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Note

Influence of a proton gradient on the transport kinetics of the H^+/amino acid cotransporter PAT1 in Caco-2 cells

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Abstract

The recently cloned proton-coupled amino acid transporter 1 (PAT1) not only accepts several amino acids as substrates but also pharmaceutically relevant L-proline or GABA derivatives such as *cis*-4-hydroxy-L-proline, L-azetidine-2-carboxylic acid (LACA), 3-amino-1-propanesulfonic acid, nipecotic acid, and the antituberculotic agent D-cycloserine. Because human intestine expresses hPAT1 at the brush border membrane, the transporter may serve as a new oral drug delivery route. Using the human intestinal cell line Caco-2, we have investigated the influence of an inwardly directed proton gradient on the kinetic parameters of L-proline uptake. H^+ altered only the apparent affinity of L-proline transport and not the maximal transport velocity. Similarly, treatment of the cells with diethylpyrocarbonate (DEPC), known to chemically modify histidyl residues and block their function, affected only the K_t value of L-proline transport. Both increasing pH and DEPC treatment strongly increased the inhibition constants (K_i) of several drugs at hPAT1. It is concluded that H^+ stimulates hPAT1 primarily by increasing the substrate affinity with no detectable influence on the maximal transport velocity of the transporter.

Keywords: H^+/amino acid cotransporter; Caco-2 cells; Proton gradient; DEPC; Histidyl residues; Drug delivery

1. Introduction

The proton-coupled amino acid transporter PAT1 is a member of the SLC36 family of mammalian membrane transporters. It has been recently cloned from mouse intestine [1] and from Caco-2 cells [2]. The system corresponds to the transporter LYAAT1 cloned earlier from rat brain [3]. The transporter is identical to the H^+/amino acid cotransporter functionally described by Thwaites et al. 13 years ago [4]. Immunolocalization studies demonstrated the localization of hPAT1 at the apical membrane of intestinal Caco-2 cells and normal intestine [2]. PAT1 preferentially transports small unbranched L-α-amino acids and some β-amino acids with a free negatively charged carboxy group [1,5]. Prototype substrates are L-proline, glycine, β-alanine, GABA, and α-(methylamino)-isobutyric acid (MeAIB). Their inhibition constants (K_i) are in the range of 1.6–7 mM [1,2,6]. Interestingly, the system also transports the antituberculotic agent D-cycloserine [7]. We have recently shown that pharmaceutically relevant L-proline or GABA derivatives such as *cis*-4-hydroxy-L-proline, L-azetidine-2-carboxylic acid (LACA), and 3-amino-1-propanesulfonic acid are also substrates for hPAT1 [6]. The structures of representative substrates are shown in Fig. 1. Serotonin, tryptophan, and tryptamine inhibit transport function but are not transported by PAT1 [8]. PAT1 mediated transport depends on an inwardly directed proton gradient rather than a sodium gradient as many other amino acid transporters [1,2,4,6]. The stoichiometry of amino acid/proton symport is 1:1 [1,2]. The binding occurs in an ordered mechanism where H^+ binds first, followed by the amino acid [9].

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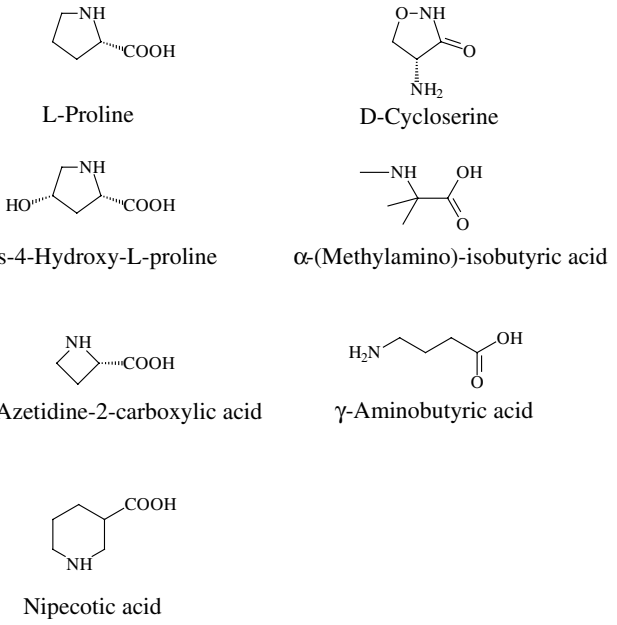

Fig. 1. Chemical structures of representative hPAT1 substrates.

