0026-895X/05/6703-749–756$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 67:749–756, 2005

Vol. 67, No. 3
8375/1196087
Printed in U.S.A.

MOLECULAR PHARMACOLOGY

A Novel Strategy for the Enhancement of Drug Absorption Using a Claudin Modulator

Masuo Kondoh, Akane Masuyama, Azusa Takahashi, Nagayoshi Asano, Hiroyuki Mizuguchi, Naoya Koizumi, Makiko Fujii, Takao Hayakawa, Yasuhiko Horiguchi, and Yoshiteru Watanbe

Department of Biopharmaceutics and Pharmaceutics, Showa Pharmaceutical University, Tokyo, Japan (M.K., A.M., A.T., N.A., N.K., M.F., Y.W.); Project III, National Institute of Health Sciences, Osaka Branch, Fundamental Research Laboratories for Development of Medicine, Osaka, Japan (H.M.); National Institute of Health Sciences, Tokyo, Japan (T.H.); and Department of Bacterial Toxicology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan (Y.H.)

Received October 17, 2004; accepted December 14, 2004

ABSTRACT

Claudin, a tight junction integral membrane protein and a family of proteins, forms the actual sealing element of the tight junction. There are more than 20 members of the claudin family with different tissue-specific expression and barrier functions. Thus, a family of claudin may be a target for modifying the absorption of drugs. Here, we examined whether modulation of claudin could be used to enhance drug absorption. In the current studies, we used a C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) as a modulator of claudin-4. The absorption of dextran was assessed in an in situ loop assay in rats to evaluate the absorption-enhancing effects of C-CPE. Treatment with C-CPE dose-dependently enhanced the absorption of dextran (mol. wt. 4000). These effects were not accompanied by injury of the intestinal mucosa as assessed by leakage of lactose dehydrogenase and histological observation. C-CPE was over 400-fold more potent at enhancing dextran absorption than capric acid, a clinically used enhancer of absorption. C-CPE interacted directly with claudin-4, and C-CPE lacking a part the C terminus neither bound claudin-4 nor enhanced absorption in the rat jejunum. These results suggest that C-CPE enhances the absorption of dextran in rat jejunum, apparently through interactions with claudin-4, and this effect may represent an effective novel strategy for enhancing the absorption of drugs.

Recent progress in genomics and proteomics has made it possible to use not only low molecular weight organic compounds but also high molecular weight peptides, proteins, and DNA as drugs for clinical therapy. Efficient delivery is a key issue in the clinical use of these higher molecular weight drugs. Passing across the epithelia is a first key step for absorption of a drug, irrespective of oral, intravenous, and interdermal administration (Powell, 1981). There are two pathways for absorption in epithelia: transcellular and paracellular. The former pathway is mediated by transporters, which are membrane proteins that regulate the cellular influx and efflux of endogenous substrates, including amino acids and fatty acids. Some drugs that mimic endogenous transporter substrates can be taken up through these proteins (Adibi, 1997; Rao et al., 1999; Edwards, 2001). Uptake via the paracellular pathway, on the other hand, is attractive route for the absorption of high molecular weight and large-sized drugs. Conventional methods for taking up molecules via the paracellular pathway include chelating divalent cations and treatment with medium chain fatty acids, including capric acid (C10). These can be toxic in the treated region, and, moreover, the specificity of the absorption-enhancing effects are very poor (Yamamoto et al., 1996). Thus, the clinical use of these absorption enhancers remains limited.

The primary barrier to the transport of solutes from the apical to the basal side are the tight junctions (TJs), specialized membrane domains located at the most apical region of polarized epithelial and endothelial cells (Mitic et al., 2000; Anderson, 2001). The TJ is a complex of various proteins, including junctional adhesion molecule (JAM), occludin, and claudin. JAM is a junction-associated membrane protein

This work was partly supported by a grant-in-aid from the Ministry of Education, Science and Sports, Japan.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.008375.

ABBREVIATIONS: TJ, tight junction; CPE, *Clostridium perfringens* enterotoxin; C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin; JAM, junctional adhesion molecule; CPE-R, receptor for *Clostridium perfringens* enterotoxin; FD, fluorescein isothiocyanate-dextran; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; AUC, area under the plasma concentration-time curve; LDH, lactose dehydrogenase; N-CPE, N-terminal fragment of *Clostridium perfringens*.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on August 27, 2017

with a molecular mass of 40 kDa (Martin-Pardura et al., 1998), and its involvement in the barrier function of the TJ remains unclear. Occludin is an integral membrane protein bearing four transmembrane domains and with a molecular mass of 65 kDa. It is predicted to play a role not only in the structure but also in the barrier function of the TJ (Furuse et al., 1993; Balda et al., 1996; McCarthy et al., 1996; Chen et al., 1997; Wong and Gumbiner, 1997). However, deletion of occludin does not result in disruption of the TJ barrier function (Balda et al., 1996; Saitou et al., 1998), and whether occludin is a major constituent of TJs remains uncertain. In contrast, claudin is now believed to be a major constituent of TJ (Morita et al., 1999; Sonoda et al., 1999; Tsukita and Furuse, 2000; Tsukita et al., 2001; Furuse et al., 2002; Nitta et al., 2003). Claudins, which have molecular masses of ~23 kDa, comprise a multigene family consisting of more than 20 members (Morita et al., 1999; Tsukita et al., 2001). TJ strands are copolymers of heterogeneous claudin species and occludin, and heterogeneous claudin species constitute the backbone of TJ strands in situ (Furuse et al., 1999). It is interesting that the localization and barrier function of each member of the claudin varies among tissues. For example, loss of function of the blood brain-barrier is observed in claudin-5-deficient mice (Nitta et al., 2003), whereas epidermal barrier function is disrupted in claudin-1-deficient mice (Furuse et al., 2002). In addition, in claudin-11-deficient mice, TJs are absent in the Sertoli cells in the testis (Gow et al., 1999). These findings indicate that disruption of claudin function with an inhibitor is a promising means for the tissue-specific enhancement of absorption.

