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L-PROLINE TRANSPORT SYSTEMS OF STARFISH PYLORIC CAECA

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Summary

Purified brush-border membrane vesicles (BBMV) of starfish [*Pycnopodia helianthoides* (Brandt)] pyloric caecal epithelium were prepared by magnesium precipitation in order to characterize the possible role of this organ in amino acid transport. L-[^{3}H]proline uptake by these vesicles was Na^+-dependent and greater at pH 7.5 than at pH 5.5. L-Pipecolate was a competitive inhibitor of L-proline influx into these BBMV, exhibiting a K_i value of 0.02 mmol l^{-1}. The amino acid inhibitors, L-pipecolate, L-alanine and L-leucine were used as test substrates to block L-proline influx by the IMINO, NBB and L transport systems, respectively, in order to estimate the contribution of each process to total L-proline entry into pyloric caecal cells. The carrier-mediated transport constants for L-proline transfer by these three systems were: K_t=0.18 mmol l^{-1} (IMINO), 0.13 mmol l^{-1} (NBB) and 0.21 mmol l^{-1} (L); J_{max}=1310 pmol mg^{-1} protein 30 s^{-1} (IMINO), 360 pmol mg^{-1} protein 30 s^{-1} (NBB) and 470 pmol mg^{-1} protein 30 s^{-1} (L). L-Proline influxes through both the IMINO and NBB systems were sigmoidal functions of the external [Na^+], while transfer by the L system was Na^+-independent. Multiple sodium ions (e.g. 2 or 3 Na^+/L-proline) appear to be associated with L-proline transport by both Na^+-dependent transport systems, but the nature of this association (i.e. activation or energization) is unclear. Results suggest that starfish pyloric caecal epithelium possesses a similar array of L-proline transport proteins to those found in similar cell types of mammalian intestine or kidney, providing tentative support for an absorptive function for this organ.

Introduction

Cellular transport of the amino acid L-proline has been extensively investigated in a wide variety of eukaryotic organisms, including yeasts (Horak and Rihova, 1982; Jayakumar *et al.* 1979), invertebrates (Giordana *et al.* 1989; Pajor and Wright, 1989; Behnke *et al.* 1990), fish (Vilella *et al.* 1988) and mammals (Christensen, 1975; Hayashi *et al.* 1980; Stevens *et al.* 1982; Stevens and Wright, 1985). These studies have shown, using inhibitors that selectively block specific

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carrier mechanisms, that L-proline is transported across most plasma membranes by the combination of Na^+-dependent and Na^+-independent processes.

Starfish do not possess an intestine for nutrient absorption as do other types of echinoderms (e.g. sea cucumber, sea urchins), but instead display an array of digestive diverticula of the pyloric stomach called pyloric caeca. These are blind-ended, epithelium-lined tubules believed to be involved in a variety of functions, including the absorption of food after digestion in the stomach (Lawrence, 1982; Lawrence and Lane, 1982; Ferguson, 1979, 1982; Jangoux, 1982). Until recently, the only means of assessing the functional properties of this remarkable organ was indirectly by cytological and histological techniques (Anderson, 1953, 1979; Jangoux and Perpeet, 1972*a*,*b*; Jangoux, 1981, 1982). Recent application of purified epithelial brush-border membrane vesicle (BBMV) techniques to the pyloric caecum of the starfish *Pycnopodia helianthoides* provided preliminary information suggesting that the cells of this organ possess amino acid transport mechanisms with several properties in common with those of more thoroughly studied mammalian nutrient absorptive epithelial cells (Ahearn, 1990).

The present investigation is a continuation of our earlier preliminary study (Ahearn, 1990) of amino acid transport by starfish pyloric caecum. In this paper, selective inhibitory compounds, effective in characterizing amino acid transport in mammalian cells, are used to define the brush-border carrier components of L-proline transport in starfish cells and to estimate their individual contributions to total transmembrane movements of this amino acid.

Materials and methods

Starfish (*Pycnopodia helianthoides*) were collected from June to August 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 initially possessing 15–20 arms and the dissected diverticula 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 (Kessler *et al.* 1978) and crustacean (Ahearn *et al.* 1985; Behnke *et al.* 1990) epithelia.

The pooled pyloric caecal sample was homogenized in hypotonic buffer and mixed with 10 mmol l^{-1} $MgCl_2$ for selective precipitation of most cellular membranes except the brush border. This was followed with purification by centrifugation at 3000 *g* and 27 000 *g* and homogenization in additional hypotonic buffer using a glass homogenizer. A further sequence of precipitation, purification and homogenization in the proper internal buffer was then carried out, followed by centrifugation at 27 000 *g*. The resulting purified sample of brush-border membrane was resuspended in a small volume of internal medium by passage 10–15 times through a syringe fitted with a 22-gauge needle. This final vesicle suspension exhibited a total protein content of approximately 18 mg ml^{-1} (Bio-Rad protei

assay). Using this preparative method, previous studies with gastrointestinal and renal organs from such diverse organisms as mammals and crustaceans produced final purified vesicle suspensions that exhibited significant enrichments of brush-border enzyme markers such as alkaline phosphatase, sucrase and leucine aminopeptidase, 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) (Kessler *et al.* 1978; Ahearn *et al.* 1985; Behnke *et al.* 1990).

