Chlorpheniramine Increases Paracellular Permeability to Marker Fluorescein Lucifer Yellow Mediated by Internalization of Occludin in Murine Colonic Epithelial Cells

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Ions, small molecules, and drugs are absorbed in the intestinal epithelium mediated by transcellular and paracellular pathways. The function of various transporters expressing in the apical and basolateral membranes of intestinal epithelial cells has been well characterized. In contrast, claudins and occludin, components of the tight junctions (TJs), determine the paracellular permeability to ions and low molecular weight compounds, but the properties for permeability have not been clarified in detail. In the present study, we examined the effects of anti-histamine drugs, chlorpheniramine and diphenhydramine, on transepithelial electrical resistance (TER) and permeability to lucifer yellow (LY), a marker of paracellular permeability, using murine colonic MCE301 cells. Chlorpheniramine significantly decreased the steady state of TER and increased permeability to LY, whereas the effects of diphenhydramine were not significant. The mRNAs of occludin and claudin-1-claudin-8 except for claudin-5 were expressed in MCE301 cells. Both anti-histamine drugs did not change solubility of claudins to 0.5% Triton X-100 solution. In contrast, the detergent solubility and intracellular localization of occludin were significantly increased by chlorpheniramine. These results indicate that occludin is dissociated from the TJs by chlorpheniramine. Chlorpheniramine increased protein phosphatase-2A (PP-2A) activity, which was inhibited by cantharidin, a potent PP-2A inhibitor. Furthermore, the changes of TER, permeability to LY, and de-phosphorylation and tight junctional localization of occludin caused by chlorpheniramine were recovered by cantharidin. These results suggest that chlorpheniramine could increase paracellular permeability to low molecular weight compounds mediated by the activation of PP-2A and internalization of occludin in the colonic epithelial cells.

Key words colon; claudin; occludin; anti-histamine drug

Orally administered drugs are absorbed in the intestinal epithelium. Lipophilic compounds diffuse across lipid membrane and are transported from the gastrointestinal tract to blood, whereas hydrophilic compounds are absorbed using a specific carrier transport system to permeate the lipid membrane. Intestinal and colonic epithelial cells express multiple drug transporters on their luminal brush border and basolateral membranes. The absorption of lipophilic compounds is affected by the physicochemical parameters and the properties of specific transporters. In addition to the transcellular route, a passive permeation to low molecular weight compounds can occur through a paracellular route. The absorption of high molecular weight compounds (>1000 Da) is very limited, but they can be passively transported through the paracellular route.

At the apical pole of the intercellular junction of the lateral membrane, epithelial cells form the tight junctions (TJs), which compose a large complex of proteins including the membrane integral proteins such as claudins and occludin, and the scaffolding proteins such as zonula occludens (ZO)-1, -2, and -3. The TJs separate the apical and basolateral epithelial compartments to produce their polarization. Although the TJs connect neighboring epithelial cells, they also form charge-and size-selective channel and control the diffusion of solutes across the epithelial sheet. The properties of tight junctional ion permeability are characterized using knockout mice and renal and colonic cell lines. However, the molecular mechanism of absorption of low molecular weight compounds has not been fully elucidated.

Claudins are the most important structural and functional components of the TJs. Claudins comprise a family of over 20 members and bear common structure of four transmembrane domains with a short cytoplasmic N-terminus, two extracellular loops (ECLs) and a C-terminal cytoplasmic domain. The first ECL containing several negatively or positively charged amino acids may be critical for determining the paracellular ion permeability and the second ECL contributes to homophilic and/or heterophilic trans-interaction of claudins. Different combinations of claudins can confer different paracellular ion permeability to epithelial cells. Occludin, another four transmembrane protein, may not be directly involved in the determination of ion selective permeability, but regulates the diffusion of small hydrophilic compounds in a size-selective manner.

Many Japanese OTC drugs contain first generation anti-histamine drugs, chlorpheniramine and diphenhydramine. Both are used for self-medication for the treatment of cold, nasal catarrh, nasal congestion, and motion sickness. The accumulation of diphenhydramine in Caco-2 cells, derived from human colonic adenocarcinoma, is inhibited by chlorpheniramine. Furthermore, diphenhydramine and chlorpheniramine inhibit the uptake of phenylethylamine. These drugs may
be absorbed via a common transport carrier expressing in the luminal membrane of Caco-2 cells. On the other hand, the effects of chlorpheniramine and diphenhydramine on paracellular permeability to low molecular weight compounds have not been examined.