Clostridium perfringens enterotoxin (CPE) is a 35-kDa protein that causes food poisoning in humans (McClane, 1994). The C terminus of CPE (C-CPE) is involved in the binding of CPE to the target cells, whereas the N terminus is involved in its cytotoxicity (Kokai-Kun and McClane, 1997). Katahira et al. (1997) identified the receptor for CPE (CPE-R) and found that CPE-R has significant similarity to the rat androgen withdrawal apoptosis protein RVP1. The biological function of CPE-R and RVP1 remained unknown until Morita et al. (1999) found that CPE-R is claudin-4 and that RVP1 is claudin-3. CPE is cytotoxic to claudin-3- and -4-positive cells, but this effect is lost if its N terminus is removed. It is noteworthy that treatment of the cells with C-CPE reduces the transepithelial electrical resistance, a typical measurement of the barrier function of the TJ (Sonoda et al., 1999). These findings indicate that C-CPE may be a promising lead compound for the development of a novel absorption-enhancing agent. In this study, we investigated whether inhibition of claudin by C-CPE can enhance the absorption of dextran in an in situ loop assay and whether this is mediated by claudin-4.

Materials and Methods

Animals, Cells, and Reagents. Wistar male rats (250–280 g) were obtained from Animal and Material Laboratories, Inc. (Tokyo, Japan). The rats were maintained in an environmentally controlled room (23 ± 1.5°C) with a 12-h light/12-h dark cycle and allowed access to standard rodent chow and water ad libitum. The rats were allowed a period of at least 1 week to adapt. Fluorescein isothiocyanate-dextran (FD) with molecular weights of 4000 (FD-4), 10,000 (FD-10), 20,000 (FD-20), and 40,000 (FD-40) were obtained from Sigma-Aldrich (St. Louis, MO). Anti-claudin-1, -2, -3, -4, and -5 antibodies were purchased from Zymed Laboratories (South San Francisco, CA), and horseradish peroxide-labeled secondary antibodies and anti-His-tag antibody were obtained from Cell Signaling Technology Inc. (Beverly, MA) and Novagen (Madison, WI), respectively. Ni-agarose resin and a Ni-agarose column were obtained from Invitrogen (Carlsbad, CA). All reagents were of research grade.

Purification of C-CPE. C-CPE was purified as described previously (Katahira et al., 1997). In brief, the DNA fragment corresponding to amino acid residues 184 to 319 of CPE was cloned into pET16b vector (Novagen). The C-CPE plasmid (pET16bHis$_{10}$C-CPE) was transfected into *Escherichia coli*. BL21 (DE3) strains and the synthesis of C-CPE were stimulated by addition of isopropyl β-D-thiogalactopyranoside (Wako Pure Chemicals, Osaka, Japan). The cells were harvested, resuspended in buffer A [10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride, and 1 mM β-mercaptoethanol], and then lysed by sonication. The lysates were applied to the Ni-column, and C-CPE bound to the Ni-resin was eluted with a 0 to 400 mM imidazole gradient in buffer A. The solvent for C-CPE was changed to phosphate-buffered saline (PBS) by gel filtration using PD-10 column (Amersham Biosciences Inc., Piscataway, NJ), and the resulting C-CPE solution was stored at −80°C until use. The concentration of C-CPE was estimated by a protein assay kit using bovine serum albumin as a standard (Bio-Rad, Hercules, CA). The presence of C-CPE was confirmed by Western blotting with a His-Tag antibody (data not shown).

Preparation of C-Terminal Truncated C-CPE. C-CPE lacking the C-terminal amino acids 290 to 319 (C-CPE289) or 304 to 319 (C-CPE303) were prepared as follows. The C-terminal-deficient C-CPEs were cloned by polymerase chain reaction amplification using pET16bHis$_{10}$C-CPE plasmid as a template, a common sense primer (5′-ctcgagagatgtgttttaacagttcca-3′, underline indicates the XhoI site), and an antisense primer for C-CPE289 (5′-ggatcctatatatcaacataatgatcttt-3′, underline indicates the BamHI site) or C-CPE303 (5′-ggatcctaattagctttcattacaagaac-3′, underline indicates the BamHI site). The resulting C-terminal C-CPE fragment was subcloned into the pGEM T-Easy Vector (Promega, Madison, WI), and the sequences of C-CPE289 and C-CPE303 were confirmed. The XhoI/BamHI fragment of the pTA/C-CPE289 or pTA/C-CPE303 was cloned into the pET16b vector at the XhoI/BamHI site. His-tagged C-CPE289 and C-CPE303 were then purified as described previously with some modifications (Katahira et al., 1997). In brief, pET16b plasmid with C-CPE289 and C-CPE303 was introduced into the *E. coli* BL21 (DE3) strain, and expression of the C-CPE289 and C-CPE303 was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (Wako Pure Chemicals). The *E. coli* cells were harvested and lysed in buffer A containing 8 M urea. The lysates were centrifuged, and the resulting supernatants were applied to a Ni-NTA resin (Invitrogen). The His-tagged proteins were eluted in a gradient of 0 to 2 M imidazole in buffer A. The purification of the His-tagged C-CPE289 and C-CPE303 were confirmed by SDS-PAGE followed by staining the gels with Coomassie Brilliant Blue and by Western blotting with a His-tag antibody (data not shown).

In Situ Loop Assay. The experimental protocol for in situ loop assay was approved by the ethics committee of Showa Pharmaceutical University. Intestinal absorption of FDs was examined by in situ loop assay as follows. Rats were anesthetized with thiamylal sodium (Mitsubishi Pharma Co. Ltd., Osaka, Japan). A midline abdominal incision was made, and the lumen of the jejunum or colon was washed with saline. A jejunal or colonal loop (5 cm in length) was prepared by closing both ends with sutures. Test samples were dissolved in 200 μl of PBS and then administered into the jejunal or colonal loop. Blood was collected from the carotid vein at the indicated periods. The plasma concentration of FDs was determined with a fluorescence spectrophotometer (Fluoroskan Ascent FL; Thermo Electron Corp., Waltham, MA). The area under the plasma concentration-time curve from 0 to 4 h (AUC_{0-4}) was calculated by the trapezoidal method.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on August 27, 2017

Side Effects of C-CPE on Rat Intestine. After 4 h of administration of C-CPE into the jejunum, the PBS buffer was recovered for determination of lactose dehydrogenase (LDH) leakage. The release of LDH was examined using a commercially available kit (Wako Pure Chemicals). Histological observation of the intestinal mucosa of the C-CPE-administered rat was also carried out. In brief, the rat jejunum was removed, fixed with formalin, and stained with hematoxylin and eosin, and the mucosa was observed under a microscope. The histological damage was scored by the indices as described in Table 1.

