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Influence of proton and essential histidyl residues on the transport kinetics of the H^+/peptide cotransport systems in intestine (PEPT 1) and kidney (PEPT 2)

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Abstract

The mechanism by which H^+ alters the kinetics of the H^+-coupled peptide transporters PEPT 1 and PEPT 2 was investigated in two different cell lines which differentially express these transporters, namely Caco-2 cells (PEPT 1) and SKPT cells (PEPT 2). The effects of H^+ on the affinity and the maximal velocity of Gly-Sar uptake were analyzed in these cells under identical conditions. In both cells, H^+ influenced only the maximal velocity of uptake and not the apparent affinity. The effects of H^+ on the IC_{50} values (i.e., concentration necessary to cause 50% inhibition) of the cationic dipeptide Ala-Lys and the anionic dipeptide Ala-Asp for inhibition of Gly-Sar uptake were also investigated. H^+ did not change the IC_{50} value for Ala-Lys but did decrease the IC_{50} value for Ala-Asp considerably. The influence of diethylpyrocarbonate (DEP) on the kinetic parameters of PEPT 1 and PEPT 2 was then studied. Histidyl residues are the most likely amino acid residues involved in H^+ binding and translocation in H^+-coupled transport systems and DEP is known to chemically modify histidyl residues and block their function. DEP treatment altered the maximal velocity of Gly-Sar uptake but had no effect on its K_t (Michaelis-Menten constant) or the IC_{50} values of Ala-Lys or Ala-Asp for the inhibition of Gly-Sar uptake. It is concluded that H^+ stimulates PEPT 1 and PEPT 2 primarily by increasing the maximal velocity of the transporters with no detectable influence on the substrate affinity.

Keywords: H^+/peptide cotransporter; Caco-2 cell; SKPT cell; Proton; Histidyl residue

1. Introduction

Ion-coupled solute transport across biological membranes is one of the fundamental principles in biology. Many nutrients are transported into the cells by carriers which are driven by transmembrane ion gradients. The ions are cotransported with substrates and the downhill movement of the ions is energeti-

Abbreviations: Gly-Sar, glycylsarcosine; Ala-Lys, alanyllysine; Ala-Asp, alanylaspartate; DEP, diethylpyrocarbonate; Hepes; 4(-2-hydroxyethyl)-1-piperazine ethanesulfonic acid; Mes, 2-(N-morpholino) ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine tetraacetic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BCECF-AM, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester.

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cally coupled to the uphill movement of the substrates. Na^+ and H^+ have been identified as the most important coupling ions for transport systems which are capable of active transport. Na^+ serves as the cotransportable ion for a variety of transport systems involved in the uphill transport of glucose, amino acids, vitamins, and neurotransmitters. H^+-coupled solute transport systems were first described in bacteria but later also found to occur in animal cells. Examples of H^+-coupled solute transport systems in animal cells include those involved in the transport of small peptides [1–3], monocarboxylates [4], and certain amino acids [5–7].

The ion-coupled transport systems are activated by their respective coupling ions due, in most cases, to the ability of the ions to increase the affinity of the transport systems for their substrates. This has been documented in animal cells, for example, in the following cases: Na^+/proline cotransporter [8], Na^+/glucose cotransporter [9], Na^+/succinate cotransporter [10], Na^+/glycine cotransporter [11], Na^+/carnitine cotransporter [12], Na^+ plus Cl^--coupled neurotransmitter transporters [13,14], and H^+/leucine cotransporter [15]. Several H^+-coupled transport systems in bacteria have also been shown to be activated by the coupling ion by a similar mechanism [16]. Surprisingly, in the case of the intestinal H^+/peptide cotransporter, the cotransported ion H^+ was found to activate the transport system by increasing the maximal velocity with no significant effect on the affinity [17]. Similar results were obtained with the renal H^+/peptide cotransporter when histidyl residues, the most likely amino acid residues to be involved in the binding and translocation of H^+, were chemically modified with diethylpyrocarbonate (DEP) [18]. In the following years, however, contradictory results have been published regarding the influence of H^+ and/or DEP on the kinetic parameters of the intestinal H^+/peptide cotransporter. Kato et al. [19] have reported that modification of histidyl residues with DEP in rabbit intestinal brush border membranes decreases the V_{max} without affecting the K_t for cephardine, a zwitterionic substrate for the H^+/peptide cotransporter. A similar conclusion was drawn by Thwaites et al. [20] who found that H^+ activates the peptide transport system in the Caco-2 intestinal cell line by increasing the V_{max} with no effect on K_t for the dipeptide substrate Gly-Sar. In contrast, Kramer et al. [21] found that DEP treatment of rabbit intestinal brush border membranes increases the K_t for cephalexin, also a zwitterionic substrate for the H^+/peptide cotransporter, without influencing the V_{max}. Corroborating the conclusions of Kramer et al. were the findings that an acidic pH increases the affinity of the intestinal H^+/peptide cotransporter for its substrates glycylglycine [22] and cefixime [23]. Therefore, it is not clear at this time whether H^+ activates the peptide transport system in the intestine by increasing the substrate affinity or by increasing the maximal velocity. Interestingly, there appears to be no such controversy with regard to the renal H^+/peptide cotransporter. Renal brush border membranes possess two different peptide transport systems, one with low-affinity and the other with high-affinity for peptide substrates [24,25]. Daniel et al. [25] have elegantly shown that H^+ activates both these systems by increasing the V_{max} without affecting the substrate affinity. These results have been recently reproduced with the high-affinity peptide transporter cloned from rabbit kidney [26].

Recent cloning studies have clearly established that the intestinal peptide transporter is structurally and functionally distinct from the high-affinity peptide transporter expressed in the kidney [26–34]. We have identified cell culture model systems to individually study these two transporters [35–37]. The human colon carcinoma cell line Caco-2 expresses the intestinal low-affinity H^+/peptide cotransporter (PEPT 1) and the rat proximal tubular cell line SKPT expresses the renal high-affinity H^+/peptide cotransporter (PEPT 2). In the present investigation, we studied the influence of the coupling ion H^+ on the kinetic parameters of PEPT 1 and PEPT 2 using these two cultured cell lines under identical experimental conditions. This was done by measuring the maximal velocity and substrate affinity for structurally diverse substrates at two different H^+ concentrations and also with or without modifying the histidyl residues in PEPT 1 and PEPT 2 by DEP treatment.