Transport studies using these pyloric caecal brush-border membrane vesicles (BBMV) were conducted at 18°C using the Millipore filtration technique of Hopfer *et al.* (1973). At the beginning of a transport experiment a volume (e.g. 20 μl) of membrane vesicles was added to a volume of radiolabelled medium (e.g. 160 μl) containing L-[2,3,4,5-^{3}H]proline (New England Nuclear, Corp.). Following incubation periods of 15 s, 1, 2, 5, 10, 20 or 120 min a known volume of this reaction mixture (20 μl) was withdrawn and plunged into 2 ml of ice-cold stop solution (composition varying with experiment, see Figure legends). The resulting suspensions were rapidly filtered through Millipore filters (0.45 μm pore diameter) to retain the vesicles and washed with another 5 ml of stop solution. Filters were added to ICN Ecolume scintillation cocktail and counted for radioactivity in a Beckman LS-9000 scintillation counter. Proline uptake was expressed (using the specific activity of amino acid in the external medium) as pmol mg^{-1} protein $filter^{-1}$. Each experiment was repeated at least twice using membranes from different animals, yielding qualitatively similar results from each experiment. Within a given experiment each point was analyzed with 3–5 replicates and values are presented in figures as means±S.E.M.

Results

Effect of external pH on uptake of L-[^{3}H]proline

To estimate the effects of external pH on L-proline transport by starfish BBMV, a long-term experiment of the uptake of radiolabelled amino acid was conducted in the presence of an inwardly directed NaCl gradient with internal and external media of pH 7.5 and 5.5. Vesicles were loaded with 300 mmol l^{-1} mannitol and either 20 mmol l^{-1} Hepes–Tris (pH 7.5) or 20 mmol l^{-1} Mes–Tris (pH 5.5) and were subsequently incubated in media containing 0.1 mmol l^{-1} L-[^{3}H]proline and 150 mmol l^{-1} NaCl at either pH 5.5 or pH 7.5. One group of vesicles at each pH was incubated in external medium including 10 mmol l^{-1} unlabelled L-proline to serve as an inhibitor of carrier-mediated radiolabelled L-proline uptake.

Fig. 1 shows that L-[^{3}H]proline uptake was greater in medium at pH 7.5 than at pH 5.5. In addition, uptake at the higher pH led to a transient accumulation of the amino acid to a concentration exceeding that at equilibrium (120 min incubation). Only equalizing transport of the amino acid occurred at the lower pH. Addition of 10 mmol l^{-1} unlabelled L-proline to the external medium at each pH significantly reduced the uptake of the amino acid, suggesting that carrier-mediated entry of

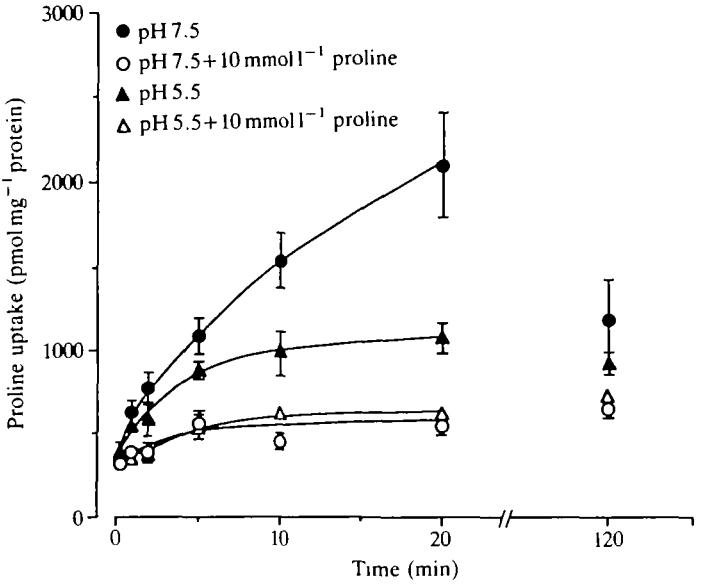

Fig. 1. Effect of external pH on the time course of uptake of 0.1 mmol l^{-1} L-[^{3}H]proline by brush-border membrane vesicles (BBMV) of *Pycnopodia helianthoides* pyloric caeca. Vesicles were loaded with 300 mmol l^{-1} mannitol buffered at either pH 7.5 or 5.5 (see text) and were incubated either with or without 10 mmol l^{-1} unlabelled L-proline in media that contained the labelled amino acid and 150 mmol l^{-1} NaCl buffered to either pH 7.5 or 5.5. Stop solution: 150 mmol l^{-1} KCl buffered to either pH 7.5 or 5.5. In all the figures, values are mean±S.E.M.; N=3–5. Where error bars are absent they were too small to extend beyond the symbols.

the organic solute accounted for much of the BBMV uptake observed over the incubation interval. These results suggest that L-proline undergoes concentrative transport at pH 7.5 by at least one carrier process using the transmembrane Na^+ gradient to transfer the amino acid temporarily against a concentration gradient.

Effect of L-pipecolate on carrier-mediated influx of L-[^{3}H]proline

Stevens and Wright (1985) showed that the compound L-pipecolate is a strong competitive inhibitor of the IMINO carrier system for L-proline transport in mammalian intestinal brush-border membrane. To determine the extent to which starfish pyloric caeca transport L-proline by this carrier protein, a similar experiment was conducted in the present investigation. Preliminary time course experiments showed that the uptake of this amino acid by starfish BBMV was a relatively slow process and remained a linear function of time for at least 1 min at both high (5 mmol l^{-1}) and low (0.05 mmol l^{-1}) concentrations. Therefore, for estimation of L-proline influx under a variety of experimental treatments either 1-min or 30-s incubations were used to ensure unidirectional movements of the radiolabelled substrate.

To ascertain the potential inhibitory effect of L-pipecolate on L-proline influx, vesicles were loaded with 300 mmol l^{-1} mannitol, 50 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes–Tris (pH 7.5) and 25 μg ml^{-1} valinomycin (potassium ionophore) and were then incubated for 1 min in external media at the same pH containing 150 mmol l^{-1} NaCl, 50 mmol l^{-1} KCl, one of the following concentrations of L-pipecolate: 0, 0.01, 0.025, 0.05, 0.10, 0.25 or 0.50 mmol l^{-1}, and either 0.05 or 0.10 mmol l^{-1} L-[^{3}H]proline. Non-specific isotope binding to vesicles and filters was subtracted from resulting uptake values.