Interaction of Claudin-4 and C-CPE. The mucosa of the jejunum or colon was collected with a scraper and washed twice with PBS. The mucosa was lysed in lysis buffer (1% Triton-X-100, 0.2% SDS, 150 mM NaCl, 10 mM HEPES, pH 7.4, 2 mM EDTA, and 1% protease inhibitor cocktail; Sigma-Aldrich). The lysate was mixed with C-CPE at 4°C for 2 h, and then Ni-resin was added. After a 2-h incubation at 4°C, the mixture was centrifuged at 5000*g* for 1 min, and the sedimented resin and supernatant were recovered. The resin was washed with the lysis buffer, and the supernatant was mixed with SDS-sample buffer and boiled for 3 min. The SDS-solubilized samples were subjected to 15% SDS-PAGE, and Western blot analysis was performed using antibodies against claudin and His-Tag. Lysate of the jejunum or colon was also used for Western blotting for claudin family proteins. The immunoreactive bands were identified by reaction with horseradish peroxidase-labeled secondary antibodies, followed by visualization with enhanced chemiluminescence reagents (Amersham Biosciences Inc.).

Statistical Analysis. Differences were statistically evaluated using one-way analysis of variance followed by Dunnett's method. The level of significance was set at $p < 0.05$.

Results

Effect of C-CPE on Intestinal Absorption in Rats. To investigate the absorption-enhancing effects of C-CPE, we performed an in situ loop assay using fluorescein isothiocyanate-dextran with a molecular mass of 4000 Da (FD-4) as a model of drug absorption in the rat. FD-4 is absorbed via the paracellular, rather than intracellular, pathway (Sallee et al., 1972). The in situ loop assay allows investigation of the absorption of substances from the rat intestine to the systemic blood flow. Substances are administered into the rat intestine ex vivo and returned to the intestine in the abdomen. C-CPE dose-dependently enhanced the absorption of FD-4 (12.6- and 23.6-fold at 0.05 and 0.1 mg/ml, respectively; Fig. 1, A and B). Injection of C10 (40 mg/ml), a clinically used enhancer of absorption, caused a 21.3-fold increase of absorption. Thus, C-CPE is 400-fold more potent than C10 at enhancing the absorption of FD-4. Next, we investigated the dependence of the absorption-enhancing effects of C-CPE on the molecular weight of the dextran. As shown in Fig. 2, A and B, treatment with C-CPE enhanced the absorption of dextran with molecular masses of up to 20,000 Da (16.4-, 15.0-, and 2.3-fold for FD-4, -10, and -20, respectively). There was a big break in C-CPE-induced absorption kinetics between FD-10 versus FD-20, and dextran with molecular mass of 40,000 Da was not absorbed.

C-CPE consists of the C terminus of CPE from amino acids 184 to 319 (Katahira et al., 1997). To assess the toxicity of C-CPE in rat jejunum, we investigated LDH leakage into the intestinal lumen (Fig. 3A) and performed histological observations (Fig. 3, B and C; Table 1). The level of LDH leakage in C-CPE-treated jejunum was similar to that in vehicle-treated jejunum, and histochemical analyses did not show evidence of toxicity. Thus, the enhancement of absorption in the rat jejunum by C-CPE is not caused by a toxic effect.

Involvement of Claudin in Absorption-Enhancing Effect of C-CPE. Because Rahner et al. (2001) reported the expression of claudin-4 in rat jejunum, we investigated the interaction of claudin-4 and C-CPE. Extract from rat jejunum was incubated with C-CPE, and C-CPE and bound proteins were precipitated with Ni-resin. As indicated in Fig. 4B, claudin-4 and C-CPE were detected in the fraction precipitated by Ni-resin, suggesting that C-CPE interacts with claudin-4 in the rat jejunum.

The C-terminal 30-amino acids of CPE289–319 are responsible for the interaction of CPE-R with CPE (Hanna et al., 1991). We sought to determine whether this region is sufficient to promote absorption. We therefore produced C-CPE289 and C-CPE303, which lack the C-terminal 30 and 21 amino acids, respectively (Fig. 4A), and we investigated their ability to enhance absorption and to bind claudin-4. Neither C-CPE289 nor C-CPE303 (0.2 mg/ml) enhanced FD-4 absorption (Fig. 4, C and D) or bound claudin-4 (Fig. 4B). In contrast, treatment with C-CPE (0.2 mg/ml) resulted in an 83-fold increase in absorption of FD-4 (data not shown). These findings suggest that the absorption-enhancing effects of C-CPE are dependent on the interaction of the C terminus of C-CPE with claudin-4.

TABLE 1.
Score for histological evaluation of mucosa

Score	Appearance
0	Normal appearance
1	Epithelium intact, columnar enterocytes, goblet cells present, slight edema, slight increase of inflammatory cells in lamina propria and submucosa
2	Epithelium intact, increase of number of cuboidal enterocytes, disappearance of goblet cell vacuoles, slight increase of inflammatory cells in lamina propria and submucosa
3	Localized detachment of enterocytes from the surface, increase of number of cuboidal enterocytes, disappearance of goblet cell vacuoles, localized edema, increase of inflammatory cells in lamina propria and submucosa
4	Localized detachment of enterocytes from the surface, increase of number of cuboidal enterocytes, disappearance of goblet cell vacuoles, generalized edema, increase of inflammatory cells in lamina propria and submucosa
5	Localized detachment of enterocytes from the surface, increase of number of cuboidal enterocytes, disappearance of goblet cell vacuoles, generalized edema, increase of inflammatory cells in lamina propria and submucosa and external muscular layers
6	Generalized detachment of enterocytes and goblet cells, generalized edema, increase of inflammatory cells in lamina propria and submucosa
7	Generalized detachment of enterocytes and goblet cells, generalized edema, increase of inflammatory cells in lamina propria, submucosa and external muscular layers
8	Generalized detachment of enterocytes and goblet cells, destruction of lamina propria, generalized edema, increase of inflammatory cells in lamina propria, submucosa and external muscular layers