2. Materials and methods

2.1. Materials

The human colon carcinoma cell line Caco-2 was obtained from the American Type Culture Collection

(Rockville, MD, USA). The rat renal proximal tubular epithelial cell line SKPT-0193 Cl.2 [36] was established by SV40 transformation of isolated cells from the proximal tubule and was provided by Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH, USA). Minimum Essential Medium (MEM) with Earle's salts and L-glutamine was purchased from Mediatech (Washington D.C., USA). Dulbecco's Modified Eagle Medium:Nutrient Mixture F12 (Ham) 1:1 (DMEM/F12) with Hepes (15 mM) and L-glutamine, MEM non-essential amino acids, penicillin (10 000 units/ml)-streptomycin (10 000 μg/ml), and trypsin were obtained from Life Technologies (Gaithersburg, MD, USA). Ala-Lys, Ala-Asp, DEP, EDTA, dexamethasone, apotransferrin and fetal bovine serum were purchased from Sigma (St. Louis, MO, USA). Recombinant insulin was from Novo Nordisk (Denmark) and epidermal growth factor from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Cephalexin was a generous gift from Dr. T. Hoshi, University of Shizioka, Shizioka, Japan. [2-^{14}C]Glycyl[1-^{14}C]sarcosine (specific radioactivity 108 mCi/mmol) was obtained from Cambridge Research Biochemicals (Cleveland, UK).

2.2. Cell culture

Caco-2 cells (passage 95 to 127) were maintained in 75 cm^2 culture flasks at 37°C and 5% CO_2. MEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% MEM non-essential amino acids was used as culture medium. Cells were grown to confluence and released by treatment with PBS/EDTA for 5 min followed by trypsin treatment (0.25% trypsin and 0.5 mM EDTA in PBS) for <10 s and subcultured in 35-mm disposable Petri dishes. With a starting cell density of 1×10^6 cells/dish, the cultures reached confluence the next day. Medium was changed every day. Uptake experiments were carried out four days after seeding because our previous studies have shown that the peptide transporter activity, expressed as Gly-Sar uptake/mg of protein, was maximal under these culture conditions [35].

SKPT cells at passage number 87 to 101 were maintained in 75-cm^2 culture flasks at 37°C in a humidified atmosphere with 5% CO_2. They were cultured as described previously [36] in DMEM/F12 medium supplemented with fetal bovine serum (10%), penicillin-streptomycin (1%), insulin (5 μg/ml), dexamethasone (4 μg/ml), apotransferrin (5 μg/ml), and epidermal growth factor (10 ng/ml). Cells grown to confluence were released by incubation in PBS/EDTA for 10 min followed by trypsinization (0.25% trypsin and 0.5 mM EDTA in PBS) for 10 s and subcultured in 35-mm disposable Petri dishes. The medium was replaced every day. With a starting cell density of 1×10^6 cells per dish, the cultures reached confluence within 20 h. Uptake was measured in these cells four days after seeding.

2.3. Cell treatment

Confluent monolayers of Caco-2 cells or SKPT cells were treated with DEP and these treatments were done at room temperature for 10 min in pH 7.5 buffer. DEP was mostly added at a final concentration of 0.4 mM to Caco-2 cells and 0.1 mM to SKPT cells. The vehicle for DEP used for the controls was ethanol. Medium for DEP-treatment was prepared fresh for each single dish due to concerns regarding the stability of DEP.

2.4. Uptake measurement

Uptake of Gly-Sar was determined as described previously [35–37]. The uptake medium was 25 mM Mes/Tris (pH 6.0), or 25 mM Hepes/Mes/Tris (pH 7.0), containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose. The pH 7.0 uptake buffer was prepared by mixing the Mes/Tris pH 6.0 uptake buffer with Hepes/Tris pH 7.5 uptake buffer to a final pH of 7.0.

Uptake was initiated by removing the medium or the treatment buffer, and washing the monolayers for 1 min in the respective uptake buffer (pH 6.0 or pH 7.0). After that, 1 ml of uptake medium containing radiolabeled Gly-Sar was added. The concentration of radiolabeled Gly-Sar in most experiments was 10 μM for Caco-2 cells and 5 μM for SKPT cells. In addition, the uptake medium contained increasing concentrations of Gly-Sar, Ala-Lys, Ala-Asp or cephalexin. After dissolving the peptides, the pH of medium was corrected before preparing the required dilutions. After incubation for 10 min, the uptake buffer was removed and the monolayers were quickly washed four times with ice-cold uptake buffer, dis-

solved with 1 ml of 0.2 M NaOH containing 1% SDS and transferred to scintillation vials for quantitation of radioactivity by liquid scintillation spectrometry. For each experiment, the samples for protein measurement were prepared and measured as described earlier [35,36].

For the calculation of the kinetic parameters of Gly-Sar uptake, the non-saturable uptake component was determined by measuring the uptake of radiolabeled Gly-Sar in the presence of an excess amount of unlabeled Gly-Sar (50 mM in Caco-2, 10 mM in SKPT cells) and also by analysis of total Gly-Sar uptake according to a transport model describing one saturable system plus one non-saturable system. Both methods gave virtually identical values for the non-saturable component. This component was subtracted from the total uptake to calculate the saturable (carrier-mediated) component. The values for the saturable component were used for construction of Eadie-Hofstee plots.

2.5. Assessment of Gly-Sar hydrolysis

Caco-2 cells and SKPT cells were incubated for 10 min at pH 6.0 in the presence or in the absence of 1 mM Gly-Sar. At the end of the incubation, the medium was aspirated and saved for amino acid analysis. The cell monolayers were washed once with the uptake buffer, following which 0.5 ml of 4% sulfosalicylic acid was added to the dish to lyse the cells and to precipitate cellular proteins. The lysates were centrifuged and the supernatants saved for amino acid analysis. Concentrations of Gly-Sar, glycine and sarcosine in the samples were determined using an amino acid analyzer (Beckman, Model 7300).

