Effect of Charge on Oligopeptide Transporter-Mediated Permeation of Cyclic Dipeptides Across Caco-2 Cell Monolayers

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INTRODUCTION

The intestinal oligopeptide transporter(s) is involved in the absorption of natural di/tripeptides and peptidomimetic drugs (1–5). Recent studies using di/tripeptides and cephalosporins have provided considerable insight into the kinetics and H^+-dependency (6–9) as well as the substrate specificity (10–12) of the apical and basolateral oligopeptide transporters in Caco-2 cells, an *in vitro* model of the intestinal mucosa. In addition to the studies carried out with di/tripeptides and cephalosporins, Hidalgo *et al.* (10) have demonstrated the structural requirements for the binding of cyclic dipeptides to the apical oligopeptide transporter in Caco-2 cells by determining their ability to inhibit the uptake of [^{3}H]cephalexin. They concluded that cyclization of neutral dipeptides (e.g. Gly-Pro, Ala-Ala) abolished their ability to interact with the oligopeptide transporter. In contrast, dipeptides containing a net negative charge on the side chain (e.g., Glu-Glu, Asp-Asp) when cyclized were reported to retain their ability to bind to the transporter.

In earlier studies (11–12) using linear di/tripeptides, we have shown that binding characteristics to the apical oligopeptide transporter in Caco-2 cells do not necessarily predict the transepithelial transport of the peptides. Therefore, in this study, we have determined whether a negatively charged cyclic dipeptide [*cyclo*(Asp-Asp)], which was reported to bind to the oligopeptide transporter (10), could be taken up into Caco-2 cells and whether this cyclic peptide undergoes transepithelial transport mediated by the oligopeptide transporter(s). For comparative purposes, we have also studied the transport characteristics of a neutral [*cyclo*(Ala-Ala)] and a basically [*cyclo*(Ala-Lys)] charged cyclic dipeptide, as well as carnosine, which is a known substrate for the oligopeptide transporter (11).

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ABBREVIATIONS: Ala, alanine; Asp, aspartic acid; Gly, glycine; Glu, glutamic acid; Lys, lysine; HBSS, Hanks' balanced salt solution; MES, 2-(N-morpholino) ethanesulfonic acid; Pro, proline; TFA, trifluoroacetic acid; Val, valine.

MATERIALS AND METHODS

Materials

[^{3}H]Cephalexin (3.7 μCi/mmol) was synthesized by the Department of Synthetic Chemistry, SmithKline Beecham Pharmaceuticals (King of Prussia, PA). [^{14}C]Mannitol (55 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). *Cyclo*(Ala-Ala), *cyclo*(Ala-Lys) and *cyclo*(Asp-Asp) were purchased from Bachem Feinchemikalien AG (Switzerland). Carnosine, Gly-Pro, and 2-(N-morpholino)-ethanesulfonic acid (MES) were purchased from Sigma Chemical Co. (St. Louis, MO). Transwell® clusters, PVP free, 24.5 mm in diameter (4.71 cm^2 surface area), and 3.0 μm pore size were purchased from Costar Corporation (Bedford, MA). Acetonitrile was of HPLC grade. Other chemicals were used as received.

Caco-2 Cell Culture

Caco-2 cells were grown on Transwell® polycarbonate membranes (3.0 μm pore size) that had been previously coated with collagen according to previously published procedures (11–13). All cells used in this study were between passages 50 and 60 and monolayers were used within 18–23 days postseeding. The integrity of the monolayer was controlled by measuring the flux of [^{14}C]mannitol. Typically, monolayers showed <1% [^{14}C] mannitol flux per h (Papp $< 5 \times 10^{-7}$ cm/s).

Uptake Studies

The uptake of [^{3}H]cephalexin (0.1 mM), carnosine (1 mM) and the cyclic dipeptides (1 mM) was determined according to previously published procedures (11, 12). Uptake was expressed as nmol/mg protein. Total protein content of cells cultured on polycarbonate filters for various days after seeding was previously determined (14).