Following the characterization of primary structure, tissue distribution, mechanism, and substrate specificity of PAT1, research in this field will focus on the binding site and the specific amino acid residues essential for substrate recognition, transport, and regulation. Histidyl residues are the most likely amino acid residues involved in H^+ binding and translocation in H^+-coupled transport systems. At the PAT1 protein, the histidyl residues H54 and H92 located in the first and second transmembrane domain may function as acceptors/donors for the cotransported proton [10].

For the Na^+/proline cotransporter [11] and the folate transporter in intestinal brush border membrane [12] it has been described that they are activated by their respective coupling ions by increasing the affinity to their substrates. In contrast, for the H^+/peptide cotransporters, PEPT1 and PEPT2, it has been reported that H^+ increases the maximal transport velocity with no effect on substrate affinity [13,14]. This has been determined by measuring transport activity at different outside pH values and after treating the cells with diethylpyrocarbonate (DEPC). This compound is known to block the H^+ acceptor/donor function of histidyl residues of proteins by producing *N*-carbethoxyhistidyl residues [15]. Subsequently, in site-directed mutagenesis studies it has been shown specifically that His-57 is essential for the catalytic activity of human PEPT1 [16].

For hPAT1 at intestinal cells, such studies have not yet been performed. Using *Xenopus laevis* oocytes expressing mPAT1 an inwardly directed proton gradient was found to influence the uptake of glycine by decreasing K_m with no significant influence on I_{max} [9].

In the present study, we investigated the influence of H^+ on the catalytic function of hPAT1 in Caco-2 cells using four different substrates by two different approaches, namely by altering the extracellular H^+ concentration and by blocking histidyl residues.

2. Materials and methods

2.1. Materials

The cell line Caco-2 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). L-[2,3-^{3}H]Proline (specific radioactivity 42 Ci/mmol) was obtained from Amersham Biosciences (Little Chalfont, UK). Cell culture reagents were purchased from Invitrogen (Karlsruhe, Germany). L-Proline, DEPC, GABA, LACA, and MeAIB were from Sigma (Taufkirchen, Germany).

2.2. Cell culture

Caco-2 cells (passages: 5–32; 78–112) were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acid solution, and gentamicin (45 μg/ml) as described [6,8]. Cells were released by trypsination and subcultured in 35 mm disposable petri dishes (Sarstedt, Nümbrecht, Germany). With a starting cell density of 0.8×10^6 cells per dish cultures reached confluence within 24 h. Uptake was measured on the 7th day after seeding.

2.3. Cell treatment and uptake measurement

Caco-2 cell monolayers were washed twice with buffer, pH 7.5 (25 mM Hepes/Tris) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. Incubation with DEPC was done at room temperature for 10 min in buffer at pH 7.5. DEPC was freshly added to each single dish at a final concentration of 0–10 mM as described [14]. Control buffer without DEPC contained 1% ethanol.

To study the effect of H^+ and DEPC treatment on L-[^{3}H]proline uptake, cells were incubated in buffer at pH 6.0 (25 mM Mes/Tris) or 7.5 (25 mM Hepes/Tris) containing 140 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 15 nM L-[^{3}H]proline. Uptake experiments were performed as described [6]. Samples were prepared for liquid scintillation spectrometry (Tri-Carb 2100TR, Packard Instrument Company, Meriden, Australia) by suspending the cells in 1 ml lysis buffer and 2.8 ml scintillation cocktail. Protein was determined according to the method of Bradford.

