Proton-Driven Dipeptide Uptake in Primary Cultured Rabbit Conjunctival Epithelial Cells

Sujit K. Basu,1 Ian S. Haworth,1 Michael B. Bolger,1 and Vincent H. L. Lee1,2


PURPOSE. To characterize proton-driven carrier-mediated dipeptide uptake in primary cultured conjunctival epithelial cells of the pigmented rabbit using β-alanyl-L-histidine (L-carnosine) as a model dipeptide substrate.

METHODS. Uptake of tritiated L-carnosine was monitored using conjunctival epithelial cells on days 6 through 8 in culture on a filter support. The structural features of dileucine stereoisomers and cephalaxin contributing to interaction with the dipeptide transporter were evaluated by computer modeling and inhibition of tritiated L-carnosine uptake.

RESULTS. Uptake of L-carnosine by primary cultured conjunctival epithelial cells in the presence of an inwardly directed proton gradient showed directional asymmetry (favoring apical uptake by a factor of five), temperature dependence, and saturability correlated with substrate concentration, with a Michaelis-Menten constant (K_m) of 0.3 ± 0.03 mM and a maximum uptake rate (V_max) of 22.0 ± 1.0 picomoles per milligram protein per minute. L-Carnosine uptake was optimal at pH 6.0 and was reduced by 60% and 35%, respectively, by 50 μM p-trifluoromethoxyphenylhydrazone (a proton ionophore) and by acid preloading with 50 mM NH_4Cl. The constituent amino acids did not inhibit L-carnosine uptake. L-Carnosine uptake was inhibited, however, from 50% to 80% by other dipeptides and structurally similar drugs such as bestatin, β-lactam antibiotics, and angiotensin-converting enzyme inhibitors. The LL, LD, or DL forms of the dipeptide Leu-Leu inhibited tritiated L-carnosine uptake by approximately 60%, 40%, and 70%, respectively. By contrast, the DD form did not inhibit uptake. Results from computer modeling suggest that an appropriate dipeptide N-terminal to C-terminal distance and a favorable orientation of the side chains may be important for substrate interaction with the conjunctival dipeptide transporter.

CONCLUSIONS. Uptake of the dipeptide L-carnosine in primary cultured pigmented rabbit conjunctival epithelial cells is probably mediated by a proton-driven dipeptide transporter. This transporter may be used for optimizing the uptake of structurally similar peptidomimetic drugs. (Invest Ophthalmol Vis Sci. 1998;39:2365–2373)

Dipeptide transporters are proton-coupled transport systems that mediate the absorption of dipeptides and tripeptides in the intestinal1–2 and renal3,4 epithelial cells. Since the isoforms PepT1,5–6 PepT2,7–8 and PHT19 were cloned, dipeptide transporters have been the focus of intensive research in drug delivery. This interest is because of their essential role in the absorption of several important peptidomimetic drugs, including β-lactam antibiotics,5,10,11 angiotensin-converting enzyme (ACE) inhibitors,12,13 renin inhibitors,14 and the antitumor drug bestatin.15,16 Several of these drugs may also be important in the treatment of such diseases as ocular bacterial infections17 and glaucoma.18

Work in our laboratory has revealed that the rabbit conjunctiva is permeable to a variety of molecules, including didoforiv,19 β-adrenergic antagonists,20 arginine vasopressin,21 and fluorescein isothiocyanate-labeled dextrans ranging from 4,400 to 167,000 in molecular weight.22 Moreover, the conjunctiva seems to possess transporter systems for nucleosides,23 arginine derivatives (nitric oxide synthase inhibitors),24,25 and monocarboxylates.26 In 1995, Sun et al.27 reported that β-alanyl-L-histidine (L-carnosine), a naturally occurring hydrolysis-resistant dipeptide that has been used to characterize dipeptide uptake in the small intestine28,29 and in the kidney,4,29 was transported across the conjunctival tissue by a carrier-mediated, pH-dependent process. The purpose of the present study was to demonstrate the functional existence of a dipeptide transporter and to delineate dipeptide uptake characteristics in primary cultured rabbit conjunctival epithelial cells. This cell culture model has been shown to exhibit drug transport characteristics comparable with those in the isolated tissue, insofar as β-adrenergic antagonists are concerned.20 Among the uptake characteristics evaluated were directionality, pH dependence, temperature dependence, concentration dependence, stereoselectivity, and substrate specificity. The experimental results of stereoselective uptake were...
MATERIALS AND METHODS

