^{-1}$ had a slight, but non-significant ($P > 0.05$), effect on the accumulation of the radiolabel. The inhibitory actions of amiloride and 1 $mmol\ l^{-1}$ Zn^{2+} were very similar and suggest that they may have influenced the same Ca^{2+} transfer process. Lack of a significant inhibition by 0.1 $mmol\ l^{-1}$ Zn^{2+} suggests that this Zn^{2+} concentration was too low to influence the transmembrane transfer of $^{45}Ca^{2+}$ under the conditions employed in this experiment.

Nature of the inhibitory effect of Zn^{2+} on $^{45}Ca^{2+}/H^+$ exchange

Fig. 5 indicated that 1 $mmol\ l^{-1}$ Zn^{2+} significantly reduced $^{45}Ca^{2+}/H^+$ exchange in starfish pyloric cecal BBMVs with an inhibitory effect similar to that of 3 $mmol\ l^{-1}$ amiloride. In order to characterize the nature of the inhibitory action of this heavy metal ion on the antiport process, an experiment was conducted in which short-circuited vesicles possessing an outwardly directed proton gradient ($pH_i=5.5$; $pH_e=8.5$) were incubated for 8 s (unpublished observations suggested that this was an appropriate exposure interval to estimate unidirectional entry of the divalent cation) in media containing either 0.1 or 0.5 $mmol\ l^{-1}$ calcium gluconate and one of the following Zn^{2+} concentrations: 0.25, 0.5, 1.0, 1.5 or 2.0 $mmol\ l^{-1}$.

Fig. 6 is a Dixon analysis showing that increasing external Zn^{2+} concentration had a marked inhibitory effect on 8 s uptake of $^{45}Ca^{2+}$ at both Ca^{2+} concentrations. Both external Ca^{2+} concentrations resulted in Dixon plots with single slopes over the range of external Zn^{2+} concentrations used and the lines intersected at a point to the left of the vertical axis and below the horizontal axis, implying a mixed inhibitory interaction between the cations during Ca^{2+} transport (Segel, 1975). The Zn^{2+} inhibitory constant, K_i , derived from this graphical treatment of the data was 0.92 $mmol\ l^{-1}$.

Effect of external cations on $^{45}Ca^{2+}$ uptake

Because Zn^{2+} proved to be a powerful inhibitor of $^{45}Ca^{2+}$ uptake in pyloric cecal BBMVs, the effects of other external

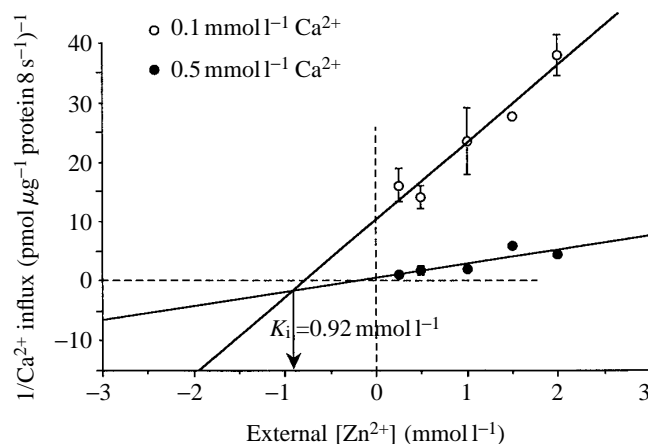


Fig. 6. The inhibitory effect of external Zn^{2+} on $^{45}Ca^{2+}$ influx in pyloric cecal BBMVs. Short-circuited vesicles were loaded as in Fig. 2 at pH 5.5 and were incubated for 8 s in media containing either 0.1 or 0.5 $mmol\ l^{-1}$ $^{45}Ca^{2+}$, 100 $mmol\ l^{-1}$ potassium gluconate, 100 $mmol\ l^{-1}$ mannitol, 25 $mmol\ l^{-1}$ Hepes/Tris and concentrations of $ZnCl_2$ from 0.25 to 2.0 $mmol\ l^{-1}$. Data shown on the Dixon plot have been corrected for the portion of Ca^{2+} influx that was independent of Zn^{2+} inhibition. Lines were drawn by linear regression analysis.