Fig. 2A shows the effect of increasing concentrations of L-pipecolate in the external incubation medium on 0.05 and 0.10 mmol l^{-1} L-proline influxes in starfish BBMV. This figure indicates that L-pipecolate acted as a potent inhibitor of L-proline entry at each amino acid concentration. Maximal L-proline influx inhibition occurred when vesicles were incubated in medium with 0.5 mmol l^{-1} L-pipecolate. Even at the highest concentration of the inhibitor, significant amounts of radiolabelled L-proline entered BBMV by processes not inhibited by L-pipecolate. To estimate the contribution of L-pipecolate-sensitive carrier transport to total L-proline influx, it was assumed that at 0.5 mmol l^{-1} L-pipecolate total inhibition of L-proline influx by the L-pipecolate-sensitive system occurred. L-proline entry at 0.5 mmol l^{-1} L-pipecolate was subtracted from the amino acid influxes at all other L-pipecolate concentrations and the remainder plotted in the Dixon plot (Fig. 2B). The results of this Dixon analysis indicate that L-pipecolate acted as a potent competitive inhibitor of L-proline influx in starfish BBMV,

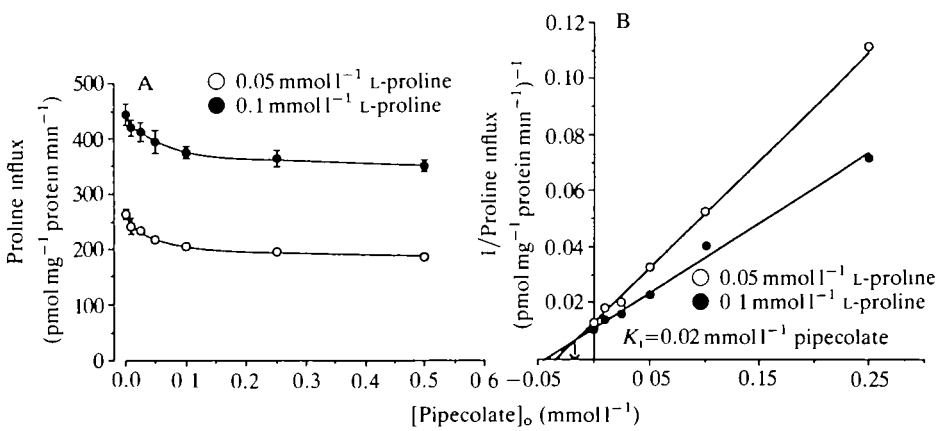

Fig. 2. Effect of various external concentrations of L-pipecolate on L-[^{3}H]proline influx in starfish BBMV. Vesicles were loaded at pH 7.5 with 300 mmol l^{-1} mannitol, 50 mmol l^{-1} KCl and 25 μg ml^{-1} valinomycin and were incubated in external media at the same pH containing 150 mmol l^{-1} NaCl, 50 mmol l^{-1} KCl, one of the following L-pipecolate concentrations: 0, 0.01, 0.025, 0.05, 0.10, 0.25 or 0.50 mmol l^{-1}, and either 0.05 or 0.10 mmol l^{-1} L-[^{3}H]proline. Data in B were obtained from values given in A, as described in the text. Straight lines shown in B were obtained by linear regression analysis. Stop solution: 200 mmol l^{-1} KCl adjusted to pH 7.5.

yielding a K_i value of 0.02 mmol l^{-1}. From this relationship it is likely that L-proline entry by the L-pipecolate-sensitive process is analogous to transfer by the IMINO system in mammalian epithelia (Stevens and Wright, 1985).

Carrier components of L-[^{3}H]proline influx

The contribution of different carrier systems to total L-proline influx was determined by incubation of the labelled amino acid in varying concentrations of compounds known to inhibit L-proline transport by the mammalian IMINO, NBB (neutral brush-border) and L (L-leucine preferring) systems (Stevens *et al.* 1982, 1984; Stevens and Wright, 1985). To make this estimation, starfish BBMV were loaded with 300 mmol l^{-1} mannitol, 50 mmol l^{-1} KCl and 25 μg ml^{-1} valinomycin at pH 7.5 and incubated for 1 min in media of the same pH containing either 150 mmol l^{-1} NaCl or 150 mmol l^{-1} choline chloride, and 0.05 mmol l^{-1} L-[^{3}H]proline, 50 mmol l^{-1} KCl and a series of L-pipecolate, L-leucine and L-alanine concentrations.

Fig. 3 shows the effects of the above inhibitors on the influx of a fixed concentration of L-proline. In the absence of any inhibitors, 0.05 mmol l^{-1}