2.6. Monitoring of intracellular pH

The intracellular pH (pH_i) was monitored in SKPT cells using BCECF as described by Paradiso et al. [38]. Cells were loaded with 2.5 μM of BCECF-AM and the video images of BCECF fluorescence at 490 nm and 440 nm were monitored by a camera. The emission bandpass was fixed at 520–550 nm. The ratio of fluorescence at 490 nm to fluoroscence at 440 nm was taken as a measure of pH_i. The ratio was continuously recorded when the cells were exposed to the uptake buffer (pH 7.5) for 5 min, then to the uptake buffer (pH 6.0) for 5 min and then to the uptake buffer (pH 6.0) containing 10 μM nigericin for 10 min.

2.7. Statistics

Experiments were routinely made in duplicate and each experiment was repeated two to three times. The results are expressed as means $\pm$ S.E.M. The kinetic constants were calculated by non-linear regression methods using the *Fig. P* 6.0 program (Biosoft, Cambridge, UK) and confirmed by linear regression of the Eadie-Hofstee plot. The calculated parameters K_t and V_{max} are shown with their S.E.M. IC_{50} values (i.e., concentration of the unlabeled compound necessary to inhibit 50% of radiolabeled glycylsarcosine carrier-mediated uptake) were also determined by non-linear regression methods using the equation for an asymmetric sigmoid (allosteric Hill kinetics; linear dose vs. effect): $y = Min + (Max\text{-}Min)/(1 + ((X/IC_{50})^{-P}))$. 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

3.1. Effect of an inwardly directed H^+ gradient on the kinetic parameters of Gly-Sar uptake in intestinal and renal cells

It has been shown in earlier studies that in both Caco-2 cells [35] and SKPT cells [36] the Gly-Sar uptake is driven by an inwardly directed H^+ gradient, is linear for at least up to 10 min, and is mediated by a single uptake system in each cell type. However, the peptide transport system in Caco-2 cells is the low-affinity system PEPT 1 and the peptide transport system in SKPT is the high-affinity system PEPT 2. We have established by reverse transcription-coupled PCR and by Northern blot analysis that Caco-2 cells do not express PEPT 2 and that SKPT cells do not express PEPT 1 [37].

Gly-Sar is widely used as a substrate in peptide transport studies because it is highly resistant to hydrolysis to plasma membrane peptidases. We investigated whether this dipeptide is also resistant to intracellular peptidases in Caco-2 cells as well as in

SKPT cells. The cells were incubated in the absence and in the presence of 1 mM Gly-Sar at pH 6.0 for 10 min. Intracellular and medium concentrations of glycine, sarcosine, and Gly-Sar were then measured. There was no evidence of hydrolysis of Gly-Sar in the medium because glycine and sarcosine were undetectable following the incubation. The intracellular concentrations of glycine, sarcosine, and Gly-Sar are given in Table 1. It is clear that Gly-Sar, following transport into the cells, remained mostly intact, as evidenced from minimal difference in glycine and sarcosine concentrations between the cells incubated in the absence of Gly-Sar and the cells incubated in the presence of Gly-Sar.

Using Caco-2 cells and SKPT cells as model systems, we performed in the present study detailed kinetic analyses to determine unequivocally the kinetic nature of the H^+ gradient-dependent activation of peptide transport activity mediated by PEPT 1 and PEPT 2. Uptake of Gly-Sar was measured for 10 min over a concentration range of 0.1–5 mM in Caco-2 cells and 0.01–0.5 mM in SKPT cells at an outside pH of either 6.0 or 7.0. The diffusional component of uptake was determined as described in Section 2. This component was subtracted from total uptake to calculate mediated uptake. The rate of mediated uptake was linear in both cells over the incubation period of 10 min, indicating that initial uptake rates were measured under the experimental conditions.

Table 1
Intracellular content of glycine, sarcosine, and Gly-Sar in Caco-2 cells and SKPT cells after exposure to Gly-Sar

Experimental condition	Concentration of amino acids/dipeptide (μM)		
	Glycine	Sarcosine	Gly-Sar
Caco-2 cells			
Without exposure to Gly-Sar	13 ± 0	ND	0.8 ± 0.2
With exposure to Gly-Sar	20 ± 1	ND	339 ± 15
SKPT cells			
Without exposure to Gly-Sar	396 ± 10	ND	1.5 ± 0
With exposure to Gly-Sar	415 ± 4	ND	84 ± 3

Cells were incubated for 10 min at pH 6.0 in the absence or in the presence of 1 mM Gly-Sar. At the end of incubation, the cell monolayers were washed and then lysed with 4% sulfosalicylic acid. The lysates were centrifuged to remove precipitated proteins and the supernatants were used for amino acids analysis. ND, not detectable.

