*J. Kor. Pharm. Sci.*, Vol. 24, No. 3, S49-S57 (1994)

# Studies on Permeation Enhancers for Ocular Peptide Delivery Systems : Pz-peptide as a Novel Enhancer of Ocular Epithelial Paracellular Permeability in the Pigmented Rabbit

**Youn-Bok Chung† and Vincnet H.L. Lee***

*Chungbuk National University, College of Pharmacy, Chungbuk 360-763, Korea*

**University of Southern California, School of Pharmacy, Los Angeles, CA 90033, U.S.A.*

(Received September 5, 1994)

The objective of this study was to determine whether Pz-peptide, an enhancer of hydrophilic solute permeability in the intestine, could elevate the paracellular permeability of the cornea and conjunctiva in the pigmented rabbit. The *in vitro* penetration of four hydrophilic solutes, mannitol (MW 182), fluorescein (MW 376), FD-4 (FITC-dextran, 4 KDa), and FD-10 (FITC-dextran, 10 KDa) across the pigmented rabbit cornea and conjunctiva was studied either in the presence or absence of 3 mM enhancers. Drug penetration was evaluated using the modified Ussing chamber. The conjunctiva was more permeable than the cornea to all four markers. EDTA and cytochalasin B showed higher effects on marker transport than Pz-peptide, but Pz-peptide elevated the corneal transport of mannitol, fluoresein, and FD-4 by 50%, 26%, and 50%, respectively, without affecting FD-10 transport. Possibly due to the leakier nature of the conjunctiva, 3 mM Pz-peptide elevated the transport of only FD-4 by about 45%, without affecting the transport of other markers. Furthermore, the transport of Pz-peptide itself across the cornea and conjunctiva increased with increasing concentration in the 1-5 mM range, suggesting that Pz-peptide enhanced its own permeability, possibly by elevating paracellular permeability. Effects of ion transport inhibitors on Pz-peptide transport were then investigated. Pz-peptide penetration was not changed by mucosal addition of 10 μM amiloride or 10 μM hexamethylene amiloride, inhibiting serosal $Na^+$ exit by 100 μM ouabain, or replacing $Na^+$ with choline chloride in the mucosal side buffer. These results seggested that Pz-peptide enhanced the paracellular permeability of rabbit cornea and conjunctiva and further indicate that ion transporters were not involved in the Pz-peptide induced elevation of paracellular marker permeability.

**Keywords**—Pz-peptide, Enhancer, Permeability, Ocular, Mannitol, Fluoresein, Transport

## Introduction

The design of ocular drug delivery systems is undergoing a gradual transition from an emperical to a rational base. This is partly due to a better understanding of the constraints on drug disposition in the eye and partly due to improved approaches of ocular drug delivery systems. Interest in the broad area of ocular drug delivery has increased in recent years due to an increased understanding of a number of ocular physiological processes and pathological conditions with a parallel increase in the number of drugs. Several of the constraints exerted by the eye on drug delivery are the same protective mechanism that aid the eye to serve its primary function of ensuring proper vision. These protective mechanisms include solution drainage, lacrimation, diversion of exogenous chemicals into the systemic circulation via the conjunctiva, and a highly selective corneal barrier to exclude these compou-

†To whom correspondance should be addressed.

nds from the internal eye.

Undoubtedly, corneal and conjunctival epithelia serve as barriers of the ocular surface like other surface-lining mucosa (Fig. 1). The relatively impermeability of corneal epithelium,[1] as compared to other epithelial tissues,[2, 3] is important for maintaining the homeostasis of fluid and solutes between the intracellular milieu and precorneal tear film. Most drug or ion transported across various epithelia are investigated via the transcellular and paracellular pathways (Fig. 2). The transcellular pathway involves both simple diffusion and active ionic transport utilizing metabolic energy.[4] The paracellular transport of a nonionic tracer through the lateral intercellular space usually depends on passive diffusion by the concentration gradient across the membrane.[5] Transcellular ionic transport in the rabbit cornea and conjunctiva has been studied intensively.[6, 7] However, little is known about the permeability of the paracellular pathway.[8]

