Effect of Size and Charge on the Passive Diffusion of Peptides Across Caco-2 Cell Monolayers via the Paracellular Pathway

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Received September 4, 1996; accepted December 4, 1996

Purpose. To evaluate the effect of size and charge on the permeation characteristics of peptides across the intestinal mucosa.

Methods. The lipophilicities of neutral, positively and negatively charged capped amino acids (Asn, Lys, Asp), tripeptides (Ac-Gly-X-Ala-NH_2; X = Asn, Lys, Asp) and hexapeptides (Ac-Trp-Ala-Gly-Gly-X-Ala-NH_2; X = Asn, Lys, Asp) were estimated using an immobilized artificial membrane. The diffusion coefficients used to calculate the molecular radii were measured by NMR. The transport characteristics of the model peptides were determined across Caco-2 cell monolayers.

Results. When model compounds having the same charge were compared, permeation was highly size-dependent (capped amino acids > tripeptides > hexapeptides), suggesting transport predominantly via the paracellular route. For example, the flux of the negatively charged Asp amino acid (P_{app} = 10.04 ± 0.43 × 10^{-8} cm/s) was 3 times greater than that observed for the Asp-containing hexapeptide (P_{app} = 3.19 ± 0.27 × 10^{-8} cm/s). When model compounds of the same size were compared, permeation across the cell monolayer was charge-dependent (negative < positive ≤ neutral). For example, the neutral, Asn-containing tripeptide (P_{app} = 25.79 ± 4.86 × 10^{-8} cm/s) was substantially more able to permeate the Caco-2 cell monolayer than the negatively charged Asp-containing tripeptide (P_{app} = 7.95 ± 1.03 × 10^{-8} cm/s) and the positively charged Lys-containing tripeptide (P_{app} = 9.86 ± 0.18 × 10^{-8} cm/s). The permeability of the cell monolayer to peptides became less sensitive to net charge as the size of the peptides increased.

Conclusions. A positive net charge of hydrophilic peptides enhances their permeation across the intestinal mucosa via the paracellular pathway. With increasing molecular size, molecular sieving of the epithelial barrier dominates the transport of peptides, and the effect of the net charge becomes less significant.

KEY WORDS: peptide delivery; size; charge; Caco-2 cells; membrane permeability.

INTRODUCTION

Oral delivery of peptide drugs is restricted by their susceptibility to extensive enzymatic metabolism (1–3) and their unfavorable physicochemical properties, which limit their membrane permeation (3–5). In an effort to overcome the metabolic barrier of the intestinal mucosa, various chemical strategies [e.g., design of peptidomimetics (6) and peptide prodrugs (7)] have been developed to prepare pharmacologically active compounds having greater enzymatic stability. The permeability of epithelial barriers, including the cornea (8), the alveolar epithelium (9), and the intestinal mucosa (4,5) to these metabolically stable peptides, has been studied more systematically over the past years. Nevertheless, the structural diversity of the compounds selected for these studies makes it impossible to draw general conclusions about the structural features that control the transport of peptides/peptidomimetics across a biological barrier via different pathways.

Permeation of molecules across the gastrointestinal epithelium is restricted to paracellular or transcellular pathways and can be influenced by polarized efflux systems (3). In general, it is accepted that the physicochemical properties of a permeant (i.e., size, charge and lipophilicity) govern the pathways of transport (3). In recent years, a considerable body of information has been presented on the structural features of peptides traversing the intestinal barrier via the transcellular pathway (4,5,10,11). However, the interplay between structural characteristics and transport via the paracellular pathway is still not well understood (3).

To investigate the effect of size and charge on the passive diffusion of peptides across the intestinal mucosa via the paracellular route, we selected a series of metabolically stable model compounds derived from a naturally occurring hexapeptide (H-Trp-Ala-Gly-Gly-Asp-Ala-OH) that has been studied extensively in our laboratory (12–14). In this study, we describe the transport properties of neutral, positively and negatively charged analogs (i.e., capped amino acids, tripeptides, and hexapeptides) of this natural hexapeptide. Transport characteristics were determined in an *in vitro* cell culture model (Caco-2) of the human intestinal mucosa (15–17). The results are discussed in light of the physicochemical properties of the permeants and the physical barrier characteristics of the gastrointestinal epithelium.

