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Extensive Hepatic Uptake of Pz-peptide, a Hydrophilic Proline-Containing Pentapeptide, into Isolated Hepatocytes Compared with Colonocytes and Caco-2 Cells

Tae Ha Shin, Pung Sok Lee, Oh-Seung Kwon[1], and Youn Bok Chung

College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea and [1]Toxicology Lab., Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, Seoul 136-790, Korea

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The objective of the present study was to investigate the uptake process of 4-Phenylazobenzoxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (Pz-peptide), a hydrophilic and collagenase-labile pentapeptide, by isolated hepatocytes. For comparison, the uptake of Pz-peptide by Caco-2 cells and colonic cells, two known paracellular routes of Pz-peptide, was also evaluated. A simple and sensitive reversed-phase HPLC assay method using UV detection has been developed. The coefficient of variation for all the criteria of validation were less than 15%. The method was, therefore, considered to be sutable for measuring the concentration of Pz-peptide in the biological cells. Pz-peptide was extensively uptaked into hepatocytes. The initial velocity of Pz-peptide uptake assessed from the initial slope of the curve was plotted as Eadie-Hofstee plots. The maximum velocity (V_{max}) and the Michaelis constant (K_m) were 0.190±0.020 nmol/min/10^6 cells and 12.1±3.23 μM, respectively. The permeability-surface area product (PS_{influx}) was calculated to be 0.0157 ml/min/10^6 cells. V_{max} and K_m values for Caco-2 cells were calculated to be 6.22±0.930 pmol/min/10^6 cells and 82.8±8.37 μM, respectively, being comparable with those of colonocytes (6.04±1.03 pmol/min/10^6 cells and 87.8±13.2 μM, respectively). PS_{influx} values for Caco-2 cells and colonocytes were calculated to be 0.0751 μl/min/10^6 cells and 0.0688 μl/min/10^6 cells, respectively. The more pronounced uptake of Pz-peptide by hepatocytes, when compared with Caco-2 cells and colonocytes, is probably due to its specific transporter. In conclusion, Pz-peptide, a paracellularly transported pentapeptide in the intestine and ocular epithelia, was uptaked into hepatocytes extensively. Although Pz-peptide is able to be uptaked into the Caco-2 cells and colonocytes, it is less pronounced when compared with hepatocytes. PS_{influx} values of Caco-2 cells and colonocytes for unbound Pz-peptide under linear conditions were less than 0.4% when compared with that of hepatocytes.

Key words: Pz-peptide, Uptake, Hepatocytes, Caco-2 cells, Colonic cells

INTRODUCTION

4-Phenylazobenzoxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (Pz-peptide) is a hydrophilic (log PC = -0.88), collagenase-labile pentapeptide with a molecular weight of 777 daltons that appears to opt for the paracellular pathway for transport across rabbit intestinal segments and Caco-2 cell monolayers (Yen *et al.*, 1994; Yen *et al.*, 1995). This peptide facilitates its own transport and that of paracellular markers up to 4,000-5,000 daltons by triggering opening of tight junctions in a transient, reversible manner (Yen *et al.*, 1994). Pz-peptide was found to stimulate transepithelial Na^+ flux across the colonic segments at the level of the amiloride-sensitive Na^+ channel, thereby triggering intracellular biochemical changes that ultimately resulted in tight junctional opening and enhanced paracellular solute transport (Yen *et al.*, 1995).