A hydrophilic pentapeptide, 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (Pz-peptide, MW=777, Fig. 3), is the most frequently used synthetic substrate for screening collagenase as well as other collagenase-like enzyme. Advantages of using Pz-peptide over other collagenase substrates are as follows. First, Pz-peptide meets several criteria for circumventing the enzymatic barrier by chemical modification, i.e., this peptide contains a D-configurational amino acid arginine at the C-terminus to protect the peptide against exopeptidases and a protecting group at the N-terminus to protect it against amino-peptidases. Second, Pz-peptide is a proline-containing oligopeptide which is resistant to brush border and pancreatic proteases but is highly susceptible to collagenase degradation. A pilot study indicated that Pz-peptide was specific for collagenase and was not degraded by enzymes trypsin, chymotrypsin, thermolysin and other metalloproteases. It is therefore a good candidate for screening for collagenase activity and evaluating its influence on peptide penetration.

Earlier work revealed that, in spite of its hydrophilicity (n-octanol/buffer coefficient=4) and susceptibility to intracellular collagenase action in the intestinal epithelial cells, Pz-peptide penet-

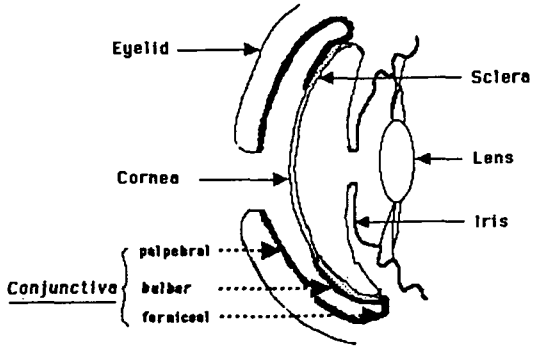


Figure 1—Sectional view of the anterior segment of the eye.

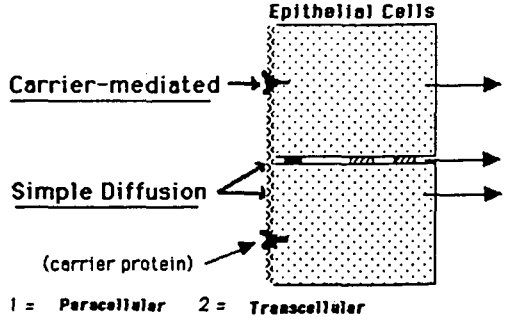


Figure 2—Chemical structure of 4-phenylazobenzoxycarbonyl-Pro-Leu-Gly-Pro-D-Arg ($P_z$-peptide).

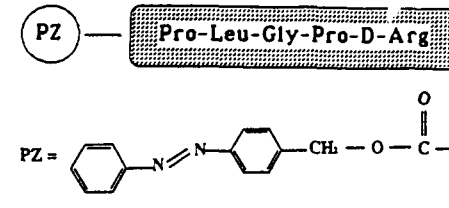


Figure 3—Chemical structure of 4-phenylazobenzoxycarbonyl-Pro-Leu-Gly-Pro-D-Arg (Pz-peptide).

rated the colonic mucosa exceedingly well than more lipophilic, metabolically stable non-peptide drugs.[9] Such good penetration characteristics of Pz-peptide were mainly due to its transport by the paracellular pathway and its action on the junctional permeability. This possibility was further confirmed by Pz-peptide induced increase in transport of hydrophilic markers mannitol and atenolol in the colon.

Enhanced penetration characteristics of Pz-peptide in vitro were also observed in the in situ absorption of Pz-peptide from both the duodenum and ascending colon of the albino rabbit into its portal vein. Although Pz-peptide was well-absorbed into the portal vein from the duodenum and ascending colon following in situ administration in the albino rabbit, less than 12% of the absorbed dose reach the systemic circulation. Low systemic bioavailability following in situ absorption suggested that hepatic first pass and biliary excretion may be partly responsible for the low bioavailability of Pz-peptide. Furthermore, in spite of its susceptibility to intracellular collagenase and its low distribution coefficient, the amount of Pz-peptide absorbed into portal circulation was 10 times higher than its hydrolytic product, Pz-Pro-Leu, and was comparable to those of metabolically stable beta-blockers. Thus, consistent with the in vitro penetration results, these findings suggest that Pz-peptide is mainly transported in the liver by the paracellular route.

Presumably, Pz-peptide was transported across the mucosa mainly as a result of its action on the tight junctions. These findings set the stage for further studies to elucidate the basis for the excellent penetration characteristics of Pz-peptide, thereby providing important information for future design of peptides that would opt for the paracellular pathway for transport.