Materials

[^3H]L-carnosine (5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Unlabeled L-carnosine, cephalaxin, bestatin, captopril, and enalapril were purchased from Sigma (St. Louis, MO). Enalaprilat was a generous gift from Merck Research Laboratory (Whitehouse Station, NJ). Cyclacillin and cefadroxil were kindly provided by Takeda Chemical Industries (Osaka, Japan) and Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ), respectively. Cell culture reagents and supplies were obtained from Life Technologies (Grand Island, NY). PC-1, a low protein defined medium used for cell growth, was obtained from BioWhittaker (Walkersville, MD). Tissue culture-treated wells (Transwells; 0.4 μm, 12 mm outer diameter) were obtained from Costar (Cambridge, MA). All other chemicals were of the highest purity available commercially.

Animals

Male Dutch-belted pigmented rabbits, weighing 2.5 to 3.0 kg, were purchased from Irish Farms (Norco, CA). The investigations using rabbits described in this report conformed to the Guiding Principles in the Care and Use of Animals (DHEW, NIH 80-23) and the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Primary Culture of Rabbit Conjunctival Epithelial Cells

The protocol developed by Saha et al.30 was used. Briefly, the conjunctiva was excised and incubated in 0.2% protease XIV (Sigma) at 37°C for 90 minutes. Epithelial cells were scraped off, suspended in a minimum essential medium (S-MEM) containing 10% fetal bovine serum and 0.5 mg/ml deoxyribonuclease I (Sigma), and centrifuged at 200g for 10 minutes at room temperature. The cells were washed twice with S-MEM containing 10% fetal bovine serum, filtered through a 40-μm cell strainer, pelleted at 200g for 10 minutes, and resuspended in PC-1 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 1 μg/ml fungizone. The isolated cells were plated in wells coated with a mixture of 15 μg/cm² rat tail type I collagen and 5 μg/cm² fibronectin (both obtained from Collaborative Bio-Medical Products, Bedford, MA), at a density of 1.5 × 10⁶ cells/cm² (day 0) and cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The volumes of apical and basolateral culture media were 0.5 ml and 1.5 ml, respectively. Potential difference (PD) and transepithelial resistance (Rₑ) were monitored from day 4 onward, to assess viability and barrier tightness. The cell layers were used for uptake experiments after reaching peak bioelectric parameters (Rₑ > 1.0 kΩ/cm²; PD > 10 mV) from day 6 through day 8.

Uptake of L-Carnosine by Primary Cultured Conjunctival Epithelial Cell Layers

Unless otherwise specified, all uptake experiments were performed in a humidified atmosphere of 5% CO₂ and 95% air at
### Table 1. Directionality, Temperature Dependence, and Energy Dependence of the Conjunctival Dipeptide Transport System

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Uptake (pmoles/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical, 37°C</td>
<td>2.47 ± 0.21 (100)</td>
</tr>
<tr>
<td>+ 100 μM DNP</td>
<td>1.01 ± 0.08 (41% ± 3)†</td>
</tr>
<tr>
<td>+ 50 μM FCCP</td>
<td>0.90 ± 0.07 (36% ± 3)†</td>
</tr>
<tr>
<td>+ 50 mM NH₄Cl</td>
<td>1.66 ± 0.28 (67% ± 11)†</td>
</tr>
<tr>
<td>Apical, 4°C</td>
<td>0.14 ± 0.02 (6% ± 1)†</td>
</tr>
<tr>
<td>Basal, 37°C</td>
<td>0.45 ± 0.04 (18% ± 2)†</td>
</tr>
</tbody>
</table>

*In the presence of an inwardly directed proton gradient (apical pH 6.0, basolateral pH 7.4) from the apical side. Before each experiment, the cell layers were washed with bicarbonate Ringer’s solution at an osmolality of 300 mosm/kg. The buffer contained 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH₂PO₄, 25 mM NaHCO₃, 1.80 mM CaCl₂, 0.81 mM MgSO₄, 5.55 mM glucose, and 15 mM HEPES (pH 4.0-5.0). Uptake was initiated using 10 μM L-carnosine spiked with 20 μCi/ml [³H]-carnosine. Values are mean ± SEM with percentages in parentheses; n = 6–8 from two cultures.† Statistical significance determined by Student’s t-test by comparing with the apical uptake at 37°C. P < 0.05 was considered statistically significant.