We have reported that Pz-peptide is able to penetrate the cornea and the conjunctiva at an efficiency of 15% and 83% that of propranolol (log PC = 3.21), respectively (Chung *et al.*, 1998). As is the case in the intestine, its transport pathway is probably also paracellular. Such a possibility is supported by (a) the less than 10% of Pz-peptide being in the metabolite form following corneal and conjunctival transport, and (b) enhancement in the transport of polar solutes up to approximately 4,000 daltons in the cornea

Correspondence to: Youn Bok Chung, College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea
E-mail: chungyb@cbucc.chungbuk.ac.kr

and 10,000 daltons in the conjunctiva. Indeed, Pz-peptide enhances its own transport across the cornea 2.9 times and the conjunctiva 2.2 times over the 1-5 mM concentration range. This contrasts with the 1.5-2.0-fold increase in the intestine (Yen *et al.*, 1995). Furthermore, the generally lower potency of Pz-peptide as an enhancer in the cornea and the conjunctiva relative to EDTA and cytochalasin B is similar to the situation in the intestine (Chung *et al.*, 1993).

Stimulation of transepithelial Na^+ transport through either Na^+/H^+ exchanger or the amiloride-sensitive Na^+ channel, an element in Pz-peptide's paracellular enhancement mechanism in the intestine (Brown *et al.*, 1990), did not appear to be involved in the Pz-peptide transport across the cornea and conjunctiva. It is conceivable that the amiloride-sensitive Na^+ channel is not known to exist in either the cornea or the conjunctiva (Kompella *et al.*, 1993). Nevertheless, we cannot rule out the involvement of other ion transport processes on the tear side of the cornea and the conjunctiva that may be affected by Pz-peptide.

Recently, we have found the interesting results that Pz-peptide was mainly eliminated into the bile from the blood circulation *in vivo*. The more than 60% of Pz-peptide as parent form is eliminated in the bile after its intravenous bolus injection at the dose of 0.5-8 mg/kg in rats (unpublished data).

The present study was, therefore, conducted to determine whether the hepatobiliary transport of Pz-peptide is behaved differently as its penetration across the intestinal or ocular epithelia. Thus, the objectives of this study was to determine the uptake of Pz-peptide by isolated hepatocytes. For comparison, the uptake of Pz-peptide by Caco-2 cells and colonic cells, two known paracellular routes of Pz-peptide, was also evaluated.

MATERIALS AND METHODS

Materials

Male S.D. rats (240-280 g) and male albino rabbits were purchased from SamTac (Kyunggi-Do, Korea). 4-Phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of either analytical or HPLC grade.

HPLC assay of Pz-peptide

Pz-peptide was assayed by reverse phase HPLC on a Beckman ODS C_{18} column (Beckman Instruments, Fullerton, CA, USA) that was interfaced with a Jasco HPLC system. This system consisted of a model PU-980 pump, an auto-injector model AS-950-10, an UV-VIS detector, and a LC-Net II control borwin integrator (Jasco Co. Ltd., Japan). The mobile phase was a mixture of acetonitrile and 0.1 % phosphoric acid in doubly deionized water (pH 3.0). The flow rate was 1 ml/min. The column was first equilibrated with 40% acetonitrile for 4 min, followed by a linear increase of acetonitrile to 60% for the next 5 min and holding it at 60% for the final 10 min. Thereafter, the column was reequilibrated with 40% acetonitrile for 5 min before the next injection. Pz-peptide in the eluate was monitered spectrophotometrically at 318 nm.

Caco-2 cell culture

Caco-2 cells were grown in plastic T-75 (75 cm^2) culture flasks at 37°C in a humidified 5% CO_2 incubator. Cells were seeded at a density of 1×10^4 cells/cm^2. The medium contained 4.5 g/l D-glucose, 584 mg/l L-glutamine, 5% non-essential amino acids, 1% penicillin-streptomycin and 10% fetal calf serum. The medium was changed every other day. Nine to 10-day postconfluent cells (16-17 days postplating) were used for studies.

Preparation of Caco-2 cell suspensions

Nine to 10-day postconfluent cells (16-17 days postplating) were washed twice with phosphate buffer (PBS) without Ca^{2+} and Mg^{2+}. The cells were removed from the flask by incubating the monolayer with 0.1% trypsin in 1 mM EDTA for 20 min at 37°C. Cell viability was assessed by 0.4% trypan blue exclusion and was greater than 90% in all preparations. The cells were then diluted with Hank's balanced salt solution (HBSS) to a final concentration of 1.7×10^6 cells/ml.

