

An L-proline-dependent proton flux is located at the apical membrane level of the eel enterocytes

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Ingrosso, L., S. Marsigliante, V. Zonno, C. Storelli, and S. Vilella. An L-proline-dependent proton flux is located at the apical membrane level of the eel enterocytes. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R1619–R1624, 2000.—This study has demonstrated the existence of an L-proline-dependent (Na independent) proton flux at the apical membrane level of the eel intestinal absorbing cells. Using isolated eel enterocytes and the pH-sensitive fluorescent dye 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF), it was shown that a 20 mM concentration of the imino acid L-proline in the extracellular medium determined an intracellular acidification of ~0.28 pH units. However, neither sucrose nor other amino acids were able to significantly acidify the resting intracellular pH. A hyperbolic relationship between extracellular proline concentration and intracellular proton accumulation was observed. Using both isolated brush-border and basolateral membrane vesicles, it was demonstrated that this proline-proton cotransport mechanism was located at the apical membrane level only. In addition, the existence of a coupling mechanism between proline and proton fluxes was demonstrated by the observation that, in brush-border membrane vesicles, the presence of a pH gradient ($pH_{in} > pH_{out}$) stimulated the uptake of L-proline.

brush-border membrane vesicle; intestine

LITTLE INFORMATION IS AVAILABLE in fish about the role played by protons on the carrier-mediated (both Na^+ dependent and Na^+ independent) intestinal absorption of substrates (amino acids, sugars, dipeptides, etc.). Few data are concerned with the existence of proton-dependent (Na independent) cotransport systems. In the intestine of the herbivorous tilapia (*Oreochromis mossambicus*) as well as in the intestine and pyloric ceca of the carnivorous rockfish (*Sebastes caurinus*), the uptake of a dipeptide (glycyl-sarcosine) at the apical membrane level was found to be stimulated by an inwardly directed transmembrane proton gradient (12). Similarly, in the intestine of the euryhaline teleost *Anguilla anguilla*, proton-dependent dipeptide transporters have been characterized (5, 17). However, a facilitated transport system for glycyl-L-phenylalanine has been characterized in brush-border mem-

brane vesicles (BBMV) isolated from tilapia intestinal absorbing cells (6).

If few data are concerned with the effect of protons on peptide transporters in fish intestine, no data are available on the effect of protons on the amino acid transporters localized in the fish intestinal absorbing cells. The role of proton electrochemical gradient in the transepithelial absorption of amino acid in the intestine has been studied by using human intestinal Caco-2 cell monolayer (15). BBMV have been used to study transepithelial absorption of amino acid in kidneys isolated from rabbit (2, 8, 9) and fish (4).

We previously demonstrated that the uptake of L-proline at the apical membrane of the eel enterocyte occurs via 1) an Na^+ -dependent carrier-mediated mechanism and 2) a simple diffusional component (18). The kinetic characteristics of the symporter have been described (18, 19), but no data are available on the effect of protons on its functionality. In this study, we demonstrate the existence of an Na^+ -independent proline-proton cotransport of protons located at the apical membrane level of eel enterocytes.

MATERIALS AND METHODS

Materials

European yellow eels (*Anguilla anguilla*) (150–200 g) were collected from Acquatina lagoon (Lecce, Italy) and kept unfed in a seawater aquarium under environmental conditions of photoperiod and controlled water temperature (16–20°C) for at least 2 wk before death.

All chemicals, reagent grade, were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO), except for acridine orange (AO), which was obtained from Eastman Kodak (Rochester, NY) and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF), which was obtained from Molecular Probes (Eugene, OR); L-[3H]proline was bought from New England Nuclear (Boston, MA).

Methods

Isolation of intestinal cells. Intestinal epithelial cells were prepared as previously described (20). Briefly, eels were killed by decapitation and the intestines were rapidly re-

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moved, placed in ice-cold NaCl solution (1.1% wt/vol), and then rinsed several times with the same buffer to remove food particles, mucus, and other contaminants. The intestinal mucosa was stripped off from the other tissues using fine forceps.

Cells were isolated at room temperature by gently stirring the intestine pieces with a glass rod for 10 min in a sodium citrate buffer (in mM: 27 Na-citrate, 96 NaCl, 5.6 NaH₂PO₄, and 1.5 KCl adjusted to pH 7.6 with Tris·HCl). This procedure was repeated twice.