MATERIALS

The capped Asn amino acid, the tripeptides (Ac-Gly-X-Ala-NH_2; X = Asn, Lys, Asp) and the hexapeptides (Ac-Trp-Ala-Gly-Gly-X-Ala-NH_2; X = Asn, Lys, Asp) were prepared by solid phase synthesis using standard Fmoc chemistry (18). Purification was performed by preparative reversed-phase HPLC, and the compounds were characterized by analytical reversed-phase HPLC (>97%) and FAB^+-MS. Capped Asp and Lys amino acids were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Dulbecco's phosphate buffered saline and Hanks' balanced salts (modified) were purchased from Sigma Chemical Co. (St. Louis, MO). L-Glutamine 200 mM (100×), penicillin (10,000 U/ml), streptomycin (10,000 μg/ml), and non-essential amino acids 10 mM (100×) in 0.85% saline were obtained from Gibco BRL, Life Technologies (Grand Island, NY). Dulbecco's modified Eagle medium was purchased from JRH Biosciences (Lenexa, KS). Rat tail collagen (type I) was obtained from Collaborative Biomedical Products (Bedford, MA), and fetal bovine serum from Atlanta Biologicals (Norcross, GA). D-1[^{14}C]Mannitol (spec. act. = 2.07 GBq/mmol) was purchased from Moravek Biochemicals (Brea, CA). All other chemicals and solvents were of high purity or analytical grade and used as received.

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ABBREVIATIONS: HBSS, Hanks' balanced salt solution; AP, apical; BL, basolateral; M_r, molecular radius.

0724-8741/97/0200-0164$12.50/0

METHODS

Lipophilicity Determination

The lipophilicities of the peptides were estimated by determining their partitioning between 0.02 M phosphate buffer, pH 7.4, and an immobilized artificial membrane (IAM.PC.DD column, 10 cm × 4.6 mm I.D., Regis Technologies, Inc., Morton Grove, IL) as described earlier (12). Aliquots (10–15 μl) of the peptide solutions (~20 μg/ml, in running buffer) were injected on the column (flow-rate 1.0 ml/min), and solutes were detected with a UV detector (λ = 220 nm) or a fluorescence detector as described below.

Molecular Size Determination

Diffusion coefficients of the peptides at 25°C were measured experimentally by NMR spectroscopy as described elsewhere (19,20). The molecular radii were calculated according to the Stokes-Einstein equation using η = 1.0951 cP (21) as the viscosity of D_2O at 25°C.

Cell Culture

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) at passage 18 and cultured as described previously (12). Cells used in this study were between passage 56 and 65.

Transport Studies

Caco-2 cell monolayers grown on collagen-coated polycarbonate filters (Transwells®) for 21 to 28 days were used for transport experiments. The integrity of each batch of cells was first tested by measuring the flux of [^{14}C]mannitol in representative cell monolayers (n = 3). AP-to-BL flux for this paracellular marker never exceeded values of 0.4%/hr ($P_{app} \leq 1.27 \times 10^{-7}$ cm/s). The transport of peptides across Caco-2 cell monolayers was performed in triplicate as described previously (12). Briefly, the peptide solution (90–140 μM in HBSS) was applied to the AP donor compartment (1.5 ml) of the cell monolayer and HBSS was added to the receiver compartment (2.6 ml). Samples (120 μl, receiver side; 20 μl, donor side) were removed from both sides at various times up to 180 min. The volume removed from the receiver side was always replaced with fresh, prewarmed HBSS. Samples were immediately frozen in a dry ice/acetone bath and stored at −80°C until HPLC analysis.