Preparation of colonic cell suspensions

Preparation of colonic cell suspensions was adopted from the method described by Gibson-D'Ambrosio *et al.* (Gibson-D'Ambrosio *et al.*, 1986) with minor modifications. Briefly, 4 male albino rabbits were sacrificed and the descending colon was collected. The intestinal segments were washed with ice-cold Hank's balanced salt solution (HBSS) containing 137 mM NaCl, 3.3 mM Na_2HPO_4, 5.3 mM KCl, 0.44 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 0.49 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.095 mM $CaCl_2$ and 5.5 mM D-glucose to remove the food residues. The tissues were washed three times with Ca^{2+}- and Mg^{2+}-free phosphate buffer (PBS) containing 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.6 mM KCl and 1.4 mM KH_2PO_4 (pH 7.2) and cut into 1 to 2 cm^3 pieces. While mincing the tissues, a 0.25 ml digestive solution containing 0.1 mg/ml trypsin and 1 mM EDTA was added to every 1 cm^3 of tissue to prevent tissue aggregation. Thereafter, the miced tissues were transferred to 240 ml of digestive enzyme solution and stirred by hand with tissue homogenizer at 150 strokes per min for 15 to 20 min at 25°C. The enzyme reaction was inactivated by adding 40 ml of ice-cold bovine serum. After mixing by pipetting, a 520 ml of ice-cold HBSS containing 5% serum was added

to the 280 ml of cell suspensions. The cell suspensions were passed through a tissue sieve (100 mesh) to remove tissue fragments. After sieving, the cells were collected by centrifugation at 750 g for 15 min at 4°C. Cell pellets were resuspended in HBSS and counted for viability using 0.4% trypan blue dye exclusion. About 90% viability and a cell concentration of 10^7 cells/ml was routinely obtained by the above procedure. The cells were diluted to 1.7×10^6 cell/ml for experimental purpose.

Preparation of isolated hepatocytes suspentions

Hepatocytes were isolated from male±S.D. rats (240-280 g) by the procedure of Baur *et al.* (Baur *et al.*, 1975; Iga *et al.*, 1979). After isolation, hepatocytes were suspended (1.7×10^6 cell/ml) at 0°C in the albumin-free Krebs-Henseleit buffer supplemented with 12.5 mM HEPES (pH 7.4). The viability of isolated cells was determined by 0.4% trypan blue exclusion test. The value obtained usually ranged from 95% to 98%.

Uptake of Pz-peptide by isolated hepatocytes

To determine the uptake rate of Pz-peptide, isolated hepatocytes (1.7×10^6 cell/ml) were incubated at 37°C with various concentrations of Pz-peptide. After 5 min of preincubation of the cells at 37°C, an aliquot (50 μl) of Pz-peptide was added to start uptake. Initial concentrations of Pz-peptide ranged from 10 μM to 50 μM. The incubation medium (albumin-free) in the uptake experiment contained 137 mM NaCl, 5.4 mM KCl, 1.25 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.8 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 10 mM Hepes, and 5 mM glucose (pH 7.4).

An aliquot of the cell suspension (100 μl) was taken at the indicated times (0.5, 1, 2, 3 and 5 min), and laid on top of a two-phase system in microfuge tubes. The bottom phase consisted of glycerol (100 μl). This was overlaid with 500 μl of silicone mineral oil (density 1.015). The tubes were then centrifuged for 20 s in a table-top microfuge (Beckman Instruments, Fullerton, CA, USA). Three ml of ethanol was added to the glycerol solution. After centrifugation of the mixtures at 3,000 rpm for 15 min at 4°C, Pz-peptide amounts in the organic phase were determined by HPLC as described in the above. Amounts taken up by hepatocytes were corrected for the adherent film (2.2 μl/mg protein). Adherent water volume was determined with [^{14}C] inulin and ^3H_2O (Yamazaki *et al.*, 1993). Protein was determined by protein assay kits (Bio-Rad Co. Ltd.). Bovine serum albumin was the standard.