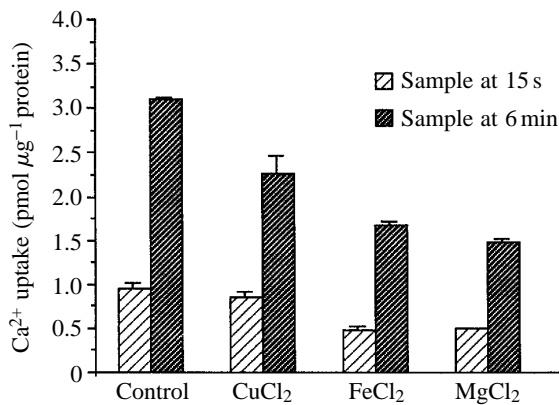


Fig. 7. Effects of the divalent cations Fe²⁺, Cu²⁺, Mg²⁺ or Mn²⁺ on ⁴⁵Ca²⁺/H⁺ exchange in short-circuited vesicles possessing an outwardly directed proton gradient. Vesicles were loaded with the same internal medium as in Fig. 2 at pH 5.5 and were incubated in media at pH 8.0 containing 0.1 mmol l⁻¹ ⁴⁵Ca²⁺ gluconate, 100 mmol l⁻¹ mannitol, 100 mmol l⁻¹ potassium gluconate (control) or with one of the following salts at 1 mmol l⁻¹: CuCl₂, FeCl₂, MgCl₂ or MnCl₂.

cations on 15 s or 6 min ⁴⁵Ca²⁺ uptakes by pyloric cecal BBMV were examined in short-circuited vesicles which had been loaded with 100 mmol l⁻¹ potassium gluconate, 100 mmol l⁻¹ mannitol, 25 mmol l⁻¹ Mes/Tris and 50 μmol l⁻¹ valinomycin at pH 5.5 and were exposed to media at pH 8.0 containing 100 mmol l⁻¹ potassium gluconate, 100 mmol l⁻¹ mannitol, 25 mmol l⁻¹ Hepes/Tris and one of the cations listed in Fig. 7.

Fig. 7 indicates that all cations added to the external medium significantly ($P < 0.05$) reduced the uptake of ⁴⁵Ca²⁺ by these membrane preparations compared with that displayed by control vesicles. The most inhibitory cation used was Mn²⁺, while the least inhibitory was Cu²⁺. Although the nature of the interaction between these external cations and radiolabelled calcium is not clear, it is apparent that the cations were generally effective inhibitors at both short (15 s) and more extended (6 min) exposure intervals. These results suggest the possibility that a variety of external cations, including heavy metal ions, may gain access to pyloric cecal epithelial cells by way of one or more Ca²⁺ transport pathways.

Effects of lobster brush-border antibody on the time course of ⁴⁵Ca²⁺ uptake

Previous results obtained from work on starfish pyloric ceca (Ahearn and Franco, 1991) suggested that the electrogenic 2Na⁺/1H⁺ exchanger characterized for crustacean epithelia also occurs in echinoderm cells and may be a widely distributed antiporter. A monoclonal antibody raised against protein elements of the electrogenic cation exchanger of lobster hepatopancreatic brush-border membranes, produced in mice, has been reported specifically to inhibit 2Na⁺/1H⁺ or 2Na⁺/1Ca²⁺ exchange activity of hepatopancreatic BBMV without influencing other Na⁺-dependent transport processes of the same membrane (de Couet *et al.* 1993). In order to

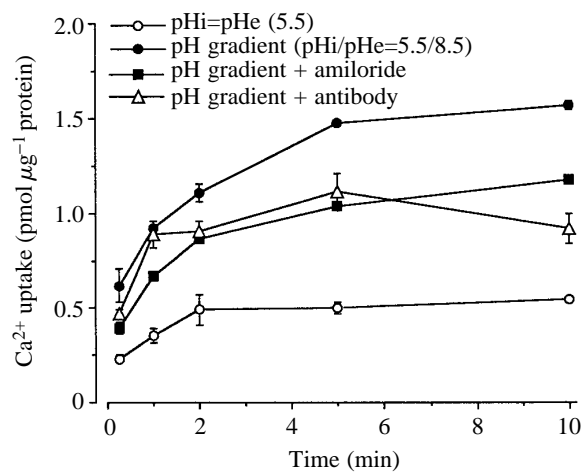


Fig. 8. Effects of crustacean monoclonal antibody on ⁴⁵Ca²⁺/H⁺ exchange in short-circuited pyloric cecal BBMV. Vesicles were loaded as in Fig. 2 at pH 5.5 and were incubated in media at pH 8.5 containing 0.1 mmol l⁻¹ ⁴⁵Ca²⁺ gluconate, 100 mmol l⁻¹ potassium gluconate and 100 mmol l⁻¹ mannitol. One series of vesicles exhibiting an outwardly directed proton gradient was exposed to 3 mmol l⁻¹ amiloride, while another was exposed to the crustacean monoclonal antibody at a final dilution of 1:100 in external medium (initial antibody concentration, 20 mg ml⁻¹). After 20 min, A23187 was added to all vesicles.

evaluate the possible molecular similarity of the electrogenic cation antiporters of lobster hepatopancreas and starfish pyloric ceca, the time course of ⁴⁵Ca²⁺ uptake by pyloric cecal BBMV was investigated in the presence and absence of amiloride or the lobster monoclonal antibody.

