Structural Requirements for Interaction with the Oligopeptide Transporter in Caco-2 Cells

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INTRODUCTION

Prior studies have demonstrated that the intestinal oligopeptide transporter is distinct from the transport system involved in the uptake of amino acids and that this transporter is responsible for the uptake of oligopeptides and peptidomimetics (1–5). This transporter has been extensively studied *in vitro* with intestinal tissues and with the human intestinal cell lines, Caco-2 and HT-29 (1,4–6). Recently, molecules have been rationally designed to target the intestinal oligopeptide transporter in an attempt to enhance oral absorption (7,8). However, for this approach to attain its full potential, a greater understanding of the structural features required for interaction with the intestinal oligopeptide transporter is required. In this study, structural features required for interaction with the intestinal oligopeptide transporter have been assessed through evaluation of the ability of dipeptides to inhibit uptake of cephalexin by Caco-2 cells grown as confluent monolayers.

MATERIALS AND METHODS

Cells. Caco-2 cells obtained from American Type Tissue Culture Collection (Rockville, MD) were used between passages 60 and 70. The cells were grown as previously described (1).

Inhibition of [^{3}H]cephalexin Uptake. [^{3}H]cephalexin uptake studies were conducted as described previously (1). For inhibition studies, cells were rinsed and incubated with buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES) in Hank's balanced salt solution (HBSS), pH 6.0 on the apical surface and 10 mM N-2-hydroxyethylpiperazine -N′-2-ethanesulfonic acid in HBSS, pH 7.4 on the basolateral surface) at 37°C for 15 min. Following this incubation period, the apical surface was replaced with 10 mM MES/HBSS containing [^{3}H]cephalexin (0.1 mM) alone or in combination with the appropriate concentrations of inhibitor. Cells were incubated for 15 min at 37°C, removed from the incubation media and washed four times with ice cold HBSS, pH 7.0. Cells and filters were then dissolved in Ready-Safe scintillation cocktail and radioactivity determined in a Liquid Scintillation Counter (LS-6000, Beckman Instruments Inc., Fullerton, CA). Percent inhibition of [^{3}H]cephalexin uptake (expressed as pmol/mg protein/min) due to inhibitors was calculated compared to controls containing no inhibitors. Results are means and standard deviations of at least three experiments for each condition.

Chemicals. [^{3}H]cephalexin was prepared as described previously (4). Gly-Pro, Glu-Lys, Asp-Lys, Val-Lys, Lys-Val, Lys-Asp, Val-Glu, Glu-Val, Gly-Lys, Asp-Asp, Lys-Lys, Glu-Glu, BOC-Val, BOC-D-Val-D-Val, Val benzyl and methyl ester hydrochlorides were purchased from Bachem Bioscience Inc. (Philadelphia, PA). Valinol (Val-OL), dicyclohexyl carbodiimide (DCC), hydroxy benzotriazole (HOBt) and diisopropyl ethyl amine (DIEA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dimethylformamide (DMF) and methylene chloride were purchased from Mallinckrodt Speciality Chemicals Co. (St. Louis, MO).

The dipeptides Val-Val, Val-D-Val, D-Val-D-Val and D-Val-Val were synthesized using similar procedures. Typically, 2.17 g (1 mM) of BOC-Val, 2.67 g (1.1 mM) Val-OBenzyl HCl were dissolved in 50 ml DMF. The solution was chilled in an ice bath followed by addition of 1.9 ml DIEA, 2.06 g DCC and 1.53 g HOBt. The mixture was allowed to warm to room temperature and stirred overnight. DMF was evaporated on a rotovap and the residue resuspended in ethyl acetate (500 ml). The solution was washed with 1N HCl, water, 1N NaOH, water and saturated NaCl solution. The organic layer was dried over $MgSO_4$ and evaporated. No further purification was performed and the yield was ~95%. The protected peptide (200 mg) was treated with liquid HF at 0°C to remove the protecting groups (BOC and Benzyl ester). HF was evaporated and the deprotected peptide was dissolved in 0.1% TFA and the solution was washed with ether. The aqueous layer was lyophilized. The crude peptide was purified using reverse phase HPLC (C-18). Structures were confirmed by FAB MS, NMR and chiral GC amino acid analysis.

Valylvalinol (Val-Val-OL) was synthesized as follows. BOC-Val, 5.26 g (2 mM) was dissolved in 100 ml methylene chloride. DCC, 2.5 g (1.25 mM) was added and the solution stirred at room temperature for 30 min. The precipitates (N,N′ dicylohexyl urea) were filtered off and methylene chloride was evaporated. The residue was dissolved in 50 ml DMF and chilled to 0°C. One gram of Val-OL was added. After 30 min 1.36 ml of DIEA was added. After stirring