Uptake of Pz-peptide by Caco-2 cells and colonocytes

To determine the uptake rate of Pz-peptide by Caco-2 cells or colonocytes, cell suspensions (1.7×10^6 cell/ml) were incubated at 37°C with various concentrations of Pz-peptide. After 5 min of preincubation of the cells at 37°C, an aliquot (50 μl) of Pz-peptide was added to start uptake. Initial concentrations of Pz-epetide ranged from 0.05 mM to 10 mM. An aliquot of the cell suspension (100 μl) was taken at the indicated times (2, 4 and 8 min), immediately filtering with 0.45 μm membrane filter in a vacuum system. The cells were then washed with ice-cold HBSS three times. The cells were removed and added into cornical tubes containing 3 ml of ethanol. After vortexing and centrifugation of the cell mixtures at 3,000 rpm for 15 min at 4°C, amounts taken up by the cells were determined by HPLC as described in the above.

Kinetic analysis

The initial uptake rate of Pz-peptide was obtained by the regression of the linear portion of the uptake time course (hepatocytes: 3 min, Caco-2 cells and colonocytes: 8 min). The uptake data at various Pz-peptide concentrations were plotted as a Eadie-Hofstee plot. The data for the Pz-peptide uptake were fitted to the Michaelis-Menten equation, and the uptake parameters were calculated with a nonlinear least squares method (Yamaoka *et al.*, 1981).

$$v = \frac{V_{max}\cdot[s]}{K_m+[S]}$$

v represents the initial uptake velocity of Pz-peptide. [s] is the initial concentration of Pz-peptide in the medium, and V_{max} and K_m represent the maximal uptake velocity and Michaelis constant for Pz-peptide, respectively. Furthermore, we determined the permeability-surface area product (PS_{influx}) for unbound Pz-peptide under linear conditions given by the ratio of V_{max} to K_m value (V_{max}/K_m).

RESULTS AND DISCUSSION

HPLC assay of Pz-peptide

A simple and sensitive HPLC method has been used to measure Pz-peptide and its metabolite in the cells. It involved extraction of the cell suspensions with ethanol, followed by reversed-phase HPLC using UV detection at 318 nm. The retention times of propranolol (internal standard) and Pz-peptide were 5.4 ± 0.6 min, 8.6 ±0 .7 min, respectively. The standard curves were linear from 20 ng/ml (limit of quantitation) to 5000 ng/ml for Pz-peptide. The coefficient of variation for all the criteria of validation were less than 15%. The extraction recoveries obtained for Pz-peptide were about more than 90%. The method was, therefore, considered to be sutable for measuring the concentration of Pz-peptide in the biological cells.

Pz-peptide uptake by hepatocytes

Pz-peptide was extensively uptaked into hepatocytes by 5 min at 37°C, whereas the uptaked amount by hepatocytes

was not increased at 4°C (Fig. 1). The initial velocity of Pz-peptide uptake at 37°C assessed from the initial slope of the curve was plotted as Eadie-Hofstee plots, being fitted to a straight curve (Fig. 2). The maximum velocity (V_{max}) and the Michaelis constant (K_m) were calculated to be 0.190±0.020 nmol/min/10^6 cells and 12.1±3.23 M, respectively (Table I). The permeability-surface area product (PS_{influx}) for unbound Pz-peptide under linear conditions is given by the ratio of V_{max} to K_m value, and was calculated to be 0.0157 ml/min/10^6 cells. Considering that 1 gram of liver contains 1.8×10^8 cells, the V_{max} value was extrapolated to that per gram of liver (Yamazaki *et al.*, 1993; Lin *et al.*, 1982). On the basis of the above extrapolation, PS_{influx} was calculated to be 2.83 ml/min/g liver.

