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# SHORT COMMUNICATION

# PROLINE TRANSPORT BY BRUSH-BORDER MEMBRANE VESICLES OF STARFISH PYLORIC CAECA

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Starfish, unlike other echinoderms such as sea cucumbers and sea urchins, lack an effective intestine for nutrient absorption. Instead, they possess digestive diverticula called pyloric caeca which extend from the stomach wall into each arm and branch laterally into numerous blind-ended, epithelial-lined tubules. These tubules exhibit a number of physiological activities including the secretion of extracellular proteolytic enzymes (Lawrence, 1982), the storage of carbohydrates and lipids as energy reservoirs (Lawrence and Lane, 1982) and the absorption of low molecular weight organic solutes resulting from digestive activities in the diverticulum lumen (Ferguson, 1979, 1982; Jangoux, 1982; Lawrence, 1982). While it is clear from these studies that starfish pyloric caeca participate in nutrient absorption, there has been no direct physiological characterization of a transapical or transcellular transport process for amino acids or sugars by the epithelial cells of this organ. The present investigation is the first to show, using purified brush-border membrane vesicles from asteroid pyloric caeca, that amino acids can be transported across this membrane by at least one carrier-mediated process that displays properties in common with gastrointestinal absorptive organs of animals from a variety of phyla.

Individuals of the starfish *Pycnopodia helianthoides* were collected during the months of May and June 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. Four to five arms were removed from an individual initially possessing more than 15 arms and the dissected diverticula were pooled. The procedure for producing purified vesicles from this pooled tissue sample was generally the same as the magnesium precipitation technique previously used to make a similar membrane preparation from mammalian (Kessler *et al.* 1978) and crustacean epithelia (Ahearn *et al.* 1985; Behnke *et al.* 1990).

Briefly, 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 by purification using

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centrifugation spins at 3000 *g* and 27000 *g*, and homogenization in additional hypotonic buffer using a glass homogenizer. A further sequence of precipitation, purification and homogenization was then carried out, followed by centrifugation at 27000 *g*. The resulting purified sample of brush-border membrane was resuspended in a small volume of external 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 protein assay). Using this preparative method, previous studies using 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) (Ahearn *et al.* 1985; Behnke *et al.* 1990; Kessler *et al.* 1978).

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 $\mu$l) of membrane vesicles was added to a volume of radiolabelled medium (e.g. 160 $\mu$l) 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 (e.g. 20 $\mu$l) was withdrawn and plunged into 2 ml of ice-cold stop solution (150 mmol $l^{-1}$ KCl, 20 mmol $l^{-1}$ Hepes–Tris buffered to pH 7.0). The resulting suspensions were rapidly filtered through Millipore filters (0.45 $\mu$m) 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 as pmol (using specific activity of amino acid in medium) per mg protein per filter. Each experiment was repeated twice using membranes prepared from different animals, yielding qualitatively similar results from each experiment. Within a given experiment each point was analysed in triplicate and values are presented in figures as means±S.E.

Fig. 1 shows the effects of transmembrane ion gradients on the time course of L-[$^3$H]proline uptake by pyloric caecal BBMV. In this experiment vesicles were loaded with 300 mmol $l^{-1}$ mannitol and 20 mmol $l^{-1}$ Hepes–Tris adjusted to pH 7.0 and were incubated in one of four different external media at the same pH: (1) 150 mmol $l^{-1}$ NaCl; (2) 150 mmol $l^{-1}$ KCl; (3) 300 mmol $l^{-1}$ mannitol; or (4) 150 mmol $l^{-1}$ NaCl plus 10 mmol $l^{-1}$ L-proline. The results indicate that proline uptake was maximal in vesicles incubated in the presence of a transmembrane NaCl gradient (1.06±0.07 nmol $mg^{-1}$ protein $min^{-1}$), and that proline transiently accumulated to a concentration approximately twice that exhibited at equilibrium (overshoot phenomenon). This is a characteristic organic solute uptake pattern demonstrated by a $Na^+$-dependent cotransport process energized by the cation gradient. When the NaCl gradient was replaced by a similar gradient of KCl or by