HPLC Analysis

The HPLC system (Shimadzu, Inc., Tokyo, Japan) consisted of two LC-10AS pumps, a SCP-6 controller, a SPD-10A UV detector, and a RF-535 fluorescence detector connected to LCI-100 integrators (Perkin-Elmer, Norwalk, CT). Chromatographic analyses were carried out using a Dynamax C_{18} reversed-phase column (5 μm, 300 Å, 25 cm × 4.6 mm I.D., Rainin Instruments, Woburn, MA) equipped with a guard column, or a Vydac strong cation-exchange column (5 μm, 7.5 cm × 50 mm I.D., Vydac, Hesperia, CA). Chromatographic conditions and the respective retention times of the peptides are summarized in Table 1. Capped amino acids and tripeptides were detected by their UV absorbance at λ = 220 nm, and the hexapeptides were analyzed by the fluorescence of the Trp residue at emission λ = 345 nm (excitation λ = 285 nm).

Data Analysis

Permeability coefficients (P_{app}) of the capped amino acids and the model peptides were calculated according to Eq. 1:

$$P_{app} = \frac{\Delta Q/\Delta t}{A \cdot c(0)} \quad (1)$$

where $\Delta Q/\Delta t$ = linear appearance rate of mass in the receiver solution, A = cross-sectional area (i.e., 4.71 cm^2), and c(0) = initial solute concentration in the donor compartment at t = 0. The results of experiments performed in triplicate are presented as mean ±SD.

RESULTS

Physicochemical Properties

The molecular radii of the peptides were calculated from their diffusion coefficients at 25°C determined by NMR spectroscopy. Table 2 shows that the hexapeptides were the largest permeants with an average molecular radius (M_r) of ~3Å, followed by the tripeptides (M_r ~ 2.5Å) and the capped amino acids (M_r ~ 2Å). In general, the neutral Asn-containing compounds appeared to be smaller than the positively or negatively charged molecules. The lipophilicities of the peptides were determined by IAM chromatography, and the results are shown in Table 2. The calculated $\log k'_{IAM}$ values indicate significantly stronger interactions between the hexapeptides and the immobi-

Table 1. Chromatographic Conditions for HPLC Analysis of Capped Amino Acids, Tripeptides, and Hexapeptides

Compound	Stationary phase[a]	Mobile phase[b]	Retention time [min]
Ac-Asp-NH_2	RP	20 mM PB, pH 7.0 + 0.5 mM TPAB	4.6
Ac-Gly-Asp-Ala-NH_2	RP	H_2O + 0.1% TFA	6.0
Ac-Trp-Ala-Gly-Gly-Asp-Ala-NH_2	RP	H_2O/ACN (88:12) + 0.1% TFA	12.9
Ac-Lys-NH_2	SCX	10 mM PB, pH 4.0	6.1
Ac-Gly-Lys-Ala-NH_2	SCX	10 mM PB, pH 4.0	5.1
Ac-Trp-Ala-Gly-Gly-Lys-Ala-NH_2	RP	H_2O/ACN (88:12) + 0.1% TFA	13.0
Ac-Asn-NH_2	n.d.	n.d.	n.d.
Ac-Gly-Asn-Ala-NH_2	RP	H_2O	5.8
Ac-Trp-Ala-Gly-Gly-Asn-Ala-NH_2	RP	H_2O/ACN (88:12) + 0.1% TFA	11.3

[a] Stationary phases: RP = reversed-phase C_{18}, SCX = strong cation-exchange.
[b] Solvent systems: ACN = acetonitrile; PB = phosphate buffer; TFA = trifluoroacetic acid; TPAB = tetrapentylammonium bromide; n.d. = not determined.