2.4. Data analysis

The results are expressed as means ± SEM. The Michaelis–Menten constant (K_t) and the maximal velocity of transport (V_{max}) were calculated by linear regression of the Eadie–Hofstee plot and confirmed by non-linear regression of the Michaelis–Menten plot (Sigma Plot 8.0, SPSS

Inc., Chicago, USA). Inhibition constants (K_i) (i.e., concentration of unlabeled substrates necessary to inhibit 50% of carrier-mediated L-[^{3}H]proline uptake) were determined as described [6,8]. Statistical analysis was done by the non-parametric two-tailed *U*-test and a p value of less than 0.05 was considered statistically significant.

3. Results and discussion

To determine the effect of H^+ on the kinetic parameters K_t and V_{max} of the L-proline uptake into Caco-2 cells, L-[^{3}H]proline uptake was measured at increasing concentrations of unlabeled L-proline (0–50 mM) and in the absence (pH 7.5) and presence (pH 6.0) of an inwardly directed pH gradient (Fig. 2). L-[^{3}H]Proline uptake into Caco-2 cells is strictly linear for 30 min [6]. A 10-min uptake time was chosen for these experiments. The K_t values of L-proline uptake calculated from the Eadie–Hofstee plots were 1.8 ± 0.2 mM at pH 6.0 and 22 ± 6 mM at pH 7.5 (Table 1). The V_{max} value at pH 6.0 (88.5 ± 19.9 nmol/10 min/mg of protein) was not significantly different from the V_{max} value measured at pH 7.5 (70.9 ± 3.5 nmol/10 min/mg of protein). Therefore, H^+ stimulates the L-proline transport via hPAT1 by increasing the substrate affinity without affecting the maximal transport velocity. This is in contrast to the H^+ cotransporters PEPT1 and PEPT2 where H^+ affects only the maximal velocity of transport [14]. It can be ruled out that this pronounced pH effect on L-proline uptake is due to a different degree of ionization of L-proline because at both pH values >99.9% of molecules exist in their zwitterionic form.

We then investigated the effect of H^+ on the inhibition constants of the hPAT1 substrates LACA, GABA, and MeAIB. At an extracellular pH of 6.0 the L-[^{3}H]proline uptake was about 6-fold higher (785 ± 46 fmol/10 min/mg of protein) than at pH 7.5 (121 ± 12 fmol/10 min/mg of protein). From the inhibition curves obtained at increasing inhibitor concentrations (Fig. 3) the corresponding K_i values were calculated (Table 1). At pH 6.0, the K_i values of LACA, GABA, and MeAIB were 1.3, 2.1, and 3.4 mM. At pH 7.5 the inhibitory strength of these unlabeled substrates is dramatically decreased ($K_i = \sim 20$, ~ 22, and ~ 29 mM, respectively).

To elucidate the role of histidyl residues on the transport activity of hPAT1, L-[^{3}H]proline uptake was measured in

Table 1
Effect of H^+ and DEPC on the Michaelis–Menten constant (K_t) of L-proline uptake and the inhibition constants (K_i) of LACA, GABA, and MeAIB for the inhibition of L-[^{3}H]proline uptake at Caco-2 cells

Substrate	Extracellular pH		DEPC	
	6.0	7.5	−	+
L-Proline	1.8 ± 0.2	$22 \pm 6^*$	2.3 ± 0.1	$4.0 \pm 0.1^*$
L-Azetidine-2-carboxylic acid	1.3 ± 0.3	$\sim 20^*$	2.6 ± 0.1	$3.6 \pm 0.4^*$
GABA	2.1 ± 0.3	$\sim 22^*$	2.8 ± 0.5	$3.7 \pm 1.6^*$
α-(Methylamino)-isobutyric acid	3.4 ± 0.7	$\sim 29^*$	5.7 ± 0.5	$7.1 \pm 1.7^*$