Downloaded from molpharm.aspetjournals.org at ASPET Journals on August 27, 2017

Specificity of Absorption-Enhancing Effects of C-CPE. Finally, we evaluated the specificity of the absorption-enhancing effect of C-CPE by comparing its effects on rat jejunum and colon. Treatment with C10, a reagent clinically used to enhance of drug absorption in Japan, elevated the plasma FD-4 levels in both jejunum and colon (11.7- and 12.7-fold, respectively). In contrast, treatment with C-CPE enhanced absorption of FD-4 in the jejunum but not the colon (11.4- and 1.4-fold; Fig. 5, A and B). These results suggested that C-CPE has tissue-specific effects, possibly because of a difference in claudin expression. Therefore, we next examined expression of claudins in rat jejunum and colon. As shown in Fig. 5C, expression of claudin-4 was observed in both rat jejunum and colon. There was no difference in the expression levels of claudin-4 between jejunum and colon. To investigate whether interaction of C-CPE with claudin-4 differs between jejunum and colon, we performed a precipitation assay using lysates extracted from jejunum and colon. As shown in Fig. 6, claudin-4 and C-CPE were coprecipitated by Ni-resin in both jejunum and colon.

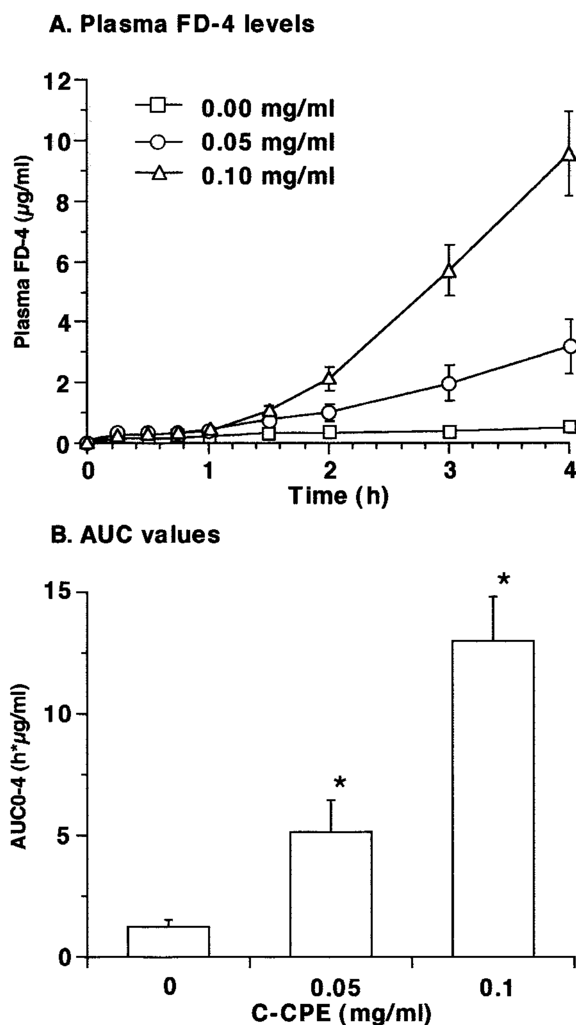


Fig. 1. Effect of C-CPE on jejunal absorption in rats. Rat jejunum was treated with FD-4 (10 mg/ml) in the presence of vehicle or C-CPE (0.05 or 0.1 mg/ml). The FD-4 levels in plasma collected from the carotid artery were determined at the indicated points (A), and the AUC_{0-4h} was calculated (B). Data are means ± S.E. ($n = 4$). The results are representative of at least three independent experiments. Significant difference from the vehicle-treated group (*, $p < 0.05$).