Fig. 8 shows that ⁴⁵Ca²⁺ uptake by starfish pyloric cecal BBMV was significantly reduced by the presence of either amiloride or the lobster monoclonal antibody in the external incubation medium compared with control uptake rates in the absence of the inhibitors. Very similar reductions in ⁴⁵Ca²⁺ uptakes occurred in vesicles incubated in either inhibitor, suggesting, but not proving, that they may have abolished Ca²⁺ entry by the same transfer process. If this is the case, these results also suggest that ⁴⁵Ca²⁺ uptake by the amiloride-insensitive transfer process was unaffected by the antibody, implying a highly specific inhibitory action of the antibody on Ca²⁺ transfer. These results suggest that the electrogenic antiporters of both lobster hepatopancreas and starfish pyloric ceca are transport proteins that may possess similar epitopes binding the monoclonal antibody used in these experiments.

Nature of the inhibitory effect of amiloride on the kinetics of ⁴⁵Ca²⁺/H⁺ exchange

Figs 2, 5 and 8 suggested that a portion of ⁴⁵Ca²⁺/H⁺ exchange was significantly inhibited by 3 mmol l⁻¹ amiloride, but the nature of this inhibition was not established. In order to assess the mechanism of amiloride action on antiporter activity, 0.1 and 0.5 mmol l⁻¹ ⁴⁵Ca²⁺ influx, at a variety of external amiloride concentrations, was measured in vesicles with an outwardly directed proton gradient. In this experiment,

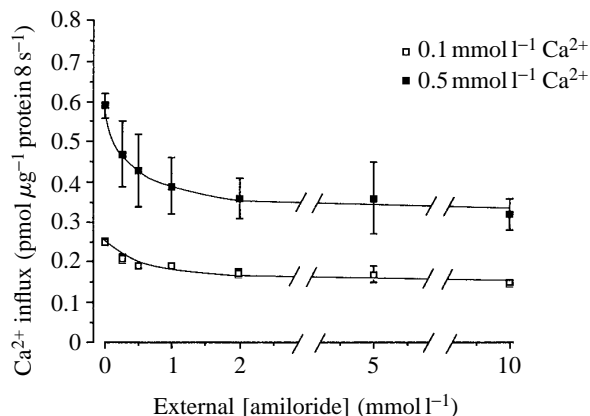


Fig. 9. Effect of external amiloride concentration on 0.1 and 0.5 mmol l⁻¹ ⁴⁵Ca²⁺ influx into pyloric cecal BBMV. Short-circuited vesicles were loaded as in Fig. 2 at pH 5.5 and were incubated for 8 s in media containing 100 mmol l⁻¹ potassium gluconate, 100 mmol l⁻¹ mannitol, 25 mmol l⁻¹ Hepes/Tris at pH 8.0 and the following concentrations of amiloride (in mmol l⁻¹): 0, 0.25, 0.5, 1, 2, 5 or 10.

vesicles were loaded at pH 5.5 (Mes/Tris) with 100 mmol l⁻¹ potassium gluconate, 100 mmol l⁻¹ mannitol and 50 μmol l⁻¹ valinomycin, and were then incubated at pH 8.0 (Hepes/Tris) in media with radiolabelled calcium gluconate, 100 mmol l⁻¹ potassium gluconate, 100 mmol l⁻¹ mannitol and amiloride at 0, 0.25, 0.5, 1, 2, 5 or 10 mmol l⁻¹.

Fig. 9 shows that, at both 0.1 and 0.5 mmol l⁻¹ ⁴⁵Ca²⁺, increasing amiloride concentration in the external medium led to a hyperbolic decrease in the rate of isotope influx to an asymptotic value that remained constant between 2 and 10 mmol l⁻¹ amiloride. At each Ca²⁺ concentration, the asymptotic value provided an index of amiloride-insensitive Ca²⁺ entry, while the declining influx from control levels (0 mmol l⁻¹ amiloride) was an estimate of the portion of Ca²⁺ entry sensitive to the drug.

