The Journal of Experimental Biology 202, 3003–3010 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JEB2047

EFFECTS OF ZINC ON L-[^{3}H]PROLINE UPTAKE BY LOBSTER (*HOMARUS AMERICANUS*) HEPATOPANCREATIC BRUSH-BORDER MEMBRANE VESICLES

MAHEALANI K. MONTEILH-ZOLLER[1], VINCENZO ZONNO[2], CARLO STORELLI[2] AND GREGORY A. AHEARN[1,*]

[1]*Department of Zoology, 2538 The Mall, University of Hawaii at Manoa, Honolulu, Hawaii 96822, USA* and [2]*Laboratorio di Fisiologia, Departimento di Biologia, Universita di Lecce, 173100 Lecce, Italy*

*e-mail: ahearn@zoology.hawaii.edu

Accepted 9 August; published on WWW 13 October 1999

Summary

Epithelial brush-border membrane vesicles (BBMVs) from the hepatopancreas of the lobster *Homarus americanus* were prepared using a magnesium precipitation technique and employed in transport experiments designed to demonstrate the effects of external and internal divalent cationic heavy metals on the uptake of L-[^{3}H]proline. When BBMVs were exposed to a high external concentration (2.5 mmol l^{-1}) of Cd^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} or Zn^{2+}, L-[^{3}H]proline (0.5 mmol l^{-1}) uptake was significantly ($P<0.05$) decreased by each metal. However, if a 30 min pre-incubation period with each metal was used before incubation of the vesicles with amino acid and metal, a significant ($P<0.05$) enhancement of L-[^{3}H]proline transport occurred. Zinc was the most stimulatory metal of those tested. Proline influxes (1.0 and 2.5 mmol l^{-1}) were hyperbolic functions of bilateral [Zn^{2+}], with a lower apparent zinc half-saturation constant (K_m) at the higher amino acid concentration. L-[^{3}H]proline influx was a hyperbolic function of external [L-proline] (K_m=2.10±0.26 mmol l^{-1}; J_{max}=2290±600 pmol mg^{-1} protein 10 s^{-1}) (means ± S.E.M., N=3), and bilateral exposure to zinc significantly ($P<0.05$) increased the maximal rate of influx, J_{max}, of proline (J_{max}=4890±250 pmol mg^{-1} protein 10 s^{-1}), but had no effect ($P>0.05$) on apparent L-[^{3}H]proline binding to the membranes (K_m=1.66±0.23 mmol l^{-1}) (means ± S.E.M., N=3). In the presence of 0.5 mmol l^{-1} l-pipecolate, bilateral zinc-stimulated, carrier-mediated, L-[^{3}H]proline influx was abolished. At low external concentrations of zinc alone (e.g. below 1.0 mmol l^{-1}), L-[^{3}H]proline influx was enhanced by the metal. Enhanced amino acid uptake in the presence of external zinc alone was abolished by L-pipecolate. A model accounting for external and internal zinc enhancements of L-[^{3}H]proline influx by the Na^+-dependent L-pipecolate-sensitive IMINO transport system in these membranes is proposed.

Key words: brush-border membrane vesicle, BBMV, L-proline, heavy metal, zinc, L-pipecolate, hepatopancreas, epithelium, lobster, *Homarus americanus*, IMINO system, amino acid transport.

Introduction

The plasma membrane transport sytems for the amino acid L-proline have been characterized in kidney and gastrointestinal epithelial cells of mammals (Stevens and Wright, 1985), fishes (Maffia et al., 1990; Vilella et al., 1989) and invertebrates (Ahearn and Behnke, 1991; Behnke et al., 1990). These studies indicate that this amino acid is transferred across plasma membranes by a combination of Na^+-dependent and Na^+-independent transport carriers as well as by simple diffusion. Through the judicious selection of inhibitory substrates, these studies have shown that the great majority of L-proline transport across these membranes takes place by the Na^+-dependent IMINO system, with a smaller proportion entering cells by way of the Na^+-dependent ASC system (Ahearn and Behnke, 1991; Stevens and Wright, 1985). Smaller quantities of radiolabelled L-proline are absorbed through the Na^+-independent L-system and by diffusion (Ahearn and Behnke, 1991).

Zinc is a trace element with several known biological roles that involve the activation and regulation of enzymes controlling a wide variety of functions in animal cells (Prasad, 1979; Vallee and Falchuck, 1993). Zinc is also known to activate transport proteins involved in moving such substrates as oligopeptides (Daniel and Adibi, 1995) and amino acids (Giroux and Henkin, 1972; Wapnir etal., 1983; Wapnir and Stiel, 1986) across cellular membranes by changing the binding affinity of the transporter for the substrate. The precise mechanism whereby the metal increases the binding affinity of the carrier proteins for these substrates is unclear and may vary with each transport system.

The epithelium of the crustacean hepatopancreas is a significant site of nutrient absorption, and much of its function in these animals is analogous to that of the intestine of mammals and other vertebrates (Ahearn, 1987, 1988; Ahearn

et al., 1985, 1992). Although the mechanisms responsible for L-proline transport by the gut of the starfish (*Pycnopodia helianthoides*; Ahearn and Behnke, 1991) and the kidney of the lobster (*Homarus americanus*; Behnke et al., 1990) have been described in recent years, there have been no detailed studies of epithelial transport of this amino acid in the gastrointestinal tract of crustaceans, nor have the effects of xenobiotics, such as heavy metals, upon this transport been clarified. The present study is an initial investigation of the influence of specific heavy metals, particularly zinc, on the Na^+-dependent IMINO L-proline transport system of the lobster hepatopancreatic epithelium. The results indicate that zinc may stimulate L-proline uptake *via* this transport protein by increasing the maximal rate of amino acid transport across the membrane (J_{max} effect) without producing a significant effect on amino acid binding (K_m effect).