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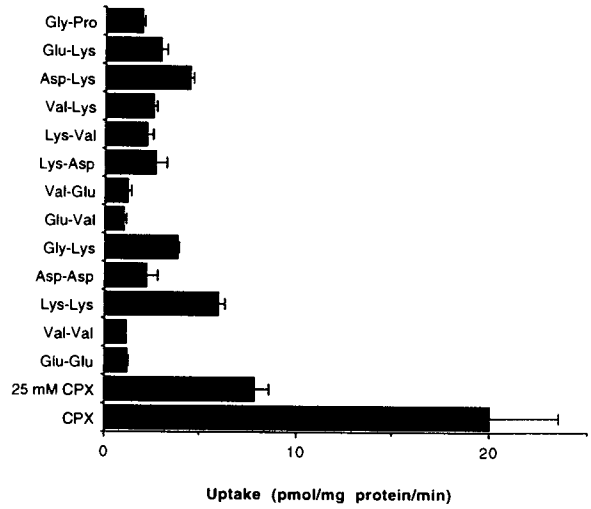

Figure 1: Uptake of [^{3}H]cephalexin (0.1 mM) in the absence or presence of cephalexin (25 mM) or linear dipeptides (20 mM). As described in the Materials and Methods section, Caco-2 cell monolayers were incubated with pH 6.0 buffer in the apical bathing solution and pH 7.4 buffer in the basolateral bathing solution. Results are means ± SD for three separate filters with each condition. Cephalexin and dipeptides all significantly inhibited rabiolabelled cephalexin uptake with a p value of 0.05 or less.

overnight at room temperature the solution was evaporated to dryness and re-suspended in ethyl acetate. The organic solution was washed with 1N HCl, water, 1N NaOH, water and saturated NaCl solution. The organic layer was dried over $MgSO_4$ and evaporated. A white solid (BOC-Val-Val-OL) 2.7 g was obtained (yield = 92%). The silica gel TLC indicated that the product was pure and no further purification was done. The BOC group was removed by stirring in 100 ml 4N HCl/dioxane. The solution was evaporated and residue was triturated with ether. A white hygroscopic solid (Val-Val-OL, 2.1 g) was obtained and was used without further purification. The structure was confirmed by FAB MS.

RESULTS AND DISCUSSION

From Figures 1–3, it can be seen that [^{3}H]cephalexin uptake at 0.1 mM ranges between 14 and 24 pmol/mg protein/min. Previously, we reported that the maximal uptake rate for cephalexin in Caco-2 cells is 1 nmol/mg protein/min (1). This compares to values of 16 and 0.44 nmol/mg protein/min for phenylalanine and α-methylglucoside uptake by Caco-2 cells, respectively (9,10). Previously, Wootton and Hazelwood reported that a net charge on the dipeptide could dramatically alter its affinity for the oligopeptide transporter (11). Figure 1 shows that linear dipeptides (all at 20 mM concentration) containing neutral, basic or acidic amino acids all inhibit [^{3}H]cephalexin uptake to the same or a greater extent than unlabelled cephalexin (25 mM). These results together with results demonstrating that the K_i's for inhibition of cephalexin uptake in the Caco-2 cell system with charged dipeptides are all less than 1 mM suggest that there is no difference in interaction with the oligopeptide transporter by linear dipeptides regardless of the net charge (12). The differences in results seen in this study compared with those reported by Wootton and Hazelwood may be due to

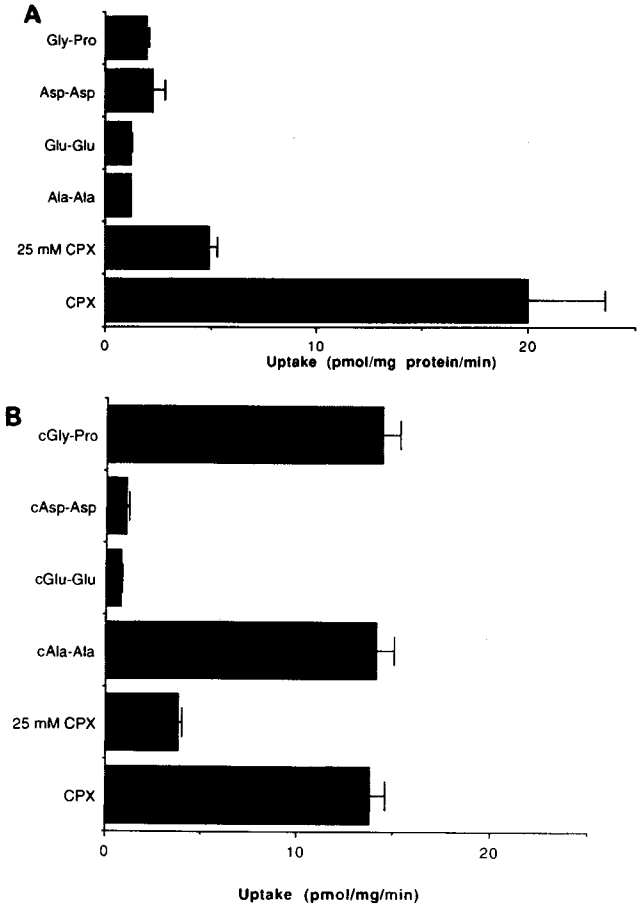

Figure 2: Uptake of [^{3}H]cephalexin (0.1 mM) in the absence or presence of cephalexin (25 mM) compared to either (2A) linear dipeptides (10 mM) or (2B) their cyclic analogues (10 mM). Results are means ± SD for three separate filters with each condition. Cephalexin and dipeptides all significantly inhibited rabiolabelled cephalexin uptake with a p value of 0.05 or less.