equal concentrations of mannitol on both vesicle surfaces, the initial uptake rate of the amino acid and the extent of vesicular accumulation were considerably reduced. L-Proline uptake in KCl medium ($0.74\pm0.03$ nmol $mg^{-1}$ protein $min^{-1}$) significantly exceeded that in mannitol ($0.59\pm0.04$ nmol $mg^{-1}$ protein $min^{-1}$) and generated a small overshoot, but this transport was considerably less than that in NaCl medium, suggesting that $K^+$ may be able partially to substitute for $Na^+$ in the cotransport process. The addition of 10 mmol $l^{-1}$ L-proline to the external vesicular surface resulted in the slowest uptake rate for the radiolabelled amino acid of all four treatments ($0.23\pm0.02$ nmol $mg^{-1}$ protein $min^{-1}$). The results in Fig. 1 are consistent with the potential occurrence of distinct $Na^+$-dependent (NaCl medium) and $Na^+$-independent (mannitol medium) carrier-mediated L-[$^3$H]proline transport processes that were both blocked by the addition of saturating L-proline concentrations (10 mmol $l^{-1}$ L-proline medium). The residual labelled amino acid uptake in the presence of the inhibitor may have largely been diffusion.

The results suggest that L-proline uptake by these vesicles was a somewhat slow process and that a 30 s incubation could be used to estimate an apparent influx rate after subtraction of non-specific isotopic binding from the vesicles. Binding was estimated by extrapolating each uptake curve in Fig. 1 to the vertical axis and determining the percentage of 30 s uptake due to this extrapolated value. For uptake in 150 mmol $l^{-1}$ NaCl medium, binding accounted for 14 % of total uptake

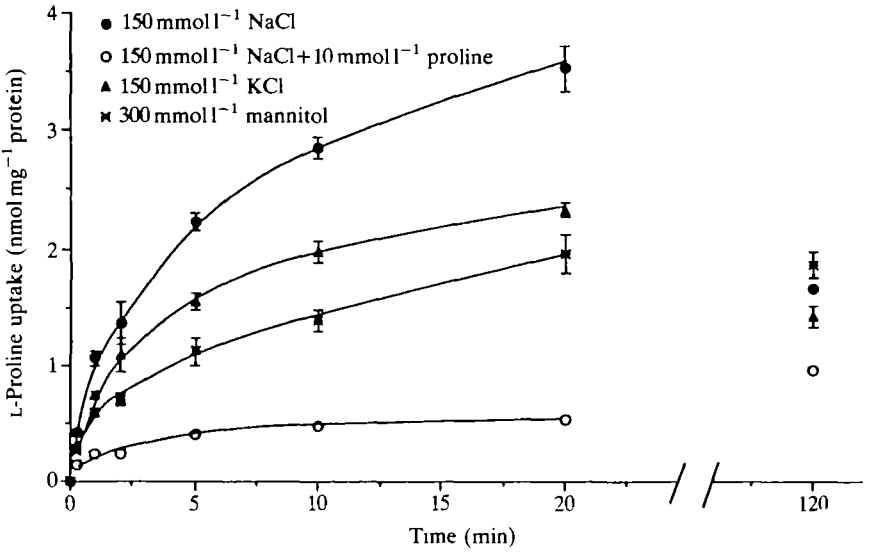


Fig. 1. Effects of transmembrane ion gradients on the time course of 0.1 mmol $l^{-1}$ L-[$^3$H]proline uptake by brush-border membrane vesicles of starfish pyloric caeca. Vesicles were loaded with 300 mmol $l^{-1}$ mannitol and 20 mmol $l^{-1}$ Hepes–Tris adjusted to pH 7.0 and were then incubated in one of the four external media shown at the same pH. Values displayed are means±S.E. (some errors are smaller than the symbols) of triplicate samples at each time point.

at 30 s, while binding in the 150 mmol $l^{-1}$ NaCl+10 mmol $l^{-1}$ L-proline medium was 59 % of the 30 s uptake. These estimates of binding were confirmed in separate experiments where 'blank vesicle uptakes' were measured by simultaneously adding membranes and isotopically labelled medium to the stop solution. In this instance only adhering activity was measured and it was not significantly different ($P<0.05$) from that estimated by the technique described above. All subsequent influx values were corrected for this degree of non-specific binding.