Materials and methods

Live Atlantic lobsters (*Homarus americanus*) were obtained from commercial dealers in Hawaii and kept at 10 °C in a filtered seawater tank for up to 7 days before experimentation.

Hepatopancreatic brush-border membrane vesicles (BBMVs) were prepared using fresh tissue from individual lobsters. Vesicle preparation utilized a single organ (approximately 25 g fresh mass) per experiment and was performed using methods developed for mammalian cells (Kessler et al., 1978) as modified for crustacean tissues (Ahearn et al., 1985). The hepatopancreas was removed from the organism and quickly transferred to 300 ml (60 ml of buffer + 240 ml of distilled water) of hypotonic buffer 1 (300 mmol l^{-1} mannitol, 12 mmol l^{-1} Tris/HCl, pH 7.4, 1.64 mmol l^{-1} EGTA and 1.0 mmol l^{-1} phenylmethylsulfonylfluoride, PMSF). Following a high-speed homogenization in a blender, the mixture was centrifuged at 27 000 ***g*** for 30 min. Soluble enzymes were removed through an additional resuspension, washing (hypotonic buffer 1) and centrifugation (27 000 ***g*** for 30 min). The resulting pellet was resuspended in 300 ml of diluted buffer 1, to which was added 4.5 mmol l^{-1} $MgCl_2$, and left on ice for 15 min. After another centrifugation at 27 000 ***g*** (30 min), the pellet was resuspended in 35 ml of hypotonic buffer 2 (60 mmol l^{-1} mannitol, 12 mmol l^{-1} Tris/HCl, 6 mmol l^{-1} EGTA at pH 7.4). The magnesium chloride precipitation step was repeated on this mixture, followed by two additional centrifugations as described above. The recovered pellet was resuspended in 10 ml of transport buffer (usually 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate, 25 mmol l^{-1} Hepes, pH 7.4) and centrifuged a final time at 27 000 ***g*** for 30 min. The resulting pellet contained purified BBMVs, as described by Ahearn et al. (1985), and was resuspended in a small volume of transport buffer.

In most experiments, hepatopancreatic BBMVs were pre-incubated for 30 min at 24 °C in different concentrations of heavy metals (Zn^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+} or Cd^{2+}) and then exposed to isotope. Hepatopancreatic BBMV uptake studies were conducted at 24 °C and were initiated by the addition of 10 µl of vesicles to 40 µl of radiolabelled transport medium (the composition of which varied with each experiment) containing L-[^{3}H]proline (as L-[2,3,4,5-^{3}H]proline; specific activity=3.70 TBq mmol^{-1}; 100 Ci mmol^{-1}) (Amersham Life Sciences). Following an incubation period of 10 s, the uptake was terminated by the addition of 2 ml of ice-cold stop solution (the composition of which varied with each experiment) lacking L-[^{3}H]proline. The resulting suspension was filtered using the Millipore filtration techniques of Hopfer et al. (1973). Filters were added to liquid scintillation cocktail (Ecolume, Beckman) and counted for radioactivity in a Beckman LS-8100 scintillation counter. 'Blank' values, where vesicles and transport media were added to the stop solution and filtered without prior mixing, were subtracted to compensate for non-specific binding of L-[^{3}H]proline to BBMVs. Uptake of L-[^{3}H]proline is expressed as pmol mg^{-1} protein. Determination of protein content was performed using the BioRad technique. Each point represents the mean of 3 replicates with their associated standard errors (S.E.M.).

Results

Effects of pre-incubation with various metals on L-[^{3}H]proline uptake

Fig. 1 illustrates the effects of various heavy metals (Cd^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+}) at 2.5 mmol l^{-1} on the uptake of 0.5 mmol l^{-1} L-[^{3}H]proline. Exposure of vesicles to radiolabelled proline and metals simultaneously, without prior metal pre-incubation, universally led to a significant reduction (P<0.05) in amino acid uptake over 10 s. The most inhibitory metal in this regard was Cd^{2+}, while the least effective in reducing L-[^{3}H]proline uptake was Fe^{2+}. When vesicles were pre-incubated for 30 min in medium containing the individual metal and then re-exposed to transport medium containing an equimolar amount of the respective metal, a stimulation of L-[^{3}H]proline uptake resulted. Although all the metals were significantly stimulatory (P<0.05), Zn^{2+} displayed the greatest difference in enhancing absorption compared with vesicles not treated with Zn^{2+} (control). Because Zn^{2+} was the most stimulatory metal for L-[^{3}H]proline uptake, additional studies were conducted with this metal to investigate the nature of its stimulatory action.

Effects of bilateral zinc pre-incubation on the time course of L-[^{3}H]proline uptake

To estimate the effects of a zinc pre-incubation on L-[^{3}H]proline transport by lobster BBMVs, a long-term experiment examining the uptake of radiolabelled amino acid was conducted in the presence and absence of Zn^{2+} in both the pre-incubation and incubation media. Vesicles were loaded with 100 mmol l^{-1} potassium gluconate, 100 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes (pH 7.4), either 0 or 10 mmol l^{-1} $ZnCl_2$ and 50 µmol l^{-1} valinomycin. Following a 30 min pre-incubation, vesicles were subsequently exposed to transport medium containing 100 mmol l^{-1} sodium gluconate, 100 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes, 0.5 mmol l^{-1}