The objective of this study was to determined whether Pz-peptide, an enhancer of hydrophilic solute permeability in the intestine, could elevate the permeability of the cornea and conjunctiva in the pigmented rabbit. For this purpose, penetration of Pz-peptide across the pigmented rabbit cornea and conjunctiva was investigated. Effect of Pz-peptide on the in vitro penetration of hydrophilic markers was also determined. Furthermore, enhanced penetration characteristics of Pz-peptide in various mucosa were discussed.

## Materials and Methods

### Materials

Male, Dutch-belted pigmented rabbits, 1.8~2.2 kg, were purchased from Irish Farm Rabbitry (Los Angeles, CA). $^{3}$H-mannitol (specific activity, 1 mCi/nmol) was purchased from New England Nuclear (Boston, MA). EDTA, cytochalasin B, fluorescein, FITC-dextran 4,000 and FITC-dextran 10,000 were obtained from Sigma (St. Louis, MO). All other reagents were of either analytical or HPLC grade.

### HPLC assay of Pz-peptide

The amount of Pz-peptide in the serosal side was assayed by reverse phase HPLC on a Beckman ODS C18 column (Beckman Instruments, Fullerton, CA) interfaced with a Shimadzu HPLC system consisting of a model LC-6A pump, an autoinjector model SIL-6A, an UV-VIS detector, and a Chromatopac model C-R3A data station (Shimadzu Instruments, Kyoto, 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 to 40% acetonitrile for 5 min before the next injection. Pz-peptide in the eluate was monitered spectrophotometrically at 318 nm. The retention times of propranolol (internal standard), Pz-peptide and Pz-product were 5.4 ± 1.2 min,

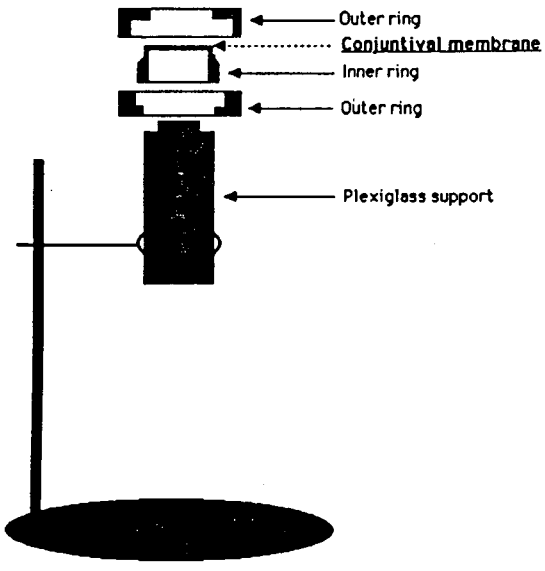


**Figure 4**—Graphical illustration of the plastic support and rings for mounting conjunctival membranes.

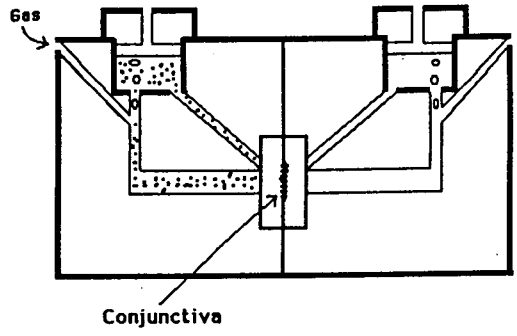


**Figure 5**—Graphical illustration of the *in vitro* transport medel-modified Ussing chamber.

8.6 ± 1.3 min and 13.4 ± 1.1 min, respectively. The inter- and intra-run variations were 3%, 6% and 5%, respectively.

**Corneal and Conjunctival penetration of Markers**

Rabbit cornea and conjunctiva were excised and mounted in modified Ussing chambers as described in Fig. 4-5.[10] Two and one-half ml of GBR (O'Brien and Edelhauser, 1977), preadjusted to pH 7.4 and 300 ± 30 mOsm/kg, were added to the serosal side. An equal volume of the same solution containing 0.5 μCi/m*l* mannitol or 0.5 mg/m*l* markers (fluorescein,. FD-4, FD-10) was then added to the mucosal side. The contents of each chamber were mixed by bubbling a 95% $O_2$-5% $CO_2$ mixture at the rate of three to four bubbles per second, and the temperature within each shamber was maintained at 37 ± 1 ℃ by a circulating water bath. Periodically up to 240 min, a 100 μ*l* aliquot was taken from the serosal side for analysis and replaced immediately by an equal volume of GBR solution.