37°C in the presence of an inwardly directed proton gradient (apical pH 6.0, basolateral pH 7.4) from the apical side. Before each experiment, the cell layers were washed with bicarbonate Ringer’s solution at an osmolality of 300 mosm/kg. The buffer contained 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH₂PO₄, 25 mM NaHCO₃, 1.80 mM CaCl₂, 0.81 mM MgSO₄, 5.55 mM glucose, and 15 mM HEPES (pH 4.0-5.0). Uptake was initiated by spiking the apical or basolateral solution with 20 μCi/ml [³H]-carnosine and an appropriate amount of unlabeled substrate. After a predetermined period, uptake was terminated by suctioning off the dosing solution and immersing the cell layers in ice-cold bicarbonate Ringer’s solution (pH 7.4). The cell layers were then solubilized in 0.5 ml 0.5% Triton X-100 solution. Ten microliters of the solution was taken for protein assay by the method of Bradford31 using a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The remainder of the sample was mixed with 5 ml scintillation cocktail (EconoSafe; Research Products International, Mount Prospect, IL) for measurement of radioactivity in a liquid scintillation counter (Beckman, Fullerton, CA).

### Computer Modeling of Dideucine Dipeptides and Cephalexin

To understand further the molecular basis of conjunctival dipeptide uptake, we performed a conformational analysis of the four diileucine dipeptides (LL, LD, DL, and DD) and of cephalexin. The equivalent study was not performed with cefadroxil, because it is structurally similar to cephalexin, the only change being the addition of a ψ-hydroxyl group on the aromatic ring (Fig. 1B). The starting structures of the dideucine dipeptide segment of cephalexin was determined using a least squares rigid body fit algorithm in the software (Quanta).

### Dipeptide Uptake in the Conjunctiva 2367

Dipeptide uptake data are presented as the mean ± SEM. Statistical significance was determined by Student’s t-test for unpaired sample assuming equal variance; P < 0.05 was considered significant. Statistical differences among multiple different groups were determined by one-way analysis of variance, and group means were contrasted for significant differences using the Fisher’s PLSD post hoc test; P < 0.05 was considered significant.

**Figure 3.** Effect of pH of apical bathing fluid on L-carnosine uptake by primary cultured pigmented rabbit conjunctival epithelial cells. (basolateral pH 7.4). Uptake was initiated using 10 μM L-carnosine spiked with 20 μCi/ml [³H]-carnosine. Each point represents the mean ± SEM value of six to eight determinations from two cultures. Statistical significance was tested by one-way analysis of variance. Group means were contrasted for significant differences using Fisher’s PLSD post hoc test; P < 0.05 was considered significant.
significant. Assumption of normal distribution and equal variance was also tested and the significance confirmed by non-parametric statistical analysis (Mann-Whitney rank sum test for comparing two groups), the Kruskal-Wallis analysis of variance on Ranks (for comparing many groups), or both, using commercial software (StatView; Abacus Concepts, Berkeley, CA).

RESULTS
Kinetcs of L-Carnosine Uptake

Directionality and Energy Dependence. L-Carnosine uptake in conjunctival epithelial cells was linear for the first 10 minutes, reaching a plateau at approximately 60 minutes (Fig. 2). Therefore, in all subsequent experiments, 10 minutes was chosen as the observation period. Apical L-carnosine uptake was five times higher than basolateral uptake ($P < 0.05$; Table 1). Moreover, apical uptake at $37^\circ C$ was 17 times higher than that at $4^\circ C$ ($P < 0.05$) and was inhibited by 60% in the presence of 100 $\mu$M 2,4-dinitrophenol, a metabolic inhibitor.

pH Dependence. Within the pH range of 4.0 to 8.5, L-carnosine uptake was maximum at pH 6.0 (Fig. 3). Uptake was reduced by more than 60% by pretreating the cells with a proton ionophore, carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone, at a final concentration of 50 $\mu$M in bicarbonated Ringer’s solution ($P < 0.05$; Table 1). Moreover, uptake was inhibited by more than 30% after intracellular acidification by preparing the conjunctival epithelial cells for 30 minutes with 50 $\mu$M NH$_4$Cl in Na$^+$- and HCO$_3^-$-free bicarbonated Ringer’s solution ($P < 0.05$; Table 1).