The released cells were filtered in sequence through nylon membranes of different pore size (225, 100, and 50 μ m, respectively) and then centrifuged at 100 *g* for 10 min in a J2-21 Beckman centrifuge. The supernatant was discarded, and the pellet was resuspended in a physiological saline solution (PSS; in mM: 4 KCl, 2 CaCl₂, 4 NaHCO₃, 1 MgSO₄, 0.5 NaH₂PO₄, 150 NaCl, 30 HEPES, 10 D-glucose, and 2 L-glutamine adjusted to pH 7.6 with Tris·HCl. The cell suspension was filtered again through a 50- μ m nylon mesh and diluted to the desired concentration with PSS.

Preparation of BBMVs. Eel intestinal BBMVs were prepared according to Storelli et al. (11). Briefly, the removed intestines were cut along their length and rinsed in a saline solution of NaCl (1.1% wt/vol). Three grams (fresh weight) of scraped intestinal mucosa were homogenized using a blender at high speed for 3 min in 30 ml of a buffer of 300 mM mannitol and 12 mM Tris·HCl adjusted to pH 7.1 with EGTA. Before homogenization, the homogenization medium was made hypotonic with the addition of 120 ml of ice-cold distilled H₂O and BBMVs were then isolated with a Mg²⁺-EGTA precipitation technique (1). All procedures were carried out at 0–4°C. The enrichment factors, obtained by dividing the enzyme activities of purified membrane pellet by those of the homogenate, were 13.9 ± 2.1 ($n = 10$; means \pm SD) for the alkaline phosphatase (EC 3.1.3.1) and 0.9 ± 0.5 ($n = 10$; means \pm SD) for the basolaterally located Na⁺-K⁺-ATPase (EC 3.6.1.3). The negligible enrichment factor found for the Na⁺-K⁺-ATPase suggests minimal contamination by basolateral plasma membranes.

Preparation of basolateral membrane vesicles. Basolateral membrane vesicles (BLMV) were prepared as described by Reshkin et al. (7). Briefly, intestinal cells were removed from the intestine as described above, and the resulting cell suspension was centrifuged for 1 min at 9,700 *g*. Pelleted cells were homogenized in Bomb Buffer (25 mM NaCl and 5 mM HEPES-Tris, pH 8.0) and centrifuged at 500 *g* for 15 min. The supernatant was centrifuged at 100,000 *g* for 30 min in a Beckman L8-55 ultracentrifuge with a swinging-bucket SW28 rotor. The fluffy white part of the resultant pellet was suspended in 50% (wt/vol) sucrose and then overlaid with 40, 30, and 20% (wt/vol) sucrose solution. This discontinuous gradient was centrifuged at 100,000 *g* for 1.5 h. The purified membrane was recovered from the 30–40% sucrose interface. The enrichment factor for the Na⁺-K⁺-ATPase was 9.5 ± 1.5 ($n = 5$; means \pm SD), while alkaline phosphatase was enriched <1.5 times.

Transport Experiments

Intracellular pH measurements using BCECF. Intracellular pH (pH_i) was measured at room temperature with the fluorescent pH indicator BCECF using an LS-50 B Perkin-Elmer spectrofluorometer connected to a personal computer. The cells were dye loaded by incubation for 45 min at room temperature in PSS containing 2.5 μ M BCECF-AM and then washed twice by centrifugation. Cells containing dye were diluted to 1 mg protein/ml. Protein concentration was mea-

sured by the Bio-Rad protein assay kit using lyophilized bovine plasma gamma globulin as standard. Fluorescence was measured alternately at 490 and 440 nm excitation (band width 5 nm) and at constant emission wavelength of 530 nm thereafter (band width 10 nm). Online calculation (FL DATA MANAGER; Perkin-Elmer, Norwalk, CT) of fluorescence ratios from these two excitation wavelengths was used to measure pH_i and gives concentration-independent, pH-sensitive fluorescence measurements (10). Cell suspensions (100 μ l, corresponding to $6.15 \pm 0.49 \times 10^6$ cells, $n = 15$) containing the BCECF were injected into a cuvette equipped with an electronic stirring system. Fluorescence ratio measurements were made every 2 s.