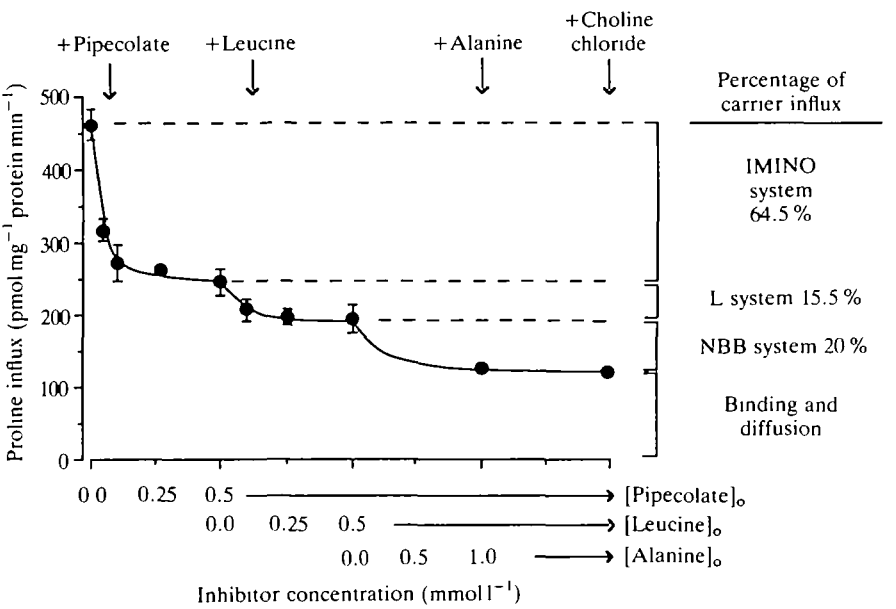

Fig. 3. Effects of external inhibitors and Na^+-free external medium on 0.05 mmol l^{-1} L-[^{3}H]proline influx by starfish BBMV. Vesicles were loaded with 300 mmol l^{-1} mannitol, 50 mmol l^{-1} KCl and 25 μg ml^{-1} valinomycin at pH 7.5 and incubated in media at the same pH containing the radiolabelled amino acid, 150 mmol l^{-1} NaCl, 50 mmol l^{-1} KCl and various concentrations of the inhibitors L-pipecolate, L-leucine and L-alanine. One additional incubation medium at pH 7.5 contained 150 mmol l^{-1} choline chloride, 50 mmol l^{-1} KCl and the following inhibitors: 0.5 mmol l^{-1} L-pipecolate, 0.5 mmol l^{-1} L-leucine and 1.0 mmol l^{-1} L-alanine. Stop solution: same as that used in Fig. 2.

L-[^{3}H]proline influx was approximately 460 pmol mg^{-1} protein min^{-1}. Increasing the external L-pipecolate concentration from 0 to 0.5 mmol l^{-1} reduced L-proline influx to approximately 250 pmol mg^{-1} protein min^{-1} as a result of the total inhibition of the IMINO transport system. Addition of L-leucine at concentrations of 0–0.5 mmol l^{-1} to an external medium already containing 0.5 mmol l^{-1} L-pipecolate further lowered L-proline influx to approximately 200 pmol mg^{-1} protein min^{-1} by blocking L-proline transport by the L system. Adding 1.0 mmol l^{-1} L-alanine to external media containing 0.5 mmol l^{-1} L-pipecolate and 0.5 mmol l^{-1} L-leucine led to a further reduction in L-proline influx to approximately 125 pmol mg^{-1} protein min^{-1} by eliminating the NBB system. Adding all three inhibitors at maximal concentration to the choline chloride medium did not cause any further reduction in L-proline entry than that observed with the NaCl medium containing all the inhibitors. This suggests that the only L-proline activity associated with BBMV in the choline chloride medium was due to non-specific binding and diffusional entry. Preliminary experiments were conducted to determine the relative selectivity of pipecolate as an inhibitor of the IMINO system by estimating the effects of this compound on the uptake of L-[^{3}H]alanine and L-[^{3}H]leucine by pyloric caecal BBMV. These experiments suggested that pipecolate had no significant inhibitory effect on the uptakes of these amino acids by this tissue, lending support to the use of this compound as a selective inhibitor of the IMINO system alone. As a result of these experiments, the contributions of the IMINO, L and NBB systems to 0.05 mmol l^{-1} L-proline influx shown on Fig. 3 can be estimated as 64.5 %, 15.5 % and 20 %, respectively.

Kinetics of L-proline influx by the IMINO, NBB and L transport systems

The kinetics of L-[^{3}H]proline influx by the IMINO, NBB and L systems were determined by measuring the entry of the radiolabelled amino acid as a function of several external concentrations of L-proline in the presence of saturating concentrations of compounds known to abolish entry by two of the three systems. In the first experiment, the kinetics of L-proline influx by the IMINO system was estimated with 0.5 mmol l^{-1} L-alanine (blocking the NBB system) and 0.5 mmol l^{-1} L-leucine (blocking the L system) added to the external media. In this experiment vesicles were loaded at pH 7.5 with 300 mmol l^{-1} mannitol, 50 mmol l^{-1} KCl and 25 μg ml^{-1} valinomycin and were incubated for 30 s in media at the same pH containing 150 mmol l^{-1} NaCl, 50 mmol l^{-1} KCl, the two saturating concentrations of the inhibitors and the following concentrations of L-proline: 0.025, 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 2.50 and 5.0 mmol l^{-1}. Non-specific binding of isotope to membranes and filters was subtracted from the total influx prior to estimation of kinetic parameters.

Fig. 4 indicates that L-proline influx under the above conditions was a biphasic function of external [L-proline]. At L-proline concentrations of 1–5 mmol l^{-1} uptake was linearly related to proline concentration, whereas at the lower substrate concentrations the relationship was curvilinear. Over the entire substrate

