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## Poly-L-arginine-Induced Internalization of Tight Junction Proteins Increases the Paracellular Permeability of the Caco-2 Cell Monolayer to Hydrophilic Macromolecules

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We investigated whether poly-L-arginine (PLA) enhances the paracellular permeability of the Caco-2 monolayer to hydrophilic macromolecules and clarified the disposition of tight junction (TJ) proteins. The transepithelial electrical resistance (TEER) and fluorescein isothiocyanate (FITC)-dextran (FD-4) permeation were determined after treatment with PLA. TJ proteins were visualized using immunofluorescence microscopy after PLA exposure and depletion, and their expression levels were determined. The barrier function of TJs was also evaluated by measuring the alterations in the TEER and in the localization of TJ proteins. PLA induced an increase in hydrophilic macromolecule, FD-4, permeation through Caco-2 cell monolayers and a decrease in the TEER in a concentration-dependent manner, without any significant impact on the cell viability. This increased paracellular permeability induced by PLA was found to be internalized of claudin-4, ZO-1, tricellulin and mainly occludin from cell–cell junction to the subcellular space. ZO-1 appeared to play an important role in the reconstitution of TJ strand structures following PLA depletion. These results indicate that the PLA led to the internalization of TJ proteins to the subcellular space, subsequently increasing the permeability of the Caco-2 cell monolayer to FD-4 *via* a paracellular route.

**Key words** poly-L-arginine; absorption enhancer; drug delivery; hydrophilic-macromolecule; tight junction

Various types of absorption enhancers (*e.g.*, surfactants, bile salts, and fatty acid salts) have so far been studied to improve the absorption of hydrophilic macromolecules, such as bioactive peptides and proteins, from the local mucosa. Unfortunately, most absorption enhancers cause irreversible damage to the mucosa, even though they can promote the absorption of hydrophilic macromolecules through the nasal epithelium.<sup>1–3)</sup>

Natsume *et al.* found that polycationic poly-L-arginine (PLA) has the potential to promote the trans-nasal delivery of macromolecules without causing mucosal disorders.<sup>4)</sup> It has been reported that PLA enhances effectively the nasal absorption of fluorescein isothiocyanate (FITC) dextrans with molecular weight of up to 20 kDa and recombinant human granulocyte colony stimulating factor (rhG-CSF) in *in vivo* rats.<sup>5,6)</sup> In addition, we and others have also shown that PLA improved the paracellular permeability of the nasal mucosa, pulmonary mucosa, alimentary canal mucosa and Calu-3 cell monolayers to water-soluble polymers, regardless of the type of mucosa.<sup>7–9)</sup>

Ohtake *et al.* reported that PLA predominantly increases the paracellular permeability to hydrophilic macromolecules across rabbit excised nasal mucosa, and that this involves the transport of tight junction (TJ) and adherens junction (AJ) proteins into the cytoplasm through the cell–cell junctions.<sup>10)</sup> PLA enhances the paracellular permeability *via* the serine/threonine phosphorylation of ZO-1 and tyrosine dephosphorylation of occludin, as shown in experiments using kinase and phosphatase inhibitors.<sup>11)</sup> However, in the rabbit nasal epithelium, it was difficult to further analyze the mechanism

underlying the TJ disassembly induced by PLA because of the lack of antibody crossover between rabbits and other animals and the low amount of TJ proteins that could be detected by an immunoblot analysis. Furthermore, data had only been obtained for ZO-1 and occludin, but not for any other TJ proteins.<sup>10,11)</sup>

TJs are found between adjacent cells, and the occludin and claudin family proteins, which includes four transmembrane domain proteins, are distributed throughout TJs.<sup>12,13)</sup> Occludin and claudin play an important role in the rate-limiting step of the permeation of ions and other solutes through the paracellular space.<sup>14,15)</sup> Recently, it was reported that a four-transmembrane-domain protein called tricellulin was located at the intersection where three adjacent cells meet (tricellular tight junctions).<sup>16)</sup> These TJ structural proteins form TJ strands cooperatively. Thus, by altering the distribution of claudin and tricellulin, PLA may also be able to enhance the permeability of cell layers to macromolecules, in addition to its known effects on occludin and ZO-1.