Calibration of the BCECF fluorescence signal. At the end of each experiment, a calibration of the fluorescent signal was performed by monitoring the 490/440 nm excitation ratio at various pH_i values. pH_i was set approximately equal to predetermined extracellular pH values (7.0, 7.25, 7.5, and 8.0) using the K⁺-nigericin technique (13). Briefly, dye-loaded cells containing 5 μ M nigericin were mixed with K⁺-clamp solutions at the above mentioned pH values. After 10 min, aliquots of these cell suspensions (100 μ l, containing 100 μ g of protein) were injected into a cuvette containing 1,900 μ l of buffer at a different pH (7.0, 7.25, 7.5, and 8.0), and the fluorescence ratio was measured.

Measurements of intravesicular acidification using the pH-sensitive fluorescent dye AO. The intravesicular acidification due to proton entry was measured by monitoring the fluorescence quenching of AO. Excitation and emission wavelengths were 498 and 530 nm, and both slit widths were set to 5 nm. Aliquots (10 μ l) of BBMVs (150–200 μ g protein) or BLMVs (30–50 μ g protein) were injected in a cuvette containing 10 μ l of 0.6 mM dye solution, 10 μ l of 1 mM valinomycin, and 1,970 μ l of appropriate buffer.

The experiments were carried out by using an LS-50 B Perkin-Elmer spectrofluorometer connected to a personal computer.

L-[³H]proline uptake measurement. L-[³H]proline uptake was measured at room temperature by mixing 10 μ l (80–100 μ g protein) of apical membrane vesicles with 90 μ l of incubation medium containing 0.5 mM of L-[³H]proline and different salts (see Fig. 5 for details). L-[³H]proline uptake was stopped by injecting 3 ml of ice-cold stop solution. BBMVs were immediately filtered onto a Millipore filter (0.45 μ m) and washed twice with another 3 ml of ice-cold stop solution. Filters, containing the vesicles and their associated radiolabeled solute, were placed in Beckman Ready Solv EP scintillation cocktail and counted in a Beckman LS-1801 scintillation counter. All isotope transport values were corrected for a "blank" obtained by adding the incubation medium and the vesicles directly to the stop solution before filtering.

In both fluorescence-based and radioactivity-based transport procedures, intravesicular and extravesicular buffers had the same ionic strength, pH, anion concentration, and osmolarity.

Statistics

Each experiment was repeated at least three times, leading to identical qualitative results. Individual experiments are presented throughout this article. Experimental points represent the mean \pm SD of 3–5 replicates of the same experiment. SD bars are shown wherever they exceed the size of the symbols. The statistical significance of differences was tested by unpaired Student's *t*-test (**P* < 0.05, ***P* < 0.01; absence of asterisks indicates the lack of statistical significance).

RESULTS

Effect of L-Proline on pH_i

The effect of L-proline on the pH_i of isolated eel enterocytes was tested both in the presence and absence of Na ions. Results are shown in Fig. 1.

An aliquot (100 μ l) of BCECF-loaded intestinal cell suspension was added to 1,900 μ l of PSS buffer with or without Na ions. After a baseline trace (resting pH_i under this experimental condition), 40 μ l of a 1.02 M solution of L-proline were added to the cuvette buffer (final concentration 20 mM). This experimental condition determined an intracellular acidification of ~ 0.28 pH units both in the presence (Fig. 1, trace b) or absence (Fig. 1, trace c) of Na^+ . This acidification was related to L-proline, because the addition of an equal amount of sucrose only slightly altered (0.07 pH units) the resting pH (Fig. 1, trace a).

The specificity of the proline-dependent intracellular acidification was also confirmed by the results shown in Fig. 2, in which the ability of different amino acids (glycine, L-lysine, L-glutamic acid, and L-proline) to affect pH_i was tested. Results clearly demonstrate that only L-proline significantly ($P < 0.01$) increased the rate of the intracellular acidification with respect to sucrose, whereas the addition of other amino acids had no effect. The addition of glycyl-L-proline, a dipeptide cotransported in association with proton substrate in eel enterocytes (5), also determined a higher rate of intracellular acidification. Similar qualitative and quantitative results were obtained both in the presence and absence of Na ions (Fig. 2). These results suggest that the proline-dependent intracellular acidification is not associated with the movement of the Na ion. The