In order to evaluate the nature of the inhibitory action of amiloride on ⁴⁵Ca²⁺ influx in these membrane preparations, a Dixon analysis of the data in Fig. 9 was performed (Fig. 10). This analysis indicated that amiloride was a competitive inhibitor of Ca²⁺ influx, apparently interacting with cation transport at a single binding site (i.e. there was a single slope at each Ca²⁺ concentration) and yielded a K_i value of 0.66 mmol l⁻¹ amiloride.

Kinetics of ⁴⁵Ca²⁺ influx: amiloride-sensitive and amiloride-insensitive entry processes

Experiments were conducted to evaluate the kinetic properties of amiloride-sensitive and amiloride-insensitive entry processes for ⁴⁵Ca²⁺ in starfish BBMV. In order to make this evaluation, short-circuited vesicles were loaded with 100 mmol l⁻¹ potassium gluconate, 25 mmol l⁻¹ Mes/Tris, 100 mmol l⁻¹ mannitol and 50 μmol l⁻¹ valinomycin at pH 5.5 and were incubated for 8 s in media at pH 8.0 with or without 5 mmol l⁻¹ amiloride containing 100 mmol l⁻¹ potassium gluconate, 25 mmol l⁻¹ Hepes/Tris, 100 mmol l⁻¹ mannitol

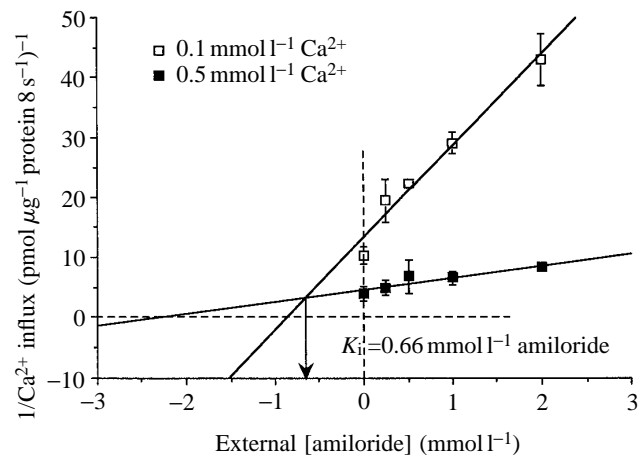


Fig. 10. Dixon plot of the data presented in Fig. 9 after subtraction of amiloride-insensitive ⁴⁵Ca²⁺ influx at 10 mmol l⁻¹ amiloride from influx values at all remaining drug concentrations. Lines were drawn by linear regression analysis.

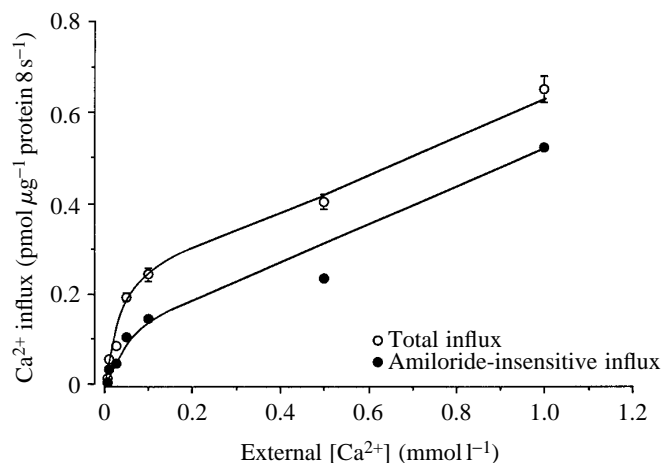


Fig. 11. Effects of external [Ca²⁺] on 8 s ⁴⁵Ca²⁺ influx into short-circuited pyloric cecal BBMV in the presence and absence of 5 mmol l⁻¹ amiloride. Vesicles were loaded as in Fig. 2 at pH 5.5 and incubated at pH 8.0 in media with or without amiloride containing various ⁴⁵Ca²⁺ gluconate concentrations (0.005–1.0 mmol l⁻¹), 100 mmol l⁻¹ potassium gluconate and an appropriate concentration of mannitol for osmotic balance.