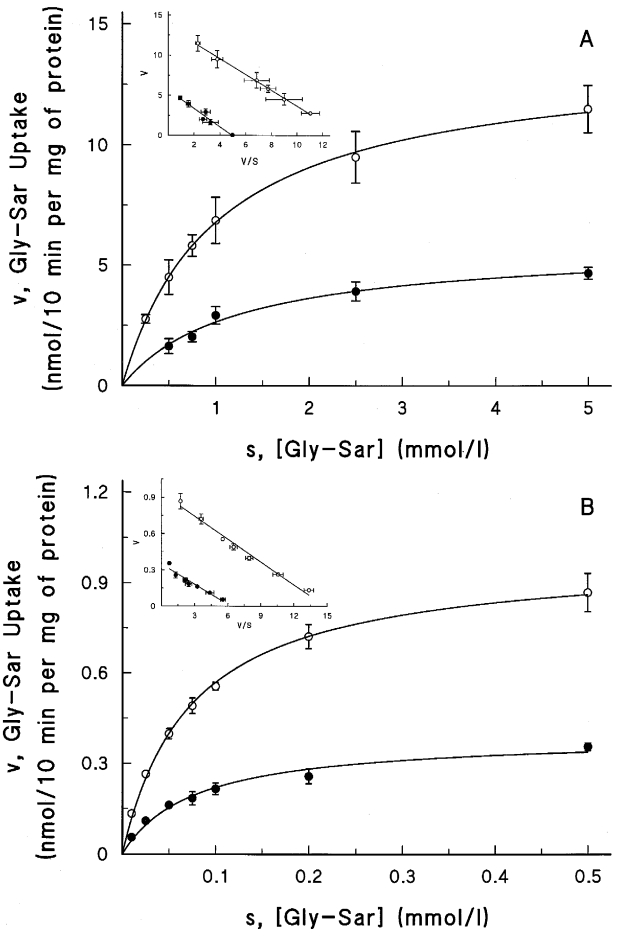

Fig. 1. Effect of a H^+ gradient on the saturation kinetics of Gly-Sar uptake in Caco-2 cells (A) and SKPT cells (B). Uptake of Gly-Sar (concentration range, 0.25 to 5 mM in Caco-2 and 10 to 500 μM in SKPT; uptake time, 10 min) was measured in confluent cell cultures at an outside pH of 7.0 (●) or pH 6.0 (○). The values are means ± S.E.M. for 4–6 determinations. They represent saturable uptake values after correction for non-saturable component. If not shown, error bars are smaller than the symbols. Insets: Eadie-Hofstee transformations of the data.

The relationship between the rate of mediated uptake and substrate concentration was found to be hyperbolic under both pH conditions in Caco-2 cells (Fig. 1A) as well as in SKPT cells (Fig. 1B). The experimental data were found to fit best for an uptake model describing a single transport system. When expressed as Eadie-Hofstee plots (uptake rate/substrate concentration versus uptake rate), the data (Fig. 1A,B, insets) yielded straight lines ($r^2 > 0.95$). Determination of kinetic parameters from the data indi-

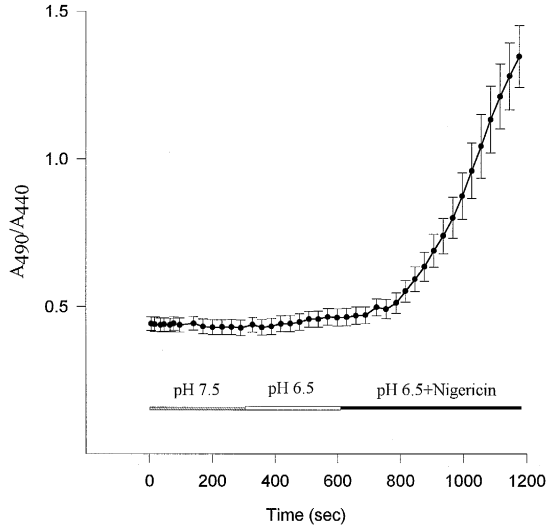

Fig. 2. Monitoring of intracellular pH in SKPT cells. Cells were loaded with the acetoxymethyl ester of BCECF (2.5 μM) and the fluorescence was monitored at 490 nm and 440 nm with the emission bandpass fixed at 520–550 nm. The florescence measurements were made at four different areas of the cell monolayer and the ratio of fluorescence at 490 nm to fluorescence at 440 nm (mean $\pm$ S.E.M. for the four different areas) is given as a measure of intracellular pH. The measurements were made continuously when the cell monolayer was exposed to the uptake buffer (pH 7.5) for 5 min, then to the uptake buffer (pH 6.0) for 5 min and then to the uptake buffer (pH 6.0) containing 10 μM nigericin for 10 min.

cated clearly that increasing the H^+ concentration outside the cells resulted in an increase of the maximal velocity of Gly-Sar transport without changing significantly the affinity of the carrier for Gly-Sar. In Caco-2 cells, the apparent Michaelis-Menten constant (K_t) for the uptake process was 1.0 ± 0.1 mM at pH 6.0 and 1.2 ± 0.2 mM at pH 7.0 (Table 2). The maximal velocity (V_{max}) of Gly-Sar uptake, however, was 13.7 ± 0.3 nmol/10 min per mg of protein at pH 6.0 and 5.8 ± 0.3 nmol/10 min per mg of protein at pH 7.0. Similar results were obtained in SKPT cells. In these renal cells, the apparent Michaelis-Menten constant (K_t) for the uptake process was 74.0 ± 3.2 μM at pH 6.0 and 76.8 ± 11.4 μM at pH 7.0 (Table 2). The maximal velocity (V_{max}) of Gly-Sar uptake, however, was 0.99 ± 0.02 nmol/10 min per mg of protein at pH 6.0 and 0.39 ± 0.02 nmol/10 min per mg of protein at pH 7.0. Therefore, these data give no evidence for an effect of H^+ on the affinity of the carrier protein to its substrate Gly-Sar in the intestinal as well as in the renal cell line.

Monitoring of pH_i in BCECF-loaded cells indicated that changing the extracellular pH from 7.5 to 6.0 did not alter pH_i (Fig. 2). The experimental technique was validated by the observed acidification of pH_i as evidenced from the increase in the ratio of fluorescence at 490 nm to fluorescence at 440 nm when the cells were exposed to pH 6.0 in the presence of nigericin.

3.2. Effect of an inwardly directed H^+ gradient on the IC_{50} values of Ala-Lys and Ala-Asp for inhibition of Gly-Sar uptake in intestinal and renal cells

Gly-Sar is a neutral dipeptide which exists predominantly as a zwitterion in the pH range 6.0–7.0.