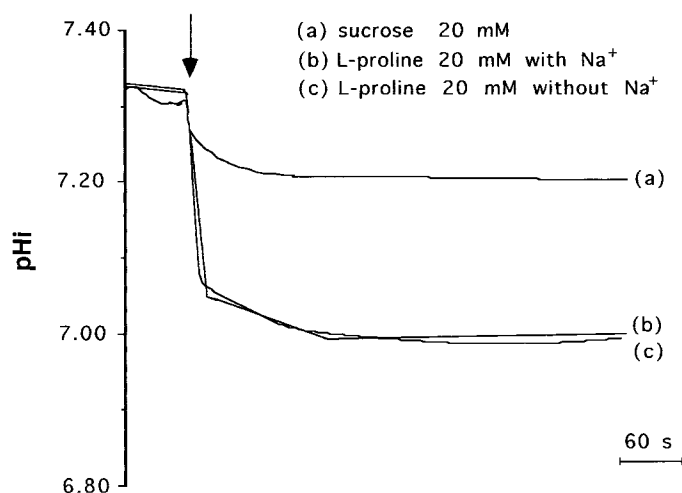


Fig. 1. Effect of L-proline on the intracellular resting pH (pH_i) of eel enterocytes. Samples of cell suspension (100 μ l) prepared in physiological saline solution (PSS) buffer with or without Na ions were injected into a cuvette containing 1,900 μ l of PSS buffer with (trace b) or without Na ions (trace c). After obtaining a baseline trace (resting pH_i determination), samples were supplemented with L-proline at a final concentration of 20 mM (traces b and c) or sucrose replacing L-proline isosmotically (trace a). The intracellular acidification obtained with L-proline (with or without sodium) was greater than that obtained with sucrose.

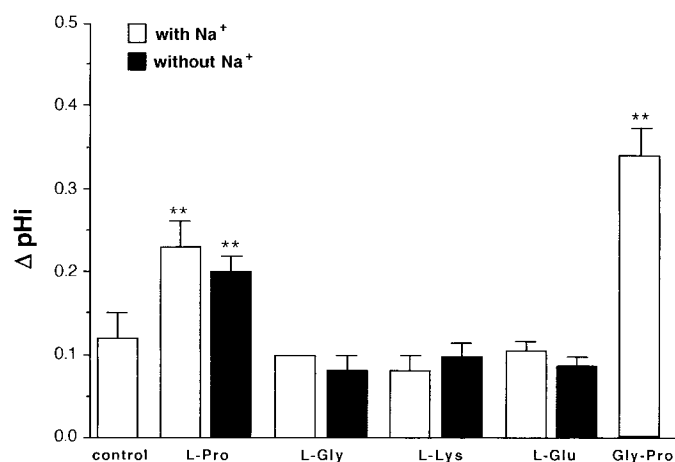


Fig. 2. Effect of different amino acids on the pH_i of eel enterocytes. The experimental conditions were the same as in Fig. 1. Samples were supplemented with 40 μ l each of a 1.02 M stock solution of L-proline, glycine, L-lysine, L-glutamic acid, and glycyl-L-proline, and the intracellular acidification was measured in the presence and absence of Na ions. Sucrose was used as a control. $**P < 0.01$.

conclusion that the pH_i acidification of the eel enterocytes was associated with the proline movement was also demonstrated by the results of the experiment depicted in Fig. 3 in which the initial rate of the proline-dependent (Na independent) intracellular acidification was measured in the presence of increasing extracellular L-proline concentrations and in the absence of Na^+ .

A hyperbolic relationship between the initial rate of pH_i variations and L-proline concentration was observed, suggesting that the intracellular acidification was attributable to the operation of a carrier-mediated system whose activity was related to the extracellular L-proline concentration. The kinetic parameters (calcu-

