Metabolism, Uptake, and Transepithelial Transport of the Stereoisomers of Val-Val-Val in the Human Intestinal Cell Line, Caco-2

Kiyoshi Tamura,[1] Chao-Pin Lee,[2] Philip L. Smith,[2] and Ronald T. Borchardt[1,3]

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Purpose. The purpose of this study was to determine the stereospecificity of the apical oligopeptide transporter(s) for the stereoisomers of Val-Val-Val and to determine whether the interaction of these molecules with this transporter(s) could be correlated with their cellular uptake and/or transepithelial transport.

Methods. The interactions of these stereoisomers with this transporter(s) were evaluated by determining their ability to inhibit [^{3}H]cephalexin uptake into Caco-2 cells. The metabolism of these stereoisomers was determined in a homogenate of Caco-2 cells and in the apical bathing solution over Caco-2 cell monolayers. The cellular uptake and transepithelial transport properties of these stereoisomers were studied using the Caco-2 cell monolayers.

Results. The L-L-L tripeptide was totally degraded within 1 h in the Caco-2 cell homogenate and within 2 h when applied to the apical side of a Caco-2 cell monolayer. In contrast, 36.7 ± 1.3% and 69.7 ± 0.9% of L-Val-L-Val-D-Val remained after 2 h in the cell homogenate and in the apical bathing solution, respectively. The other six stereoisomers of Val-Val-Val were completely stable in the Caco-2 cell homogenate. Five of the stereoisomers (L-L-L, L-L-D, L-D-L, D-L-L, D-D-L) significantly inhibited the cellular uptake of [^{3}H]cephalexin (91%, 62%, 14%, 45%, 16%, respectively). The other stereoisomers had no effect on the [^{3}H]cephalexin uptake. When the cellular uptake of the stereoisomers was determined, the D-L-L and L-D-L tripeptides showed the highest intracellular concentrations (1.32 ± 0.25 and 0.62 ± 0.20 nmol/mg protein after a 2-h incubation, respectively). In contrast, the intracellular concentrations of the other stereoisomers were less than 0.1 nmol/mg protein. Moreover, the cellular uptake of the D-L-L and L-D-L tripeptides was inhibited by Gly-Pro by 82% and 68%, respectively, whereas Gly-Pro showed moderate to no inhibitory effect on the cellular uptake of the other stereoisomers. The permeability coefficients of the stereoisomers across the Caco-2 cell monolayers were very low (1.8 to 3.1 × 10^{-7} cm/sec) and almost identical. Gly-Pro had no effect on their transepithelial transport.

Conclusions. These results suggest that the interaction of the Val-Val-Val stereoisomers with the apical oligopeptide transporter(s) could be a good predictor of their cellular uptake. However, since the major transepithelial transport mechanism of Val-Val-Val stereoisomers is passive diffusion via the paracellular route, the binding of these molecules to the oligopeptide transporter(s) is not a good predictor of their transepithelial transport. It appears that the stereochemical requirements for the transporter that mediates permeation of the peptide across the basolateral membrane may be different from the requirements for the apical transporter that mediates cellular uptake.

KEY WORDS: oligopeptide transporter; structure-transport; metabolism; Caco-2; tripeptides; cephalexin.

INTRODUCTION

The intestinal peptide transporter(s) is involved in the absorption of natural di/tripeptides and peptidomimetic drugs (1–5). Recently, our laboratory (6) has used diastereomers of Val-Val to determine the stereochemical requirements for interaction with the apical oligopeptide transporter(s), for transporter-mediated uptake into intestinal epithelial cells, and for transporter-mediated transepithelial transport. Based on these studies (6), it appears that the stereochemical requirements for dipeptide binding to the transporter(s) in the apical membrane of intestinal epithelial cells may be different from the stereochemical requirements for interacting with the transporter(s) in the basolateral membrane. This conclusion is based on the observation that Val-Val dipeptides containing one D-amino acid could inhibit the apical uptake of [^{3}H]cephalexin, indicating that they bind to the apical transporter(s). In addition, they could be taken up into the cells by a transporter-mediated process. However, these same peptides containing one D-amino acid did not readily translocate across the intestinal mucosal cell, suggesting they did not effectively serve as substrates for the basolateral peptide transporter(s).