To determine the influence of H^+, uptake of L-[^{3}H]proline (15 nM) was measured at pH 6.0 or pH 7.5 for 10 min. To measure the effect of DEPC on L-[^{3}H]proline uptake, cells were incubated with or without DEPC (1.5 mM) for 10 min and L-[^{3}H]proline (10 nM) uptake was measured at pH 6.0 (10 min). Concentrations of unlabeled substrates were 0–50 mM. K_t values (± SEM) were determined from linear regression of the data shown in Figs. 2 and 4. K_i values (± SEM) were determined by non-linear regression analysis of the dose–response data shown in Fig. 3.
* $p < 0.05$.

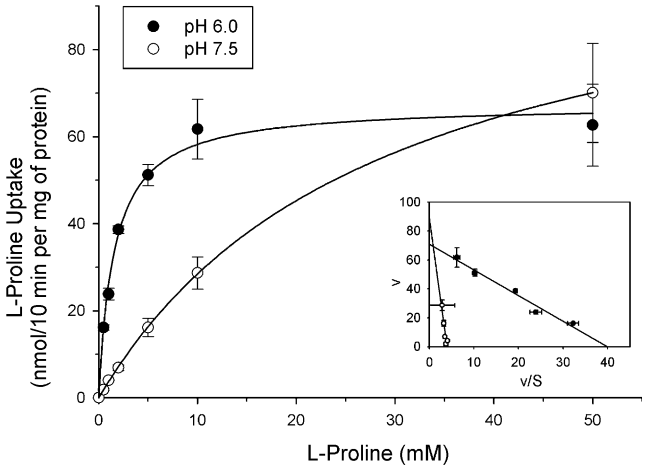

Fig. 2. Effect of a H^+ gradient on the saturation kinetics of L-proline uptake in Caco-2 cells. Uptake of L-[^{3}H]proline (15 nM) was measured at an outside pH of 7.5 (open circle) or 6.0 (closed circle) in the presence of increasing concentrations of unlabeled L-proline (0–50 mM). The values are means ± SEM, $n = 4$–6. They represent uptake values after correction for non-saturable transport component which was calculated by non-linear regression of the data. Inset: Eadie–Hofstee transformation of the data.

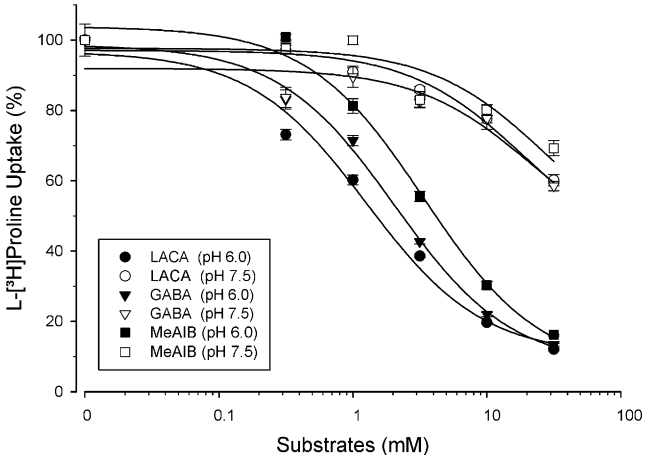

Fig. 3. Effect of a H^+ gradient on the inhibition of L-[^{3}H]proline uptake in Caco-2 cells by LACA, GABA, and MeAIB. Uptake of L-[^{3}H]proline (15 nM) was measured for 10 min at an outside pH 7.5 (open symbols) where 100% correspond to 121 ± 12 fmol/10 min/mg of protein, or pH 6.0 (closed symbols) where 100% correspond to 785 ± 46 fmol/10 min/mg of protein, in the presence of increasing concentrations of LACA, GABA, and MeAIB. The unsaturable transport component was determined using an excess amount of 100 mM L-proline. The value was 11% of total uptake, independent of pH and set as Y_{min} value during non-linear regression of the data. The values are means ± SEM, $n = 4$–6.