The concentration of mannitol was determined by a liquid scintillation counter (Beckman LS1801, Fullerton, CA) after mixing with 5 ml of liquid scintillation cocktail (Ecosint, Nationalo Diagnostics, Manville, NJ). The amount of fluorescein, FD-4, and FD-10 was measured in a fluorescence spectrophotometer (Perkin-Elmer, 10 S fluorescence spectrophotometer, Norwalk, CT) at an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

The apparent permeability coefficient ($P_{app}$, cm/sec) was calculated from the following equation.

$$P_{app} = Flux/A \times Co \times 60 \quad (1)$$

where flux (nmol/min) is the slope of the linear portion of a plot of amount of drug accumulated vs. time, A is the surface area of the cornea (1.089 $cm^2$) or conjunctiva (0.95 $cm^2$), Co if the initial drug concentration in the donor compartment (nmol/m*l*), 60 is the factor for conversion from minutes to seconds.

**Effect of Ion Transport Blockers on the Penetration of Pz-peptide**

After 2.5 m*l* of GBR were added to the serosal side, an equal volume of the same solution containing 3 mM Pz-peptide with or without 10 μM amiloride or hexamethylene amiloride was added to the mucosal side. The $Na^+$ free GBR in mucosal side was used to investigate Na effect on Pz-peptide penetration. Ouabain (100 μM) was added to serosal side, and the same solution containing 3 mM Pz-peptide or 0.5 mg/m*l* mannitol was added

to mucosal side. Papp values of Pz-peptide and mannitol were calculated as the same way des cribed in the above.

**Statistical Analysis**

Comparison between two means was performed using the unpaired Student's t-test. One-way analysis of variance was used to test for significant difference between groups. Statistical significance was defined as $P \leq 0.05$.

## Results

**Pz-peptide and Pz-product Transport across Cornea and Conjunctiva**

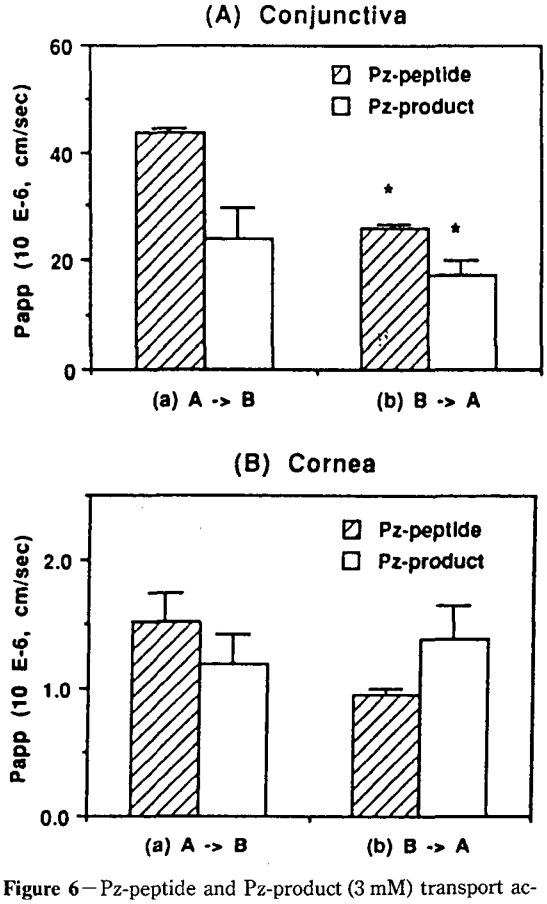


**Figure 6**—Pz-peptide and Pz-product (3 mM) transport across conjunctiva (A) and cornea (B). Trnsport from apical to basolateral side (A->B) or basolateral to apical side (B ->A) was determined.