Since it is unclear whether the same proteins are involved in transporting both dipeptides and tripeptides, we undertook studies using stereoisomers of Val-Val-Val. Here we describe how the eight stereoisomers of Val-Val-Val (L-L-L, L-L-D, L-D-L, D-L-L, D-D-L, D-L-D, L-D-D, and D-D-D) interact with the apical oligopeptide transporter(s), their cellular uptake, their transepithelial transport, and their lability to metabolism. For these studies, we have used Caco-2 cell monolayers, a well-established model of the intestinal mucosa (7, 8) that contains the di/tripeptide transporter(s) (6).

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). Gly-Pro, 2-(N-morpholino)ethanesulfonic acid (MES), and Dulbecco's phosphate buffer solution (D-PBS; powder form) were purchased from Sigma Chemical Co. (St. Louis, MO). N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonate] (Hepes), Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), and non-essential amino acids (NEAA) were obtained from JRH Biosciences (Lenexa, KS). Fetal bovine serum (FBS) was from Intergen Company (Cambridge, MA), and rat tail collagen (Type I) was from Collaborative Research (Lexington, MA). Penicillin and

[1] Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66047.

[2] Department of Drug Delivery, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406.

[3] To whom correspondence should be addressed.

ABBREVIATIONS: D-PBS, Dulbecco's phosphate buffer solution; DMEM, Dulbecco's modified Eagle medium; EBSS, Earle's balanced salt solution; FBS, Fetal bovine serum; HBSS, Hanks' balanced salt solution; Hepes, (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonate]); MES, 2-(N-morpholino)ethanesulfonic acid; NEAA, non-essential amino acids; Val, Valine.

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streptomycin were obtained as a mixture from Irvine Scientific (Santa Ana, CA). 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.

Syntheses of the Stereoisomers of Val-Val-Val

The syntheses of Val-Val-Val stereoisomers were performed on an ATC 200 peptide synthesizer (Advanced ChemTech, Louisville, KY) by means of solid phase techniques using p-benzyloxybenzyl alcohol resin and Fmoc-protected amino acids. The peptides were purified by preparative HPLC and were shown to be >95% pure by analytical HPLC. Structures were confirmed by fast atom bombardment mass spectrometry (FAB-MS) and by chiral GC amino acid analysis.

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 (6, 7). All cells used in this study were between passages 76 and 87 and monolayers were used 18–21 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 ($P_{app} < 5 \times 10^{-7}$ cm/s).

Uptake Studies

The uptake of [^{3}H]cephalexin and Val-Val-Val stereoisomers (1 mM) was determined in Caco-2 cells in the presence or absence (controls) of competitors using procedures recently published by our laboratory (6). Buffers (pH 6.0 on the apical side and pH 7.4 on the basolateral side) consisting of Earle's balanced salt solution (EBSS) containing 25 mM glucose and 10 mM MES were used to create the H^+ gradient that is required for the optimal activity of the oligopeptide transporter. After a preincubation period of 10 min at 37°C, the monolayers were incubated for 15 min at 37°C with [^{14}C]cephalexin (0.1 mM) or the stereoisomers of Val-Val-Val (1 mM) in the absence or presence of competitors (10 mM of the Val-Val-Val stereoisomers or GlyPro). The incubation medium was removed and the cells were washed 3 times with ice-cold pH 7.4 buffer to stop further uptake and to remove unbound peptide.