Table 2. Physicochemical Properties of Capped Amino Acids, Tripeptides, and Hexapeptides

Compound	MW	Radius[a] [Å]	Lipophilicity[b] [log k'_{IAM}]	Net charge
Ac-Asp-NH_2	174	2.3	−1.48	negative
Ac-Gly-Asp-Ala-NH_2	302	2.5	−1.62	negative
Ac-Trp-Ala-Gly-Gly-Asp-Ala-NH_2	616	3.3	−0.82	negative
Ac-Lys-NH_2	187	1.8	−0.92	positive
Ac-Gly-Lys-Ala-NH_2	315	2.8	−0.97	positive
Ac-Trp-Ala-Gly-Gly-Lys-Ala-NH_2	629	3.0	−0.11	positive
Ac-Asn-NH_2	173	1.7	−1.20	neutral
Ac-Gly-Asn-Ala-NH_2	301	2.6	−1.24	neutral
Ac-Trp-Ala-Gly-Gly-Asn-Ala-NH_2	615	3.1	−0.37	neutral

[a] Stokes-Einstein radius calculated from the diffusion coefficient in D_2O (see Materials and Methods).
[b] Capacity factor determined from partitioning of the solute between 0.02 M phosphate buffer, pH 7.4 and an immobilized artificial membrane of phosphatidylcholine analogs (IAM.PC.DD).

lized phosphatidylcholine analogs than were found for the tripeptides and the capped amino acids. However, it should be noted that the $\log k'_{IAM}$ data suggest that all these model peptides are hydrophilic.

Transport Across Caco-2 Cell Monolayers

The ability of the compounds to permeate a biological barrier was studied using Caco-2 cell monolayers, an *in vitro* model of the intestinal mucosa. When the peptides were applied to the AP side of the cell monolayer, linear fluxes were observed for all model compounds to the receiver compartment (i.e., BL side of the monolayer). At the end of the 180 min incubation period, the mass balance for all model peptides used in this study was ≥86.9%. Figure 1A shows the time courses of the positively charged, Lys-containing compounds. The apparent permeability coefficient (P_{app}) of the capped Lys amino acid was approximately 2 times greater than that determined for the Lys-containing tripeptide ($9.86 \pm 0.18 \times 10^{-8}$ cm/s, Table 3). The larger positively charged hexapeptide, however, was significantly less able to traverse the cell monolayer ($P_{app} = 4.94 \pm 0.33 \times 10^{-8}$ cm/s). Within the series of hexapeptides, P_{app} values of the neutral and the positively charged molecules appeared to be slightly greater than the respective value determined for the negatively charged Asp-containing hexapeptide.

With the tripeptides, the permeation of the neutral Asn-containing compound was dramatically greater than that determined for the corresponding charged peptides (Fig. 1B). The negatively charged tripeptide exhibited the lowest P_{app} in this series ($7.95 \pm 1.03 \times 10^{-8}$ cm/s, Table 3). The smallest molecules under investigation were the capped amino acids (Table 2). Both the negatively and positively charged representative of this group were able to permeate Caco-2 cell monolayers to a substantially greater degree than any charged peptide used in this study (Table 3). Differences in the P_{app} values obtained for the positively and negatively charged solutes ($P_{app} = 17.16 \pm 1.58 \times 10^{-8}$ cm/s and $10.04 \pm 0.43 \times 10^{-8}$ cm/s, respectively) emphasize the effect of charge on the permeation characteristics since those two molecules are approximately of the same molecular weight and size. Unfortunately, it was impossible to analyze the samples of the transport studies performed with the capped Asn amino acid due to its insufficient retention characteristics on various HPLC stationary phases.