Transepithelial Transport Studies

The transepithelial transport of the cyclic dipeptides (1 mM) and carnosine (1 mM) was determined in Caco-2 cells at 37°C in the presence or absence (controls) of Gly-Pro (10 mM) according to previously published procedures (11, 12).

The permeability coefficients (P_{app}) were calculated according to the following equation:

$$P_{app} = \frac{V \cdot dC}{A \cdot C_0 \cdot dt}$$

where $V \cdot (dC/dt)$ is the steady-state rate of appearance of the apically applied peptide in the receiver chamber after initial lag time; C_0 is the initial peptide concentration in the donor chamber; and A is the area of the Transwell® polycarbonate membrane. Percent inhibition of transepithelial transport was calculated by comparing the amount of peptide transported in the receiver chamber during a 2-h incubation in the presence and absence of inhibitors.

Metabolism Studies

The metabolism of the cyclic dipeptides was determined in both the Caco-2 cell homogenate and the apical bathing

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solution over the Caco-2 cell monolayers at 37°C according to previously published procedures (11, 12).

HPLC Analysis

A C18 column (4.6 × 250 mm, Vydac, Hesperia, CA) was used to detect *cyclo*(Ala-Ala), *cyclo*(Ala-Lys) and carnosine. Mobile phase for *cyclo*(Ala-Ala) was 0.1% TFA solution. Mobile phase for *cyclo*(Ala-Lys) and carnosine was 70 mM phosphate buffer (pH 3.5) containing 10 mM heptane sulfonic acid and 0–5% acetonitrile. The flow rate of the mobile phase was 1 ml/min. The wavelength of the detector was set at 210 nm. The retention times of *cyclo*(Ala-Ala), *cyclo*(Ala-Lys), and carnosine were approximately 10, 22 and 8 min, respectively.

A strong anion exchange column (300VHP, Vydac, Hesperia, CA) was used to detect *cyclo*(Asp-Asp). Mobile phase A was 10 mM Tris-HCl buffer (pH 7.5), and mobile phase B was 0.3 N NaCl. The flow rate of the mobile phase was 1 ml/min. Separation was carried out with a linear gradient of 0 to 50% of mobile phase B over 30 min. The detection wavelength was set at 225 nm. The retention time of *cyclo*(Asp-Asp) was approximately 9 min.

Statistical Analysis

Statistical analysis of the data was performed by one way ANOVA using Tukey's family error $P < 0.05$.

RESULTS AND DISCUSSION

The purpose of this study was to determine whether the cellular uptake and the transepithelial transport of a negatively charged cyclic dipeptide [*cyclo*(Asp-Asp)] was mediated by the oligopeptide transporter or by passive diffusion. Cellular uptake and transepithelial transport of this cyclic peptide was compared to carnosine, which is a known substrate of the oligopeptide transporter(s) (11), and to a neutral [*cyclo*(Ala-Ala)] and a basically charged [*cyclo*(Ala-Lys)] cyclic dipeptide.

In initial studies, the metabolic stability of the cyclic dipeptides was determined. All three cyclic dipeptides were determined to be stable at least 2 h in both homogenates of Caco-2 cell (~1 mg protein, pH 7.4) and when applied in the apical bathing solution over Caco-2 cell monolayers (pH 6.0) (data not shown).