Pz-peptide uptake by Caco-2 cells and colonocytes

The uptaked amount of Pz-peptide was increased linearly by 8 min in the case of both Caco-2 cells and colonic cell suspensions (Fig. 3). Thus, the initial velocity of Pz-peptide uptake assessed from the initial slope of the curve was fitted to the Michaelis-Menten equation, and the uptake parameters were calculated. When the initial velocity of Pz-peptide uptake was plotted as Eadie-Hofstee plots, a

Table I. Uptake parameters of Pz-peptide by hepatocytes, Caco-2 cells and colonocytes[a]

	V_{max}[b] (pmol/min/10^6 cells)	K_m[b] (μM)	PS_{influx}[c] (μl/min/10^6 cells)
Hepatocytes	190 ±20.0	12.1±3.23	15.7 ±3.14
Caco-2 cells	6.22± 0.930	82.8±8.37	0.0751±0.0103
Colonocytes	6.04± 1.04	87.8±13.2	0.0688±0.00844

[a] MeanS.E. of three different preparations.
[b] V_{max} and K_m values were calculated by nonlinear least squares method (Yamaoka *et al.*, 1981).
[c] PS_{influx} value was calculated from the equation (V_{max}/K_m). See details in the text.

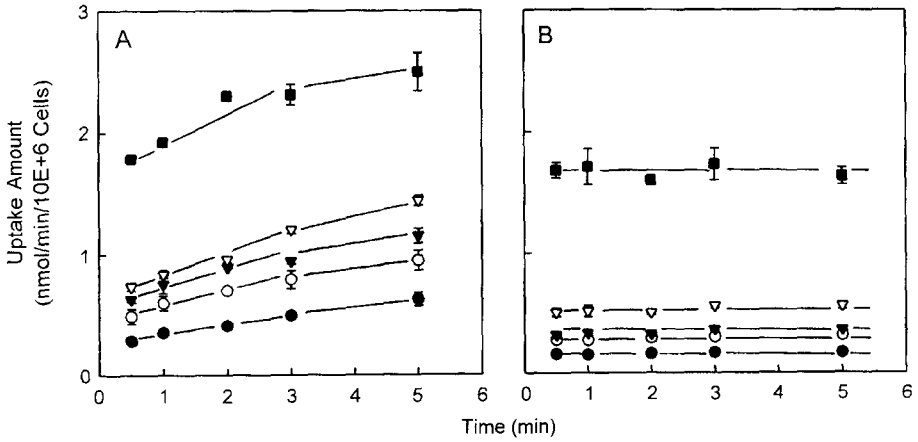

Fig. 1 The time profiles of the uptake of Pz-peptide by hepatocytes at 37°C (A) and 4°C (B). After preincubation for 5 min at 37°C, an aliquot of Pz-peptide was added to the incubation cell mixtures. An aliquot of the incubation mixture (100 μl) was taken at given times. Uptaked amount was determined with reversed phase HPLC. See details in the text. Each point represents the mean±S.E. of three preparations. Key; (●), 0.01 mM; (○), 0.03 mM; (▼), 0.05 mM; (▽), 0.1 mM; (■), 0.5 mM.