and one of the following ⁴⁵Ca²⁺ concentrations: 0.005, 0.01, 0.025, 0.05, 0.1, 0.5 or 1.0 mmol l⁻¹. After correction for non-specific isotope binding as discussed above, the results in Fig. 11 indicate that ⁴⁵Ca²⁺ influx both in the presence and in the absence of 3 mmol l⁻¹ amiloride was a biphasic function of external Ca²⁺ concentration, exhibiting a hyperbolic relationship at the lower substrate levels and a linear function above 0.1 mmol l⁻¹ Ca²⁺. However, addition of the drug significantly ($P < 0.05$) reduced Ca²⁺ entry at every external divalent cation concentration. These results suggest that both amiloride-sensitive (AS) and amiloride-insensitive (AIS) carrier-mediated ⁴⁵Ca²⁺ influx processes were present in the pyloric cecal brush-border membrane in addition to a linear

process that may represent diffusion of the cation through an aqueous pathway. These relationships both in the presence and in the absence of amiloride followed a combination of a Michaelis–Menten saturable relationship plus a non-saturable process, as described by the equation:

$$J^{Ca} = \left\{ \frac{J_{max}[Ca^{2+}]}{K_t + [Ca^{2+}]} \right\} + K_d[Ca^{2+}], \quad (1)$$

where J^{Ca} is the $^{45}Ca^{2+}$ influx by AS or AIS transporters and diffusion, J_{max} is the apparent maximal divalent cation uptake by either carrier mechanism, K_t is the respective apparent half-saturation constant, $[Ca^{2+}]$ is the external Ca^{2+} concentration and K_d is the apparent diffusion constant of the membrane to Ca^{2+} .

In order to estimate values for the Michaelis–Menten kinetic constants for both AS and AIS carrier processes and the apparent diffusional permeability of the membrane to Ca^{2+} , $^{45}Ca^{2+}$ influx measurements obtained in the presence of the drug were subtracted from those obtained in its absence. The resulting relationships between the variables under both conditions are shown in Fig. 12, which was generated using an iterative computer curve-fitting program to estimate the best fits to the data: (AS) $K_t = 66 \pm 2 \mu\text{mol l}^{-1}$; $J_{max} = 0.173 \pm 0.002 \text{ pmol } \mu\text{g}^{-1} \text{ protein } 8 \text{ s}^{-1}$; (AIS) $K_t = 18 \pm 0.3 \mu\text{mol l}^{-1}$; $J_{max} = 0.100 \pm 0.0007 \text{ pmol } \mu\text{g}^{-1} \text{ protein } 8 \text{ s}^{-1}$; $K_d = 0.337 \pm 0.0087 \text{ pmol } \mu\text{g}^{-1} \text{ protein } 8 \text{ s}^{-1} \text{ l mmol}^{-1}$. No apparent effect of amiloride was observed on the non-saturable Ca^{2+} entry process in these experiments. These results suggest that considerable differences occurred in the kinetic constants between the two carrier processes, with the AS system exhibiting a 3.5-fold lower apparent binding affinity and twice the apparent maximal transport velocity of the AIS system.

Equilibrium shift experiment demonstrating transmembrane cation exchange

Previous experiments in this study showing the effects of external cations on $^{45}Ca^{2+}/H^+$ exchange in starfish pyloric cecal BBMV's suggested that a variety of substances were able to inhibit the uptake of labelled Ca^{2+} . Even though Ca^{2+} entry into these membrane preparations was reduced in these experiments, there was no suggestion that the inhibiting agent was transported across the membrane in place of Ca^{2+} 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 Ca^{2+} , a series of equilibrium shift experiments was conducted. In these experiments, $^{45}Ca^{2+}$ was first equilibrated across short-circuited vesicle membranes and then the equilibrated vesicles were abruptly exposed to an external medium with the same isotopic specific activity but also containing a relatively high concentration of an unlabelled potential exchange substrate. If radiolabelled Ca^{2+} and the test external substrate were capable of antiport through a common mechanism, the addition of the test solute to the external medium would drive $^{45}Ca^{2+}$ out of the equilibrated vesicles against a concentration gradient, by exchange, and thereby reduce the level of activity associated with the membranes.