Caco-2 cells form a monolayer and have been widely used for the functional analysis of the permeation of drugs across a cell sheet to mimic the intestinal epithelium. Therefore, Caco-2 cells could be useful for analyzing whether the PLA-enhanced paracellular permeability to macromolecules involves alterations in the TJ proteins.

In the present study, we first confirmed that PLA increased the paracellular permeation of a Caco-2 cell monolayer to FD-4, a model drug, and led to the disappearance of TJ proteins (ZO-1, occludin, claudin-4, tricellulin) as well as rabbit excised nasal mucosa.<sup>10)</sup> Claudin-4 is the most important member of the claudin family and provides the barrier function in epithelial cell sheets. Second, following PLA exposure,

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the dispositions of TJ proteins and AJ proteins (E-cadherin,  $\beta$ -catenin) were determined in Caco-2 monolayers by immunostaining and an immunoblot analysis. Finally, following PLA depletion, the relocalization and disposition of TJ proteins were evaluated.

## MATERIALS AND METHODS

**Materials** Poly-L-arginine (molecular weight (MW) 44300) and fluorescein isothiocyanate dextran (FD-4, MW 3850) were obtained from Sigma Aldrich (St. Louis, MO, U.S.A.). Sodium dodecyl sulfate (SDS), Triton-X, and sodium deoxycholate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The cell culture reagents and supplies were purchased from Invitrogen (Carlsbad, CA, U.S.A.). The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Dojindo (Kumamoto, Japan), and the protease inhibitors cocktail was purchased from Roche (Mannheim, Germany). All other reagents were of analytical grade.

**Antibodies** Mouse monoclonal anti-occludin (WB 1:1000, IF 1:250), anti-claudin-4 (WB 1:1000, IF 1:250), anti-E-cadherin (WB 1:1000, IF 1:250), rabbit polyclonal anti-ZO-1 (WB 1:500, IF 1:125), anti-tricellulin (WB 1:1000, IF 1:250), anti- $\beta$ -catenin (WB 1:500, IF 1:125), Alexa fluor-488 anti-rabbit immunoglobulin G (IgG), and Alexa fluor-594 anti-mouse IgG (IF 1:500) were purchased from Invitrogen. Rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), horse-radish peroxidase (HRP)-conjugated anti-mouse IgG, and anti HRP-conjugated anti-rabbit IgG (WB 1:1000) were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). Each antibody was used at the dilutions described above in the Western blot analyses and for immunofluorescence microscopy. Nonspecific signals were not detected in Western blot analyses and immunofluorescent microscopic observation.

**Cell Culture** Caco-2 cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.) and were maintained in Dulbecco's modified Eagle's medium (DMEM) (10% fetal bovine serum (FBS), 1% NEAA, 2% Gluta-MAX, 2% Antibiotic-antimycotic) and used for experimental purposes between passages 60–70. The cells were subcultured by partial digestion with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA). A total of  $1 \times 10^5$  cells per  $\text{cm}^2$  were seeded on polycarbonate Transwell membranes (24mm; Corning, Lowell, MA, U.S.A.), and experiments were performed 21–28d after seeding.

**Transepithelial Electrical Resistance (TEER) Measurement** The TEER was measured using a Millicell-ERS (Millipore, Billerica, MA, U.S.A.) according to the manufacturer's protocol, and then was calculated in  $\text{ohms cm}^2$  by multiplying the value by the surface area of the monolayer. Only the cell monolayers that provided a TEER of  $>500 \text{ ohms cm}^2$  were used for experiments as described by Ferruzza *et al.*<sup>17)</sup> The resistance of the polycarbonate membrane in the Transwells was subtracted from all readings. Caco-2 cell monolayers were washed twice with Hank's balanced salt solution (HBSS), and the DMEM was replaced with HBSS. PLA was added to the apical side of the Caco-2 cell monolayer for 2h. The TEER was measured at 0, 15, 30, 45, 60, 90, and 120min after treatment with PLA. The TEER was expressed as a percentage of

the initial values.