For the [^{3}H]cephalexin uptake experiments, cells and filters were dissolved in a Ready-Safe scintillation cocktail and radioactivity was determined in a Beckman LS6000IC liquid scintillation counter. For Val-Val-Val stereoisomers, cells were scraped from the polycarbonate filters into ice-cold pH 7.4 buffer. After briefly washing the cells and sonication as described by Tamura *et al.* (6), the peptide was extracted from the cells by a modification (6) of the method of Wessel and Flugge (9). After acidification of the samples with 0.04 N HCl, the samples were analyzed by HPLC (see HPLC Analysis section below).

Uptake was expressed as nmol/mg protein. Total protein content of cells cultured on polycarbonate filters for various days after seeding was previously determined (10). The percent inhibition of cellular uptake was calculated by comparing the amount of uptake in the presence and absence of inhibitors.

Transepithelial Transport Studies

The transepithelial transport of Val-Val-Val stereoisomers (1 mM) was determined in Caco-2 cells at 37°C in the presence or absence (controls) of Gly-Pro (10 mM) using procedures recently published by our laboratory (6). Again, pH 6.0 and 7.4 buffers were used on the apical and basolateral sides of the monolayer, respectively, to maintain a H^+ gradient. Samples (200 μl) were removed at designated times from the basolateral chamber and replaced with fresh pH 7.4 buffer. The samples were then acidified by addition of 0.08 N HCl (100 μl) and analyzed by HPLC (see HPLC Analysis section below).

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®. 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 Val-Val-Val stereoisomers was determined in the Caco-2 cell homogenate at 37°C using procedures recently published by our laboratory (6). Caco-2 cells were scraped into ice-cold pH 7.4 buffer, homogenized, and the peptide (1 mM) of interest added. Aliquots (200 μl) were removed at various times, and the reaction was quenched by the addition of 200 μl of ice-cold 0.04 N HCl. Samples were analyzed by HPLC (see HPLC Analysis section below).

The metabolism of the Val-Val-Val stereoisomers in the apical bathing solution over the Caco-2 cell monolayers was also determined using procedures recently published by our laboratory (6). The stereoisomers (1 mM) were applied to the apical side of Caco-2 cell monolayers in pH 6.0 buffer. Aliquots (200 μl) were removed after a 2-h incubation at 37°C. The reaction was quenched by the method described above, and the amounts of stereoisomers remaining were determined by HPLC (see HPLC Analysis section below).

HPLC Analysis

The analysis conditions for the Val-Val-Val stereoisomers were as follows: column, C18 (Vydac, 4.6 × 250 mm, Hesperia, CA); isocratic mobile phase, 70 mM phosphate buffer (pH 3.5) containing 10 mM heptane sulfonic acid and 13–20% acetonitrile; detection, 210 nm; flow rate, 1 ml/min. The retention times of L-Val-L-Val-L-Val, L-Val-L-Val-D-Val, L-Val-D-Val-L-Val, D-Val-L-Val-L-Val, D-Val-D-Val-L-Val, D-Val-L-Val-D-Val, L-Val-D-Val-D-Val and D-Val-D-Val-D-Val were approximately 10, 11, 10, 12, 13, 10, 11, and 10 min, respectively.

Statistical Analysis

Statistical analysis of the data was performed by one-way ANOVA using Tukey's family error $P < 0.05$. The software used was Minitab™.

RESULTS

Metabolism of the Val-Val-Val Stereoisomers

When L-Val-L-Val-L-Val was added to Caco-2 cell homogenates, it degraded very rapidly, with no tripeptide detectable after a 1-h incubation at 37°C (Figure 1A). L-Val-L-Val-D-Val degraded more slowly, with 36.7 ± 1.3% of the peptide remaining in the reaction solution after a 2-h incubation (Figure 1A). The other stereoisomers were stable up to 2 h of incubation.