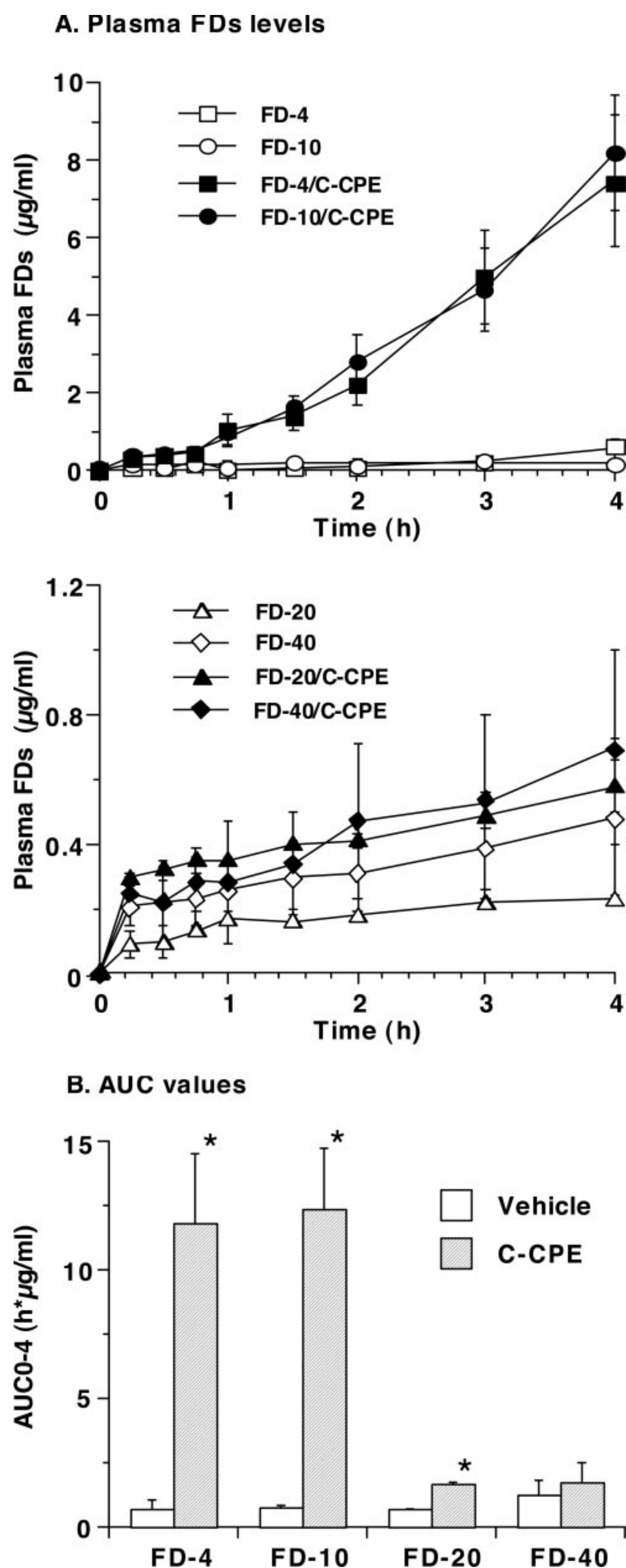


Fig. 2. Dependence of the enhancement of absorption by C-CPE on the molecular mass of dextran. Rat jejunum was treated with FD-4, -10, -20, or 40 (10 mg/ml) in the presence of vehicle or C-CPE (0.1 mg/ml). FD levels in plasma collected from the carotid artery was determined at the indicated points (A), and the AUC_{0-4h} was calculated (B). Data are means ± S.E. ($n = 4$). The results are representative of at least three independent experiments. Significant difference between vehicle and C-CPE-treated group (*, $p < 0.05$).

Downloaded from molpharm.aspetjournals.org at ASPET Journals on August 27, 2017

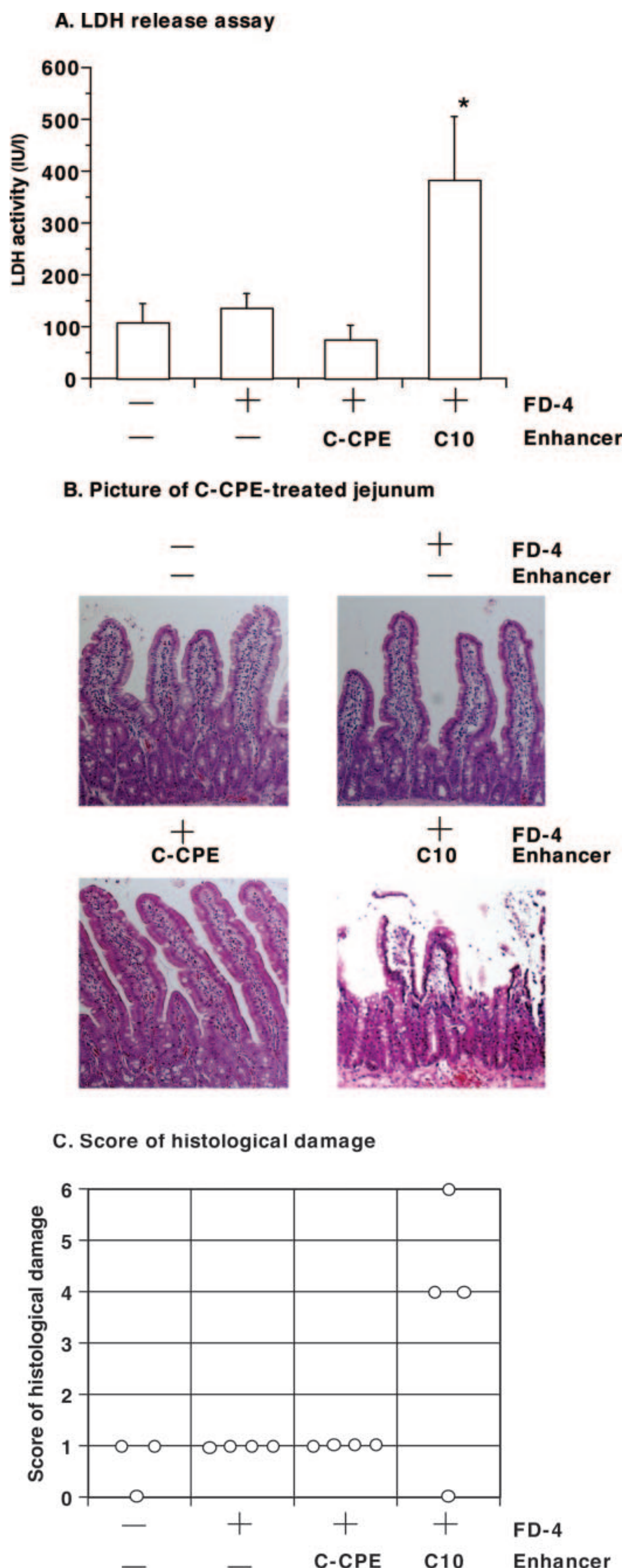


Fig. 3. Toxicity of C-CPE against jejunum in rats. A, LDH leakage from the jejunum treated with C-CPE. After a 4-h treatment with C-CPE (0.1 mg/ml) or C10 (40 mg/ml), PBS was added to the jejunum, and the buffer was recovered for the determination of LDH leakage. Data are means ± S.E. (n = 4). Significant difference from vehicle-treated group (*, $p <$ 0.05). B and C, histological analysis of the C-CPE-treated jejunum. After a 4-h treatment with C-CPE (0.1 mg/ml), the jejunum was fixed with formalin and stained with hematoxylin & eosin, and the stained jejunum was observed under a microscope (B). The results are representative of at least three independent experiments. The grade of histological damage in C-CPE-treated jejunum was scored by the indices described in Table 1 (C).

Thus, interaction of C-CPE with claudin-4 was observed in both jejunum and colon.

Discussion

In this study, we proposed a novel strategy for the enhancement of drug absorption by the inhibition of claudin, a component of TJs. Claudins are a family of more than 20 tetraspan membrane proteins that create the TJ, a selective barrier between epithelia and endothelia (Mitic et al., 2000). C-CPE is the only known inhibitor of the barrier function of claudin-4. Treatment of Madin-Darby canine kidney cells with C-CPE has been shown to specifically inhibit claudin-4, opening TJs, thus elevating the paracellular permeability to drugs (Sonoda et al., 1999). Focusing on this report, we investigated the possibility of claudin as a molecular target for the enhancement of absorption using C-CPE. We found that, in rat jejunum, C-CPE enhanced the absorption of dextran with molecular masses up to 20,000 Da. C-CPE was over 400-fold more potent at enhancing absorption than C10, an enhancer of drug absorption that is clinically used in Japan, Denmark, and Sweden. Highly effective absorption enhancers, such as C10, often cause damage to the intestinal mucosal membrane, and the degree of absorption enhancement parallels the damage (Yamamoto et al., 1996). However, treatment with C-CPE did not induce intestinal damage in the current study.