Table 2
Effect of H^+ and DEP on the IC_{50} values for different peptides for the inhibition of [^{14}C]glycylsarcosine uptake in Caco-2 cells and SKPT cells

Inhibitor	Caco-2: IC_{50} (mM)				SKPT: IC_{50} (μM)			
	pH		DEP		pH		DEP	
	6.0	7.0	−	+	6.0	7.0	−	+
Glycylsarcosine	1.01 ± 0.04	1.21 ± 0.17	0.83 ± 0.1	0.76 ± 0.09	74.0 ± 3.2	76.8 ± 11.4	64.1 ± 9.9	62.3 ± 4.5
Alanyllysine	0.21 ± 0.02	0.20 ± 0.03	0.24 ± 0.01	0.23 ± 0.05	13.7 ± 0.7	10.0 ± 1.1	10.3 ± 3.3	6.30 ± 0.8
Alanylaspartate	0.19 ± 0.02	1.62 ± 0.33	0.18 ± 0.01	0.24 ± 0.03	15.6 ± 3.3	26.3 ± 2.8	10.6 ± 0.6	8.4 ± 1.4
Cephalexin	–	–	15.0 ± 4.3	11.7 ± 6.6	–	–	72.6 ± 0.8	66.4 ± 8.8

Uptake (10 min) of [^{14}C]glycylsarcosine (10 μM in Caco-2, 5 μM in SKPT cells) was measured, in one set of experiments, at pH 6.0 or pH 7.0 and, in another set of experiments, at pH 6.0, but after a 10-min treatment with or without DEP in pH 7.5 buffer at a concentration of 0.4 mM (Caco-2) or 0.1 mM (SKPT). Concentration of unlabeled compounds was varied between 0 and 10 mM. The values given ($\pm$ S.E.M.) for IC_{50} were determined from the dose–response data by nonlinear regression analysis of the data shown in Figs. 1, 3, 5 and 6.

To determine whether the observed lack of effect of the H^+ gradient on K_t is unique only to neutral peptide substrates or is also true for other structurally diverse peptide substrates, we investigated the potency of a cationic dipeptide (Ala-Lys) and an anionic dipeptide (Ala-Asp) to inhibit Gly-Sar uptake at two different extracellular pH values, 6.0 and 7.0. We could not determine the affinity of the peptide transporters for these peptides directly by measuring their uptake because of nonavailability of these peptides in radiolabeled form. Instead, we analyzed the ability of these peptides to inhibit the uptake of radiolabeled Gly-Sar. By this approach, we were able to estimate the IC_{50} values (concentration of the peptides necessary to inhibit 50% of Gly-Sar uptake) and K_i values (inhibition constant) of these peptides as indicators of their respective affinity to the peptide transport systems. As shown in Fig. 3A,B, the Gly-Sar uptake is two- to three-fold higher at pH 6.0 than at pH 7.0. Moreover, Ala-Lys and Ala-Asp are substrates with a much higher affinity than Gly-Sar for both PEPT 1 and PEPT 2. In the intestinal cell line, where the K_t value for Gly-Sar uptake is 1 mM, the IC_{50} values for Ala-Lys and Ala-Asp at pH 6.0 are 0.21 and 0.19 mM respectively (Table 2). Although the potency of these two peptides to inhibit Gly-Sar uptake in SKPT cells is several-fold higher than in Caco-2 cells, the relative difference between the affinity for Gly-Sar and the affinity for these peptides is similar in both cell types. In the SKPT cell line, the K_t for Gly-Sar is 75 μM, and the IC_{50} values for Ala-Lys and Ala-Asp at pH 6.0 are 14 and 16 μM respectively (Table 2). More important, however, is the effect of the H^+ gradient on these IC_{50} values. As shown Table 2, the H^+ gradient does not change the IC_{50} values for Ala-Lys in Caco-2 cells and in SKPT cells, indicating that the inwardly directed H^+ gradient does not influence the affinity of PEPT 1 and PEPT 2 for the cationic dipeptide Ala-Lys. In the case of Ala-Asp, however, decreasing the H^+ concentration outside the cells has dramatic consequences for the inhibition constants both in intestinal as well as in renal cells. In Caco-2 cells, the IC_{50} value increases by 8-fold and in SKPT cells by 2-fold when the pH is changed from 6.0 to 7.0. There are two possible explanations for the results obtained with Ala-Asp. An inwardly directed H^+ gradient may influence the affinity of the peptide transporter for this peptide. But