Colonic epithelial MCE301 cells were established from normal mice by expressing simian virus 40 large T oncogene. The in vivo establishment by Tabuchi et al. (1998) was accomplished by isolating intestinal mucosal monolayer tissue from immuno-deficient BALB/c-nu/nu mice at 15–20 days postpartum. MCE301 cells were cultured for 21–25 days. Extractions were performed by overlaying the cells with CSK-1 buffer (0.5% Triton X-100, 100 mM NaCl, 300 mM sucrose, and 10 mM Tris–HCl, pH 7.4) for 10 min at 4°C on a gentle rocking platform. The extracts were then collected by low-speed centrifugation (Triton X-100-soluble fractions) and the residue was dissolved in a RIPA buffer. The supernatants were sonicated for 20 s and centrifuged at 10000 × g for 5 min. The supernatants were referred to as Triton X-100-insoluble fractions.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

For the immunoprecipitation assay, the cells were lysed in a radioimmune precipitation assay (RIPA) buffer containing 150 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 50 mM Tris–HCl (pH 8.0), protease inhibitor mixture (Sigma-Aldrich) and then sonicated for 20 s. After centrifugation at 10000 × g for 5 min, the supernatant was collected. The aliquots (400 µg) were incubated with a protein G-Sepharose and anti-occludin antibody (1:1000 dilution) at 4°C with gently rocking. After centrifugation at 6000 × g for 1 min, the pellet was washed 3 times with RIPA buffer. The immunoprecipitates, detergent soluble (10 µg), and detergent insoluble (10 µg) fractions were applied to SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated with each primary antibody (1:1000 dilution) at room temperature for 1.5 h. Finally, the blots were incubated in EzWestLumi plus (ATTO Corporation, Tokyo, Japan) or ImmunoStar Basic (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and scanned with a C-Dig Blot Scanner (LI-COR Biotechnology, Lincoln, NE, U.S.A.). Band density was quantified with ImageJ software (National Institute of Health software).

Measurement of PP-2A Activity Cytosolic fractions

Table 1. Primers for PCR Amplification

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Claudin-1 sense</td>
<td>5'-GTCTTCGGATTCCTTGGCTGAA-3'</td>
</tr>
<tr>
<td>Claudin-1 antisense</td>
<td>5'-CCCTGCGCAAAAATCTACAAGTCT-3'</td>
</tr>
<tr>
<td>Claudin-2 sense</td>
<td>5'-TGACGACACACACAGGGACTGCA-3'</td>
</tr>
<tr>
<td>Claudin-2 antisense</td>
<td>5'-CAAGAAGACACGGCGAGATGAA-3'</td>
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<tr>
<td>Claudin-3 sense</td>
<td>5'-CATCTGGTCTGGCCCTCTGC-3'</td>
</tr>
<tr>
<td>Claudin-3 antisense</td>
<td>5'-GGATTAGTGGTGTTTGCAGTTAC-3'</td>
</tr>
<tr>
<td>Claudin-4 sense</td>
<td>5'-TCGTGGTGTTGCTCCTGGAATGTC-3'</td>
</tr>
<tr>
<td>Claudin-4 antisense</td>
<td>5'-GGCACTTGACGTGGACTGC-3'</td>
</tr>
<tr>
<td>Claudin-5 sense</td>
<td>5'-GAGGAATGGTACCTGTGAGTGG-3'</td>
</tr>
<tr>
<td>Claudin-5 antisense</td>
<td>5'-GACGCAAGACCCAGCAACAGCGA-3'</td>
</tr>
<tr>
<td>Claudin-6 sense</td>
<td>5'-ACCCCTCTCATTGCTCTGTTG-3'</td>
</tr>
<tr>
<td>Claudin-6 antisense</td>
<td>5'-CCACACAGACAGGAAATGAGGT-3'</td>
</tr>
<tr>
<td>Claudin-7 sense</td>
<td>5'-GGGCACTCCGACTTAAATGTGG-3'</td>
</tr>
<tr>
<td>Claudin-7 antisense</td>
<td>5'-CGTGGCCAGGACAAAGAGATG-3'</td>
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<tr>
<td>Claudin-8 sense</td>
<td>5'-CATGCCAACATCAGCAGTTAC-3'</td>
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<tr>
<td>Claudin-8 antisense</td>
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<td>β-Actin sense</td>
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<tr>
<td>β-Actin antisense</td>
<td>5'-CCAGAGGCACATACAGGGCA-3'</td>
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Fig. 1. Effects of Chlorpheniramine and Diphenhydramine on Paracellular Permeability

MCE301 cells were incubated in the presence and absence of 50 µM chlorpheniramine (CHL) or 50 µM diphenhydramine (DIP) for 1h. (A) Paracellular permeability to ions was estimated using volt-ohmmeter. (B) LY flux from the apical to basal chambers was measured using transwell inserts. LY (0.2 mM) was added into the filter well and incubated at 4°C for the periods indicated. **p<0.01 significantly different from control (CNT). NS, not significantly different.