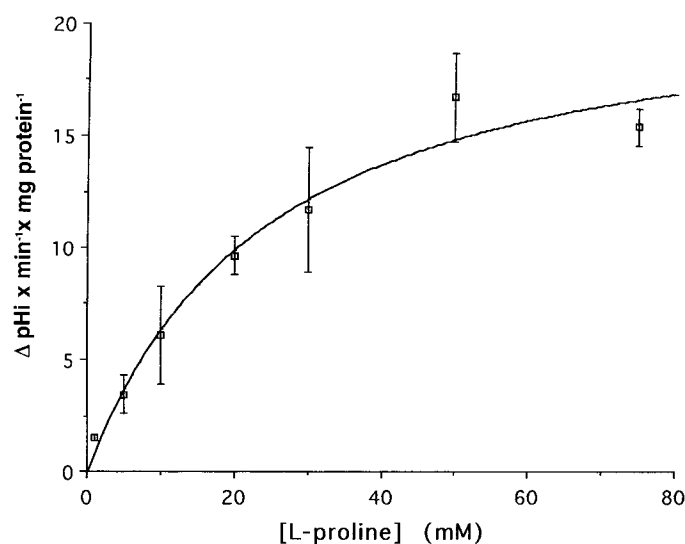


Fig. 3. Effect of increasing concentrations of L-proline on the pH_i acidification rate in the eel enterocytes. Experiments were performed according to a protocol similar to that described in Fig. 1. Cell suspension (100 μ l) was injected into 1,900 μ l of cuvette buffer (lacking Na ions) containing increasing concentration of L-proline (from 1 to 75 mM with sucrose replacing proline isosmotically).

lated by nonlinear regression analysis of the experimental data) determined on three different cell preparations were $21.37 \pm 4.76 \Delta\text{pH} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for maximal flux and $24.03 \pm 11.11 \text{ mM}$ for apparent affinity constant (K_{app}).

Localization of the L-Proline-Dependent Proton Transport

To directly demonstrate the exact localization of this L-proline-dependent (Na independent) proton cotransport mechanism on the enterocyte plasma membrane, experiments have been carried out following the movement of the pH-sensitive dye AO through the isolated apical (Fig. 4A) and BLMV (Fig. 4B) of the eel entero-

cytes. BBMVs (or BLMVs) prepared in a medium containing 100 mM KCl at pH 7.4 were injected in a cuvette buffer with or without 100 mM of KCl. The potassium ionophore valinomycin was always present in the cuvette buffer to generate or to clamp the transmembrane potential difference. Under these experimental conditions (presence or absence of membrane potential), the effect of L-proline on the intravesicular acidification was tested. Results reported in Fig. 4A demonstrate that, in the presence of a transmembrane potential difference, the addition of L-proline in the extravesicular medium determined an intravesicular acidification rate ($676.89 \pm 97.30 \Delta\text{F} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) significantly higher ($P < 0.05$) than that observed in its absence ($378.22 \pm 10.62 \Delta\text{F} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$). Similar qualitative (but not quantitative) results were obtained under short-circuited membrane potential conditions.

Results of similar experiments performed on BLMV and reported in Fig. 4B demonstrate that in the same experimental conditions, a proline-dependent proton transport could not be observed at this level. Anyway, the ability of the BLMVs to accumulate protons was demonstrated by the observation that when BLMV prepared in a buffer containing 100 mM potassium were injected in a medium lacking potassium and containing the K^+/H^+ exchanger nigericin, an intravesicular acidification was observed.

Altogether, these results argue for the existence at the apical membrane level of the eel enterocytes of a proline-dependent proton translocation mechanism whose activity does not depend on the Na ion.

Effect of pH Gradient on L-[^3H]Proline Uptake in BBMVs

To further demonstrate the existence of a coupled translocation between proline and proton at the apical membrane level of the eel enterocytes, experiments have been performed to evaluate the effect of the pH gradient (out < in) on the Na-independent L-[^3H]proline uptake. The uptake of L-[^3H]proline was measured in brush border prepared in 60 mM mannitol, 100 mM choline chloride, 100 mM KCl, 0.005 mM valinomycin, 0.5% ethanol, 20 mM HEPES, adjusted to pH 7.4 with Tris·HCl, and incubated in a medium (60 mM mannitol, 100 mM KCl, 100 mM choline chloride, 0.005 mM valinomycin, 0.5% ethanol) supplemented with 20 mM HEPES and 0.5 mM L-[^3H]proline, pH 7.4 with Tris ($\Delta\text{pH} = 0$), or supplemented with 20 mM MES and 0.5 mM L-[^3H]proline buffered at pH 5.4 with Tris ($\Delta\text{pH} \neq 0$) and in the presence or absence of KCl-valinomycin to generate or to clamp the membrane potential difference (see Fig. 5).