Hidalgo *et al.* (10) have reported that negatively charged cyclic dipeptides [e.g., *cyclo*(Asp-Asp)] have affinity for the oligopeptide transporter(s) expressed on Caco-2 cells. In order to confirm these results and to determine the affinity of *cyclo*(Ala-Ala) and *cyclo*(Ala-Lys) for this transporter, the ability of all three cyclic dipeptides to inhibit [^{3}H]cephalexin uptake into Caco-2 cells was determined. As shown in Figure 1, 10 mM of *cyclo*(Ala-Lys) inhibited cellular uptake of [^{3}H]cephalexin (0.1 mM) by 30%, whereas *cyclo*(Ala-Ala) and *cyclo*(Asp-Asp) had no effect on the [^{3}H]cephalexin uptake. Under identical experimental conditions, carnosine inhibited cellular uptake of [^{3}H]cephalexin by >95% (11). These results suggest that *cyclo*(Ala-Lys) has low affinity for the oligopeptide transporter(s), while the other two cyclic dipeptides have no affinity for this transporter. The results reported here for the dipeptide *cyclo*(Ala-Ala) are consistent with those reported earlier by Hidalgo *et al.* (10). However, the lack of affinity of *cyclo*(Asp-Asp) for the oligopeptide transporter observed in this study is in sharp contrast to the high affinity for this cyclic dipeptide reported previously by Hidalgo *et al.* (10). Upon closer examination, we observed that when *cyclo*(Asp-Asp) is added at a concentration of 10 mM to the uptake buffer (Hanks' balanced salt solution containing 25 mM glucose and 10 mM MES, pH 6.0), the pH drops to 3.5 and must be adjusted to pH 6.0 with

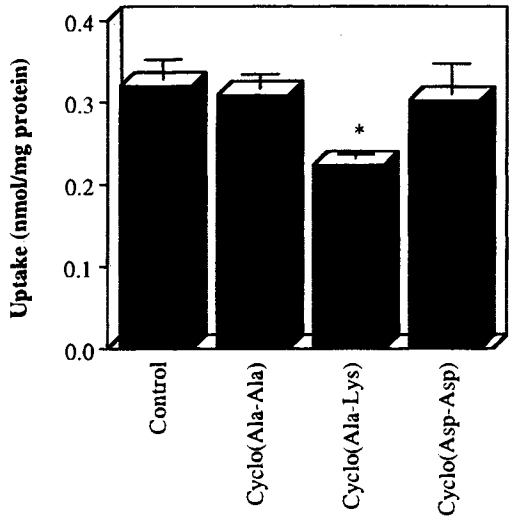

Fig. 1. Uptake of [^{3}H]cephalexin (0.1 mM) in the presence or absence of the cyclic dipeptides (10 mM) for 15 min at 37°C. Caco-2 cell monolayers were incubated with pH 6.0 buffer on the apical side and pH 7.4 buffer on the basolateral side. The buffers consisted of Hanks' balanced salt solution (HBSS) containing 25 mM glucose and 10 mM MES. Results are the means ± SD for three separate filters. The asterisks (*) indicate that the differences from the control level were statistically significant ($P < 0.05$) according to a one-way ANOVA test.

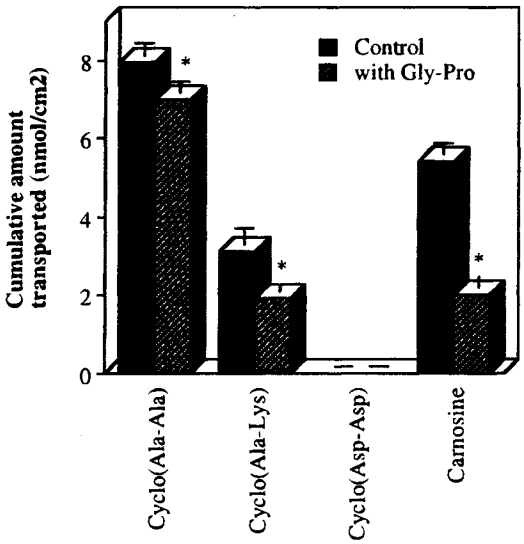

Fig. 2. Effect of Gly-Pro (10 mM) on the transepithelial transport of the cyclic dipeptides (1 mM) and carnosine (1 mM) at 37°C. Caco-2 cell monolayers were incubated with pH 6.0 buffer on the apical side and pH 7.4 buffer on the basolateral side. The total amount of each peptide transported into the basolateral chamber during a 2-h incubation is compared. Results are the means ± SD for three separate filters. The asterisks (*) indicate that the differences from the control levels were statistically significant ($P < 0.05$).