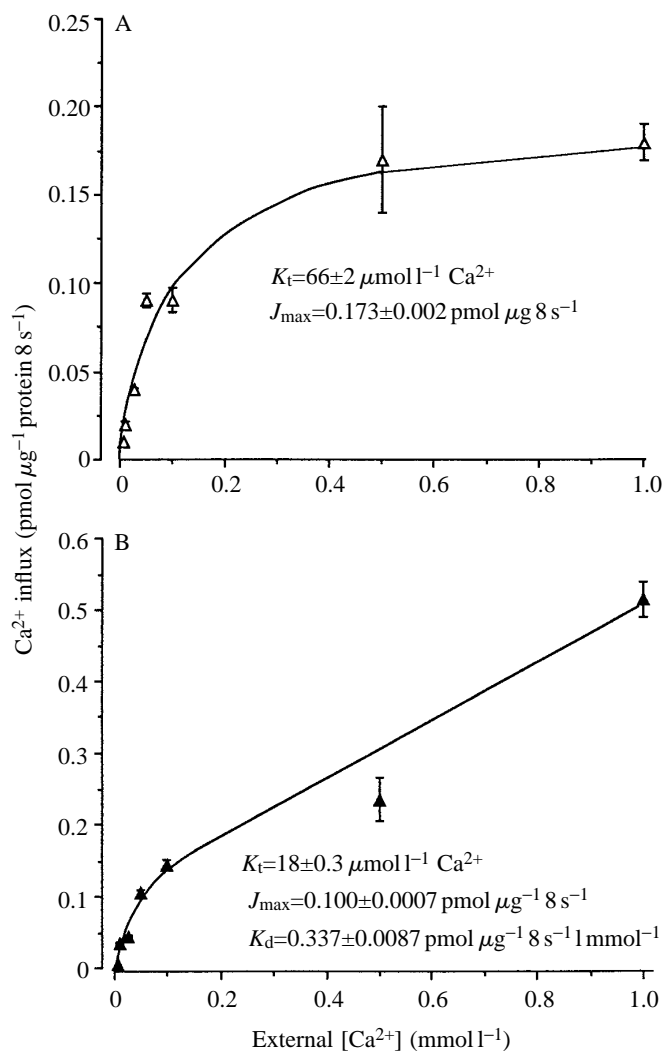


Fig. 12. Amiloride-sensitive and amiloride-insensitive components of $^{45}Ca^{2+}$ influx into pyloric cecal BBMV's. (A) Results obtained by subtracting amiloride-insensitive influx from total influx in Fig. 11 to produce an estimate of amiloride-sensitive calcium influx. The curve drawn through the data and the estimated kinetic variables were obtained using a curve-fitting computer program and the Michaelis–Menten equation for carrier transport. (B) Results taken directly from Fig. 11 showing amiloride-insensitive flux; the curve was fitted using the same computer program and a combination of Michaelis–Menten function plus diffusion according to equation 1 in the text.

This type of experiment would demonstrate two phenomena. First, countertransport of labelled Ca^{2+} by the test solute would suggest that both substances were able to use the same carrier protein for exchange and, second, these results would imply that *in vivo* the test solute could gain access to the intracellular compartment of pyloric cecal cells by way of this membrane protein.

Short-circuited vesicles were loaded with 150 mmol l^{-1} KCl, 5 mmol l^{-1} HEPES and $50 \mu\text{mol l}^{-1}$ valinomycin at pH 6.0 and were preincubated for 30 min in an identical medium containing 0.1 mmol l^{-1} $^{45}Ca^{2+}$ gluconate at pH 8.0. At the end

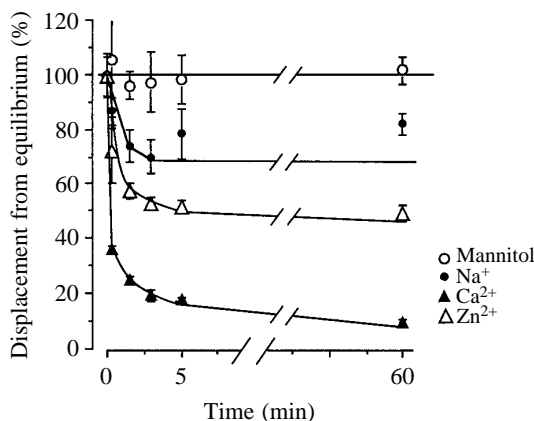


Fig. 13. An equilibrium shift experiment demonstrating *trans*-stimulation of 0.1 mmol l^{-1} $^{45}\text{Ca}^{2+}$ efflux from equilibrated pyloric cecal BBMV by external Na^+ , Ca^{2+} or Zn^{2+} (mannitol serving as a control). Vesicles were preincubated and exposed to the various external substrates as described in the text.

of this preincubation period, triplicate samples of reaction mixture were taken to estimate the vesicular isotope content. Subsequently, a small volume of highly concentrated solutions of mannitol, Na^+ , Ca^{2+} and Zn^{2+} (final concentrations in the reaction mixture were 10 mmol l^{-1} each) was added to the remaining reaction mixtures to initiate the countertransport process. Triplicate samples of these reaction media were subsequently taken at 30 s, 1.5, 3, 5 and 60 min, added to ice-cold stop solution, filtered and counted for radioactivity content.