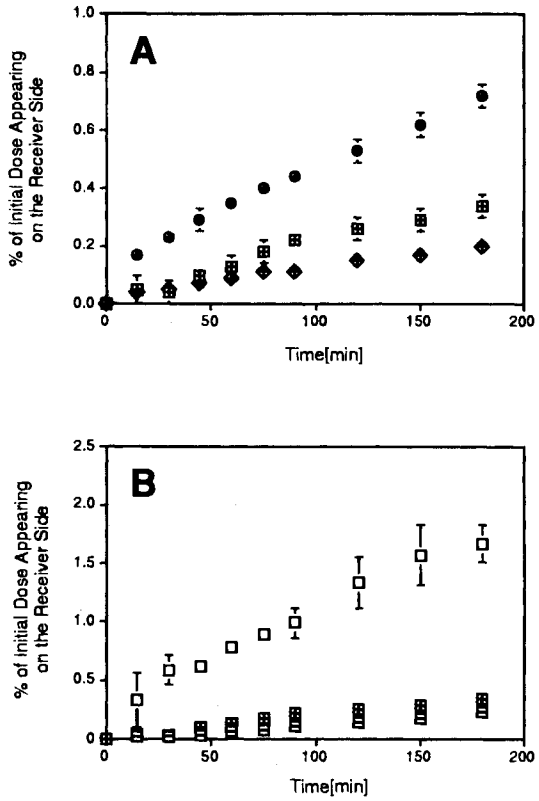

Fig. 1. Effect of size and charge on the transport of metabolically stabilized peptides across Caco-2 cell monolayers. Panel A shows the appearance of the positively charged Lys-containing capped amino acid (⊕), tripeptide (⊞), and hexapeptide (⧁). In Panel B, the time profiles of the negatively charged (⊟), positively charged (⊞), and neutral (□) tripeptides in the receiver compartment (i.e., BL side) are presented. Experiments were performed in triplicate (average ± SD).

DISCUSSION

The pathway of permeation of a solute is determined by its physicochemical properties and the barrier characteristics of the epithelium (11,22). To confirm that the solutes used in this study are paracellular markers, we characterized their lipophilicities using IAM chromatography (Table 3). The capac-

Table 3. Transport Characteristics of Capped Amino Acids, Tripeptides, and Hexapeptides Determined Across Caco-2 Cell Monolayers

Compound	Apparent Permeability Coefficient × 10^8 [cm/s]		
	X = Asp	X = Lys	X = Asn
Ac-X-NH_2	10.04 ± 0.43	17.16 ± 1.58	n.d.[a]
Ac-Gly-X-Ala-NH_2	7.95 ± 1.03	9.86 ± 0.18	25.79 ± 4.86
Ac-Trp-Ala-Gly-Gly-X-Ala-NH_2	3.19 ± 0.27	4.94 ± 0.33	5.12 ± 0.31

[a] n.d. = not determined.

ity factors ($logk'_{IAM}$) indicate that the molecules used in this study are all hydrophilic in nature. Within the series of capped amino acids, tripeptides or hexapeptides, the positively charged Lys-containing analog showed consistently stronger interactions with the IAM.PC.DD stationary phase than did the neutral or negatively charged molecules within the same series. Vallat and colleagues (23) have calculated the polarity parameter (Λ) of the side chains of various protected and unprotected amino acids and peptides which, together with the hydrophobic forces, contributes to the overall lipophilicity of a molecule. Based on the Λ values determined for Lys (3.6), Asp (2.0) and Asn (2.0), the increased apparent lipophilicity of the Lys-containing compounds seems to be due to increased polar interactions between the solute and the stationary phase rather than to increased hydrophobic interactions.

In general, it is accepted that small hydrophilic solutes traverse the cell barrier predominantly via the paracellular route, an aqueous extracellular pathway across the epithelium (3,22). The main barrier to the paracellular diffusion of molecules and ions is the region of the tight junction, which limits the transfer of solutes according to their size and charge (24,25). The P_{app} values of our model compounds (Table 3) clearly indicate a size-restricted permeation mechanism across the Caco-2 cell monolayer (capped amino acids > tripeptides > hexapeptides). In earlier studies (25,26) performed with small organic based hydrophilic markers MW ≤ 250, the permeability of the intestinal mucosa correlated reasonably well with predictions of the diffusion-filtration theory according to Renkin (27). A plot of P_{app} vs. M_r of the model peptides is presented in Fig. 2. As