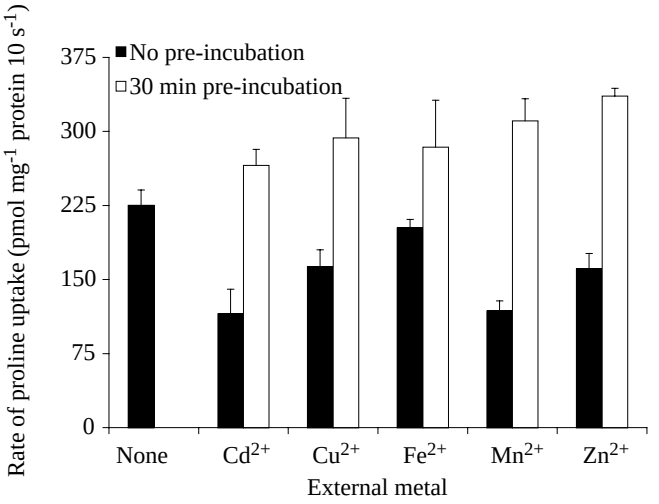

Fig. 1. Effects of bilateral pre-incubation in the presence of various heavy metals on L-[^{3}H]proline uptake by lobster hepatopancreatic brush-border membrane vesicles. Vesicles were pre-incubated for 30 min in medium containing 150 mmol l^{-1} potassium gluconate (inside-negative membrane potential), 2.5 mmol l^{-1} metal (Cd^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} or Zn^{2+}), 50 μmol l^{-1} valinomycin and 25 mmol l^{-1} Hepes/Tris at pH 7.4. Pre-incubated vesicles were incubated for 15 s in medium of the same pH containing 150 mmol l^{-1} sodium gluconate, 0.5 mmol l^{-1} L-[^{3}H]proline and 2.5 mmol l^{-1} of the metal. Vesicles that were not pre-incubated in metal were exposed for 10 s to the same external medium as the pre-incubated vesicles. Values are expressed as means + S.E.M. of three replicates.

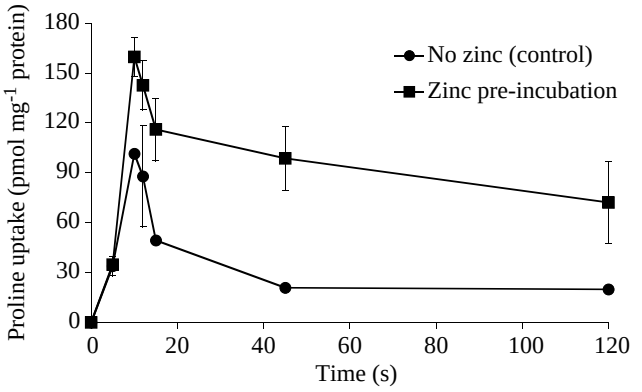

Fig. 2. Effects of bilateral zinc pre-incubation on the time course of L-[^{3}H]proline uptake by lobster brush-border membrane vesicles. Vesicles were exposed for a 30 min pre-incubation to medium containing 100 mmol l^{-1} potassium gluconate, 100 mmol l^{-1} mannitol, either 0 or 10 mmol l^{-1} Zn^{2+}, 50 μmol l^{-1} valinomycin and 25 mmol l^{-1} Hepes/Tris at pH 7.4. Transport was initiated by a second exposure (5–120 s) to external medium containing 100 mmol l^{-1} sodium gluconate, 100 mmol l^{-1} mannitol, 0.5 mmol l^{-1} L-[^{3}H]proline, 0 or 10 mmol l^{-1} Zn^{2+} and 25 mmol l^{-1} Hepes/Tris at pH 7.4. Values are expressed as means ± S.E.M. of three replicates.

L-[^{3}H]proline and either 0 or 10 mmol l^{-1} $ZnCl_2$ for 10 s. Fig. 2 shows L-[^{3}H]proline uptake to be significantly greater in vesicles pre-incubated and incubated in medium containing $ZnCl_2$ than in medium lacking the metal. Furthermore, the overshoot phenomenon, illustrating its maximal value at 15 s incubation and often observed in carrier-mediated transport, was increased by approximately 60 % when zinc pre-incubation/incubation was imposed on hepatopancreatic BBMV preparations. From Fig. 2, 10 s incubations were chosen to estimate apparent L-[^{3}H]proline influxes in all subsequent experiments.

Effects of varying bilateral concentrations of zinc on L-[^{3}H]proline influx

The effects of various concentrations of Zn^{2+} on the influx of 1.0 and 2.5 mmol l^{-1} L-[^{3}H]proline were measured to assess the nature of the interaction between proline and zinc. In this experiment, vesicles were preloaded with 100 mmol l^{-1} potassium gluconate, 100 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes (pH 7.4), 50 μmol l^{-1} valinomycin and 0–10 mmol l^{-1} $ZnCl_2$ and allowed to pre-incubate for 30 min. Following this pre-incubation interval, BBMVs were introduced to uptake medium composed of 100 mmol l^{-1} sodium gluconate, 100 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes (pH 7.4), either 1.0 or 2.5 mmol l^{-1} L-[^{3}H]proline and 0–10 mmol l^{-1} $ZnCl_2$ for a period of 10 s.

Fig. 3 indicates the effect of increasing concentrations of Zn^{2+} in both pre-incubation and incubation media on the influx of 1.0 and 2.5 mmol l^{-1} L-[^{3}H]proline. L-[^{3}H]proline influxes at both concentrations were hyperbolic functions of Zn^{2+} concentration. Significant vertical axis intercepts at both amino acid concentrations at 0 mmol l^{-1} Zn^{2+} were obtained, suggesting the presence of one or more zinc-independent transport processes in addition to the transport system that was enhanced by the metal. Maximal uptake values (J_{max}) for 1.0 and 2.5 mmol l^{-1} L-[^{3}H]proline influxes were 758±199 and 777±38 pmol mg^{-1} protein $10\,s^{-1}$, respectively, while K_m values of Zn^{2+} binding at 1.0 and 2.5 mmol l^{-1} L-[^{3}H]proline were 6.97±3.73 mmol l^{-1} and 0.32±0.07 mmol l^{-1} Zn^{2+} respectively. The kinetic values clearly indicate an effect on the apparent binding affinity (K_m; zinc half-saturation constant): 22 times less Zn^{2+} is required at the higher L-[^{3}H]proline concentration to achieve half-maximal uptake of the amino acid.