Fig. 13 shows that 10 mmol l^{-1} mannitol in the external medium had no effect on the amount of radiolabelled Ca^{2+} remaining in vesicles during a 60 min exchange period. In contrast, 10 mmol l^{-1} Na^+ , Ca^{2+} and Zn^{2+} all stimulated the efflux of $^{45}\text{Ca}^{2+}$ from the pre-loaded vesicles, with unlabelled Ca^{2+} being the best *trans*-stimulator. Significant countertransport of internal Ca^{2+} for external Na^+ and Zn^{2+} strongly suggests that both monovalent and divalent cations are able to employ a common carrier mechanism for exchange and to gain access to the intracellular compartment of pyloric cecal cells.

Discussion

The results of the present study indicate that the brush-border membrane of starfish pyloric ceca possesses an electrogenic $2\text{Na}^+/\text{H}^+$ antiporter that is capable of transporting the monovalent cations Na^+ and H^+ and the divalent cations Ca^{2+} and Zn^{2+} and may be inhibited in its exchange function by a monoclonal antibody produced in mice against protein elements of the analogous exchanger in crustacean hepatopancreas. Previous work with brush-border vesicles of the starfish *Pycnopodia helianthoides* indicated that $^{22}\text{Na}^+$ influx, in the presence of an outwardly directed proton gradient, was a sigmoidal function of external $[\text{Na}^+]$, and yielded a Hill coefficient of 2.6, suggesting that two or more

Na^+ exchanged with each internal proton during the transport event (Ahearn and Franco, 1991). In addition, amiloride acted as a competitive inhibitor of Na^+ binding to two external sites with markedly dissimilar apparent amiloride affinities ($K_{i1}=28 \mu\text{mol l}^{-1}$; $K_{i2}=1650 \mu\text{mol l}^{-1}$). Finally, a static-head flux analysis resulted in a $2\text{Na}^+/\text{H}^+$ exchange stoichiometry, where a balance of driving forces (e.g. no net Na^+ flux) was attained with a combination of a 10:1 Na^+ gradient and a 100:1 H^+ gradient. These experiments, taken together, suggested that the brush-border membrane of the echinoderm gastrointestinal diverticulum possessed an exchange protein with physiological properties very similar to those recently described for both the hepatopancreatic and antennal gland epithelia of crustaceans (Ahearn and Clay, 1989; Ahearn and Franco, 1990; Ahearn *et al.* 1990).

Recent work with crustacean hepatopancreas and antennal gland epithelial electrogenic $2\text{Na}^+/\text{H}^+$ antiporters has indicated that proteins in both organs were capable of binding and transporting the divalent cation Ca^{2+} as well as Na^+ (Ahearn and Franco, 1990, 1993; Zhuang and Ahearn, 1995), a property not shared by Na^+/H^+ exchangers of vertebrates (Aronson, 1985; Aronson and Igarashi, 1986). In lobster (*Homarus americanus*) antennal glands, external Ca^{2+} , like amiloride, was a strong competitive inhibitor of $2\text{Na}^+/\text{H}^+$ exchange, acting at two sites on the outer vesicular face with markedly different apparent divalent cation affinities ($K_{i1}=20 \mu\text{mol l}^{-1}$; $K_{i2}=500 \mu\text{mol l}^{-1}$) (Ahearn and Franco, 1990). In brush-border membrane vesicles from both organs, $^{45}\text{Ca}^{2+}$ influx was composed of three components: (1) an electrogenic amiloride-sensitive carrier system (the $2\text{Na}^+/\text{H}^+$ antiporter); (2) an electroneutral amiloride-insensitive carrier system ($2\text{Na}^+/\text{Ca}^{2+}$ or $2\text{H}^+/\text{Ca}^{2+}$ exchanger); and (3) a verapamil- and membrane-potential-sensitive process that may be diffusional transfer through a Ca^{2+} channel (Ahearn and Franco, 1993; Zhuang and Ahearn, 1995). A monoclonal antibody raised against protein elements of the lobster hepatopancreatic brush-border membrane strongly inhibited the amiloride-sensitive $2\text{Na}^+/\text{H}^+$ and $1\text{Ca}^{2+}/\text{H}^+$ exchange functions of these membranes, but had no effect on Na^+ -dependent D-glucose transport by the same preparation (de Couet *et al.* 1993). Therefore, in the lobster, the Na^+ and Ca^{2+} transport functions of the hepatopancreas and antennal glands were closely associated and involved shared membrane transport proteins.