Pz-peptide and Pz-product (3 mM) transport across cornea and conjunctiva were shown in Fig. 6. Trnsport from apical to basolateral side was larger than that of opposite direction except Pz-product in cornea. The quantitative permeability of cornea was about 10-fold lower than that of conjunctiva, indicating that the conjunctiva was more permeable to Pz-peptide and Pz-product than cornea. The formation of metabolite, i.e., Pz-product, during 4 hr transport of Pz-peptide was shown in Fig. 7. Pz-peptide was more extensively metabolized in cornea than conjunctiva.

**Concentration Dependent Transport of Pz-peptide and Pz-product**

The transport of Pz-peptide across the cornea and conjunctiva was determined in the 1-5 mM concentration range. The permeability coefficient (Papp, cm/sec) of Pz-peptide in cornea and conjunctiva increased with increasing its concentration, suggesting that Pz-peptide enhanced its own permeability (Fig. 8). These may mainly be due to its elevating paracellular junctional permeability.

**Effect of Pz-peptide or Pz-product on the Corneal and Conjunctival Penetration of Atenolol**

Effect of Pz-peptide or Pz-product (1-5 mM) on the penetration of atenolol, a hydrophilic solute, was shown in Fig. 9. Pz-peptide or Pz-product

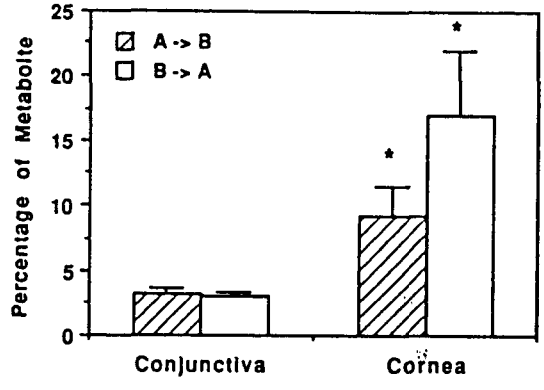


**Figure 7**—The formation of metabolite, i.e., Pz-product, during 4 hr transport of Pz-peptide (3 mM).

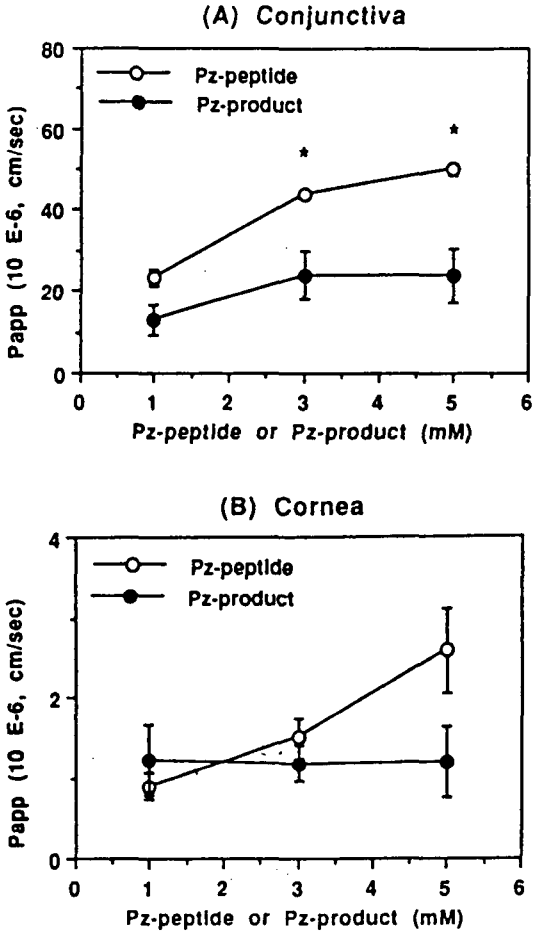


**Figure 8**—Concentration dependent transport of Pz-peptide and Pz-product across the conjunctiva (A) and Cornea (B) at the 1-5 mM concentration range.