Effects of bilateral zinc pre-incubation on the kinetics of L-[^{3}H]proline influx

To investigate further the stimulatory action of zinc on L-proline uptake by hepatopancreatic BBMVs, L-[^{3}H]proline influx over a wide range of amino acid concentrations was measured in the bilateral presence and absence of Zn^{2+}. Vesicles were loaded with 100 mmol l^{-1} potassium gluconate, 100 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes (pH 7.4), 50 μmol l^{-1} valinomycin and either 0 or 10 mmol l^{-1} $ZnCl_2$. Following a 30 min pre-incubation, vesicles were exposed for 10 s to incubation medium containing 100 mmol l^{-1} sodium gluconate, 100 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes (pH 7.4),

the same $ZnCl_2$ concentrations as those of the respective pre-incubation medium and 0–10 mmol l^{-1} L-[^{3}H]proline.

Fig. 4 shows that L-[^{3}H]proline influx was a hyperbolic function of external amino acid concentration both in the presence and in the absence of bilateral zinc. Addition of the metal to both the vesicular interior and exterior surfaces resulted in a doubling of the maximal influx velocity (J_{max}) from 2290±600 to 4890±250 pmol mg^{-1} protein 10 s^{-1} without significantly affecting the apparent binding affinity (K_m) of the transporter for the amino acid. These results suggest that the overall effect of the metal on the kinetics of amino acid uptake may be to influence either the number of L-proline transporters traversing the membrane within a given period or the transfer rate of a fixed number of these proteins. The apparent association between the amino acid and the transport system appears to be unaffected by the presence of the metal.

Effects of L-pipecolate on L-[^{3}H]proline influx in bilaterally zinc-pre-incubated BBMVs

L-Pipecolate has been shown to be a competitive inhibitor of the IMINO carrier system for L-proline transport in mammalian intestinal brush-border membranes (Stevens and Wright, 1985). To ascertain the primary target of the stimulatory effect of zinc on L-[^{3}H]proline influx in lobster hepatopancreatic BBMVs, L-pipecolate was added to the external incubation medium during the measurement of amino acid uptake. Vesicles were subjected to the usual 30 min pre-incubation as in the experiments described in Fig. 4. The incubation medium was similar to that described in Fig. 4, except that 0.5 mmol l^{-1} L-pipecolate was added to the external medium.

As shown in Fig. 5, vesicles treated with L-pipecolate and also exposed to Zn^{2+} displayed a non-saturable, diffusion-like influx of L-[^{3}H]proline over the range of amino acid concentrations used, whereas vesicles not subjected to L-pipecolate treatment, but exposed to Zn^{2+}, displayed a hyperbolic influx pattern indicative of carrier-mediated transport. These transport characteristics suggest that the L-pipecolate-sensitive IMINO L-proline carrier system is influenced by Zn^{2+} and is a likely candidate for mediating the enhanced amino acid uptake observed in the presence of the metal. In the presence of L-pipecolate, all other L-[^{3}H]proline influx pathways appear to be refractory to this heavy metal.

Effects of external zinc only on L-[^{3}H]proline uptake

To determine the locus of the stimulatory action of zinc upon L-[^{3}H]proline uptake by lobster hepatopancreatic BBMVs, the influxes of 1.0 and 2.5 mmol l^{-1} L-[^{3}H]proline were measured in the presence of several concentrations of the metal added only to the external medium during the time of transport measurement. Vesicles were loaded with 100 mmol l^{-1} potassium gluconate, 100 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes (pH 7.4) and 50 µmol l^{-1} valinomycin. Vesicles were then exposed for 10 s to transport medium containing 100 mmol l^{-1} sodium gluconate, 100 mmol l^{-1} mannitol,

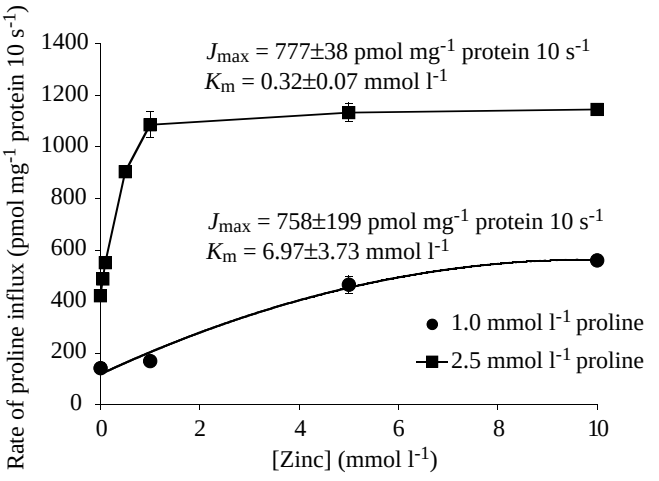

Fig. 3. Effects of bilaterally pre-incubated zinc concentration on the apparent influx of L-[^{3}H]proline into brush-border membrane vesicles of lobster hepatopancreas. Vesicles were pre-incubated (30 min) in medium containing 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate, 0–10 mmol l^{-1} Zn^{2+}, 50 µmol l^{-1} valinomycin and 25 mmol l^{-1} Hepes/Tris at pH 7.4. A second exposure for 10 s was conducted in medium containing 100 mmol l^{-1} mannitol, 100 mmol l^{-1} sodium gluconate, either 1 or 2.5 mmol l^{-1} L-[^{3}H]proline, 0–10 mmol l^{-1} Zn^{2+} and 25 mmol l^{-1} Hepes/Tris at pH 7.4. Values are expressed as means ± S.E.M. of three replicates, and kinetic constants were obtained using Sigma Plot (Jandel) curve-fitting software.