Fig. 2. Detergent Solubility of Claudins and Occludin

(A) RT-PCR was performed using primers pair of claudin-1–claudin-8, occludin, and β-actin. The PCR products were analyzed on an agarose gel. The size marker is indicated on the left. (B, C) Cells were in the presence and absence of 50 µM chlorpheniramine (CHL) or 50 µM diphenhydramine (DIP) for 1h. Detergent soluble (S) and insoluble (I) fractions were isolated using 0.5% Triton X-100 solution. After Western blotting of claudins and occludin, the expression levels in soluble fractions were represented relative to the total levels (S/T). n=3–4. **p<0.01 significantly different from control (CNT). NS, not significantly different.
were extracted using CSK-1 buffer. PP-2A activity was measured using colorimetric substrate, para-nitrophenyl phosphate, as described elsewhere.\(^{(16)}\)

**Immunocytochemistry** Cells were cultured on cover glasses. The cells were fixed with methanol for 10 min at \(-20^\circ\text{C}\), then permeabilized with 0.2% Triton X-100 for 15 min. After blocking with 4% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 30 min, the cells were incubated with anti-occludin and anti-ZO-1 antibodies for 16 h at 4°C. They were then incubated with Alexa Fluor 488- and 555-conjugated antibodies for 1.5 h at room temperature. Immunolabelled cells were visualized on LSM 700 confocal microscope (Carl Zeiss, Germany). The image area containing the signal from the TJs was manually marked using ImageJ software. The area below the TJs was defined as the cell interior. The intracellular intensity of occludin was shown as percentage of total intensity.

**Statistics** Results are presented as means±standard error of the mean (S.E.M.). Differences between groups were analyzed with a one-way ANOVA, and corrections for multiple comparison were made using Tukey’s multiple comparison tests. Significant differences were assumed at \(p<0.05\).

**RESULTS**

**Effects of Anti-histamine Drugs on Paracellular Permeability** TER was significantly decreased by chlorpheniramine, but not by diphenhydramine (Fig. 1A). Permeability to LY was significantly increased by chlorpheniramine (Fig. 1B). Diphenhydramine slightly increased permeability to LY, but the effect was not significant. These results indicate that chlorpheniramine can increase paracellular permeability to low molecular weight compounds and ions.

**Effects of Anti-histamine Drugs on Detergent Solubility of Claudins and Occludin** In semi-quantitative PCR analysis, occludin and claudin-1–claudin-8 except for claudin-5 were expressed in MCE301 cells (Fig. 2A). These results are similar to those previously reported in mice.\(^{(17)}\) MCE301 cells were treated with chlorpheniramine and diphenhydramine for 1 h followed by isolation of 0.5% Triton X-100-soluble and -insoluble fractions. The TJs proteins are connected to cytoskeletal proteins and mainly distributed in the Triton X-100-insoluble fractions.\(^{(18)}\) The detergent solubility of occludin was significantly increased by chlorpheniramine (Figs. 2B, C). In contrast, the detergent solubility of claudin-1, -2, -3, -4, -7, and -8 was not significantly changed by chlorpheniramine and diphenhydramine. The protein expression of claudin-5 and -6 was under detection limit (data not shown). These results indicate that occludin may be involved in the elevation of permeability to LY caused by chlorpheniramine.

**Effects of Anti-histamine Drugs on Intracellular Localization of Occludin** So far, it has been reported that the intracellular localization of occludin is controlled by phosphorylation.\(^{(19)}\) MCE301 cells were treated with chlorpheniramine
in the presence and absence of cantharidin, a potent PP-2A inhibitor, for 1 h. PP-2A activity was increased by chlorpheniramine, which was significantly inhibited by cantharidin (Fig. 3A). In contrast, diphenhydramine did not change PP-2A activity (data not shown). The phosphorylation level of occludin was decreased by chlorpheniramine, which was recovered by cantharidin (Fig. 3B). In addition, the amounts of occludin in the detergent-soluble fractions were significantly increased by chlorpheniramine, which was recovered by cantharidin (Fig. 3C). Immunofluorescence measurement showed that occludin is mainly distributed in the TJs concomitant with ZO-1 under the control conditions (Fig. 3D). The cytosolic localization of occludin was increased by chlorpheniramine, which was recovered by cantharidin. These results are similar to those in Western blotting.

Effects of Chlorpheniramine and Cantharidin on Paracellular Permeability The detergent solubility and tight junctional localization of occludin were recovered by cantharidin. Therefore, we examined the effects of chlorpheniramine and cantharidin on paracellular permeability. The chlorpheniramine-induced decrease in TER was significantly recovered by cantharidin (Fig. 4). Similarly, the elevation of LY flux was significantly recovered by cantharidin. TER and LY flux were unchanged by cantharidin alone (data not shown). These results indicate that the chlorpheniramine-induced increase in paracellular permeability to low molecular weight compounds may be regulated by a PP-2A-dependent internalization of occludin.