Concentration Dependence. L-carnosine uptake by the conjunctival epithelial cells was saturable within the concentration range of 0.02 mM to 2 mM (Fig. 4A). Eadie-Hofstee analysis (Fig. 4B) of the uptake data revealed a Michaelis-Menten constant ($K_v$) of 0.3 ± 0.03 mM and a maximum uptake rate ($V_{max}$) of 22.0 ± 1.0 picomoles per milligram protein per minute ($R^2 = 0.96$).

Substrate Selectivity. The substrate selectivity of dipeptide uptake by conjunctival epithelial cells was studied by measuring the inhibitory effect of unlabeled amino acids and dipeptides on the uptake of tritiated L-carnosine (Fig. 5). L-Histidine and $\beta$-alanine, the constituent amino acids of L-carnosine, at 10 mM, did not affect tritiated L-carnosine uptake ($P > 0.05$). By contrast, 10 mM unlabeled L-carnosine decreased $[^3H]L$-carnosine uptake by 80% ($P < 0.05$; Fig. 5). Gly-Sar, Gly-Phe, Gly-Val, and Leu-Leu in the L-configuration, all at 10 mM, also inhibited L-carnosine uptake by at least 60% ($P < 0.05$; Fig. 5). The peptidomimetics cyclacillin, an aminopenicillin antibiotic; cephalexin and cefadroxil, aminocephalosporin antibiotics; captopril ([25]-A r-mercapto-2-methylpropiophenone)-L-proline), ACE inhibitors; and bestatin ([S]-l-[N-(1-ethylcarboxyloxy)-3-phenylpropyl]-l-alanyl)-l-proline), ACE inhibitors, and bestatin ([25,3i?]-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine) inhibited L-carnosine uptake by 50% to 80% ($P < 0.05$; Fig. 5). By contrast, thyrotrpin-releasing hormone (TRH; $\gamma$-pyrrolglutamyl-$\gamma$-histidyl-$\gamma$-prolinamide), a tripeptide without a free carboxylic acid group, did not inhibit the uptake of L-carnosine ($P > 0.05$; Fig. 5).

Stereoselectivity. The stereoselectivity of dipeptide uptake in the conjunctival epithelial cells was studied by measuring the inhibitory effect of unlabeled Gly-Phe and Leu-Leu on the uptake of tritiated L-carnosine (Table 2). As can be seen, Gly-$\delta$-Phe was twice as inhibitory as Gly-$\gamma$-Phe. For the leucine dipeptides, Leu-$\delta$-Leu (59%) was slightly more inhibitory than Leu-$\gamma$-Leu (43%). Surprisingly, $\delta$-Leu-$\delta$-Leu was even more inhibitory (72%). By contrast, $\delta$-Leu-$\gamma$-Leu did not inhibit L-carnosine uptake ($P > 0.05$).

Rationalization of Stereoselective Dipeptide Uptake Using Computer Modeling

Li et al.$^{35}$ have reported a correlation between the dipeptide NC distance (the distance between the N and C termini) for the neutral forms of four divaline stereoisomers (LL, LD, DL, and DD) and their interaction with the dipeptide transporter. To investigate whether the same association holds true for other dipeptides, we performed computer modeling studies with dileucine stereoisomers (LL, LD, DL, and DD). Our results
suggest that each zwitterionic dileucine dipeptide exists in two conformer populations, characterized by the $\psi(1)$-$\psi(2)$ torsion angle pairs shown in Table 3 and referred to as conformer (I) and (II). The NC distance of the two conformer populations of each dileucine dipeptide is also shown in Table 3. Two minimum energy conformers were also obtained for cephalexin (for $\omega(1)$, $\omega(2)$, and $\omega(3)$ torsion angles, see note to Table 3).

The cephalexin conformers are probably similar to the conformation recognized and transported by the dipeptide transporter, because deformation of the rigid cephalexin would be energetically expensive. Therefore, we computed the similarity in shape of cephalexin N- and C-terminal dipeptide segments with each dileucine conformer. The LL dileucine conformer (I) closely overlays the N-terminal dipeptide fragment (from the $\alpha$-amino group to the carbonyl group of the $\beta$-lactam ring) of cephalexin (Table 3, Fig. 6). Although the DD dileucine conformer (I) has an NC distance comparable with that of the LL dileucine conformer (I), it poorly overlays the same region of cephalexin. Overlay of all other preferred dileucine dipeptide conformers on the N- and the C-terminal fragment of cephalexin produced much higher root mean square deviations than that of the LL dileucine conformer (I) (Table 3). The overlay of all the dileucine dipeptide conformer (I) structures on the N-terminal fragment of cephalexin showed that the best fit was for the LL conformer and least fit with the DD conformer, with the fit for DL and LD conformers being intermediate (Fig. 6).