When L-Val-L-Val-L-Val and L-Val-L-Val-D-Val were added to the apical side of the Caco-2 cell monolayers, degradation of both peptides was observed; 0% of L-Val-L-Val-L-Val and 69.7 ± 0.9% of L-Val-L-Val-D-Val were detected in the apical bathing solution after a 2-h incubation (Figure 1B). All of the Val-Val-Val stereoisomers were stable at least 12 h in pH 7.4 buffer at 37°C (data not shown).

The metabolism of L-Val-L-Val-L-Val and L-Val-L-Val-D-Val in Caco-2 cell homogenates and when applied to the apical side of the Caco-2 cell monolayers is probably mediated by aminopeptidases (11, 12).

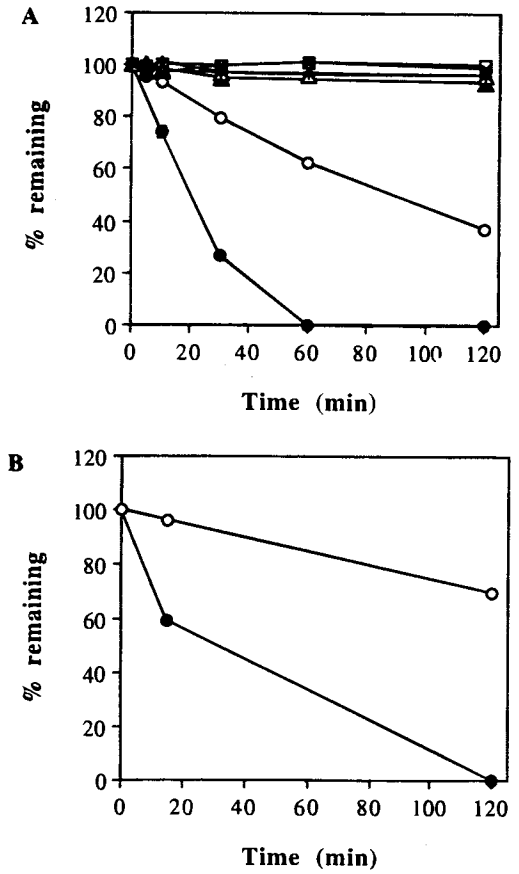

Fig. 1. Metabolism of the Val-Val-Val stereoisomers (A) in the Caco-2 cell homogenate (~1 mg protein/ml, pH 7.4) and (B) in the apical bathing solution (pH 6.0) at 37°C. (●) L-Val-L-Val-L-Val, (○) L-Val-L-Val-D-Val, (■) L-Val-D-Val-L-Val, (□) D-Val-L-Val-L-Val, (▲) D-Val-D-Val-L-Val, (△) D-Val-L-Val-D-Val, (+) L-Val-D-Val-D-Val, (×) D-Val-D-Val-D-Val. Results are the mean ± SD for three separate experiments.

Inhibition of [^{3}H]Cephalexin Uptake by Val-Val-Val Stereoisomers

Figure 2 shows the accumulative uptake of [^{3}H]cephalexin (0.1 mM) in the presence or absence of the Val-Val-Val stereoisomers (10 mM) for 15 min at 37°C. The order of inhibitory potency of these stereoisomers was: L-Val-L-Val-L-Val > L-Val-L-Val-D-Val > D-Val-L-Val-L-Val >> L-Val-D-Val-L-Val = D-Val-D-Val-L-Val. The tripeptides D-Val-L-Val-D-Val, L-Val-D-Val-D-Val and D-Val-D-Val-D-Val had no effect on [^{3}H]cephalexin uptake under these conditions.