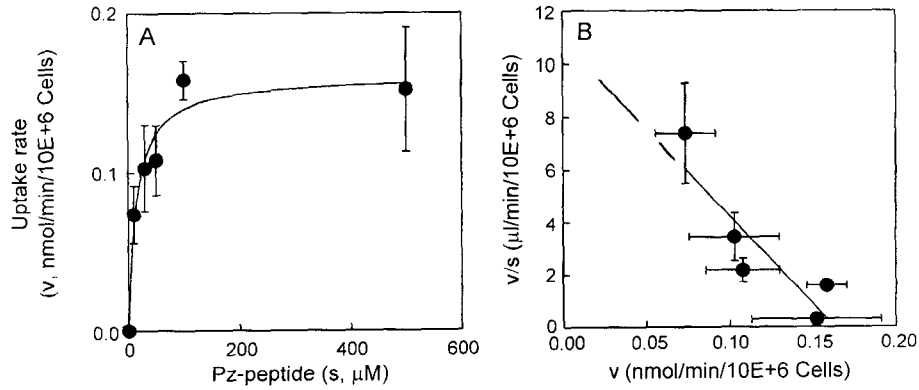

Fig. 2. Pz-peptide uptake rate (A) and Eadie-Hofstee plots (B) of Pz-peptide uptake by hepatocytes at 37°C. The initial velocity of Pz-peptide uptake was calculated by measuring the initial slop of the curve shown in Fig. 1A (within 3 min). The solid line represents the best fit to the data based on the Michaelis-Menten equation. Each point represents the mean±S.E. of three preparations.

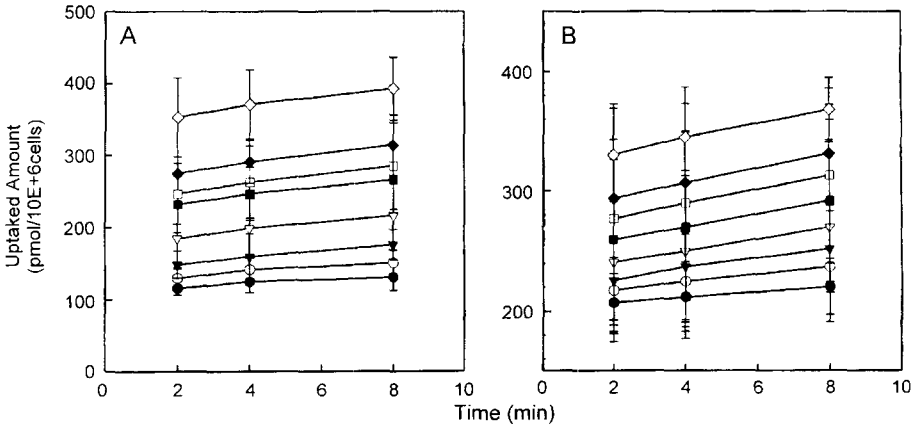

Fig. 3. The time profiles of the uptake of Pz-peptide by Caco-2 cells (A) and colonic cell suspentions (B) at 37°C. After preincubation for 5 min at 37°C, an aliquot of Pz-peptide was added to the incubation cell mixtures. An aliquot of the incubation mixture (100 μl) was taken at given times. Uptaked amount was determined with reversed phase HPLC. See details in the text. Each point represents the mean±S.E. of three preparations. Key; (●), 0.05 mM; (○), 0.1 mM; (▼), 0.2 mM; (▽), 0.5 mM; (■), 1 mM; (□) 3 mM; (◆), 5 mM; (◇), 10 mM