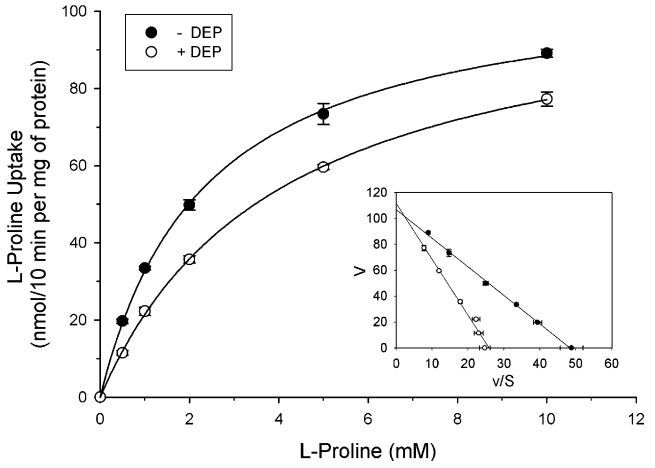

Fig. 4. Effect of DEPC on the saturation kinetics of L-proline uptake. Uptake of L-proline (0–10 mM, L-[^{3}H]proline 10 nM) was measured at pH 6.0 after treatment without (closed circle) or with (open circle) DEPC (1.5 mM) for 10 min at pH 7.5. The values (means ± SEM, $n = 4$–6) represent uptake values corrected for non-saturable transport component which was calculated by non-linear regression of the data. Inset: Eadie–Hofstee transformation of the data.

the presence of increasing concentrations of DEPC (0–10 mM). An incubation time of 10 min at pH 7.5 was shown to be sufficient. The L-[^{3}H]proline uptake was almost completely abolished by 10 mM DEPC. From the dose–response relationship an IC_{50} value of 1.4 ± 0.1 mM was calculated (data not shown). Therefore, in the following experiments we treated the Caco-2 cells with 1.5 mM DEPC. First, the influence of DEPC on the K_t and V_{max} values of L-proline uptake into Caco-2 cells was determined in saturation studies. As shown in Fig. 4, the effect of DEPC on the kinetic parameters was similar to the effect of a pH gradient. The K_t value of L-proline uptake determined by linear regression of the data shown as Eadie–Hofstee plot (inset; $r^2 > 0.98$) was 2.3 ± 0.1 mM in control cells and 4.0 ± 0.1 mM after DEPC treatment. The V_{max} values were 109.1 ± 1.4 nmol/10 min/mg of protein in control cells and 108.2 ± 1.3 nmol/10 min/mg of protein, respectively. Hence, treatment with DEPC at a concentration close to its IC_{50} value affected only the Michaelis–Menten constant of L-proline uptake.

Similar to the experiments described above for the effect of H^+, we then performed competition assays to determine the influence of DEPC on the K_i values of LACA, GABA, and MeAIB for L-[^{3}H]proline uptake inhibition. As shown in Table 1, DEPC treatment caused a very significant increase of the K_i values of the substrates.

We conclude that the amino acid and drug transport at hPAT1 constitutively expressed in intestinal cells is stimulated by extracellular H^+ by increasing the substrate affinity with no effect on the maximal transport velocity. It can be speculated that the interaction of one H^+ with a histidyl residue at or very near to the substrate binding pocket of the transporter protein changes the binding strength for one substrate molecule. The membrane topology model of hPAT1 based on hydropathy analysis predicts nine putative transmembrane domains, the amino terminus facing the cytoplasmic side [2]. Both mPAT1 and hPAT1 contain 10 histidyl residues. At least five of them are located in the extracellular loops of the transport protein. H54, H92, and H134 are conserved in the PAT proteins (PAT1, PAT2, and PAT3) [10]. It can be assumed that H54 and H92 are accessible for H^+ binding [10]. Obviously, more work is needed to clearly define the role of such histidyl residues for H^+ coupling, e.g., by site-directed mutagenesis studies.

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