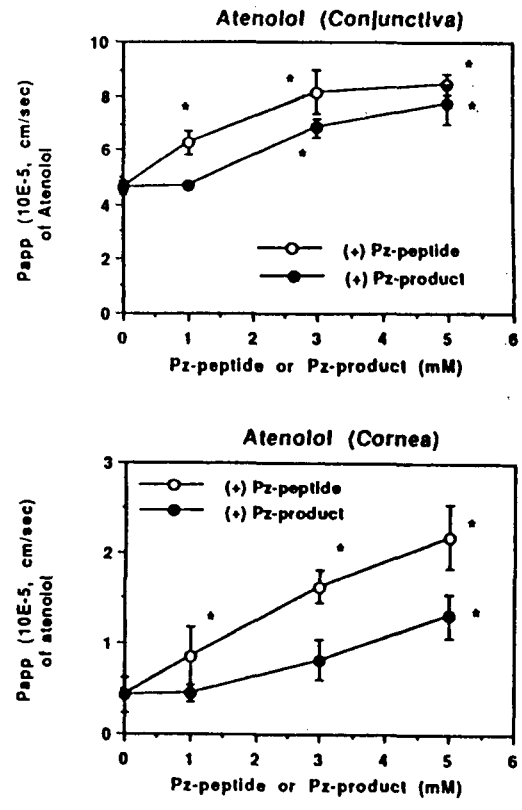


**Figure 9**—Effect of Pz-peptide or Pz-product (1-5 mM) on the penetration of atenolol across conjunctiva (A) and Cornea (B).

increased the permeability of atenolol across the cornea and conjunctiva in a concentration dependent manner, suggesting that Pz-peptide was transported across the cornea and conjunctiva mucosa mainly as a result of its action on paracellular permeability and effects on the tight junctions.

**Effect of Paracellular Transport Enhancers on the Corneal and Conjunctival Penetration of Hydrophilic Markers**

Four hydrophilic paracellular markers with different molecular radii were used to determined the extent of enhancement of Pz-peptide. Pz-peptide at 3 mM elevated the corneal tranport of mannitol (3.6 A, MW 182), fluorescein (5.5 A, MW 376), and FD-4 (14 A, 4 K) by 50%, 26%, and 50%, respectively, while no enhancement was found in FD-10 (22 A, 10 K). Possibly due to the leakier nature of the conjunctiva, Pz-peptide elevated the transport of only FD-4 by about 45%, without affecting the transport of other markers. The extent of enhancement by Pz-peptide was further compared with two other paracellular penetration enhancers EDTA, an extracellular calcium chelator, and cytochalasin B (CB), a disrupting agent of actin filaments. Both EDTA and cytochalasin B, two commonly used paracellular penetration enhancers, increased marker transport to a greater degree than Pz-peptide. These results suggested that Pz-peptide may open a given tight

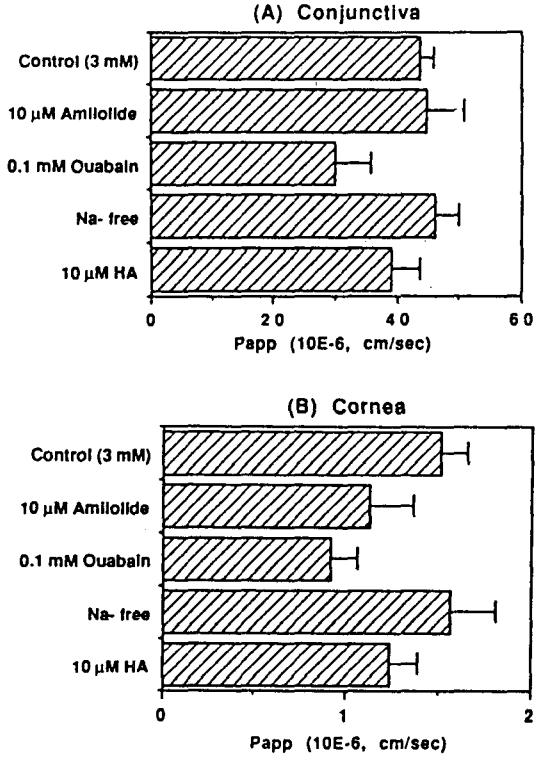


**Figure 10**—Effect of ion transport inhibitors on the conjunctival (A) and Corneal (B) penetration of Pz-peptide (3 mM).

junction to a smaller extent and it may open a smaller fraction of tight junctions than do EDTA and cytochalasin B.