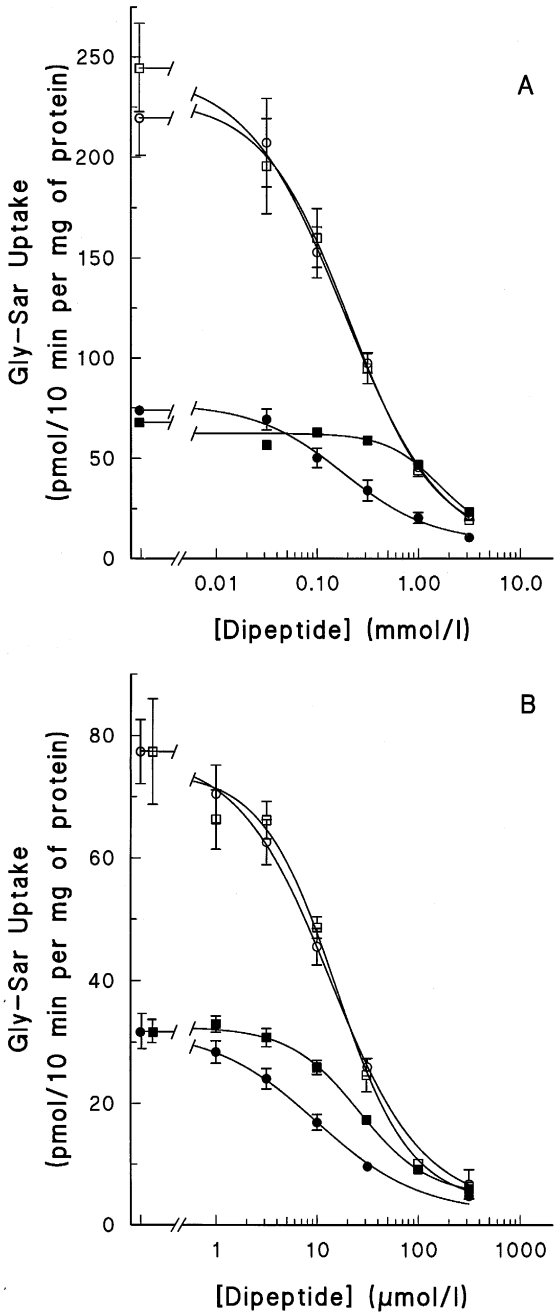

Fig. 3. Effect of a H^+ gradient on the inhibition of Gly-Sar uptake by Ala-Lys and Ala-Asp in Caco-2 cells (A) and SKPT cells (B). Uptake of radiolabeled Gly-Sar (10 μM in Caco-2, 5 μM in SKPT) for 10 min was measured in confluent cell cultures at an outside pH of 7.0 (●, ■) or pH 6.0 (○, □) in the presence of increasing concentrations of Ala-Lys (○, ●) or Ala-Asp (□, ■). The values are means ± S.E. for 3–6 determinations. If not shown, error bars are smaller than the symbols.

this is unlikely because a similar phenomenon is not apparent for the other two dipeptide substrates Gly-Sar and Ala-Lys. There is no obvious reason to believe that such an effect of the H^+ gradient on the affinity is unique to the anionic dipeptide Ala-Asp. A more plausible explanation is that the changes in the relative ratio of different ionic species of Ala-Asp which is expected to differ significantly at pH 6.0 versus pH 7.0 are responsible for the observed effects. This can happen if the peptide transporters show different affinities to different ionic species of a given peptide substrate. Given the data that the change of pH from 6.0 to 7.0 has much more dramatic effect on the IC_{50} values for Ala-Asp in Caco-2 cells than in SKPT cells, this would then mean that PEPT 2 expressed in SKPT cells is much more tolerant than PEPT 1 expressed in Caco-2 cells towards changes in the ionic nature of a given peptide substrate.

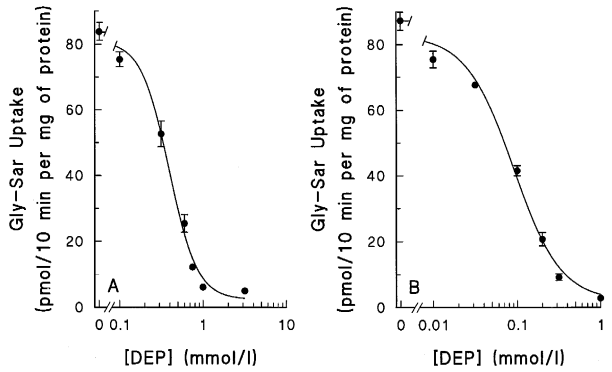

Fig. 4. Dose–response relationship of the effect of DEP on Gly-Sar uptake in Caco-2 cells (A) and SKPT cells (B). Cells were treated with DEP in pH 7.5 buffer for 10 min before uptake. Uptake of radiolabeled Gly-Sar (5 μM in both cell lines, 10 min) was measured in confluent cell cultures at an outside pH 6.0. The values are means $\pm$ S.E.M. for 3–4 determinations. If not shown, error bars are smaller than the symbols.

3.3. Inhibition of Gly-Sar uptake in Caco-2 cells and SKPT cells by DEP: dose–response relationship

Several transport systems which are energized by a transmembrane H^+ gradient possess specific histidyl residues that are critical for their catalytic activity. Examples of these transport systems include Na^+/H^+ exchanger [39,40], organic cation$/H^+$ antiporter [41], and folate transporter [42] in animal tissues. The *Escherichia coli* Lac permease, a prototypical H^+-coupled transport system has been extensively studied with regard to the role of critical histidyl residues in the binding and translocation of the cotransported H^+ [43,44]. We have shown several years ago that the renal H^+-coupled peptide transporter possesses critical histidyl residues [18]. This conclusion was based on the data that chemical modification of histidyl residues with DEP leads to inactivation of the transporter. The essential nature of histidyl residues in the intestinal H^+-coupled peptide transporter has been subsequently demonstrated [19,21]. The uniqueness of the histidyl residue resides in the *p*K value of 6.5 for the imidazole group of its side chain which is very close to the physiological pH. This characteristic renders the histidyl residues capable of easily accepting and releasing H^+ under physiological conditions. Therefore, the critical histidyl residues are the most likely amino acid residues involved in the binding and translocation of H^+ in PEPT 1 and PEPT 2. Modification of these histidyl residues with DEP makes them incapable of being a H^+ acceptor/donor. Therefore, an alternative approach to delineate the mechanism of activation of PEPT 1 and PEPT 2 by H^+ is to investigate the influence of DEP modification of histidyl residues on the kinetic parameters of these transport systems while keeping the extracellular H^+ concentration constant.

Treatment of monolayer cultures of Caco-2 cells and SKPT cells with DEP for 10 min at pH 7.5 leads to marked inhibition of Gly-Sar uptake. Studies on the time-dependence of DEP-induced inhibition have shown that maximal inhibition occurs with 10 min treatment (data not shown). Furthermore, we investigated the concentration-dependence of the DEP effect in both cell models (Fig. 4). The IC_{50} value for DEP inhibition was 0.39 ± 0.04 mM in Caco-2 cells (Fig. 4A) and 0.10 ± 0.02 mM in SKPT cells (Fig. 4B). Therefore, in the following experiments we treated the Caco-2 cells with 0.4 mM DEP and the SKPT cells with 0.1 mM DEP.