CPE is functionally separated into N- and C-terminal domains (N- and C-CPE, respectively) (Hanna et al., 1991; Kokai-Kun and McClane, 1997). N-CPE has been shown to be responsible for its cytotoxic activity, whereas C-CPE has been shown to mediate its binding to receptors, including claudin-3 and -4 (Hanna et al., 1991; Kokai-Kun and McClane, 1997; Sonoda et al., 1999). Hanna et al. (1991) fully investigated the functional region required for binding to claudin-3 and -4 and reported that amino acids between 290 and 319 are essential for binding. We therefore prepared C-CPEs lacking the C-terminal amino acids required for claudin binding. We confirmed that removal of the amino acids between 303 and 319 eliminates its binding to claudin-3 and -4, and, moreover, that this attenuates the ability of C-CPE to enhance absorption. Together, these results indicate that C-CPE elevates the absorption of drugs by binding to claudin, which is followed by inhibition of its barrier function.

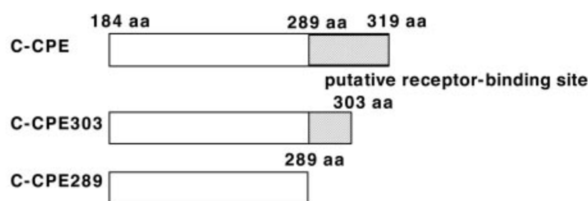
How does C-CPE enhance the absorption of drugs? As described above, the effect is mediated by its binding to claudin-4. Claudins play a barrier role in the TJ (Sonoda et al., 1999; Mitic et al., 2000; Tsukita and Furuse, 2000), and the effects of C-CPE may be caused by inhibition of the barrier function of claudin-4. Indeed, Sonoda et al. (1999) reported that treatment of Madin-Darby canine kidney cells with C-CPE inhibits the barrier function of the TJs (Sonoda et al., 1999). We found that absorption of dextran was depen-

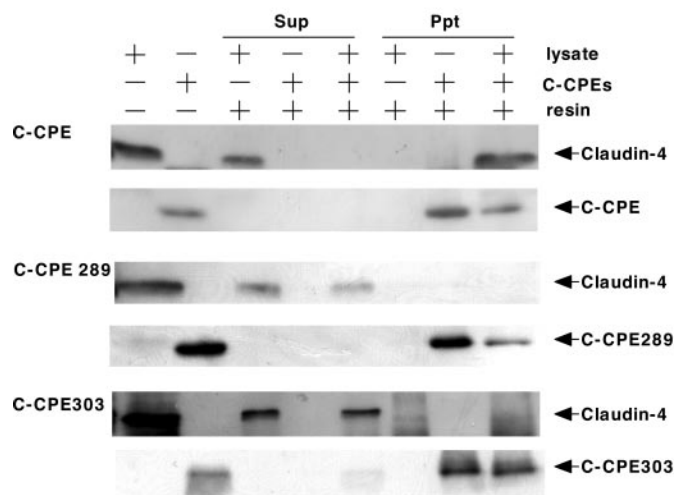
Downloaded from molpharm.aspetjournals.org at ASPET Journals on August 27, 2017

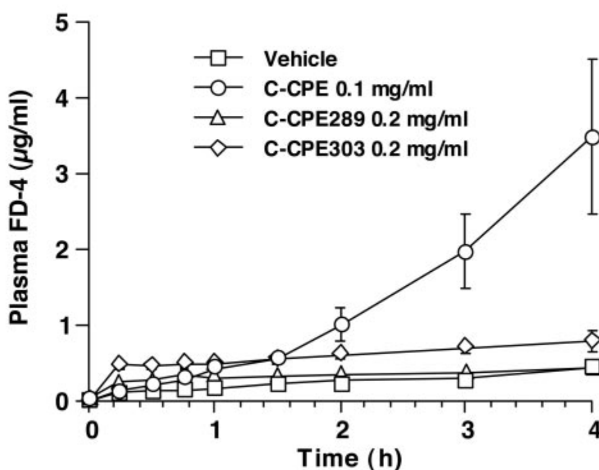
dent on its molecular mass. Dextrans with molecular masses of 4000, 10,000, and 20,000 Da were absorbed from rat jejunum, but dextran with molecular mass of 40,000 Da was not absorbed. Stokes radius of FD-4, FD-10, FD-20, and FD-40 are calculated to be 1.4, 2.3, 3.3, and 4.5 nm, respectively. The cavity of TJ is estimated to be 0.5 nm in physiological condition, and treatment of human epithelial cell lines (Caco-2 and T84 cells) with an enhancer of absorption resulted in opening TJ up to 1.5 nm (Knipp et al., 1997; Watson et al., 2001). Together, although further detailed analyses are necessary to understand the involvement of TJs in the enhancement of absorption by C-CPE, the results suggest that C-CPE can open TJs.

Various strategies for enhancing absorption via the TJ pathway have been developed. For example, fatty acids, including C10 and lauric acid, as well as nitric oxide donors and ion chelators have been used to open TJs (Lindmark et al., 1998; Ye et al., 1999 Lee and Cheng, 2004). The target of nitric oxide donors in this regard is unknown, but it may be caused by dilation of the TJs (Salzman et al., 1995; Yamamoto et al., 2001). Calcium chelators open TJs by removal of calcium ions, which are a component of TJs. These previous approaches for opening TJs have poor specificity because calcium ions are ubiquitous constituent of TJs. Furthermore, the enhancement of absorption via transporters has limited application. Claudin, on the other hand, is an ideal target molecule the enhancement of absorption because the expression and barrier functions of claudin family members are tissue-specific. For example, although expression of claudin-5 is observed in almost all endothelial cells, a deficiency in claudin-5 causes a loss of the barrier function in the blood-brain barrier (Nitta et al., 2003). Likewise, the epidermal barrier is lost in claudin-1-deficient mice (Furuse et al., 2002). Therefore, an inhibitor of claudin with specificity for a