**FD-4 Permeability Study** An FD-4 permeability study was performed as reported previously.<sup>18)</sup> In brief, Caco-2 cell monolayers were washed twice with HBSS and the DMEM culture medium was replaced with HBSS. An apical bathing solution that contained PLA (0, 10, 25, 50, or  $100 \mu\text{g/mL}$ ) and FD-4 ( $2.5 \text{ mg/mL}$ ) was applied to the monolayer. Samples were collected from the basal side at 15, 30, 45, 60, 90, and 120min after the start of PLA treatment, and the cumulative amount of FD-4 that crossed the barrier per unit surface area ( $\mu\text{g/cm}^2$ ) was calculated. The fluorescence was determined using a fluorometer RF-1500 (Shimadzu, Tokyo, Japan) with excitation wavelength of 495nm and an emission wavelength of 515nm. The apparent permeability coefficient ( $P_{\text{app}}$ , in  $\text{cm/s}$ ) of FD-4 was calculated as follows:  $P_{\text{app}} (\text{cm/s}) = dQ/dt / (A \times C_0)$ , where  $dQ/dt$  is the steady state permeation rate of FD-4 ( $\mu\text{g/s}$ ),  $A$  is the surface area of the Transwell ( $\text{cm}^2$ ), and  $C_0$  is the initial concentration of FD-4 on the apical side ( $\mu\text{g/cm}^3$ ).

**Visualization of FD-4 Transport** The investigation of the FD-4 permeation route was performed as reported previously.<sup>10)</sup> In brief, Caco-2 cell monolayers were washed twice with HBSS, and the DMEM culture medium was replaced with HBSS. An apical bathing solution of PLA ( $100 \mu\text{g/mL}$ ) and FD-4 ( $2.5 \text{ mg/mL}$ ) was applied to the cell monolayer for 120min. Thereafter, the cells were fixed in 4% paraformaldehyde-phosphate buffered saline (PBS) for 10min. The fixed cells were washed twice with PBS and mounted on cover-glasses. Fluorescent images of FD-4 were visualized using an FV1000 confocal laser scanning microscope (CLSM; Olympus, Tokyo, Japan).

**MTT Assay** The MTT assay was performed according to the protocol described by Maher and McClean.<sup>19)</sup> Briefly, Caco-2 cell monolayers were washed twice with HBSS, and the DMEM culture medium was replaced with HBSS. The PLA solution was added to the apical bathing solution, and the Caco-2 cell monolayers were exposed to the solution for 2h. Thereafter, the cells were washed twice with 1.5mL of HBSS, which was replaced with 1.5mL (apical side) and 2.6mL (basal side) of DMEM containing the MTT ( $0.5 \text{ mg/mL}$ ). After incubation for 3h, the formazan produced was released by lysing the cells with  $500 \mu\text{L}$  of DMSO. The absorbance at a wavelength of 535nm was determined using a GENios microplate reader (Tecan, Männedorf, Switzerland).

**Immunofluorescent Microscopic Observation** Immunofluorescent microscopic observation of the TJ proteins was performed as described by Seth *et al.*<sup>20)</sup> In brief, Caco-2 cell monolayers were washed twice with HBSS and fixed in acetone-methanol (1:1) at  $4^\circ\text{C}$  for 10min. Cell monolayers were blocked in TBS-T (10mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween-20) with 3% skim milk for 1h at room temperature and incubated with primary antibodies at  $4^\circ\text{C}$  overnight. The cell monolayers were washed twice with TBS-T for 15min, followed by incubation for 1h with secondary antibodies. Fluorescence images were visualized using a CLSM. The junctional fluorescence intensity of tight junction proteins was determined according to the method described by Gopalakrishnan *et al.*<sup>21)</sup> In brief, fluorescence images of samples were selected randomly, and the background of this image was calibrated using the threshold function of the Image J software program. This image was inverted and determined to be the