**TABLE 2. Stereoselectivity of the Conjunctival Dipeptide Transport System**

<table>
<thead>
<tr>
<th>Substrates* (apical, 37°C)</th>
<th>Uptake (pmoles/mg protein/min)$\dagger$</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.05 ± 0.03 (100)</td>
</tr>
<tr>
<td>+ Gly-L-Phe</td>
<td>0.18 ± 0.02 (17 ± 2)$\dagger$</td>
</tr>
<tr>
<td>+ Gly-D-Phe</td>
<td>0.34 ± 0.08 (32 ± 8)$\dagger$</td>
</tr>
<tr>
<td>+ Leu-Leu</td>
<td>0.43 ± 0.11 (41 ± 10)$\dagger$</td>
</tr>
<tr>
<td>+ Leu-D-Leu</td>
<td>0.60 ± 0.14 (57 ± 13)$\dagger$</td>
</tr>
<tr>
<td>+ D-Leu-Leu</td>
<td>0.29 ± 0.03 (28 ± 3)$\dagger$</td>
</tr>
<tr>
<td>+ D-Leu-D-Leu</td>
<td>0.91 ± 0.22 (87 ± 21)$^\text{NS}$</td>
</tr>
</tbody>
</table>

* Final substrate concentration 10 mM.
† In the presence of an inwardly directed proton gradient (apical pH 6.0, basolateral pH 7.4). Uptake was initiated using 10 μM l-carnosine spiked with 20 μCi/ml [3H]l-carnosine. Values are mean ± SEM, with percentages in parentheses; n = 6-8 from two cultures.
‡ Represents statistical significance determined by Student's $t$-test by comparing with the apical uptake at 37°C. $P < 0.05$ was considered statistically significant.
NS, Statistically not significant.

**FIGURE 5. Effect of unlabeled amino acids, excess unlabeled l-carnosine, unlabeled dipeptides, and drugs structurally similar to dipeptides and tripeptides at 10 mM final concentration on [3H]l-carnosine uptake by primary cultured pigmented rabbit conjunctival epithelial cells (apical pH 6.0; basolateral pH 7.4). Uptake was initiated using 10 μM l-carnosine spiked with 20 μCi/ml [3H]l-carnosine. Each column represents the mean ± SEM value of six to eight determinations from two cultures. Statistical significance was determined by Student's $t$-test; $^*P < 0.05$ was considered statistically significant. TRH, thyrotropin-releasing hormone; N.S., not significant.

**DISCUSSION**

A major finding in the present study is that a proton-coupled dipeptide transporter probably exists on the apical side of the conjunctival epithelial cells rabbit that mediates the transport of dipeptides and peptidomimetics. Evidence for a predominantly apical location is the fivefold higher uptake of l-carnosine from the apical than the basolateral side of the conjunctiv-
tival epithelial cells (Table 1). The first hint for the involvement of a nonpassive component in the uptake of L-carnosine (2.85 ± 0.19 picomoles per milligram protein per minute) by primary cultured conjunctival epithelial cell was the 18-fold difference compared with the uptake of the paracellular transport marker 14C-mannitol (0.16 ± 0.03 picomoles per milligram protein per minute). L-carnosine uptake was not affected by its constituent amino acids, L-His and β-Ala (Fig. 5), consistent with the substrate selectivity of a dipeptide transporter. Conjunctival dipeptide uptake was energy dependent, indicated by the 94% and 60% inhibition obtained on lowering the temperature to 4°C and on treating the cells with 100 μM of the protonophore p-trifluoro-2,4-dinitrophenol (Table 1). Moreover, L-carnosine uptake was driven by a proton gradient that was optimal at pH 6.0 (Fig. 3) and was inhibited by pretreating the conjunctival epithelial cells with 50 μM of the protonophore p-trifluoro-methoxyphenylhydrazone and by preacidifying them with 50 mM NH₄Cl (Table 1). The pH optimum for dipeptide transport has been reported to be dependent on the substrates studied. Steel et al. reported that, for PepT1 expressed in Xenopus laevis oocytes, maximum uptake for basic, neutral, and acidic dipeptides occurred at pH 6.0, 5.5, and 5.0 of the bathing fluid, respectively. The maximum uptake of L-carnosine, itself a basic dipeptide, by primary cultured rabbit conjunctival epithelial cells also occurred at pH 6.0 (Fig. 3).