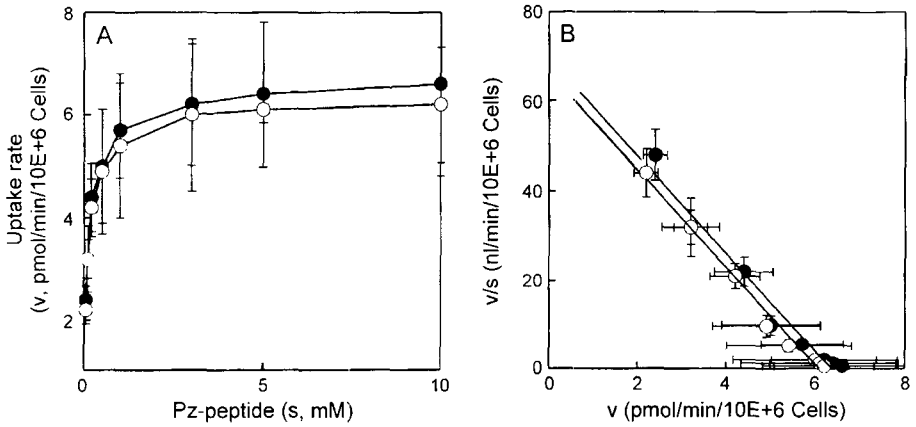

Fig. 4. Uptake rate (A) and Eadie-Hofstee plots (B) of Pz-peptide uptake by Caco-2 cells (●) and colonic cell suspentions (○) at 37°C. The initial velocity of Pz-peptide uptake was calculated by measuring the initial slop of the curve shown in Fig. 3 (within 8 min). The solid line represents the best fit to the data based on the Michaelis-Menten equation. Each point represents the mean±S.E. of three preparations.

straight curve was obtained (Fig. 4). The maximum velocity (V_{max}) and the Michaelis constant (K_m) for Caco-2 cells were calculated to be 6.22±0.930 pmol/min/10^6 cells and 82.8±8.37 μM, respectively (Table I). V_{max} and the K_m for colonocytes were comparable with those of Caco-2 cells, being calculated to be 6.04±1.03 pmol/min/10^6 cells and 87.8±13.2 μM, respectively (Table I). PS_{influx} values for Caco-2 cells and colonocytes for unbound Pz-peptide under linear conditions were given by the ratio of V_{max} to K_m value, and were calculated to be 0.0751 μl/min/10^6 cells and 0.0688 μl/min/10^6 cells, respectively.

A significant finding in the present study is that Pz-peptide (log PC = -0.88), a paracellularly transported pentapeptide in the intestine (Yen *et al.*, 1994; Yen *et al.*, 1995) and ocular epithelia (Chung *et al.*, 1998), is able to uptake into hepatocytes extensively. Such a possibility is supported by (a) the less uptake of Pz-peptide by hepatocytes at 4°C (Fig. 1B), (b) the saturation of uptake process in the Pz-peptide concentraion range of 0.01-5 mM (Fig. 2), (c) the more than 60% of Pz-peptide eliminating in the bile after its intravenous administration at the dose of 0.5-8 mg/kg in rats (unpublished data). The more pronounced uptake of Pz-peptide by hepatocytes, when compared with Caco-2 cells and colonocytes, is probably due to its specific transporter. The mechanism by which Pz-peptide uptakes into hepatocytes via transporter requires further study. Nevertheless, we cannot rule out the involvement of paracellular process in the hepatobiliary transport of Pz-peptide, because of the less than 4% of Pz-peptide being in the metabolite form following hepato-biliary transport *in vivo* (unpublished data).

Although Pz-peptide is able to be uptaked into the Caco-2 cells and colonocytes, it is less pronounced when compared with hepatocytes (Fig. 3-4, Table I). This finding should

not be surprising, since Pz-peptide has been known to be transported mainly by the paracellular routes across both intestine and Caco-2 cell monolayer (Brown *et al.*, 1990). This may be attributed to the stimulation of transepithelial Na^+ transport through either Na^+/H^+ exchanger of the amiloride-sensitive Na+ channel (Brown *et al.*, 1990). PS_{influx} values of Caco-2 cells and colonocytes for unbound Pz-peptide under linear conditions were less than 0.4% when compared with that of hepatocytes.

CONCLUSIONS

Pz-peptide, a paracellularly transported pentapeptide in the intestine and ocular epithelia, was uptaked into hepatocytes extensively. Although Pz-peptide is able to be uptaked into the Caco-2 cells and colonocytes, it is less pronounced when compared with hepatocytes. PS_{influx} values of Caco-2 cells and colonocytes for unbound Pz-peptide under linear conditions were less than 0.4% when compared with that of hepatocytes.

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