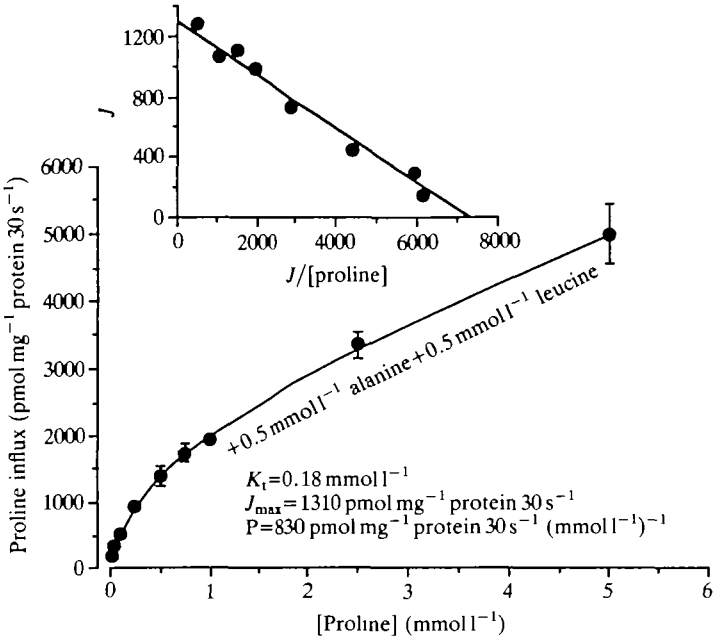

Fig. 4. Kinetics of L-[^{3}H]proline influx by the IMINO system plus diffusion in BBMV of starfish pyloric caeca. Vesicles were loaded with 300 mmol l^{-1} mannitol, 50 mmol l^{-1} KCl and 25 μg ml^{-1} valinomycin at pH 7.5 and were incubated in media of the same pH containing variable concentrations of radiolabelled L-proline, 150 mmol l^{-1} NaCl, 50 mmol l^{-1} KCl, 0.5 mmol l^{-1} L-alanine and 0.5 mmol l^{-1} L-leucine. Stop solution: same as that used in Fig. 2. The inset is a Hofstee plot used to calculate J_{max} and K_t.

range, influx followed the combination of Michaelis–Menten kinetics plus an apparent diffusion component, as defined by the equation:

$$J = \frac{J_{max}[\text{proline}]_o}{K_t + [\text{proline}]_o} + P[\text{proline}]_o, \quad (1)$$

where J is total L-proline influx in the presence of saturating concentrations of L-alanine and L-leucine, J_{max} is maximal L-proline influx by the IMINO system, K_t is the external [L-proline] resulting in half-maximal influx by the IMINO system and P is the apparent diffusional permeability coefficient of vesicles to L-proline. The apparent diffusional permeability coefficient (P) was calculated using linear regression analysis of the slope between 1 and 5 mmol l^{-1} L-proline. Subtraction of this calculated apparent diffusional influx from total entry values at each external substrate concentration provided an index of amino acid influx by the IMINO carrier alone. These calculated carrier influx estimates are shown in the Hofstee plot (inset) and quantitative values for K_t and J_{max} were obtained using linear regression analysis of these data. Mean values and their associated standard errors for K_t, J_{max} and P from this experiment are displayed in the main body of Fig. 4.

Table 1. *Kinetic constants for L-proline influx into brush-border membrane vesicles of pyloric caecal epithelial cells by independent carrier systems*

Carrier system	K_t ($mmol\,l^{-1}$)	J_{max} ($pmol\,mg^{-1}\,protein\,30\,s^{-1}$)
IMINO	0.18±0.05	1310±143
NBB	0.13±0.03	360±57
L	0.21±0.05	470±64

Values are means±S.E. and were calculated after subtraction of apparent diffusional influx from total L-proline entry in the presence of selective inhibitors.

Results shown are from representative experiments. Replicate experiments yielded similar quantitative values for each constant.

and in Table 1. Similar quantitative values for each constant were obtained in two replicate experiments.

A similar kinetic analysis was performed to estimate the kinetic parameters of L-proline influx by the NBB system. In this instance saturating concentrations of L-pipecolate ($0.5\,mmol\,l^{-1}$) and L-leucine ($0.5\,mmol\,l^{-1}$) were added to each external medium containing radiolabelled L-proline. For this analysis vesicles were loaded and incubated in the internal and external media described above, differing only in the inhibitors added so that transport by the NBB system could be independently assessed. Fig. 5 shows that influx under these conditions showed a similar biphasic pattern to that described in Fig. 4 and equation 1. Kinetic parameters for L-proline influx by the NBB system were estimated as in Fig. 4 and the resulting quantitative values for these parameters from a representative experiment are displayed in the body of Fig. 5 and in Table 1. Similar quantitative values for each constant were obtained in a replicate experiment.

The kinetic constants for L-proline influx by the L system were next assessed. In this case $0.5\,mmol\,l^{-1}$ L-pipecolate and $0.5\,mmol\,l^{-1}$ L-alanine were added to each external L-proline concentration to saturate influx by the IMINO and NBB systems, respectively. Vesicles were prepared and incubated as described above. Results from a representative experiment, shown in Fig. 6, are similar to those presented in Figs 4 and 5, suggesting that L-proline entry by the L system and diffusion follows equation 1. Estimation of the appropriate kinetic constants for this system obtained from this experiment are displayed on the figure and in Table 1. A replicate experiment provided similar quantitative values for each kinetic constant. It is apparent from reviewing Figs 4–6 and Table 1 that the majority of L-proline influx in starfish BBMV occurred by the IMINO system (J_{max} is three times that of the NBB or L systems), but that the apparent binding affinities (K_t) of the three systems were very similar.

Effect of external [NaCl] on L-proline influx

To ascertain the influence of external NaCl concentration on L-proline influx in starfish BBMV, vesicles were loaded at pH 7.5 with $800\,mmol\,l^{-1}$ mannitol,