**Effect of Ion Transport Inhibition on the Corneal and Conjunctival Penetration of Pz-peptide and Mannitol**

To investigate the indirect action of ion flux on junctional permeability, effect of the ion transport inhibitors on the corneal and conjunctival penetration of Pz-peptide was determined. Pz-peptide penetration was not changed by the mucosal addition of 10 μM amiloride or 10 μM hexamethylene amiloride, a $Na^+$ channel bloker and $Na^+/H^+$ exchange blocker, respectively (Fig. 10). Ouabain at 100 μM drcreased Pz-peptide penetration by about 30% but there was no significant difference from control value. The effect of Pz-peptide on $Na^+$ channel activity was further confirmed by mucosal addition of $Na^+$ free GBR. Replacing Na with choline chloride in the mucosal side GBR buffer did not effect on Pz-peptide penetration. These results indicated that electrogenic $Na^+$ absorption was not involved in the Pz-peptide induced elevation of paracellular marker permeability. This notion is supported by the fact that $Na^+$ channel dose not exist in conjuntival epithelial cells.

Although $Na^+$ absorption did not influence Pz-peptide penetration in the cornea and conjunctiva, its penetration was reduced by mucosal addition of oubain, $Na^+/K^+$ ATPase inhibitor. To confirm the involvement of $Na^+/K^+$ exchange in the paracellular permeability, the effect of ouabain on the mannitol penetration was determined. The Papp value of mannitol was not changed by ouabain, suggesting that ion transport was not involved in Pz-peptide paracellular permeability.

## Discussion

Pz-peptide, a collagenase labile pentapeptide, penetrated to a greater extent in the descending colon, the region with highest intracellular collagenase activity.[9] Furthermore, in spite of the low distribution coefficient for Pz-peptide, it penetrated as well as or even better than more lipophilic, metabolically stable atenolol and propranolol in both upper and lower G.I. segments.[9] Presumably, Pz-peptide was transported across the intestinal mucosa mainly by the paracellular route and may have effects on the tight junctions in the lower G.I. segments. Such a possibility was further confirmed in corneal and conjunctival epithelia in this study. These was supported by a) its enhanced penetration with increasing peptide concentration and b) Pz-peptide induced increase in penetration of hydrophilic molecule across the corneal and conjunctiva epithelia.

Pz-peptide induced transport of paracellular markers across the ocular epithelia was less pro-

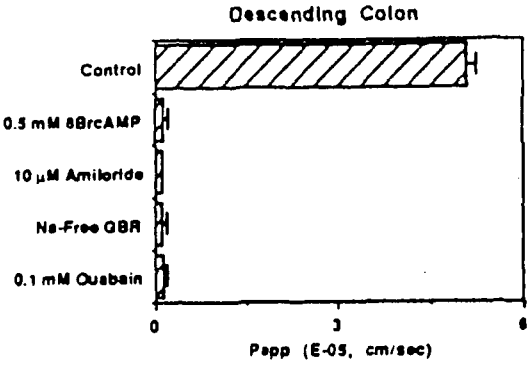


**Figure 11**—Effect of $Na^+$ modulation on 3 mM Pz-peptide penetration.

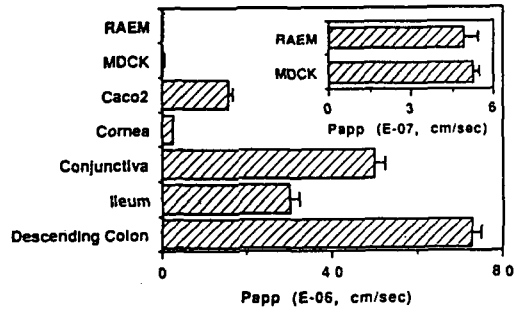


**Figure 12**—Penetration of 5 mM Pz-peptide across various epithelia.

nounced for compounds with molecular radius greater than 14 A, indicating the limitation of the tight junction pore size. In addition, for a given paracellular marker, the effect of Pz-peptide was lower than that of EDTA and cytochalasin B at the same concentration, suggesting that Pz-peptide may open tight junctions to a smaller extent and/or a smaller fraction of the tight junctions. These findings were compatible in the results in intestine and implied the importance in regulation of the degree of paracellular permeability in delivering peptide and protein drugs across the intestinal mucosa in a more size discriminat fashion.