Downloaded from molpharm.aspetjournals.org at ASPET Journals on August 27, 2017







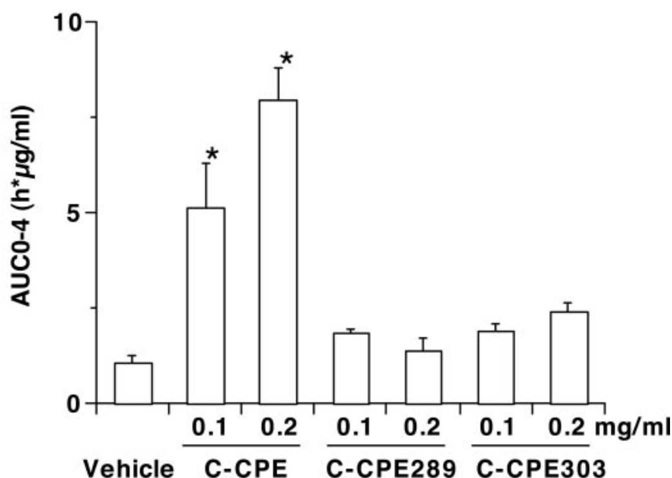


Fig. 4. Involvement of claudin-4 in C-CPE-induced jejunal absorption in rats. A, descriptions of C-CPE289 and C-CPE303. Slashed column indicates the putative CPE receptor binding site (Hanna et al., 1991). B, interaction of C-CPE, C-CPE289, or C-CPE303 with claudin-4. Jejunal mucosa was removed by a scraper and lysed in lysis buffer. The lysate was incubated with C-CPE, C-CPE289, or C-CPE303 and then mixed with Ni-resin. After a 2-h incubation at 4°C, the resulting complex bound to the Ni-resin (ppt) and the free fraction (sup) were subjected to SDS-PAGE and analyzed by Western blotting with antibodies against claudin-4 and His-tag. C and D, effect of C-CPE, C-CPE289, and C-CPE303 on absorption. Rat jejunum was treated with FD-4 (10 mg/ml) in the presence of C-CPE, C-CPE289, or C-CPE303 (0.1 or 0.2 mg/ml). FD-4 levels in plasma collected from the carotid artery were determined at the indicated points (C), and the AUC_{0-4h} was calculated (D). Data are means ± S.E. ($n = 4$). The results are representative of at least three independent experiments. Significant difference from the C-CPE-treated group ($*$, $p < 0.05$).

claudin family member could be a tissue-specific absorption-enhancer. Indeed, treatment with C-CPE enhanced absorption in rat jejunum but not in rat colon, whereas treatment with C10 enhanced absorption in both tissues. The reason for this difference remains to be determined, but one possible explanation is the difference of surface area between the jejunum and colon. Because villi develop in jejunum and not in the colon, the surface area is much larger in the jejunum. However, treatment with C10 enhanced absorption of dextran in both the jejunum and colon, and the tissue-specific effect of C-CPE cannot be fully accounted for by the surface area alone. Another possible explanation is that the expression and combination of claudins are different between the tissues. Rahner et al. (2001) indicated heterogeneity in the expression of claudin-2, -3, -4, and -5 in the rat gut (Rahner et al., 2001). The combination and mixing ratios of claudin

Fig. 5. Comparison of absorption-enhancing effects of C-CPE in rat jejunum and colon. The rat jejunum or colon was treated with FD-4 (10 mg/ml) in the presence of C10 (40 mg/ml) or C-CPE (0.1 mg/ml). FD-4 levels in plasma collected from the carotid artery were determined at the indicated points (A), and the AUC_{0-4h} was calculated (B). Data are means ± S.E. (n = 4). The results are representative of at least three independent experiments. C, detection of TJ-constituted proteins. The mucosa from the jejunum or colon was recovered, lysed in lysis buffer, and the lysates were analyzed by Western blotting. The results are representative of three independent experiments.

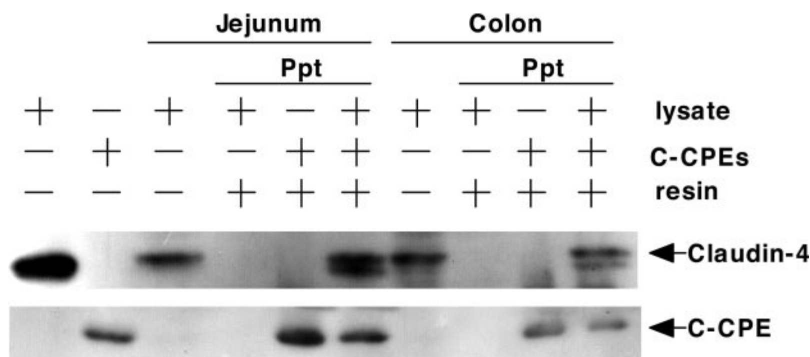


Fig. 6. Comparison of the interaction of C-CPE with claudin-4 between rat jejunum and colon. Jejunum or colon mucosa was removed with a scraper and lysed in lysis buffer. The lysate was incubated with C-CPE and Ni-resin. After a 2-h incubation at 4°C, the resulting complex bound to Ni-resin (ppt) and the free fraction (sup) were subjected to SDS-PAGE and analyzed by Western blotting with antibodies against His-tag. Data are representative of three independent experiments.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on August 27, 2017

species is thought to be an important determinant of the tightness of the TJ (Furuse et al., 2001). However, we did not observe a different pattern of claudin expression between the jejunum and colon (data not shown). Thus, the reason for the tissue specificity of C-CPE remains unclear.

In summary, we found that C-CPE can enhance jejunum absorption at least partly through its binding to claudin-4. We also found that the amino acids between residue 303 and 319 play a pivotal role in the absorption of enhancement by C-CPE. Using the information about the claudin-4 binding domain in C-CPE and phage display, new inhibitors with specificities for different claudins are being developed. This is the first report to describe the enhancement of absorption by targeting claudins. These results indicate a new possible route for the development of tissue-specific modulators of absorption.

Acknowledgments

We thank N. Akiyama and Y. Zenimoto for excellent technical assistance, and Drs. J. Sakurai and M Nagahama (Tokushima Bunri University, Tokushima City, Japan) for useful discussion.