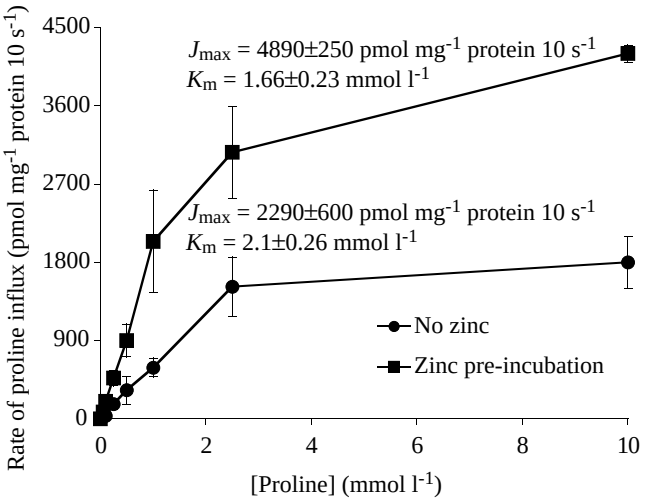

Fig. 4. Effects of external L-proline concentration on the kinetics of L-[^{3}H]proline influx in the presence and absence of bilaterally pre-incubated zinc into brush-border membrane vesicles of lobster hepatopancreas. Vesicle pre-incubation was conducted in medium containing 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate, either 0 or 10 mmol l^{-1} Zn^{2+}, 50 µmol l^{-1} valinomycin and 25 mmol l^{-1} Hepes/Tris at pH 7.4. Transport was initiated by addition of vesicles to external medium of the same pH, containing 100 mmol l^{-1} mannitol, 100 mmol l^{-1} sodium gluconate, either 0 or 10 mmol l^{-1} Zn^{2+} and 0–10 mmol l^{-1} L-[^{3}H]proline. Values are expressed as means ± S.E.M. of three replicates, and kinetic constants were obtained using Sigma Plot (Jandel) curve-fitting computer software.

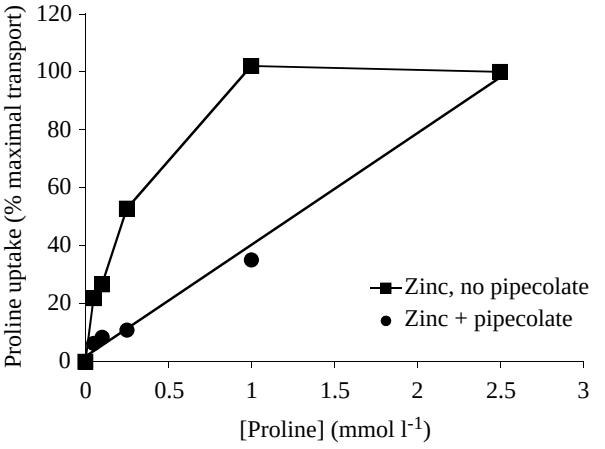

Fig. 5. Effects of L-pipecolate on L-[^{3}H]proline influx into lobster hepatopancreatic brush-border membrane vesicles bilaterally pre-incubated with zinc. Vesicles were pre-incubated (30 min) in medium containing 100 mmol l^{-1} mannitol, 100 mmol l^{-1} potassium gluconate, 10 mmol l^{-1} Zn^{2+}, 50 μmol l^{-1} valinomycin and 25 mmol l^{-1} Hepes/Tris at pH 7.4. Transport (10 s uptakes) was measured in medium of the same pH containing 100 mmol l^{-1} mannitol, 100 mmol l^{-1} sodium gluconate, 10 mmol l^{-1} Zn^{2+}, 0–10 mmol l^{-1} L-[^{3}H]proline and either 0 or 0.5 mmol l^{-1} pipecolate. Values are mean percentage values from three replicates, and lines were drawn by eye.

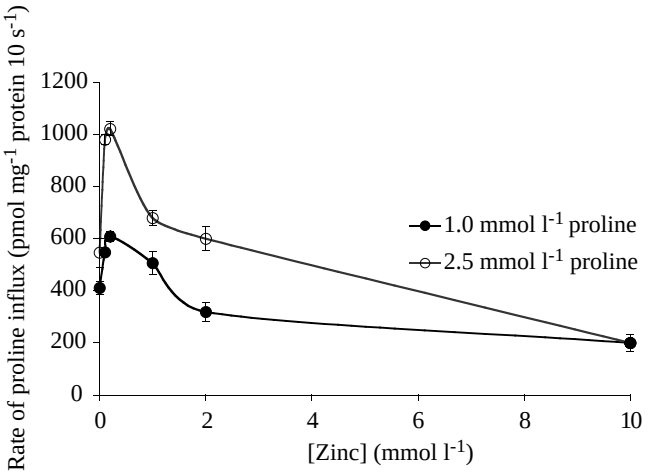

Fig. 6. Effects of external zinc alone on L-[^{3}H]proline influx into lobster hepatopancreatic brush-border membrane vesicles. Vesicles were preloaded with medium containing 100 mmol l^{-1} potassium gluconate, 100 mmol l^{-1} mannitol, 50 μmol l^{-1} valinomycin and 25 mmol l^{-1} Hepes/Tris at pH 7.4. Vesicles were then exposed (for 10 s) to transport medium containing 100 mmol l^{-1} sodium gluconate, 100 mmol l^{-1} mannitol, 1 or 2.5 mmol l^{-1} L-[^{3}H]proline, 0–10 mmol l^{-1} zinc and 25 mmol l^{-1} Hepes/Tris at pH 7.4. Values are expressed as means ± S.E.M. of three replicates.

25 mmol l^{-1} Hepes (pH 7.4), either 1.0 or 2.5 mmol l^{-1} L-[^{3}H]proline and 0–10 mmol l^{-1} $ZnCl_2$.