DISCUSSION

The mechanisms of intestinal absorption of ions and drugs are usually investigated using Caco-2 cells, derived from human colonic adenocarcinoma. Caco-2 cells are the most widely used model cells, but they form exclusively tight monolayer (900Ω×cm²), which is different from that in human small intestine (40Ω×cm²). In contrast, human small intestinal epithelial (HIEC) cells differentiated from human adult intestinal stem cells form leaky monolayer (100Ω×cm²). TER is determined by the balance of expression between ion permeable and impermeable claudin subtypes, but the cause leading to the difference in TER between Caco-2 and HIEC cells is unknown. The paracellular permeability to ions is well characterized using renal tubular Madin–Darby canine kidney (MDCK) and LLC-PK₁ cells. Claudin-2 makes cation permeable pore, whereas claudin-4, -7, and -8 make anion selective pore. In addition, claudin-1 and -3 do not form ion-permeable pore and claudin-5 blocks ions permeability. The mRNAs expression of claudin-1, -3, -4, -5, and -7 was decreased by chlorpheniramine, which was significantly inhibited by cantharidin (Fig. 4). Similarly, the elevation of LY flux was significantly recovered by cantharidin. Therefore, we examined the effects of chlorpheniramine and cantharidin on paracellular permeability. The chlorpheniramine-induced decrease in TER was significantly recovered by cantharidin (Fig. 4A). In contrast, the expression of occludin and ZO-1 is constant during postnatal development. Occludin may be regulated by a PP-2A-dependent internalization of occludin. Claudin-5 is highly expressed in the human colonic tissues and cell line including Caco-2 and HT-29/B6 cells. In contrast, our results indicate that claudin-5 is not endogenously expressed in MCE301 cells. Similarly, lower expression of claudin-5 is reported in the mouse and canine colon. Therefore, the elevation of permeability to LY caused by chlorpheniramine should not be due to the change of expression and localization of claudin-5 in MCE301 cells. Oc-
occludin is also involved in the formation of intestinal barrier. Polyphenols including theaflavins and quercetin enhance barrier function of Caco-2 through the elevation of occludin.\textsuperscript{30,31} In the present study, we found that chlorpheniramine increases paracellular permeability to LY in MCE301 cells. Chlorpheniramine increased the detergent solubility (Fig. 2) and cytosolic localization of occludin (Fig. 3D). This is the first report showing that chlorpheniramine increases paracellular permeability to LY mediated by internalization of occludin. The change of intracellular localization of occludin may also enhance the paracellular permeability to ions because the size of ion is smaller than that of LY.

The intracellular localization of junctional proteins is controlled by phosphorylation on serine and threonine residues. Occludin is dephosphorylated by PP-2A, PP1 and protein tyrosine phosphatase 1B, and distributed in the cytosolic compartments including endosomes, resulting in the elevation of paracellular permeability in MDCK cells.\textsuperscript{32,33} The function of these protein phosphatases is correlated with the activity and intracellular localization of PKC\textsubscript{ζ}, and PKC\textsubscript{ι}/. Our data clearly indicated that chlorpheniramine decreases phosphorylation of occludin and increases paracellular permeability to LY, which are inhibited by a PP-2A inhibitor cantharidin in MCE301 cells. Histamine H1, H2, and H4 receptors are expressed in various tissues including colon.\textsuperscript{34} Histamine H1 receptor couples to G\textsubscript{α}\textsubscript{q/11} proteins and activates phospholipase C, resulting in the elevation of release of inositol trisphosphate and diacylglycerol followed by activation of PKC. Histamine H2 and H4 receptors couple to G\textsubscript{s} and G\textsubscript{i}, respectively, and affect adenylate cyclase activity. Chlorpheniramine may change paracellular permeability and tight junctional localization of occludin mediated through the inhibition of H1 receptor. However, this possibility seems unlikely, because: 1) the cells are not stimulated with histamine, 2) diphenhydramine, another H1 antagonist, do not increase permeability to LY, and 3) PKC activity must be decreased by blocking of histamine H1 receptor. We need further study to clarify the mechanism of chlorpheniramine on PP-2A activity.

In conclusion, chlorpheniramine significantly increased LY flux and decreased TER in MCE301 cells. In contrast, diphenhydramine had no effect on paracellular permeability. Chlorpheniramine increased the solubility of occludin to 0.5% Triton X-100 solution without affecting those of claudins, and increased the cytosolic distribution of occludin. The chlorpheniramine-induced reduction of barrier function was significantly inhibited by PP-2A inhibitor. These results indicate that chlorpheniramine may increase internalization of occludin mediated by PP-2A-dependent mechanism and enhance paracellular flux of low molecular weight compounds in colonic epithelial cells. Chlorpheniramine is contained in many Japanese OTC medications and the drugs used concomitantly may enhance risk for dysfunction of colonic barrier function.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

3196 (2000).