L-[$^3$H]proline influx kinetics were estimated in vesicles loaded with 300 mmol $l^{-1}$ mannitol adjusted to pH 7.0 with 20 mmol $l^{-1}$ Hepes–Tris and incubated in media of the same pH containing 150 mmol $l^{-1}$ NaCl and one of the following labelled amino acid concentrations: 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, 2.50 or 5.00 mmol $l^{-1}$. A second influx estimation using identical external media was conducted, but in this instance an additional 10 mmol $l^{-1}$ unlabelled L-proline was added as an inhibitor of carrier-mediated L-[$^3$H]proline influx to each of the concentrations shown above. In control NaCl media apparent L-[$^3$H]proline influx followed a biphasic relationship with external L-proline concentration, exhibiting curvilinear and linear functions at opposite ends of the substrate range (Fig. 2). When an additional 10 mmol $l^{-1}$ L-proline was added as inhibitor to each substrate concentration and the initial specific activity of the amino acid in the absence of inhibitor

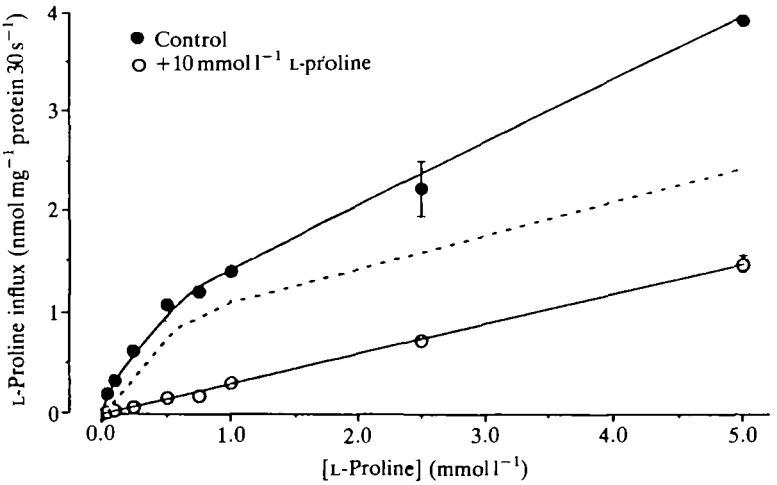


Fig. 2. Effect of external L-proline concentration on 30 s L-[$^3$H]proline influx into brush-border membrane vesicles of starfish pyloric caeca. Vesicles were loaded as in Fig. 1 and were incubated at the same pH in external media containing 150 mmol $l^{-1}$ NaCl and L-[$^3$H]proline concentrations from 0.05 to 5.0 mmol $l^{-1}$. In one group of external media an additional 10 mmol $l^{-1}$ unlabelled L-proline was added to each concentration of labelled amino acid, while the control group did not receive the added inhibitor. Values displayed are means±S.E. of triplicate samples at each concentration (where not shown, the errors are smaller than the symbol). Solid lines represent experimental points, while the dashed line shows an estimate of carrier-mediated amino acid influx after subtraction of apparent diffusion (+10 mmol $l^{-1}$ L-proline curve) from total influx.

was used to calculate influx, transport became a linear function of substrate concentration over the entire concentration range. The dashed line in Fig. 2 represents the calculated difference between influxes in control and 10 mmol $l^{-1}$ L-proline media and still describes a biphasic relationship between the variables.