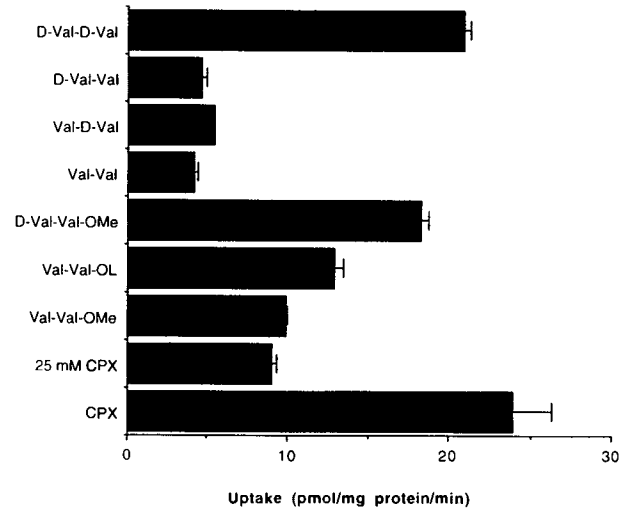

Figure 3: Uptake of [^{3}H]cephalexin (0.1 mM) in the absence or presence of cephalexin (25 mM) or analogues of Val-Val (20 mM). Results are means ± SD for three separate filters with each condition. With the exception of D-Val-D-Val and D-Val-Val-OMe, cephalexin and dipeptides all significantly inhibited rabiolabelled cephalexin uptake with a p value of 0.05 or less.

the test system employed (e.g. brush border membrane vesicles vs Caco-2 cells), the conditions of the experiments (e.g. presence of a pH gradient) or the substrate used in the competition studies (e.g. Gly-Pro vs cephalexin) (11).

The role of the α-carboxyl and α-amino groups and the conformation of the peptide backbone in constrained molecules was investigated with a series of cyclized dipeptides (Figures 2A and 2B). With Gly-Pro and Ala-Ala, cyclization abolished their ability to interact with the oligopeptide transporter. However, with dipeptides containing a net negative charge in the side chain (e.g. Glu-Glu or Asp-Asp), cyclization did not alter their ability to inhibit [^{3}H]cephalexin uptake. Thus, the inability of conformationally constrainted (e.g. cyclized) dipeptides to interact with the oligopeptide transporter can be overcome through judicious selection of amino acids in the dipeptides.

The role of the β-carboxyl group in the interaction of linear dipeptides with the oligopeptide transporter was investigated through modification of the carboxylic acid in Val-Val by esterification or reduction to the alcohol (Figure 3). The alcohol (Val-Val-OL) and the methyl ester (Val-Val-OMe) produce approximately 50% of the activity seen with Val-Val. These results suggest that the ability of dipeptides to optimally inhibit [^{3}H]cephalexin uptake is dependent on the presence of a free carboxy group. Compared to the methyl ester of Val-Val, the methyl ester of D-Val-Val (D-Val-Val-OMe) demonstrates very little activity. Whether this is due to a reduced interaction resulting from the absence of an L amino acid in the N-terminal position of D-Val-Val-OMe and/or to differences in metabolic stability of the D-Val-Val methyl ester against intestinal enzymes cannot be determined from the present studies.

Figure 3 also shows that alteration of the chirality of one amino acid in Val-Val does not alter its ability to inhibit ^{3}H-cephalexin uptake although when both amino acids are in the D-configuration, the ability of Val-Val to inhibit [^{3}H]cephalexin uptake is abolished. Thus, D-amino acids can be incorporated into dipeptides to increase metabolic stability without compromising their ability to optimally interact with the oligopeptide transporter. This may also apply to tripeptides. Evidence to support this proposal is provided by the finding that both the D- and L-isomers of the peptidomimetic, cephalexin interact with the oligopeptide transporter (13).

These studies demonstrate inhibition of cephalexin uptake by selective dipeptides implying that specific dipeptides interact with the oligopeptide transporter. However, this interaction may not result in enhanced transepithelial transport of these dipeptides. Previously, we demonstrated that benzylpenicillin is a potent inhibitor of cephalexin uptake in Caco-2 cells, but in rabbit small intestinal tissues, benzylpenicillin transport is passive (1,4). From these studies, it can be seen that for the design of molecules which interact with the oligopeptide transporter, the peptidomimetic chemist has at his disposal several possibilities. These include, incorporation of D amino acids in place of L amino acids in linear dipeptides or peptidomimetics or inclusion of acidic amino acids in cyclic peptides.

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