References

Adibi SA (1997) The oligopeptide transporter (Pept-1) in human intestine: biology and function. *Gastroenterology* **113:**332–340.
Anderson JM (2001) Molecular structure of tight junctions and their role in epithelial transport. *News Physiol Sci* **16:**126–130.
Balda MS, Whitney JA, Flores C, Gonzalez S, Cereijido M, and Matter K (1996) Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol* **134:**1031–1049.
Chen YH, Merzdorf C, Paul DL, and Goodenough DA (1997) COOH terminus of occludin is required for tight junction barrier in early *Xenopus* embryos. *J Cell Biol* **138:**891–899.
Edwards RH (2001) Drug delivery via the blood-brain barrier. *Nat Neurosci* **4:**268–274.
Furuse M, Furuse K, Sasaki H, and Tsukita S (2001) Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney I cells. *J Cell Biol* **153:**253–272.
Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, and Tsukita S (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* **156:**1099–1111.
Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, and Tsukita S (1993) Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* **123:**1777–1788.
Furuse M, Sasaki H, and Tsukita S (1999) Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* **147:**891–903.
Gow A, Southwood CM, Li JS, Pariali M, Riordan GP, Brodie SE, Danias J, Bronstein JM, Kachar B, and Lizzarini RA (1999) CNS myelin and sertoli cell tight junction strands are absent in Osp/Claudin-11 null mice. *Cell* **99:**649–659.
Hanna PC, Mietzner TA, Schoolnik GK, and McClane BA (1991) Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides. *J Biol Chem* **266:**11037–11043.
Kokai-Kun JF and McClane BA (1997) Deletion analysis of the *Clostridium perfringens* enterotoxin. *Infect Immun* **65:**1014–1022.
Knipp GK, Ho NFH, Barsuhn CL, and Borchardt RT (1997) Paracellular diffusion in Caco-2 cell monolayers: effect of perturbation on the transport of hydrophilic compounds that vary in charge and size. *J Pharm Sci* **86:**1105–1110.
Katahira J, Inoue N, Horiguchi Y, Matsuda M, and Sugimoto N (1997) Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. *J Cell Biol* **136:**1239–1247.
Lee NP and Cheng CY (2004) Nitric oxide/nitric oxide synthase, spermatogenesis and tight junction dynamics. *Biol Reprod* **70:**267–276.
Lindmark T, Kimura Y, and Artursson P (1998) Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in caco-2 cells. *J Pharmacol Exp Ther* **284:**362–369.
McClane BA (1994) *Clostridium perfringens* enterotoxin acts by producing small molecule permeability alterations in plasma membranes. *Toxicology* **87:**43–67.
Mitic LL, Vanitallie CM, and Anderson JM (2000) Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. *Am J Physiol* **279:**G250–G254.
Morita K, Furuse M, Fujimoto K, and Tsukita S (1999) Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc Natl Acad Sci USA* **96:**511–516.
McCarthy KM, Skare IB, Stankewich MC, Furuse M, Tsukita S, Rogers RA, Lynch RD, and Schneeberger EE (1996) Occludin is a functional component of the tight junction. *J Cell Sci* **109:**2287–2298.
Martin-Pardura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, et al. (1998) Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that disturbutes at intracellular junctions and modulates monocyte transmigration. *J Cell Biol* **142:**117–127.
Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, and Tsukita S (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* **161:**653–660.
Powell DW (1981) Barrier function of epithelia. *Am J Physiol* **241:**G275–G288.
Rao R, Baker RD, and Baker SS (1999) Inhibition of oxidant-induced barrier disruption and protein tyrosine phosphorylation in caco-2 cells monolayers by epidermal growth factor. *Biochem Pharmacol* **57:**685–695.
Rahner C, Mitic LL, and Anderson JM (2001) Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas and gut. *Gastroenterology* **120:**411–422.
Sallee VL, Wilson FA, and Dietschy JM (1972) Determination of undirectional uptake rates for lipids across the intestinal brush border. *J Lipid Res* **12:**184–192.
Salzman AL, Menconi MJ, Unno N, Ezzell RM, Casey DM, Gonzalez PK, and Fink MP (1995) Nitric oxide dilates tight junctions and depletes ATP in cultured Caco-2Bbe intestinal epithelial monolayers. *Am J Physiol* **268:**G361–G373.
Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, and Tsukita S (1999) *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. *J Cell Biol* **147:**195–204.
Saitou M, Fujimoto K, Doi Y, Itoh M, Fujimoto T, Furuse M, Takano H, Noda T, and Tsukita S (1998) Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. *J Cell Biol* **141:**397–408.
Tsukita S and Furuse M (2000) Pores in the wall: claudins constitute tight junction strands containing aqueous pores. *J Cell Biol* **149:**13–16.
Tsukita S, Furuse M, and Itoh M (2001) Multi-functional strands in tight junctions. *Nat Rev Mol Cell Biol* **2:**285–293.
Wong V and Gumbiner BM (1997) A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J Cell Biol* **136:**399–409.
Watson CJ, Rowland M, and Warhurst G (2001) Functional modeling of tight junctions in intestinal cell monolayers using polyethylene glycol oligomers. *Am J Physiol* **281:**C388–C397.
Yamamoto A, Tatsumi H, Maruyama M, Uchiyama T, Okada N, and Fujita T (2001) Modulation of intestinal permeability by nitric oxide donors: implications in intestinal delivery of poorly absorbable drugs. *J Pharmacol Exp Ther* **296:**84–90.
Yamamoto A, Uchiyama T, Nishikawa T, Fujita T and Muranishi S (1996) Effectiveness and toxicity screening of various absorption enhancers in the rat small intestine: effects of absorption enhancers on the intestinal absorption of phenol red and the release of protein and phospholipids from the intestinal membrane. *J Pharm Pharmacol* **48:**1285–1289.
Ye J, Tsukamora T, Sun A, and Nigam SK (1999) A role for intracellular calcium in tight junction reassembly after ATP depletion-repletion. *Am J Physiol* **277:**F524–F532.

Address correspondence to: Dr. Masuo Kondoh, Department of Pharmaceutics and Biopharmaceutics, Showa Pharmaceutical University, Machida, Tokyo, 194-8543, Japan. E-mail: masuo@ac.shoyaku.ac.jp

Downloaded from molpharm.aspetjournals.org at ASPET Journals on August 27, 2017