Fig. 6 shows that 10 s L-[^{3}H]proline influx illustrated a biphasic response to increasing concentrations of external zinc. At zinc concentrations between 0.1 and 1.0 mmol l^{-1}, significant stimulation of L-[^{3}H]proline influx was recorded, with 0.2 mmol l^{-1} zinc yielding maximum stimulation. At zinc concentrations of 2.0 mmol l^{-1} or greater, significant inhibition of L-[^{3}H]proline influx was observed. These results suggest that stimulation of L-[^{3}H]proline influx by zinc occurs not only at the exterior surface of lobster BBMVs but also within a specific range of zinc concentrations.

Effects of external zinc and L-pipecolate only on L-[^{3}H]proline influx

To assess the extent to which the IMINO L-proline transport system was stimulated by external zinc, the inhibitor L-pipecolate was added to the external medium simultaneously with the metal, and the combined effects on the time course of L-[^{3}H]proline uptake were examined. Vesicles were loaded as described in Fig. 6. L-[^{3}H]proline influxes were measured over various periods (0–60 s) in transport medium containing 100 mmol l^{-1} sodium gluconate, 100 mmol l^{-1} mannitol, 25 mmol l^{-1} Hepes (pH 7.4), 1.0 mmol l^{-1} L-[^{3}H]proline, 0 or 0.2 mmol l^{-1} Zn^{2+} and 0 or 0.5 mmol l^{-1} L-pipecolate.

As Fig. 7 illustrates, vesicles exposed to 0.2 mmol l^{-1} Zn^{2+} exhibited enhanced L-[^{3}H]proline uptake, while vesicles treated with L-pipecolate and either 0 or 0.2 mmol l^{-1} Zn^{2+} showed a nearly identical reduction in uptake. These results suggest that the L-pipecolate-sensitive IMINO L-proline transport system of lobster BBMVs is influenced by external application of zinc.

Discussion

The hepatopancreatic epithelium of the lobster *Homarus americanus* exhibits a Na^+-dependent sugar transport system (Ahearn et al., 1985) that resembles the familiar SGLT-1 transporter from vertebrates (Wright et al., 1993) and a wide variety of Na^+-dependent and Na^+-independent amino acid transport mechanisms (Ahearn et al., 1983, 1992). The details of L-proline transport by lobster hepatopancreatic epithelium have not been previously investigated. However, published L-[^{3}H]proline influx studies with echinoderm pyloric caeca (Ahearn and Behnke, 1991) have shown that, in this invertebrate gastrointestinal diverticulum, there are two Na^+-dependent L-proline transport proteins (the IMINO and ASC systems) and a single Na^+-independent L-proline carrier process (the L system). This array of L-proline transporters is similar to that described for vertebrate epithelia (Stevens and Wright, 1985) and, because of the occurrence of similar transport mechanisms across phyla, it was thought that the hepatopancreas of the lobster would exhibit similar transmembrane transfer processes for this amino acid as well. This suggestion was strengthened by a published characterization of a Na^+-dependent IMINO transport process for L-proline in the antennal gland of the lobster with similar properties to those previously described for other IMINO

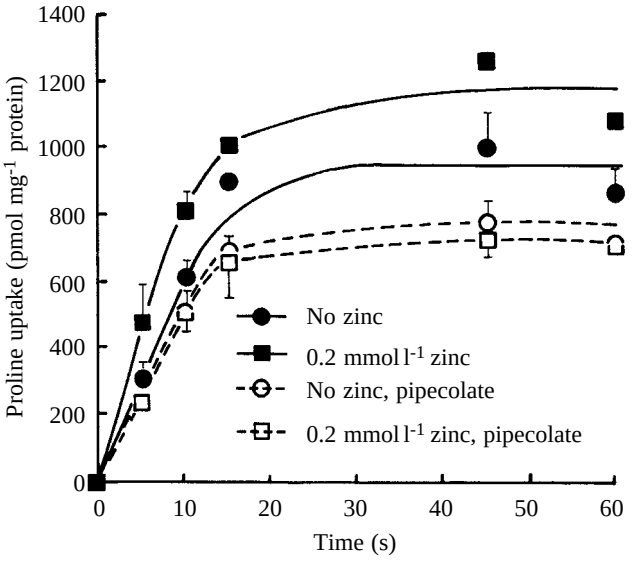

Fig. 7. Effect of external zinc and pipecolate added together to the external medium only on L-[^{3}H]proline uptake into lobster hepatopancreatic brush-border membrane vesicles. Vesicles were preloaded with medium identical to that described in Fig. 6. Transport was initiated by a second exposure (5–60 s) to an external medium containing 100 mmol l^{-1} sodium gluconate, 100 mmol l^{-1} mannitol, 1 mmol l^{-1} L-[^{3}H]proline, either 0 or 0.2 mmol l^{-1} zinc, either 0 or 0.5 mmol l^{-1} L-pipecolate and 25 mmol l^{-1} Hepes/Tris at pH 7.4. Values are expressed as means ± S.E.M. of three replicates.