Results shown in Fig. 5 demonstrate that in BBMVs, the presence of an inwardly directed proton gradient significantly ($P = 0.00156$) stimulated the L-proline uptake measured at 15 s. This effect was significantly increased when an inside negative membrane potential difference was also present ($P = 0.0079$). As shown in Fig. 5, the equilibrium values measured at 60 min

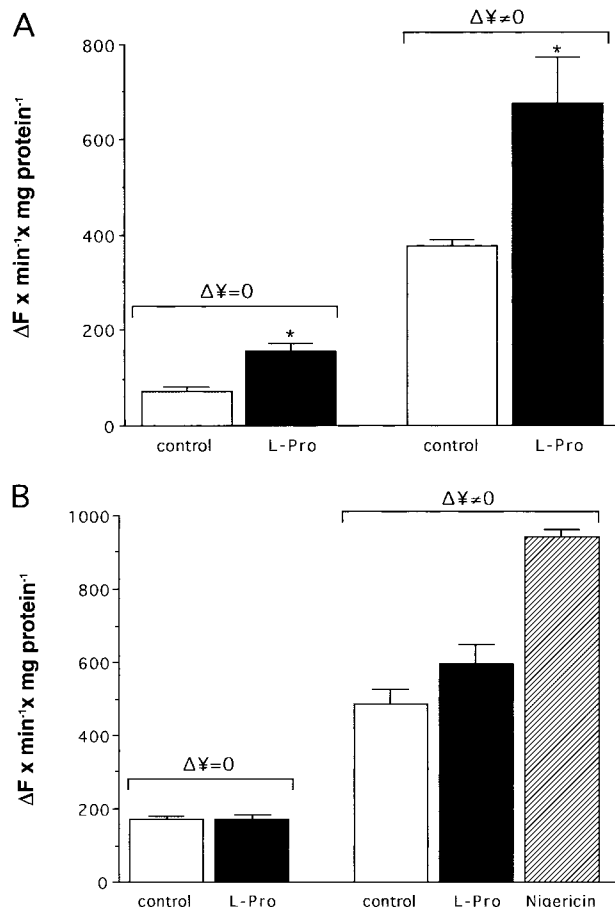


Fig. 4. A: effect of L-proline on the brush-border membrane intravesicular acidification measured in the presence or absence of transmembrane electrical potential difference. Brush-border membrane vesicle (BBMV; 10 μl) suspension, prepared in (in mM) 160 mannitol, 100 KCl, 20 HEPES, adjusted to pH 7.4 with Tris·HCl, was injected into 1,990 μl of cuvette buffer containing (in mM) 0.005 valinomycin, 0.003 of acridine orange, 0.5% ethanol, 20 HEPES, buffered at pH 7.4 with Tris and 160 mannitol, 100 KCl (control, $\Delta\psi = 0$) or 60 mannitol, 100 KCl, and 100 L-proline (L-Pro, $\Delta\psi = 0$) or 160 mannitol and 100 choline chloride (control, $\Delta\psi \neq 0$) or 60 mannitol, 100 choline chloride, and 100 L-proline (L-Pro, $\Delta\psi \neq 0$). * $P < 0.05$. B: effect of L-proline on the basolateral membrane intravesicular acidification measured in the presence or absence of transmembrane electrical potential difference. The experimental conditions were similar to those reported in A. In addition, the data of the intravesicular acidification obtained in the presence of nigericin are shown.

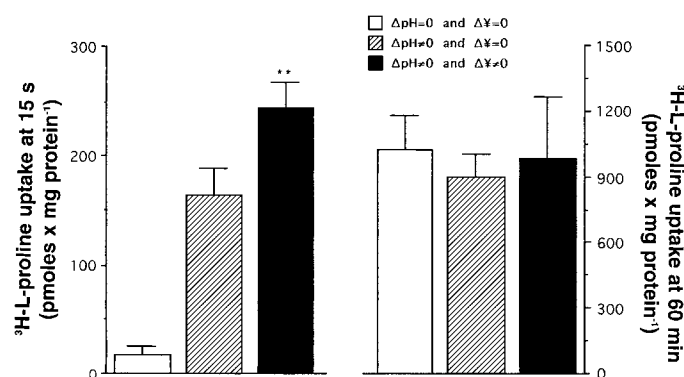


Fig. 5. Effect of pH gradient on the L-[³H]proline uptake in the presence or absence of transmembrane electrical potential difference. BBMVs suspension was prepared in a buffer containing 60 mM mannitol, 100 mM choline chloride, 100 mM KCl, 0.005 mM valinomycin, 0.5% ethanol, 20 mM HEPES, adjusted to pH 7.4 with Tris·HCl. An aliquot (10 μ l) was mixed with 90 μ l of incubation buffer (60 mM mannitol, 100 mM KCl, 100 mM choline chloride, 0.5 mM L-[³H]proline, 0.005 mM L-[³H]proline, 0.005 mM valinomycin, 0.5% ethanol), and 20 mM HEPES pH 7.4 with Tris (Δ pH = 0) or 20 mM MES pH 5.4 with Tris (Δ pH \neq 0). When the effect of membrane potential was tested, the extravesicular KCl was replaced isosmotically with choline chloride. The L-[³H]proline uptake was stopped at 15 s and at 60 min; ** P < 0.01 indicates the significance of the test bar 2 vs. 1.