Uptake of the Val-Val-Val Stereoisomers and the Effect of Gly-Pro

Figure 3 shows the apical uptake of the Val-Val-Val stereoisomers (1 mM) during a 2-h incubation at 37°C in the presence or absence of Gly-Pro (10 mM). Intracellular accumulations of L-Val-L-Val-D-Val, L-Val-D-Val-L-Val, D-Val-L-Val-L-Val, D-Val-D-Val-L-Val, D-Val-L-Val-D-Val, L-Val-D-Val-D-Val and D-Val-D-Val-D-Val were 0.04 ± 0.01, 0.62 ± 0.20, 1.32 ± 0.25, 0.09 ± 0.04, 0.02 ± 0.01, 0.02 ± 0.02, and 0.04 ± 0.01 nmol/mg protein, respectively. Gly-Pro strongly inhibited the cellular uptake of L-Val-D-Val-L-Val and D-Val-L-Val-L-Val (68% and 82%, respectively). Gly-Pro had moderate to no effect on the cellular uptake of the other stereoisomers. L-Val-L-Val-L-Val was not detected in the cells in either the presence or absence of Gly-Pro.

Transepithelial Transport of the Val-Val-Val Stereoisomers and the Effect of Gly-Pro

Table I shows the permeability coefficients (P_{app}) of the Val-Val-Val stereoisomers (1 mM) in the Caco-2 cell monolayers determined during a 2-h incubation. L-Val-L-Val-L-Val was not detected in the basolateral chamber. The P_{app} values of the other stereoisomers were nearly identical (1.8 to 3.1 ×

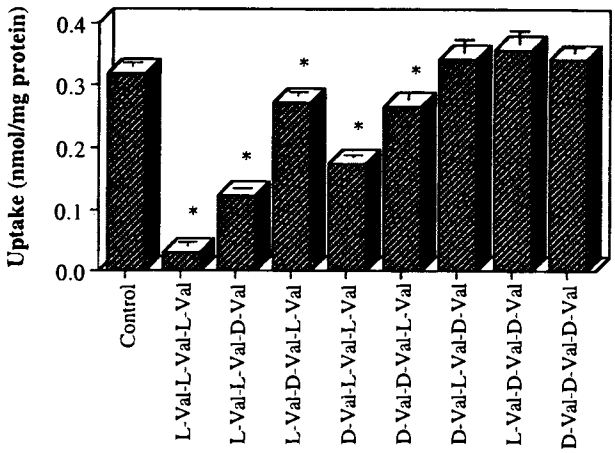

Fig. 2. Uptake of [^{3}H] cephalexin (0.1 mM) in the presence or absence of the Val-Val-Val stereoisomers (10 mM) for 15 min at 37°C. Caco-2 cell monolayers were incubated with pH 6.0 buffer on the apical side and pH 7.4 buffer on the basolateral side. Results are the means ± SD for three separate filters.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

Table I. Permeability Coefficients of the Val-Val-Val Stereo Isomers Across the Caco-2 Cell Monolayers

Tripeptides	$P_{app} \times 10^7$ (cm/sec)
L-Val-L-Val-L-Val	Not detected
L-Val-L-Val-D-Val	1.84 (0.34)[a]
L-Val-D-Val-L-Val	2.54 (0.40)
D-Val-L-Val-L-Val	3.09 (0.51)
D-Val-D-Val-L-Val	2.76 (0.25)
D-Val-L-Val-D-Val	2.04 (0.29)
L-Val-D-Val-D-Val	2.48 (0.34)
D-Val-D-Val-D-Val	1.89 (0.08)

[a] Mean (±SD).

10^{-7} cm/sec) and Gly-Pro (10 mM) did not show a significant effect on their transepithelial transport (data not shown).

DISCUSSION

Recently, our laboratories used diastereomers of Val-Val to determine the stereochemical requirements for interaction with the apical oligopeptide transporter(s) and whether this affinity for the transporter(s) could be correlated with their cellular uptake and transepithelial transport (6). Since it is unclear whether the same proteins are involved in transporting both dipeptides and tripeptides, we report here a similar study using stereoisomers of Val-Val-Val. For these studies we have used Caco-2 cells that spontaneously undergo differentiation into enterocyte-like cells (7) and exhibit morphological and biochemical characteristics similar to those of intestinal epithelial cells (10–17).