In the present investigation with starfish pyloric cecal brush-border membrane vesicles, $^{45}\text{Ca}^{2+}$ uptake was stimulated by an outwardly directed proton gradient and this stimulation was enhanced by the addition of an induced membrane potential (inside negative; $\text{K}^+/\text{valinomycin}$) (Figs 2, 3). External amiloride and the crustacean monoclonal antibody both inhibited approximately half of the proton-gradient-stimulated $^{45}\text{Ca}^{2+}$ uptake, suggesting that Ca^{2+} may be transported by the electrogenic antiporter and that the crustacean antibody was inhibitory to its exchange function in echinoderms (Figs 2, 5, 8, 9–12). Ca^{2+} was transported by two apparently carrier-mediated transfer mechanisms, distinguishable on the basis of

their amiloride sensitivity (Figs 11, 12). Ca^{2+} transfer across the pyloric cecal brush-border membrane also appeared to take place by diffusion through a proton- and nifedipine-sensitive channel, which appeared to be responsive to the imposition of a transmembrane electrical potential.

Zn^{2+} , Fe^{2+} , Mn^{2+} , Cu^{2+} and Mg^{2+} inhibited $^{45}\text{Ca}^{2+}/\text{H}^{+}$ exchange by starfish pyloric cecal brush-border vesicles (Figs 5–7). Zn^{2+} was a mixed inhibitor of carrier-mediated $^{45}\text{Ca}^{2+}$ influx, exhibiting a K_i of 0.92 mmol l^{-1} (Fig. 6), suggesting the possibility that both divalent cations were able to use a common exchanger for uptake into the cells. Equilibrium shift experiments verified the ability of Ca^{2+} , Na^{+} and Zn^{2+} to exchange with each other by way of a common transport protein and thereby strengthen the argument that each had the potential to gain access to the pyloric cecal intracellular compartment *in vivo* (Fig. 13).

Considerable work has been conducted on the time course of heavy metal ion uptake, sequestration and detoxification in marine invertebrates (Coombs and George, 1978; Mason and Nott, 1981; Viarengo and Nicotera, 1991; Viarengo and Nott, 1993), but the physiological mechanisms by which these divalent cations enter animal cells prior to the initiation of intracellular detoxification processes has largely remained unclear. The results of the present study, for the first time, suggest that Zn^{2+} , and perhaps other related heavy metal ions, is transferred across epithelial cell membranes in invertebrates by carrier proteins that are also responsible for the regulation of other cellular constituents, such as Na^{+} , H^{+} and Ca^{2+} . These data also suggest that one possible mechanism for acute or chronic heavy metal toxicity in animals living in environments with significant metal concentrations might be the inability to regulate cellular Ca^{2+} levels because of competitive interactions between the metal ions and this important divalent cation. Obvious physiological activities that could be impaired by reduced Ca^{2+} uptake in marine species include calcification processes (e.g. skeletal growth, shell formation, molting, etc.) and a large variety of biochemical events that must be regulated by precise intracellular Ca^{2+} activities.

In order for an environmental heavy metal ion to be of significance as a possible inhibitor of Ca^{2+} uptake, the transport mechanism shared by the metal ion and Ca^{2+} must have an apparent binding affinity for the metal ion that is similar to, or higher than, that for Ca^{2+} . Because metal ions and Ca^{2+} act as competing substrates at the same carrier binding sites, the cation with the highest affinity will be more likely to associate with the transporter (at equal environmental concentrations) and to enter the cell. Sea water has a Ca^{2+} concentration near 10 mmol l^{-1} , whereas environmental Zn^{2+} concentrations are generally many times lower than this (Forstner and Wittmann, 1979). The present investigation reported the apparent Ca^{2+} binding affinities (K_t) of starfish amiloride-sensitive and amiloride-insensitive transporters in pyloric cecal epithelium to be $66 \pm 2\ \mu\text{mol l}^{-1}$ and $18 \pm 0.3\ \mu\text{mol l}^{-1}$, respectively (Fig. 12), while the inhibitor constant (K_i) for Zn^{2+} was $920\ \mu\text{mol l}^{-1}$ (Fig. 6). Because the shared carrier exhibited a considerably higher apparent binding affinity for Ca^{2+} than for

Zn^{2+} , and because environmental concentrations of Ca^{2+} far exceed those for Zn^{2+} , 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 may contain much higher concentrations of Zn^{2+} , or other potential heavy metal inhibitors of Ca^{2+} transport, as a result of bioaccumulation and sequestration mechanisms in the cells of the prey. Under these conditions, it is possible that gastrointestinal heavy metal ion concentrations could be much higher than those found in the water column or sediments and could therefore pose a potentially far more serious physiological problem. Information concerning relative Ca^{2+} inhibitory constants for each heavy metal, and realistic dietary concentrations of these agents, are needed to determine which metal ions are likely to pose the greatest physiological threat to Ca^{2+} regulation.

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