As shown in Table 2, the conjunctival dipeptide transporter can tolerate one amino acid of a dipeptide in the D-configuration. To investigate further the role of chirality of the constituent amino acids on the ability of a dipeptide substrate to interact with the transporter, Leu-Leu was chosen because all four chiral compounds are commercially available. Alteration of the chirality of one amino acid in Leu-Leu did not abolish its ability to interact with the dipeptide transporter. However, when both amino acids were in the D-configuration, the ability of Leu-Leu to interact with the dipeptide carrier system was abolished (Table 2). These data are consistent with the stereoselectivity of the dipeptide transporter in the human intestinal epithelial cell line Caco-2 for the dipeptide Val-Val, the tripeptide Val-Val-Val, and the peptidomimetic cephalixin.

Detailed structural information about the substrate binding domain of the dipeptide transporter is unlikely to be forthcoming in the near future because of the inherent complexity of determining the structure of a transmembrane protein. As an alternative, we used a computer modeling approach to gain insight into the stereoselective inhibition of L-carnosine uptake by diuretic dipeptides and especially the exclusion of the DD diuretic from interaction with the conjunctival dipeptide transporter. We found that each of the diuretic dipeptides and cephalixin have two preferred conformer populations. Superimposition of the preferred conformers of each diuretic dipeptide with the N-terminal dipeptide segment of cephalixin revealed lower root mean square deviations for the LL zwitterionic diuretic conformer (I) but not for the DD conformer (I) with a comparable NC distance, which suggests that the dipeptide NC distance alone cannot account for the differential uptake of these molecules. This finding agrees with that of Lj et al., who found that the NC distance alone could not explain the potential for a tripeptide (Val-Val-Val) to interact with the intestinal dipeptide transporter. Therefore, it is possible that an appropriate NC distance and a favorable orientation of the side chains are needed for the recognition of a substrate by the dipeptide transporter.

In addition to the Gly- and Leu-containing dipeptides, peptidomimetics such as bestatin, β-lactam antibiotics, and ACE inhibitors also inhibited the uptake of L-carnosine by primary cultured conjunctival epithelial cells to varying extents (Fig. 5). The involvement of the dipeptide transporter in the uptake of bestatin, itself a leucine derivative, by rabbit intestinal dipeptide transporter. We found that the stereoselectivity of the dipeptide transporter in the human intestinal epithelial cell line Caco-2 for the dipeptide Val-Val was abolished (Table 2). These data are consistent with the stereoselectivity of the dipeptide transporter in the human intestinal epithelial cell line Caco-2 for the dipeptide Val-Val, the tripeptide Val-Val-Val, and the peptidomimetic cephalixin.

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In addition to the Gly- and Leu-containing dipeptides, peptidomimetics such as bestatin, β-lactam antibiotics, and ACE inhibitors also inhibited the uptake of L-carnosine by primary cultured conjunctival epithelial cells to varying extents (Fig. 5). The involvement of the dipeptide transporter in the uptake of bestatin, itself a leucine derivative, by rabbit intestinal brush border membranes has been reported. Tomita et al. have shown that bestatin inhibits cephradine uptake in a competitive manner. Inui et al. have shown that the uptake of bestatin itself is inhibited by cephalosporins and dipeptides but not by amino acids. Of the three ACE inhibitors studied, enalapril was 1.5 times more inhibitory than captopril (Fig. 5). This finding is consistent with the observation made by Kita-gawa et al., in that ACE inhibitors significantly inhibited the uptake of cephradine uptake by rabbit small intestinal brush border membrane vesicles in the order of captopril < enalapril. Moreover, Thrwates et al. have found that the inhibition offered by enalapril is approximately 1.5 times more than that of captopril for [14C]Gly-Sar uptake into Caco-2 cell monolayers. Furthermore, the active ACE inhibitor enalapril failed to inhibit l-carnosine uptake, whereas the prodrug enalapril offered significant inhibition (Fig. 5). This finding is consistent with the results of Swaan et al., who have reported that

### Table 3. Preferred Conformers of Zwitterionic Dileucine Dipeptides and Their Root Mean Square Deviation from Cephalexin