Since apical uptake of cephalexin is mediated mainly by oligopeptide transporter(s) (1), we evaluated the interaction of the Val-Val-Val stereoisomers with the apical transporter(s) by determining their effects on [^{3}H]cephalexin uptake. The data from these inhibition studies suggest that substitution of a D-amino acid into a tripeptide lowers the affinity of the molecule for the apical oligopeptide transporter(s) (Figure 2). The decrease in affinity depends on the number of D-amino acid incorporated into the tripeptide and their location in the molecule. Since L-Val-L-Val-L-Val is metabolically unstable in the apical bathing solution (Figure 1B), it is difficult to assess the actual affinity of this tripeptide. Probably, the strong inhibition of [^{3}H]cephalexin uptake observed when L-Val-L-Val-L-Val was included into the incubation mixture arises from both the tripeptide and its metabolite, L-Val-L-Val. Although L-Val-L-Val-D-Val also degrades in the apical bathing solution, the percentage of degradation after 15 min (the time used for the [^{3}H]cephalexin uptake studies) is insignificant (<5%) (Figure 1B). Thus, it is assumed that the inhibition of [^{3}H]cephalexin uptake seen with L-Val-L-Val-D-Val is primarily due to the interaction of the tripeptide with the apical transporter(s). With the other stereoisomers, metabolism was not a problem (Figure 1A). Substitution of a D-amino acid at the N-terminal and or C-terminal end of the tripeptide slightly reduces the affinity for the apical transporter(s). More significant loss of affinity was observed if the D-amino acid was inserted in the middle of the tripeptide or if two D-amino acids were incorporated at residues 1 and 2 in the sequence. If the D-amino acids are the 1st and 3rd or the 2nd and 3rd residues, the peptides show no affinity for the apical transporter(s). A similar loss of affinity was observed if all the amino acids were D. Therefore, in addition to L-Val-L-Val-L-Val, L-Val-L-Val-D-Val and D-Val-L-Val-L-Val showing significant affinity for the apical oligopeptide transporter(s), L-Val-D-Val-L-Val and D-Val-D-Val-L-Val also appear to retain some affinity for this transporter(s).

When the cellular uptake of the Val-Val-Val stereoisomers was evaluated, L-Val-L-Val-L-Val and L-Val-L-Val-D-Val showed very low or no intracellular accumulation, probably due to their metabolic lability. D-Val-L-Val-L-Val, which interacts with the apical oligopeptide transporter(s) strongly and is metabolically stable in the Caco-2 cell system, showed significant cellular accumulation (Figure 3). Moreover, strong inhibition of D-Val-L-Val-L-Val uptake by Gly-Pro suggests that the major cellular uptake process of this tripeptide is transporter-mediated. L-Val-D-Val-L-Val and D-Val-D-Val-L-Val were expected to accumulate to identical intracellular concentrations based on their similar inhibitory effects on [^{3}H]cephalexin uptake and their metabolic stability. Instead, L-Val-D-Val-L-Val accumulated in the Caco-2 cells about six times more than did D-Val-D-Val-L-Val (Figure 3). Moreover, Gly-Pro inhibited L-Val-D-Val-L-Val uptake by 68%, whereas the inhibition of D-Val-D-Val-L-Val uptake by Gly-Pro was 26%. These data suggest that L-Val-D-Val-L-Val is taken up mainly by the apical oligopeptide transporter(s), whereas the binding of D-Val-D-Val-L-Val to the transporter(s) may include higher non-specific binding that does not lead to its cellular uptake. The other three stereoisomers, which do not bind to the apical transporter(s), showed very low intracellular concentrations (1/60 to 1/30 of that of D-Val-L-Val-L-Val). In addition, Gly-Pro had no effect on their cellular uptake, suggesting that their cellular accumulation is by passive diffusion. Therefore, the binding of the Val-Val-Val stereoisomers to the apical oligopeptide transporter(s) is a good predictor of cellular uptake.