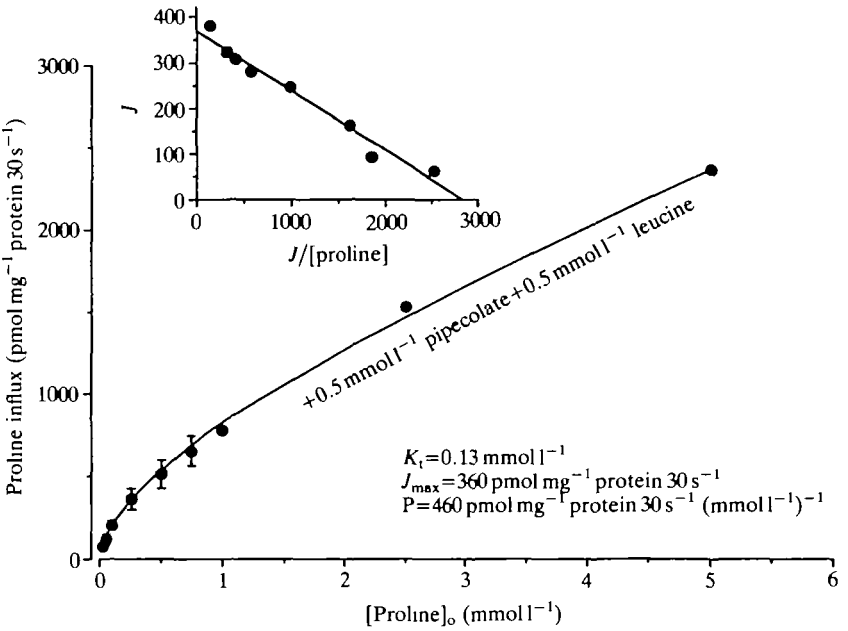

Fig. 5. Kinetics of L-[^{3}H]proline influx by the NBB system plus diffusion in BBMV of starfish pyloric caeca. Vesicles were loaded and incubated as in Fig. 4, except that in this instance the incubation media contained 0.5 mmol l^{-1} L-pipecolate and 0.5 mmol l^{-1} L-leucine. Stop solution: same as that used in Fig. 2. For further details, see Fig. 4.

50 mmol l^{-1} KCl and 25 μg ml^{-1} valinomycin and incubated for 1 min in a variety of external media, at the same pH, containing 0.05 mmol l^{-1} L-[^{3}H]proline, 50 mmol l^{-1} KCl and one of the following concentrations of NaCl: 400, 300, 250, 200, 150, 100 or 50 mmol l^{-1}. Osmotic pressure was held constant in all media with the addition of appropriate amounts of mannitol. One group of external media contained 0.5 mmol l^{-1} L-pipecolate to saturate L-proline influx by way of the IMINO system at each external [NaCl], allowing an estimate of the Na^+-dependency of the NBB system alone, while the other group lacked the inhibitor and represented the effect of external [Na^+] on L-proline entry by both IMINO and NBB systems. Non-specific isotope binding to membranes and filters, as well as L-proline influx by apparent Na^+-independent diffusion, were subtracted from uptake data at each experimental condition.

Fig. 7 indicates that the influx of 0.05 mmol l^{-1} L-proline was a sigmoidal function of external [NaCl] in the presence and absence of the inhibitor L-pipecolate, with each curve approaching saturation at the highest external NaCl concentrations used. The sigmoidal Na^+-dependency of L-proline influx suggested that the relationship followed the general Hill equation for binding cooperativity:

$$J = \frac{J_{max}[Na^+]^n}{K_{Na}^n + [Na^+]^n}, \quad (2)$$

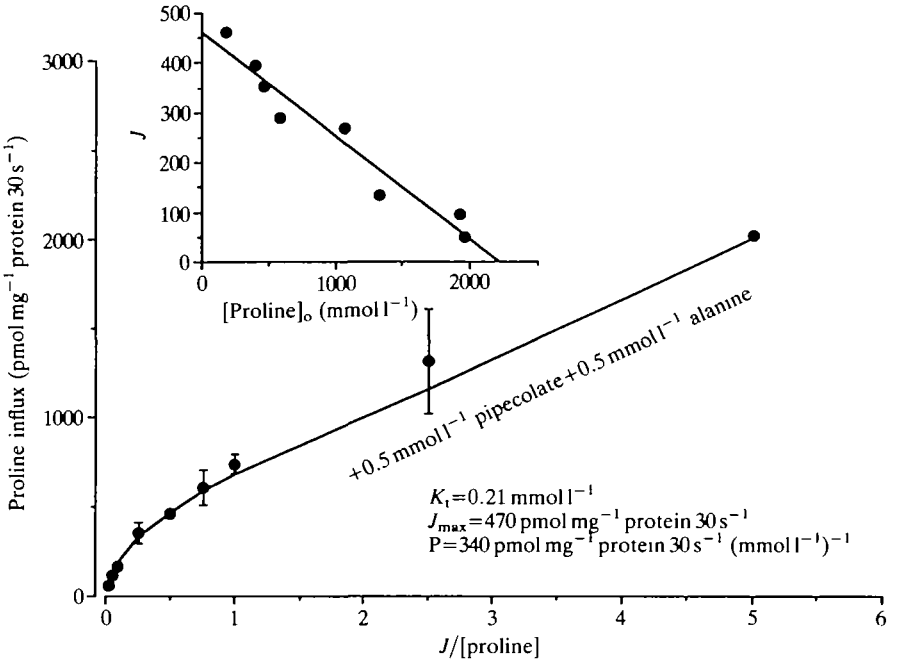

Fig. 6. Kinetics of L-[^{3}H]proline influx by the L system plus diffusion in BBMV of starfish pyloric caeca. Vesicles were loaded and incubated as in Fig. 4, except that in this case the incubation media contained 0.5 mmol l^{-1} L-pipecolate and 0.5 mmol l^{-1} L-alanine. Stop solution: same as that used in Fig. 2. For further details, see Fig. 4.

where J in this case represents total Na^+-dependent L-proline influx, J_{max} is total maximal Na^+-dependent L-proline influx by all Na^+-dependent systems, K_{Na}^n is an apparent affinity constant modified to take into account multisite interactions between two or more sodium ions binding simultaneously to all Na^+-dependent carrier systems, n is the Hill coefficient and represents an index of the number of sodium ions associated with all Na^+-dependent carrier systems and $[Na^+]$ is the external sodium concentration.