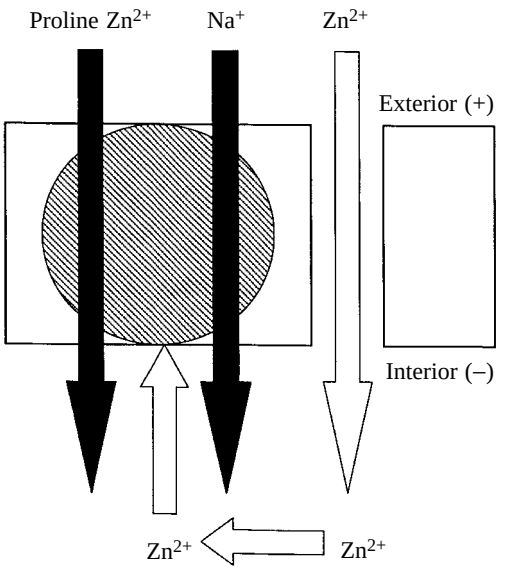

Fig. 8. Working model describing possible mechanisms by which external and internal zinc may influence the transmembrane transport of L-[^{3}H]proline by the Na^+-dependent IMINO system in the lobster hepatopancreatic epithelial brush-border membrane. The model describes the allosteric activation of the IMINO system following binding of Zn^{2+} to an internal regulator site on the transport protein after passage of the metal across the membrane by an independent route such as an ion channel. It also describes transmembrane transport of both metal and amino acid following complexation between the solutes in the external medium to produce new combined substrates that move across the membrane more rapidly than the independent native solutes.

systems in both vertebrates and invertebrates (Behnke et al., 1990).

The results of the present investigation have shown not only that a Na^+-dependent IMINO transport system exists in the hepatopancreas but also that this is apparently the L-proline transporter most influenced by zinc. As suggested by Figs 1 and 2, pre-incubation of hepatopancreatic BBMVs with heavy metals resulted in an enhanced uptake of L-[^{3}H]proline, and Zn^{2+} appeared to be the most stimulatory metal used. While pre-incubation with heavy metals resulted in a greater amino acid uptake by vesicles than if no metal were present, simultaneous incubation of BBMVs with both labelled amino acid and metal without a pre-incubation period led to significant inhibition of L-[^{3}H]proline transport (Fig. 1). The nature of this inhibition is at present unclear and was not investigated in the present study.

Insight into the mechanism by which zinc stimulated the transmembrane transfer of L-proline is gleaned from the results of Fig. 4, which shows that, in the presence of the metal, the maximal L-[^{3}H]proline transport velocity (J_{max}) was doubled, whereas the apparent binding affinity of the transporter for the amino acid (K_m) was unaffected. These data suggest either that the metal increased the number of effective L-proline carriers that transferred the amino acid across the membrane per unit of time or that there was a metal-induced enhancement of the translocation rate of a fixed number of transport proteins. At present, it is not possible to distinguish between these alternative mechanisms.

The experiments conducted in the present study were carried out in the presence of an inwardly directed Na^+ gradient. Under these conditions, all three possible identified L-proline transporters (both Na^+-dependent and Na^+-independent) could have been responsible for enhanced amino acid uptake in the presence of the metal. To determine which L-proline transporter was stimulated by pre-incubation in zinc, an experiment was conducted in the presence of an inwardly directed Na^+ gradient, a fixed concentration of metal and the inhibitor L-pipecolate. By using L-pipecolate, a known specific inhibitor of the IMINO system (Stevens and Wright, 1985), in the presence of external zinc, it was shown (Fig. 5) that carrier-mediated L-proline transport was eliminated, strongly implicating this L-proline transporter as the protein that was activated by addition of the metal.

The stimulation of amino acid transport by zinc has been reported by other investigators. Two differing models describing proposed mechanisms by which zinc enhances amino acid absorption have been published: (1) zinc may bind to the transporter protein itself and activate the transporter allosterically (Bettger and O'Dell, 1981), or (2) zinc may bind to the L-proline molecule in solution and produce a combined substrate that more readily associates with the transport system than does L-proline alone (Wapnir and Stiel, 1986; Wapnir et al., 1983). The application of these hypotheses to the current

investigation of zinc stimulation of L-[^{3}H]proline transport by lobster hepatopancreatic BBMVs is illustrated in the model shown as Fig. 8. This model proposes a possible dual function for zinc on the Na^+-dependent IMINO L-proline transport system. In one of its two roles, zinc acts as a potential allosteric activator of the transporter by binding to a putative intracellular binding site after passage through the membrane by a channel or pore independent of an L-proline transport system. The maximal transport velocity of the IMINO system is enhanced by the metal transforming the carrier protein into an activated conformation that could more readily accelerate the transmembrane transfer of the amino acid. In its second function, zinc stimulates L-proline transport through the IMINO system by complexation between the metal and amino acid in solution to form a combined substrate that more readily associates with the transport protein than does the native amino acid alone. In this instance, the more Zn^{2+}/proline complexes there are in solution, the greater will be the number of L-proline molecules transported per unit of time. As discussed below, it is likely that both these roles of zinc, operating simultaneously, could account for the enhanced L-proline influx J_{max} (Fig. 4) and for the hyperbolic stimulatory effect of zinc at a fixed amino acid concentration (Fig. 3) seen in the present study.

Support for the idea that zinc acts externally to enhance the uptake of L-[^{3}H]proline is presented in Figs 6 and 7. In these experiments, vesicles were exposed to external Zn^{2+} only during transport measurement, and the resulting enhanced amino acid uptake is interpreted as reflecting an external effect of the metal as a likely result of complexation between the substrates in solution. In Fig. 6, external zinc had two opposing actions. At low concentrations, zinc enhanced L-proline influx, while at higher concentrations Zn^{2+} inhibited L-proline influx. Stimulation of L-[^{3}H]proline influx is believed to occur at lower metal concentrations because Zn^{2+} at these concentrations produces optimal numbers of Zn^{2+}/proline complexes in solution.

Although the nature of zinc inhibition of L-[^{3}H]proline influx at higher metal concentrations is not known, a previous study of the electrogenic $2Na^+/1H^+$ antiporter of lobster hepatopancreatic BBMVs showed that metals, including zinc, inhibited $^{22}Na^+/H^+$ exchange by this transporter as a result of competitive inhibition between Zn^{2+} and Na^+ at the external cation-binding site (Ahearn, 1996; Ahearn et al., 1994). Therefore, at elevated concentrations of zinc in solution, complexation between the metal and proline may still occur, but more free zinc is available to potentially inhibit Na^+ binding to its cotransport binding site on the IMINO transport system. If Na^+ is not able to power the transmembrane transfer of L-[^{3}H]proline by way of the Na^+-dependent IMINO system because of competitive inhibition by zinc, an overall reduction in the rate of amino acid uptake in the presence of elevated external zinc concentrations would be observed. This hypothesis is also supported by the results in Fig. 1, in which simultaneous exposure of BBMVs to L-[^{3}H]proline and to 2.5 mmol l^{-1} zinc without pre-incubation led to a pronounced inhibition of amino acid influx.