1 N NaOH. Thus, it is possible that the apparent inhibition of cephalexin uptake by *cyclo*(Asp-Asp) reported by Hidalgo *et al.* (10) may be an artifact caused by a lower pH in their experiment.

When the transepithelial transport characteristics of these cyclic dipeptides were determined in Caco-2 cell monolayers in the absence and presence of Gly-Pro, a substrate of the oligopeptide transporter, the transport of *cyclo*(Ala-Lys) and carnosine was inhibited by 39% and 65%, respectively, in the presence of Gly-Pro (Figure 2). In contrast, Gly-Pro inhibited the transepithelial transport of *cyclo*(Ala-Ala) only slightly (12%), whereas *cyclo*(Asp-Asp) was not detected in the receiver chamber in the presence or absence of Gly-Pro even after a 2-h incubation. These results suggest that *cyclo*(Ala-Lys) is partially transported by the oligopeptide transporter(s), while the other two cyclic dipeptides are transported mainly by passive diffusion.

The permeability coefficient (P_{app}) value of *cyclo*(Ala-Ala) was approximately 2 times greater than that of *cyclo*(Ala-Lys) (Table 1). If one estimates the maximum P_{app} value of *cyclo*(Asp-Asp) based on the detection limit of our assay, the neutral [*cyclo*(Ala-Ala)] and the positively charged [*cyclo*(Ala-Lys)] cyclic peptides are at least 13 and 5 times more able to permeate, respectively, than is *cyclo*(Asp-Asp) (Table I).

To ascertain whether the passive transepithelial transport of the cyclic dipeptides occurs by the transcellular or paracellular route, the cellular uptake characteristics of these cyclic dipeptides (1 mM) were determined. The intracellular levels for all three cyclic dipeptides were found to be below the limit of detection of our analytical methods [the detection limits of *cyclo*(Ala-Ala), *cyclo*(Ala-Lys) and *cyclo*(Asp-Asp) were 0.1, 0.05 and 0.05 nmol/mg protein, respectively] even after a 2-h incubation (data not shown). In contrast, a concentration of 4.56 ± 0.32 nmol/mg protein of carnosine was detected in the cells after a 2-h incubation. These data suggest that the cyclic dipeptides used in this study are passively permeating the Caco-2 cell monolayers mainly via the paracellular route rather than the transcellular route.

Considering that the route of permeation of these cyclic dipeptides across Caco-2 cells appears to be predominantly paracellular (except for *cyclo*(Ala-Lys), which has a minor oligopeptide transporter-mediated component) the rank order (neutral > positive >> negative) of the P_{app} values shown in Table I is consistent with that predicted based on the charge characteristics of the cellular junctional complexes (15–18). Since these junction complexes are negatively charged, a negatively charged cyclic peptide should be less able to permeate than are neutral and positively charged cyclic peptides.

In summary, we have shown in this study that, contrary to what had been published previously, a negatively charged cyclic dipeptide [*cyclo*(Asp-Asp)] does not have affinity for the oligopeptide transporter in Caco-2 cells. In addition, we have shown that cyclic dipeptides permeate Caco-2 cell monolayers primarily via a paracellular route in the order (neutral > positive >> negative) predicted by potential charge-charge interactions with the negative charge in the cellular functional complexes.

Table I. Permeability Coefficients of the Cyclic Dipeptides and Carnosine Across the Caco-2 Cell Monolayers

Compounds	$P_{app} \times 10^7$ (cm/sec)
cyclo(Ala-Ala)	10.75 ± 0.64[a]
cyclo(Ala-Lys)	4.59 ± 0.63
cyclo(Asp-Asp)	<0.8[b]
carnosine	7.44 ± 0.49

[a] Mean (±SD).
[b] Calculated from the detection limit.

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