Best-fitting curves were generated for the data points in Fig. 7 by an iterative computer program using equation 2. It is apparent that the curve generated in the absence of the inhibitor L-pipecolate is the sum of two separate, Na^+-dependent functions representing L-proline influx by the NBB and IMINO systems. In this instance, therefore, the value displayed for J_{max} is the sum of the maximal transfer velocities for both processes operating simultaneously, that for K_{Na}^n is the average apparent affinity constant for the two systems, and n is an estimate of the total number of sodium ions interacting with both mechanisms. The curve shown for Na^+-dependent L-proline influx in the presence of L-pipecolate represents amino acid entry by the NBB system alone and the kinetic constants displayed for these data only relate to this mechanism. An analysis of Fig. 7 indicates that both Na^+-dependent carrier systems appear to exhibit similar apparent affinity constants

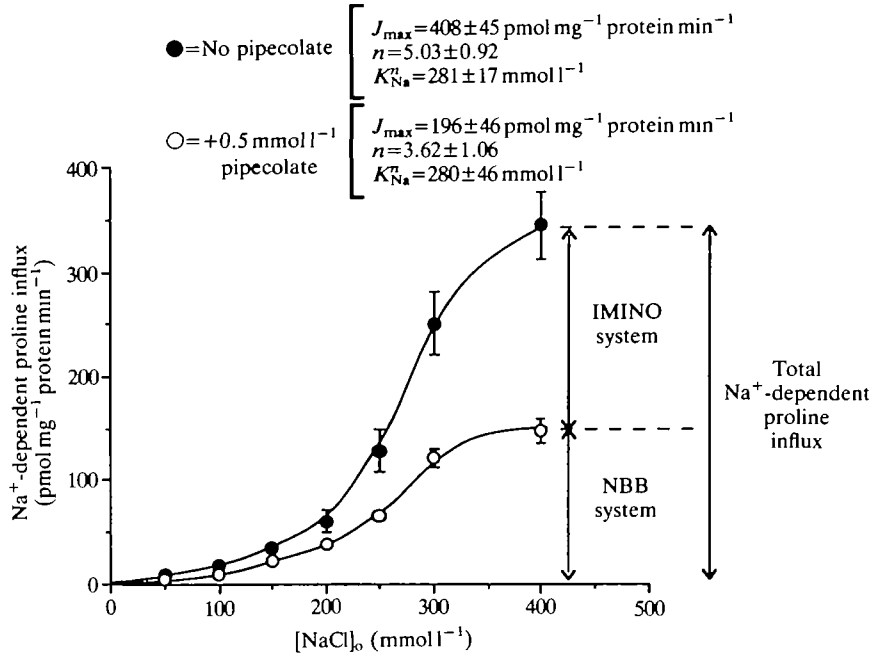

Fig. 7. Effect of external NaCl concentration on Na^+-dependent influx of 0.05 mmol l^{-1} L-[^{3}H]proline into starfish pyloric caeca BBMV. Vesicles were loaded at pH 7.5 with 800 mmol l^{-1} mannitol, 50 mmol l^{-1} KCl and 25 μg ml^{-1} valinomycin and incubated in media, at the same pH, containing the radiolabelled amino acid, 50 mmol l^{-1} KCl and one of the following NaCl concentrations: 50, 100, 150, 200, 250, 300 or 400 mmol l^{-1}. Osmotic pressure was held constant in all media by adding appropriate amounts of mannitol. One group of incubation media (●) contained 0.5 mmol l^{-1} L-pipecolate to saturate the IMINO system at each [Na^+], while the other group (○) lacked the inhibitor. Stop solution: 400 mmol l^{-1} tetramethylammonium hydroxide, 400 mmol l^{-1} gluconic acid lactone and 50 mmol l^{-1} KCl, buffered to pH 7.5.

(K^n_{Na}=280 mmol l^{-1}), maximal Na^+-dependent influx velocities (approx. 200 pmol mg^{-1} protein min^{-1} at this amino acid concentration) and Hill coefficients (n=2–3). From this graphical treatment it is clear that total Na^+-dependent L-proline influx is the sum of the transport activities of two separate Na^+-dependent carriers, each displaying Hill kinetics. Therefore, a modification to equation 2, which takes into account the separate Na^+-dependent activities of the IMINO and NBB systems, is described by:

$$J = \underbrace{\left[\frac{J_{max}[Na^+]^n}{K^n_{Na} + [Na^+]^n}\right]}_{\text{IMINO system}} + \underbrace{\left[\frac{J_{max}[Na^+]^n}{K^n_{Na} + [Na^+]^n}\right]}_{\text{NBB system}}, \quad (3)$$

where all constants have the meanings given for equation 2, except that, in this instance, those encompassed by brackets to the left are associated with the IMINO system and those in brackets to the right are assigned to the NBB system.

Discussion

The present investigation provides evidence for the occurrence of three separate carrier transport mechanisms for L-proline in the brush-border membrane of starfish pyloric caeca. From their responses to selective inhibitors, the three starfish transporters appear to be analogous to the IMINO, NBB and L carriers identified in mammalian intestine (Stevens and Wright, 1985). Like the mammalian gut transporters, the starfish IMINO and NBB carriers are Na^+-dependent, whereas the L system appears to be Na^+-independent (Stevens *et al.* 1984). In mammals, L-proline transport by the IMINO, NBB and L systems accounts for 60, 35 and 5%, respectively, of the total transmembrane transfer of this amino acid (Stevens and Wright, 1985), whereas in starfish 64.5% of L-proline transport takes place by the IMINO system, but the remaining movements are approximately evenly divided between the NBB and L systems (Fig. 3). Lastly, the apparent K_t value of the starfish IMINO system (0.18 ± 0.05 mmol l^{-1}, Fig. 4; Table 1) is not significantly different ($P>0.05$) from that reported for the same transporter in rabbit jejunum (0.23 ± 0.01 mmol l^{-1}; Stevens and Wright, 1985), providing strong support for the conservation of L-proline transport systems across phyla.