Most of the experiments in the present investigation were performed with equimolar bilateral zinc concentrations established by a 30 min pre-incubation period. In all cases, a pre-incubation in the presence of metal, even at elevated metal concentrations, resulted in stimulated L-[^{3}H]proline influx. This interesting result probably occurred because pre-incubation in the presence of metal allowed access of zinc to an intracellular allosteric metal activation site on the IMINO transporter. Consistent stimulation of L-[^{3}H]proline influx in the presence of elevated Zn^{2+} concentrations, following a pre-incubation period, suggests that intracellular allosteric activation of the IMINO system in concert with enhanced metal/amino acid complex formation in solution is able to overcome competitive inhibitory interactions between external Zn^{2+} and Na^+ acting at the external cotransport site on the carrier protein.

This investigation was supported by grants from the US National Science Foundation (IBN93-17230 and IBN97-30874) and the US Environmental Protection Agency (R-823068-01-0).

References

Ahearn, G. A. (1987). Nutrient transport by the crustacean gastrointestinal tract: recent advances with vesicle techniques. *Biol. Rev.* **62**, 45–63.

Ahearn, G. A. (1988). Nutrient absorption by the invertebrate gut. In *Advances in Environmental and Comparative Physiology*, vol. 2 (ed. R. Gilles), pp. 91–129. Berlin: Springer-Verlag.

Ahearn, G. A. (1996). The invertebrate electrogenic $2Na^+/1H^+$ exchanger: Polyfunctional epithelial workstation. *News Physiol. Sci.* **11**, 31–35.

Ahearn, G. A. and Behnke, R. D. (1991). L-Proline transport systems of starfish pyloric caeca. *J. Exp. Biol.* **158**, 477–493.

Ahearn, G. A., Gerencser, G. A., Thamotharan, M., Behnke, R. D. and Lemme, T. H. (1992). Invertebrate gut diverticula are nutrient absorptive organs. *Am. J. Physiol.* **263**, R472–R481.

Ahearn, G. A., Grover, M. L. and Dunn, R. E. (1985). Glucose transport by lobster hepatopancreatic brush border membrane vesicles. *Am. J. Physiol.* **248**, R133–R141.

Ahearn, G. A., Monckton, E. A., Henry, A. E. and Botfield, M. C. (1983). Alanine transport by lobster hepatopancreatic cell suspensions. *Am. J. Physiol.* **244**, R150–R162.

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

Behnke, R. D., Wong, R. K., Huse, S. M., Reshkin, S. J. and Ahearn, G. A. (1990). Proline transport by brush border membrane vesicles of lobster antennal glands. *Am. J. Physiol.* **258**, F311–F320.

Bettger, W. J. and O'Dell, B. L. (1981). A critical physiological role of zinc in the structure and function of biomembranes. *Life Sci.* **28**, 1425–1438.

Daniel, H. and Adibi, S. A. (1995). Selective effect of zinc on uphill transport of oligopeptides into kidney brush border membrane vesicles. *FASEB J.* **9**, 1112–1117.

Giroux, E. L. and Henkin, R. I. (1972). Competition for zinc among

serum albumin and amino acids. *Biochim. Biophys. Acta* **373**, 64–72.

Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K. J. (1973). Glucose transport in isolated brush border membrane from rat small intestine. *J. Biol. Chem.* **248**, 25–32.

Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M. and Semenza, G. (1978). A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems. *Biochim. Biophys. Acta* **506**, 136–154.

Maffia, M., Cassano, G., Marcucci, D., Vilella, S. and Storelli, C. (1990). The Na^+-dependent proline carrier of eel intestinal brush border membrane sequentially binds proline and then Na^+. *Biochim. Biophys. Acta* **1027**, 8–16.

Prasad, A. S. (1979). Clinical, biochemical and pharmacological role of zinc. *Annu. Rev. Pharmac. Toxicol.* **20**, 393–426.

Stevens, B. R. and Wright, E. M. (1985). Substrate specificity of the intestinal brush border proline/sodium (IMINO) transporters. *J. Membr. Biol.* **87**, 27–34.

Vallee, B. L. and Falchuck, K. H. (1993). The biochemical basis of zinc physiology. *Physiol. Rev.* **73**, 79–118.

Vilella, S., Cassano, G. and Storelli, C. (1989). How many Na^+-dependent carriers for L-alanine and L-proline in the eel intestine? Studies with brush border membrane vesicles. *Biochim. Biophys. Acta* **984**, 188–192.

Wapnir, R. A., Khani, D. E., Bayne, M. A. and Lifshitz, F. (1983). Absorption of zinc by the rat ileum: effects of histidine and other low molecular weight ligands. *J. Nutrit.* **113**, 1346–1354.

Wapnir, R. A. and Stiel, L. (1986). Zinc intestinal absorption in rats: specificity of amino acids as ligands. *J. Nutrit.* **116**, 2171–2179.

Wright, E. M., Hirayama, B., Hazama, A., Loo, D. D. F., Supplisson, S., Turk, E. and Hager, K. M. (1993). The sodium/glucose cotransporter (SGLT1). In *Molecular Biology and Function of Carrier Proteins* (ed. L. Reuss, J. M. Russell and M. L. Jennings), pp. 229–241. New York: Rockerfeller University Press.