3.4. Effect of DEP treatment on the kinetic parameters of Gly-Sar uptake in intestinal and renal cells

We performed detailed kinetic studies at first to determine if the inhibition of peptide uptake elicited by DEP treatment is due to a decrease in the affinity or a decrease in the maximal velocity of the transport

systems. Cells were treated at pH 7.5 for 10 min with the appropriate concentrations of DEP (0.4 mM for Caco-2; 0.1 mM for SKPT) or ethanol. Cells were then washed for 1 min with 1 ml buffer at pH 6.0. Uptake of Gly-Sar was measured for 10 min over a concentration range of 0.1–5 mM in Caco-2 cells and 0.01–0.5 mM in SKPT cells at an extracellular pH 6.0. The non-saturable transport component was again subtracted from total uptake to calculate mediated uptake as detailed in Section 2. The relationship between the rate of mediated uptake and substrate concentration was found to be hyperbolic in both cell lines and both in control cells and in DEP-treated cells (Fig. 5A,B). The experimental data were found to fit best for an uptake model describing a single transport system. When expressed as Eadie-Hofstee plots (Fig. 5, insets), the data yielded straight lines ($r^2 > 0.86$). Determination of kinetic parameters from the data indicated that DEP treatment resulted in a 50% decrease of the maximal velocity of Gly-Sar transport without changing significantly the affinity of the carrier for Gly-Sar. In Caco-2 cells, the apparent Michaelis-Menten constant (K_t) for the uptake process in control cells was 0.83 ± 0.1 mM and the corresponding value in DEP-treated cells was 0.76 ± 0.1 mM (Table 2). The maximal velocity (V_{max}) of Gly-Sar uptake, however, was 13.8 ± 0.6 nmol/10 min per mg of protein in controls and 6.4 ± 0.3 nmol/10 min per mg of protein in DEP-treated cells. In SKPT cells, the apparent Michaelis-Menten constant (K_t) for the uptake process in control cells was 64.1 ± 9.9 μM and the corresponding value in DEP-treated cells was 62.3 ± 4.5 μM (Table 2). The maximal velocity (V_{max}) of Gly-Sar uptake, however, was 0.98 ± 0.05 nmol/10 min per mg of protein in the controls and 0.39 ± 0.01 nmol/10 min per mg of protein in DEP-treated cells. These data give no evidence for an effect of DEP on the affinity of the carrier protein to its substrate Gly-Sar in the intestinal as well as in the renal cell line.

3.5. Effect of DEP-treatment on the IC_{50} values of Ala-Lys, Ala-Asp and cephalexin for inhibition of Gly-Sar uptake in intestinal and renal cells

Similar to those experiments described earlier, we performed studies with Ala-Lys, Ala-Asp and cephalexin as inhibitors of Gly-Sar uptake in control cells and in DEP-treated cells (Fig. 6A,B). These data were used to calculate IC_{50} values. As shown in Table 2, the DEP treatment does not change the IC_{50} values for Ala-Lys, Ala-Asp or cephalexin. These results show that blockade of the function of histidyl residues as a H^+ acceptor/donor with DEP treatment does not influence the affinity of PEPT 1 and PEPT 2 for their substrates. These results are similar to those obtained with reduced H^+ concentration. It should be noted that the affinity of PEPT 1 (Caco-2 cells) for

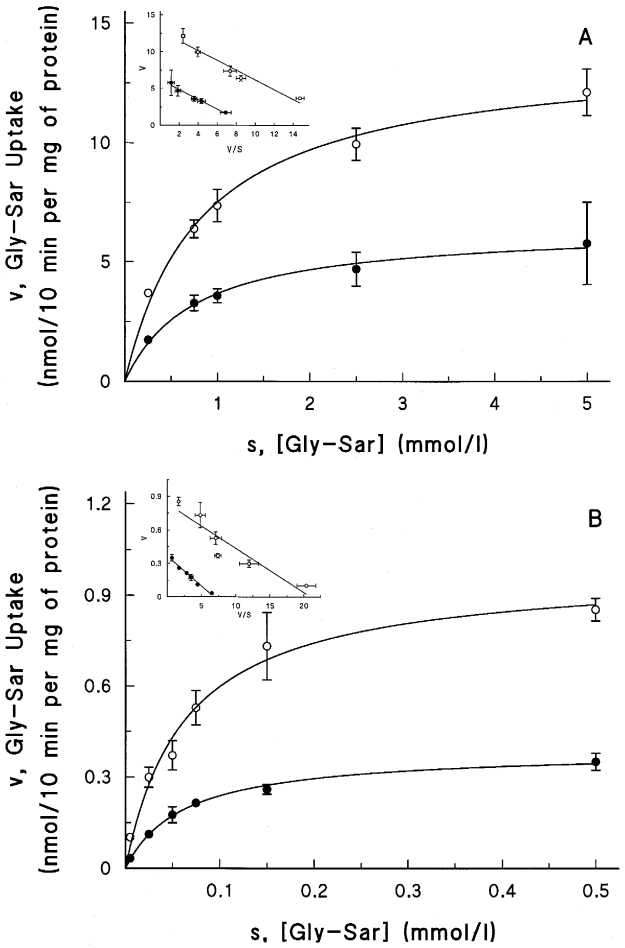

Fig. 5. Effect of DEP on the saturation kinetics of Gly-Sar uptake in Caco-2 cells (A) and SKPT cells (B). Uptake of Gly-Sar (concentration range, 0.25–5 mM in Caco-2 and 10–500 μM in SKPT, uptake time 10 min) was measured at pH 6.0 in confluent cell cultures after treatment with (●) or without (○) DEP (0.4 mM in Caco-2 cells, 0.1 mM in SKPT cells) for 10 min at pH 7.5. The values are means ± S.E.M. for 4–6 determinations. They represent saturable uptake values after correction for non-saturable component. If not shown, error bars are smaller than the symbols. Insets: Eadie-Hofstee transformations of the data.

cephalexin is 10- to 15-fold less than for Gly-Sar. In contrast, the affinity of PEPT 2 (SKPT cells) for cephalexin is comparable to the affinity for Gly-Sar. Apparently, PEPT 1 and PEPT 2 exhibit significantly different substrate recognition pattern.

Kramer et al. [21] have demonstrated that DEP treatment reduces the affinity of the intestinal H^+/peptide cotransporter for cephalexin. Our present results are in contrast to those obtained by these investigators. The reasons for the discrepancy are not known. However, our results are in agreement with those of Kato et al. [19] which show no change upon DEP treatment in the affinity of the intestinal H^+/peptide cotransporter for cephradine, also a zwitterionic aminocephalosporin similar to cephalexin. Another interesting observation from our studies with DEP is that the affinity of the peptide transporters for Ala-Asp remains the same in control cells and in DEP-treated cells. It has to be noted that these experiments were done at a fixed extracellular pH of 6.0. However, our studies with two different extracellular pH values showed that the affinity of the peptide transporters for Ala-Asp at pH 7.0 is much less than at pH 6.0. Based upon the data from studies with DEP, the apparent change in the affinity for Ala-Asp at different extracellular pH is most likely an artifact, resulting from pH-induced changes in the concentration of different ionic species of Ala-Asp.