The results in Fig. 2 support those displayed in Fig. 1 and suggest that transmembrane transport of L-proline by pyloric caecal BBMV may occur by the combination of two distinct carrier processes and diffusion, following the equation:

$$J_{pro} = \frac{J^1_{max}[proline]}{K^1_t + [proline]} + \frac{J^2_{max}[proline]}{K^2_t + [proline]} + P[proline],$$

where $J_{pro}$ represents total apparent L-[$^3$H]proline influx, $J^1_{max}$ and $J^2_{max}$ are apparent maximal influx rates by proposed $Na^+$-dependent and $Na^+$-independent carrier processes, respectively, $K^1_t$ and $K^2_t$ are apparent half-saturation constants for the two carriers, $P$ is the diffusional permeability of BBMV to labelled L-proline, and [proline] is the external amino acid concentration. In Fig. 2 diffusional influx of L-[$^3$H]proline may be approximated by the slope of the linear relationship displayed in the presence of 10 mmol $l^{-1}$ L-proline [0.60 nmol $mg^{-1}$ protein $min^{-1}$ (mmol $l^{-1}$ L-proline)$^{-1}$]. The combination of L-[$^3$H]proline influx by apparent high-affinity and low-affinity carrier-mediated transport processes is shown in Fig. 2 as the dashed line. It is likely, but unproven, that the apparent low-affinity system (the linear portion of the dashed curve) is $Na^+$-independent, while the apparent high-affinity process (the curvilinear portion of the dashed curve) occurs by cotransport with the cation. An estimation of $K^1_t$ and $J^1_{max}$ for the apparent high-affinity process was made using an iterative curve-fitting computer program applying the Michaelis–Menten equation plus a linear component to the data represented by the dashed line in Fig. 2. Calculated kinetic results of this analysis were $K^1_t = 0.22 \pm 0.08$ mmol $l^{-1}$ L-proline and $J^1_{max} = 1.96 \pm 0.28$ nmol $mg^{-1}$ protein $min^{-1}$.

Mircheff *et al.* (1982) and Stevens and Wright (1985) characterized the substrate specificities of mammalian renal and intestinal epithelial brush-border L-proline transporters and concluded that in these cases the transmembrane transfer of L-proline occurs by the combination of two $Na^+$-dependent processes (NBB and IMINO), one $Na^+$-independent process (L-system) and diffusion. Results in Fig. 2 are consistent with the mammalian pattern, except that only a single $Na^+$-dependent transporter appears to occur in starfish. However, two carriers requiring the cation, and having similar kinetic properties, could not be distinguished with the present techniques. In support of only a single $Na^+$-dependent carrier process in the starfish is the finding of $0.22 \pm 0.08$ mmol $l^{-1}$ for the estimated $K_t$ of the apparent high-affinity system shown as the curved portion of the dashed line in Fig. 2. This value is not significantly different ($P<0.01$) from that reported by Stevens and Wright (1985) for L-proline transfer by the IMINO system (0.23 mmol $l^{-1}$) in rabbit jejunum.

Previous studies have suggested that starfish pyloric caeca can absorb low

molecular weight organic solutes across the diverticulum epithelium from lumen to coelomic fluid by the combination of transport activities of undefined carrier mechanisms located in the epithelial brush-border and basolateral membranes (Ferguson, 1979, 1982; Jangoux, 1982; Lawrence, 1982). If such a vectorial transfer of digestion products were to occur across this epithelial cell layer, the first step of this process would be solute flow from lumen to cytosol across the brush-border membrane. The next step in transcellular nutrient transfer would be solute exit across the basolateral cell pole.

This study provides an initial characterization of the first step in transepithelial L-proline transport by starfish pyloric caeca by examining the properties of brush-border carrier mechanisms for this amino acid. It also shows that the magnesium precipitation technique developed by mammalian epithelial physiologists (Kessler *et al.* 1978) and recently applied to arthropod tissues (Ahearn *et al.* 1985; Behnke *et al.* 1990) can be used to produce transporting vesicles of echinoderm plasma membranes. This study suggests the possible occurrence of two distinct $Na^+$-dependent and $Na^+$-independent carrier processes for the transapical transport of the amino acid L-proline. Further studies using this membrane preparation should be able to provide additional insight into the properties of L-proline transport and to define the characteristics of other brush-border membrane carriers involved in cellular uptake of nutrients from diverticulum lumen to cytosol. These studies, in conjunction with future experiments defining organic solute transport properties of the pyloric caecal basolateral membrane, may provide a clear picture of the physiological role of the pyloric caecum in nutrient absorption.

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