<table>
<thead>
<tr>
<th>Conformer (I)</th>
<th>Conformer (II)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leucine Dipeptides</strong></td>
<td><strong>NC Distance</strong></td>
</tr>
<tr>
<td>Leu-Leu</td>
<td>5.98</td>
</tr>
<tr>
<td>Leu-β-Leu</td>
<td>5.51</td>
</tr>
<tr>
<td>D-Leu-Leu</td>
<td>5.50</td>
</tr>
<tr>
<td>D-Leu-β-Leu</td>
<td>5.83</td>
</tr>
</tbody>
</table>

* Conformation of diuretic having the longer NC distance of the two conformer populations.
† Conformation of diuretic dipeptides having the shorter NC distance.
‡ The distance in Ångstrom (Å) between the nitrogen of the α-amino group and the carbon of the carboxyl group of dipeptides.
§ Root mean square deviation in Ångstrom (Å) from the cephalixin conformer [ω (1) = 90°, ω (2) = 180°, ω (3) = 60°, E = 30.6 kcal/mol].
∥ Root mean square deviation in Ångstrom (Å) from cephalixin conformer [ω (1) = 180°, ω (2) = 180°, ω (3) = 60°, E = 30.9 kcal/mol].
NC, N-terminal to C-terminal; RMSD, root mean square deviation. ψ(1) and ϕ(2) values are in degrees.
Dipeptide Uptake in the Conjunctiva

FIGURE 6. Overlay of conformer (I) of each di-leucine dipeptide (LL in green, DL in red, LD in blue, and DD in yellow) on the N-terminal dipeptide fragment of cephalixin (white).

enalapril is transported by intestinal dipeptide transporter in a carrier-mediated manner, whereas enalaprilat is transported by passive diffusion. The reason for the difference in interaction between enalapril and enalaprilat with the dipeptide transport system is thought to be the negative influence of the second free carboxylic acid group in enalaprilat. The inhibitory results of cefadroxil and cyclacillin are particularly interesting, because they may shed some light on the relative abundance of the dipeptide transporter isoforms PepT1 and PepT2 in the conjunctival epithelial cells. Ganapathy et al. have shown that cyclacillin is approximately 9 times more selective of PepT1 than of PepT2, whereas cefadroxil is approximately 14 times more selective of PepT2 than of PepT1 based on its 50% inhibitory concentration (IC_{50}; substrate concentration required for 50% inhibition of uptake) and K_{i} (inhibition constant) values. Similar studies for conjunctival epithelial cells are under way. Yamashita et al. have recently cloned a peptide-histidine transporter PHT1 from brain and retina. L-carinosine was found, by competitive inhibition study, to interact with this transport system. Nevertheless, PHT1 was probably not involved in the conjunctival epithelial uptake of L-carinosine, because histidine, a substrate for PHT1, did not significantly inhibit L-carinosine uptake in conjunctival epithelial cells (Fig. 5).

Whether a dipeptide transporter is involved in the transepithelial transport of TRH is controversial. In the present study, we found no evidence for the interaction of TRH with the conjunctival dipeptide transport system. Thus, our finding is consistent with that of Gan et al. and of Thwaites et al., who detected predominantly non-carrier-mediated paracellular transport of TRH across Caco-2 cell monolayers. By contrast, Walter and Kissel observed saturable carrier-mediated transport of TRH in addition to passive paracellular transport in a certain clone of Caco-2. Their findings are in agreement with the observation of Tanaka et al. that TRH was taken up in rat intestinal mucosal cells by a dipeptide transporter.
In conclusion, a proton-coupled dipeptide transport system probably exists in the conjunctival epithelial cells to mediate the uptake of model dipeptide \( \text{\textalpha}-\text{carnosine} \). Gly-\( \text{\textalpha}-\text{Sar} \), Gly-\( \text{\textalpha}-\text{Phe} \), Gly-\( \text{\textalpha}-\text{Val} \), and Leu-\( \text{\textalpha}-\text{Leu} \) and peptidomimetics such as bestatin, \( \beta \)-lactam antibiotics, and ACE inhibitors that may be important in ocular therapeutics significantly inhibited conjunctival \( \text{\textalpha}-\text{carnosine} \) uptake. The present study sets the stage for confirmation of the molecular identity of the dipeptide transporter isofrom in the conjunctival epithelial cells using techniques based on molecular biology.

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References

39. Tanaka K, Bhattacharjee P, Takata JS, Lee C-P, Smith PL, Borchardt RT. Metabolism, uptake, and transepithelial transport of the diaste-