were not different, indicating that the stimulatory effect was effectively due to L-[³H]proline influx into vesicular space.

DISCUSSION

This study reports evidence about the existence of coupling between proline and proton fluxes at the apical membrane level of the eel enterocytes. Data reported here clearly showed that the presence of the imino acid L-proline in the extracellular medium determines a specific intracellular acidification by a carrier-mediated mechanism. The effect of L-proline on the resting pH_i was measured by using the pH-sensitive dye BCECF (Fig. 1). Results showed that the carrier translocates protons through the apical plasma membrane in the presence of L-proline. It is evident that the presence of L-proline determined an intracellular acidification (fluorescence quenching) significantly higher than that measured in its absence. The pH_i decrement was observed only when experiments were performed in the presence of L-proline in the extracellular space, whereas it was unaffected in the presence of other neutral, acidic, or basic amino acids. An intracellular acidification was also obtained in the presence of the proton-cotransported dipeptide glycyl-L-proline, as previously reported by Maffia et al. (5), confirming these data.

Finally, the hyperbolic relationship between extracellular L-proline concentration and pH_i changes sustained that the intracellular proton accumulation was due to a carrier-mediated mechanism (Fig. 3). The possibility that the hyperbolic behavior of the system could be due to the limit of sensibility of the method and not to the presence of a carrier-mediated system

was ruled out by a previous observation that this method can measure rates of acidification higher than those reported here (20).

All these results suggest that an L-proline-dependent cotransport mechanism operates in the plasma membrane of the eel enterocyte. This system translocates protons, and its activity does not require Na ions. Physiologically, the intestinal proline uptake is particularly interesting, because proline appears to be the only amino acid tested here whose transport depends on the extracellular protons. Even though proline is not essential, it is largely present in the body collagen at the extracellular matrix level and it represents a highly effective physiological taste stimulus also detected through olfaction (3, 16).

The existence of substrate-dependent proton fluxes is not a novel issue, because it has been observed also in human Caco-2 cell monolayer (14) and in BBMVs prepared from eel intestine (17).

To demonstrate the exact localization of the proline-proton cotransport activity on the plasma membrane, experiments were carried out with isolated plasma membrane vesicles prepared from both basolateral and apical plasma membranes. Results shown in Fig. 4A demonstrate that the presence of L-proline in the extravesicular medium greatly increased the rate of the intravesicular acidification with respect to that measured in its absence. However, no proline-dependent intravesicular acidification was observed at the basolateral level (Fig. 4B). Altogether, these results demonstrate that a proline-dependent (Na independent) proton cotransport mechanism is operative at the apical membrane level of the eel intestinal absorbing cells.

Further confirmation of the existence of a coupling between proline and proton fluxes at the apical membrane level was given by the results reported in Fig. 5. These results demonstrate the ability of the proton gradient to stimulate the proline uptake in BBMVs. It was previously reported that protons can act as a driving force for the absorption of dipeptides in the intestine of both the carnivorous rockfish (*Sebastes caurinus*) (12) and the eel (*Anguilla anguilla*) (17), but as of now no information is available about the possible role of protons as a driving force for the absorption of amino acids in fish intestine. These results are the first to demonstrate the existence of an Na-independent cotransport between amino acids and protons in fish intestine.

Perspectives

The present report adds to the body of knowledge about amino acid transport in fish intestine. There is no information on the existence of similar amino acid transport systems in fish intestine; it would, therefore, be interesting to study the characteristics of this transporter at molecular level. For this reason, the next step would involve the cloning of this proline- H^+ cotransporter.

A detailed analyses of the characteristics of this transporter could improve the basic knowledge on the

physiology of nutrient absorption in fish and this in turn could give useful information on diet formulation and feeding strategy.

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