Ionic transport inhibitors did not effect on the corneal and conjunctival penetration of Pz-peptide, suggseting that ion transport was not involved in Pz-peptide paracellular permeability (Fig. 10). On the other hand, one possible means of indirect action on junctional permeability is by changing ion fluxes across the intestine. Electrogenic $Na^+$ absorption in the colon is mediated by $Na^+$ channels on the apical membrane. Pz-peptide may activated sodium channels in the descending colon, thereby facilitating its penetration and enhancing marker transport. As shown in Fig. 11, Pz-peptide penetration was abolished by mucosal addition of 10 M amiloride, ouabain, or replacing $Na^+$ with cholin chloride in the buffer in the descending colon. Furthermore, inhibition of $Na^+$ transport reversed Pz-peptide induced enhancement of paracellular markers mannitol and FD-4. These findings indicate a novel means for improving the paracellular transport of peptide and proteins in the colon by modulation of Na+ transport.

Pz-peptide is able to penetrate across both leaky and tight epithelia. However, the degree of increase in junctional permeability by Pz-peptide is not correlated with the tightness of the junctions, being highest across the descending colon and lowest across the tight monolayer of MDCK (Madin-Darby canine kidney) and RAEM (Rat alveolar epithelial monolayers) (Fig. 12). The rank order was descending colon>conjunctiva>ileum >$CaCo_2$ (Human colon carcinoma cell line)> MDCK=RAEM. There was about a 100 times difference in Papp between the descending colon and RAEM. Its Papp's in the leaky epithelia ileum and $CaCo_2$ are lower than that in a relatively tight epithelia, the conjunctiva. Such a difference in Papp vs tightness relationship in various epithelia may be due to the difference in cellular function and endogenous regulation of junctional permeability in each tissue.

## Conclusion

We have demonstrated that Pz-peptide penetrates across the cornea and conjunctiva by para-

cellular pathway as indicated by a) its exceedingly well penetration characteristics in spite of its hydrophilic properties and susceptibility to intrace llular collagenase action and b) its ability to increase penetration of hydrophilic molecules and increase its own permeability by increasing concentration. The cellular locus where Pz-peptide may act to facilitate paracellular transport was further identified in the various mocosa including descending colon. These findings have provided important clues on the future design of peptide drugs that would opt for the paracellular pathway for transport and would act to facilitate paracellular transport in a transient, reversible and more size discriminant manner by physiologically regulating the tight junctions.

## Acknowledgements

The authors wish to express their appreciation to Dr. Yong-Hee Lee (Present adress: Lucky Co.) for his kind advice.

## References

1) S. Mishima and B.O. Hedbys, The permeability of the corneal epitheliaum and endothelium to water, *Exp. Eye. Res.*, **6**, 10 (1967).
2) I.G. Fels, Permeability of the anterior bovine lens capsule, *Exp. Eye. Res.*, **10**, 8 (1970).
3) J.E. Pederson, Fluid permeability of monky ciliary epithelium *in vivo*, *Invest. Ophthamol. Vis. Sci.*, **23**, 176 (1982)
4) P. Claud, mophological factors influencing transepithelial permeability : A model for the resistance of the zonula occludent, *J. Membr. Biol.*, **39**, 219 (1978)
5) E. Fromter and J.Diamond, Route of passive ion permeation in epithelia, *Nature (New Bio.)*, **235**, 9 (1972).
6) W.S. Marshall and S.D. Klyce, Cellular and paracellular pathway resistances in the tight Cl-secreting epithelium of the rabbit cornea, *J. Membrane Biol.*, **73**, 275 (1983).
7) S.D. Klyce and C.E. Crosson, Transport processes across the rabbit corneal epithelium. A Review, *Current Eye. Res.*, **4**, 323 (1985).
8) A.J.W. Huang, S.C.G. Tseng and K.R. Kenyon, Paracellular permeability of corneal and conjunctival epithelia, *Invest. Ophthalmol. Vis. Sci.*, **30**, 684 (1989).
9) W.C. Yen, A. Nishiura, H. Yamahara, and Vincent H.L.Lee, Influence of collagenase on the intestinal transport of proteolytically labile peptide across various epithelia, *Proceed. In tern. Symp. Contrl. Rel. Bioact. Mater.* **18**, 95-96 (1991).
10) V.H.L. Lee, D.S. Chien and H. Sasaki, Ocular keton reductase distribution and its role in the metabolism of ocularly applied levobunolol in the pigmented rabbit, *J. Pharmacol. Exp. Ther.*, **246**, 871 (1988).