In a previous preliminary publication concerning L-proline transport by starfish pyloric caeca, it was concluded that at least two carrier processes for this amino acid were present in the epithelial brush borders of this gut diverticulum. One exhibited a high apparent affinity for its substrate and displayed Na^+-dependency, while the other had a low apparent amino acid binding affinity and appeared to be unresponsive to this cation (Ahearn, 1990). An apparent K_t of the high-affinity, Na^+-dependent transporter was reported as 0.22 mmol l^{-1} L-proline, while the apparent low-affinity process was a linear function of substrate concentration over the range selected. The apparent diffusional permeability of these vesicles to L-proline was calculated to be 300 pmol mg^{-1} protein 30 s^{-1} (mmol l^{-1})$^{-1}$ L-proline. Results of the present investigation with starfish pyloric caeca support and extend these earlier conclusions. By employing L-pipecolate, L-alanine and L-leucine as inhibitors of the IMINO, NBB and L systems, respectively, the apparent affinity constant of each process was disclosed. These values were very similar (ranging from 0.13 to 0.21 mmol l^{-1}; Figs 4–6, Table 1) and were close to that reported for the single high-affinity process found in the earlier study. Without the use of inhibitors that selectively blocked the transport contributions from two of the three carrier processes, this earlier study reported an overall apparent affinity constant that represented an average of the values for the three systems. The average P value for apparent diffusion reported in the present study in Figs 4–6 is 543 ± 147 pmol mg^{-1} protein 30 s^{-1} (mmol l^{-1})$^{-1}$ L-proline, which is not very different from that reported for apparent diffusion in the earlier investigation (see above).

The Na^+-dependency of epithelial amino acid transport has been reported for a wide range of organisms, including mammals (Mircheff *et al.* 1982; Stevens *et al.* 1984; Wright and Peerce, 1984), teleosts (Vilella *et al.* 1988) and invertebrates

(Stevens and Preston, 1980; Preston and Stevens, 1982; Gerencser and Stevens, 1989; Pajor and Wright, 1989; Behnke *et al.* 1990). Among vertebrates, the transport stoichiometry for Na^+/L-proline cotransport has been reported as being either 1:1 in teleost intestine (Vilella *et al.* 1988) or 2:1 in rabbit intestine (Wright and Peerce, 1984). Multiple sodium ions (2, 3 or more) have been reported as cotransport substrates in L-proline transport in invertebrate epithelial cells from worm integument (Stevens and Preston, 1980; Preston and Stevens, 1982), mussel gill tissue (Pajor and Wright, 1989) and crustacean antennal gland (Behnke *et al.* 1990). In none of these studies was the transport stoichiometry of a specific L-proline carrier defined with the use of inhibitors that cleanly abolished the potential transport contribution from two or more simultaneously operating Na^+-dependent processes. It is, therefore, uncertain in tissues from vertebrates or invertebrates what the specific Na^+/L-proline cotransport stoichiometries are for the IMINO or NBB systems. Fig. 7 shows, for the first time, the specific Na^+/L-proline binding stoichiometries of both the IMINO and NBB systems of starfish pyloric caeca. This figure indicates that each Na^+-dependent carrier process binds two or three sodium ions with each amino acid molecule. However, this experiment does not indicate how many cations are *transported* for each molecule of L-proline. In this case, as in others pointed out in a recent publication by Gerencser and Stevens (1989), discussing the energetics of Na^+/amino acid cotransport of invertebrate carrier systems involving the binding of multiple sodium ions for each organic solute molecule, the specific number of cations involved in driving the uphill transport of the cotransported amino acid has not been determined, and therefore the cotransport stoichiometry is unknown. Some associated cations may serve as essential activators of the transport mechanism and may not themselves be transferred across the membrane, while others may accompany the amino acid from one membrane surface to the other. Additional experiments, such as applying the static head method of stoichiometric cotransport analysis developed by Turner and Moran (1982) for mammalian cells and recently successfully used with crustacean epithelia (Ahearn *et al.* 1990; Behnke *et al.* 1990; Balon and Ahearn, 1991), need to be applied to each transport system to establish its specific cotransport ratio.

Echinoderms and arthropods are organisms representing the two major divisions of the animal kingdom based on embryological development. The Protostomia, including flatworms, annelids, molluscs and arthropods, exhibit spiral determinate cleavage and the embryonic blastopore gives rise to both the mouth and anus. In contrast, the Deuterostomia are composed of the echinoderms, hemichordates and chordates and exhibit radial indeterminate cleavage, where the mouth does not develop from the embryonic blastopore, but is formed independently. Other characteristics also differentiate these two main lines of animal development. Both major groups of animals have representatives with extensive gastrointestinal diverticula extending from various portions of the gut. The results of this study, and those of the one published earlier (Ahearn, 1990), provide strong evidence for a possible nutrient absorptive role for the starfish pyloric caeca. If the

epithelium of this organ is absorptive, the first step of this process would be solute flow from lumen to cytosol across the brush-border membrane, while the next step in transcellular nutrient transfer would be solute exit across the basolateral cell pole. This study and the earlier publication (Ahearn, 1990) characterize the first step in transepithelial L-proline transport by examining the properties of brush-border carrier mechanisms. Future experiments defining organic solute transport properties of the pyloric caecal basolateral membrane and studies examining transepithelial nutrient transport by isolated cells from this organ may provide a clear picture of the absorptive nature of this gut diverticulum in echinoderms.

The L-proline transport properties of the epithelial brush border of this structure are very similar to those reported for the same amino acid in the mammalian jejunum (Stevens *et al.* 1982, 1984; Stevens and Wright, 1985), suggesting that this organ may be the major site of amino acid absorption in this group of echinoderms. This tentative conclusion agrees with results obtained over the last decade concerning the nutrient absorptive role of the crustacean hepatopancreas, another invertebrate organ that is an extensive diverticulum of the pyloric stomach (Ahearn, 1987, 1988; Ahearn and Clay, 1988; Ahearn *et al.* 1991). In the crustaceans this organ displays a wide range of absorptive and secretory processes for sugars, amino acids and ions. Because representative organisms from the two major divisions of animal development possess gastrointestinal diverticula with similar apparent absorptive capabilities, this function for a little-understood, but widely distributed, structure may be more universal than presently appreciated.

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