With respect to transepithelial transport, L-Val-L-Val-L-Val was not detected in the basolateral chamber, probably due to its

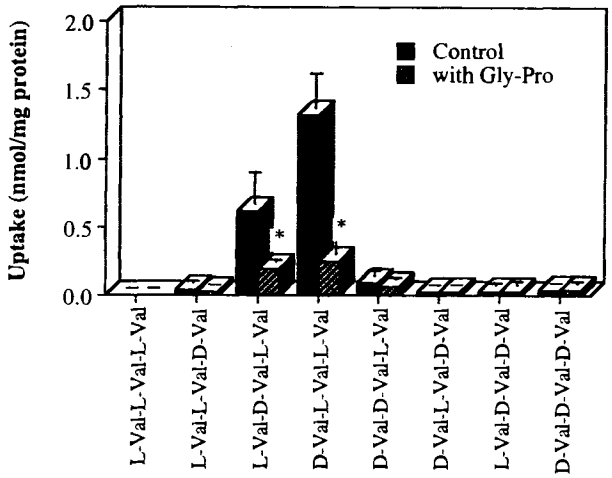

Fig. 3. Uptake of the Val-Val-Val stereoisomers (1 mM) in the presence or absence of Gly-Pro (10 mM) during a 2-h incubation at 37°C. Caco-2 cell monolayers were incubated with pH 6.0 buffer on the apical side and pH 7.4 buffer on the basolateral side. Results are the mean ± SD for three separate filters. The asterisks (*) indicate that the differences from the control levels were statistically significant ($P < 0.05$) according to a one-way ANOVA test.

metabolic lability. The transepithelial transport characteristics of the other Val-Val-Val stereoisomers are almost identical (Table I), even though the uptake properties of the stereoisomers are quite different (Figure 3). In addition, Gly-Pro did not show any inhibition of their transepithelial transport (data not shown), which suggests that the major transepithelial transport mechanism of the Val-Val-Val stereoisomers is passive diffusion via either the transcellular or paracellular route. If the major route were transcellular diffusion, D-Val-L-Val-L-Val would have shown a much higher transepithelial transport due to its higher intracellular concentration. This discrepancy implies that the major transepithelial transport pathway of the Val-Val-Val stereoisomers is paracellular.

Moreover, the observations above infer that there is little or no contribution of the basolateral oligopeptide transporter to the permeation of the Val-Val-Val stereoisomers. In contrast, the transporter(s) on the apical membrane facilitates the apical uptake of D-Val-L-Val-L-Val and L-Val-D-Val-L-Val. Therefore, the basolateral transporter may have different and much stricter structural requirements for peptides to bind and be transported than does the apical transporter(s).

Therefore, since the active transepithelial transport process of peptides or peptidomimetics involves the oligopeptide transporters on both the apical and the basolateral membranes, the binding of the Val-Val-Val stereoisomers to the apical oligopeptide transporter(s) is not sufficient to predict their transepithelial transport. It appears that the structural requirements for the transporter that mediates permeation of the peptide across the basolateral membrane may be different from the requirements for the apical transporter that mediates cellular uptake. Inui *et al.* (18,19) have suggested that the basolateral oligopeptide transporter is involved in the transport of cephalosporins and bestatin across the basolateral membrane. Furthermore, they also demonstrated that the transport of cephalosporins across the basolateral membranes are mediated by the oligopeptide transporter in a H^+ gradient-independent manner (20). In contrast Thwaites *et al.* (21) have compared the properties of the apical and basolateral oligopeptide transporter and shown both to be H^+-coupled but they exhibit different kinetic properties. These published observations and the data described in this manuscript suggest that the apical and basolateral transporters might be different proteins.

Since these observations are quite consistent with those seen in our previous studies using the diastereomers of Val-Val (6), the same oligopeptide transporters on both the apical and the basolateral membranes might be involved in transepithelial transport of both dipeptides and tripeptides.

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