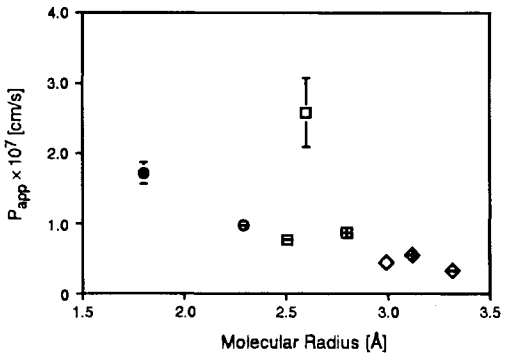

Fig. 2. Correlation between transport properties of metabolically stabilized peptides and their molecular size. Capped amino acids are represented as circles (⊕ positively and ⊖ negatively charged), tripeptides as squares (⊞ positively and ⊟ negatively charged and □ neutral), and the hexapeptides are symbolized by diamonds (◈ positively and ◈ negatively charged, and ◇ neutral). Averages ± SD are shown from three experiments.

predicted by the Renkin theory, smaller permeants traverse Caco-2 cell monolayers to a greater extent than larger molecules, emphasizing the sieving properties of the intercellular junctions.

The Asn-containing tripeptide seems to be an exception to the predicted relationship between peptide permeation characteristics across Caco-2 cell monolayers and molecular size. From the linear flux depicted for this peptide in Fig. 1B, it seems unlikely that the Asn-containing tripeptide was able to modify the physical barrier properties at the tight junctions and, thus, "self-enhancing" its own permeation across the cell monolayer. This is experimentally supported by measurements of the transepithelial electrical resistance (TEER) of Caco-2 cell monolayers in the presence and absence of this tripeptide which were not significantly different (data not shown). Therefore, the relatively high flux measured for this neutral compound suggests a different permeation pathway than passive diffusion via the paracellular route. Although the $logk'_{IAM} = -1.24$ indicates very moderate lipophilicity, the neutral Asn-containing tripeptide is a small, uncharged molecule that is able to permeate the hydrophobic core of a membrane. Assuming this transcellular pathway, comparison with the P_{app} of the neutral Asn-containing hexapeptide implies that transcellular permeation might be even more dependent on molecular size than is permeation via the paracellular pathway.

The effect of the net charge of solutes on the paracellular permeation of the intestinal mucosa has been studied systematically for a series of organic-based compounds with MW ≤ 250 (25). Protonated amines permeated the pores faster than neutral solutes of comparable size, whereas the anions of weak acids exhibited the lowest permeation characteristics. The transport behavior of the charged peptides used in this study was in accord with the fact that the paracellular route is negatively charged (28,29). Rubas and co-workers investigated the intestinal transport of cyclic RGD peptide analogs (MW between 600 and 800, with various net charges from −3 to +1) using the Caco-2 cell culture model (30). Their conclusion was that peptides with net charges of −1 and −2 are able to permeate the intestinal mucosa most efficiently. In contrast, our results indicate a favorable effect of a positive net charge for the transmucosal transport of peptides, which is in agreement with the findings of Adson and colleagues (25). However, our study shows that the effect of charge becomes less important for the transport of peptides as the molecular size of the permeants increases. At the level of hexapeptides, the contribution of net charge to the overall transport characteristics is almost negligible.

In conclusion, the experimental results presented in this paper illustrate that permeation of metabolically stable, hydro-

philic peptides is restricted by the molecular sieving properties of the intestinal mucosa. Charge selectivity for the permeation of solutes via the paracellular route (i.e., positive > negative) is only effective for smaller peptides. With increasing molecular radius, the size sieving by the pores becomes more dominant and, finally, eliminates the influence of the electric field at the junctional complex.

ACKNOWLEDGMENTS

The authors would like to thank Dr. David G. Vander Velde for his valuable help in the determination of the diffusion coefficients of the peptides. We are grateful to the Biochemical Research Service Laboratory of The University of Kansas for the synthesis of the capped tripeptides. This work was supported by research grants from Glaxo, Inc., The United States Public Health Service (GM-51633, GM-088359), Costar Corporation, and a fellowship to G. M. P. from the Swiss National Science Foundation.

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