In conclusion, we have investigated the influence of H^+ on the catalytic function of PEPT 1 and PEPT 2 using cell culture model systems by two different approaches, namely by altering the extracellular H^+ concentration and by blocking the function of essential histidyl residues as H^+ acceptor/donor. The results of the investigation demonstrate unequivocally that H^+, which is the cotransported ion for PEPT 1 and PEPT 2, activates the transporters without influencing the substrate affinity. The H^+ gradient apparently enhances the activity of PEPT 1 and PEPT 2 solely by increasing the translocation rate of the transporters during the catalytic cycle. Our recent work on the operational mechanisms of the cloned human PEPT 1 supports this conclusion [45]. This is

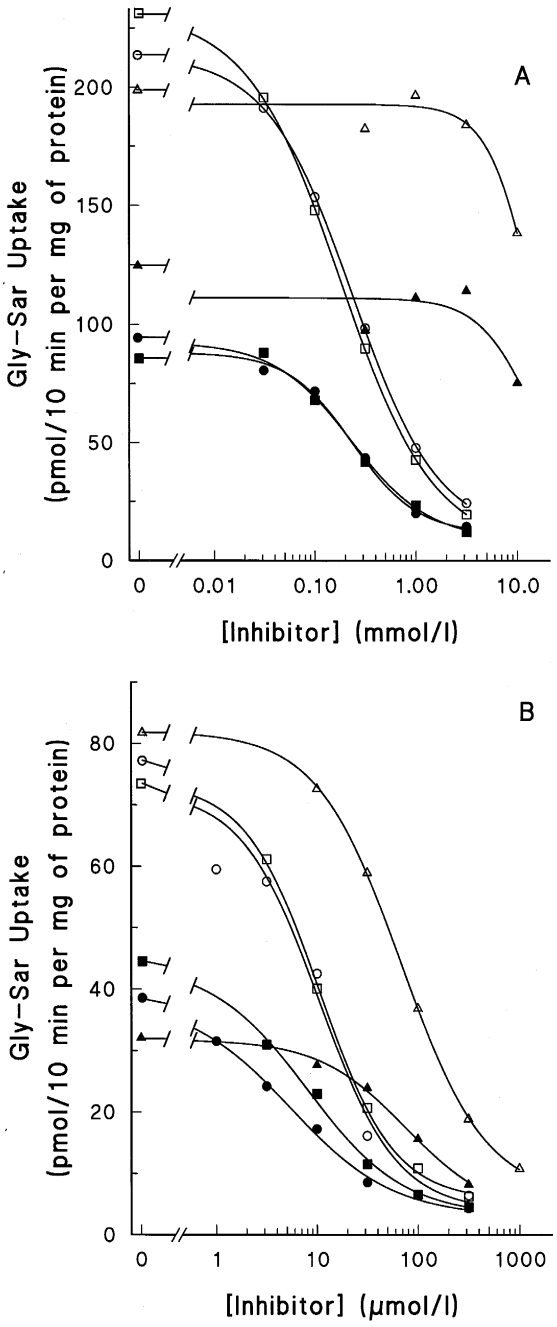

Fig. 6. Effect of DEP on the inhibition of Gly-Sar uptake by Ala-Lys, Ala-Asp and cephalexin in Caco-2 cells (A) and SKPT cells (B). Cells were treated for 10 min with (●, ■, ▲) or without (○, □, △) DEP (0.4 mM in Caco-2 cells, 0.1 mM in SKPT cells) for 10 min at pH 7.5. Uptake of radiolabeled Gly-Sar (10 μM in Caco-2, 5 μM in SKPT) for 10 min was measured in confluent cell cultures at pH 6.0 in the presence of increasing concentrations of Ala-Lys (○, ●), Ala-Asp (□, ■) or cephalexin (△, ▲). The values are means for 3–6 determinations. The standard error (not shown) was within 5–15% of the mean.

in contrast to the known role of Na^+, the other major coupling ion for active transport systems in animal tissues, in increasing the affinity of the Na^+-coupled transporters for their substrates.

There have been two recent reports on the handling of cationic and anionic peptides by peptide transporters [46,47]. Temple et al. [46] have shown using renal brush border membrane vesicles that the H^+/peptide coupling ratio differs depending on the net charge of the peptide substrate. The ratio is one for zwitterionic peptides, two for anionic peptides, and zero for cationic peptides. Since kidney expresses PEPT 1 as well as PEPT 2, it is not readily apparent whether these characteristics belong to PEPT 1 or PEPT 2. Our most recent study with the cloned human PEPT 1 has demonstrated that the H^+/peptide coupling ratio is one irrespective of the charge of the peptide substrate [48]. This may suggest that the characteristics described by Temple et al. [45] belong to PEPT 2. The present study does not address the issue of H^+/peptide coupling ratio for Ala-Asp and Ala-Lys in SKPT cells nor does it probe the possible differences in the H^+-dependence of the transport of these peptides. What is analyzed in this study is not the transport of Ala-Lys and Ala-Asp but the ability of these peptides to compete with Gly-Sar for transport. Wenzel et al. [47] have suggested that the rabbit PEPT 1 accepts only the zwitterionic species of peptidomimetic antibiotics as substrates. This means that anionic and cationic peptide substrates would have markedly different pH optima for their transport via PEPT 1. But, the substrate-induced current by the cloned human PEPT 1 in *Xenopus laevis* oocytes exhibits comparable pH dependence for cationic and anionic peptides [48]. It is obvious that more work is needed to clearly define the possible differences in the handling of peptide substrates of varied net charges by the peptide transporters.

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