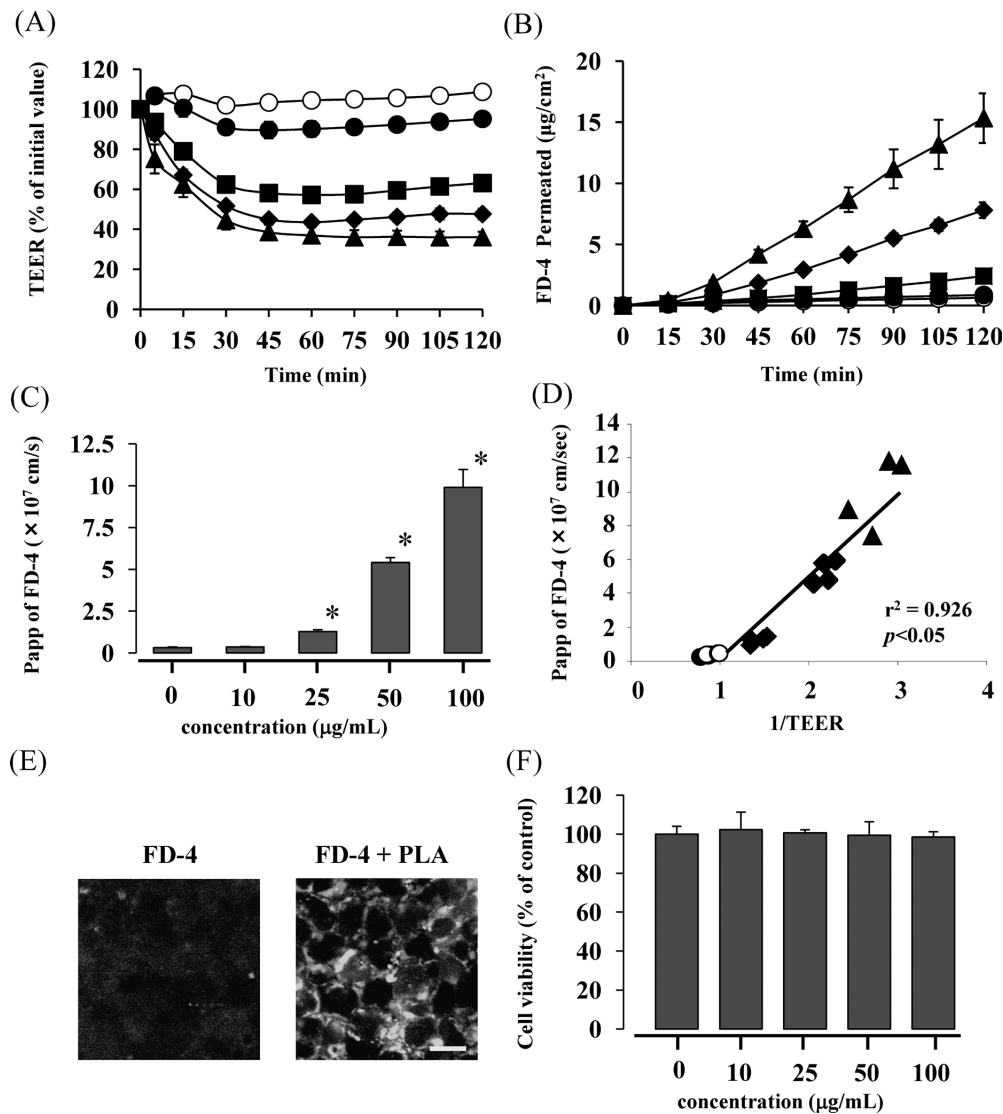


Fig. 1. Effects of PLA on the TEER and FD-4 Permeability in the Caco-2 Cell Monolayer

The change in the TEER (A), amount of FD-4 that crossed the cell layer (B) and the  $P_{app}$  of FD-4 (C) after treatment of Caco-2 monolayers with PLA (○, 0 µg/mL, ●, 10 µg/mL, ■, 25 µg/mL, ◆, 50 µg/mL, ▲, 100 µg/mL). Relationship between  $P_{app}$  of FD-4 and 1/TEER (D). The fluorescence of FD-4 120 min after PLA (100 µg/mL) exposure in Caco-2 cell monolayers (E). After treatment with PLA, the Caco-2 cell viability was evaluated by an MTT assay (F). Each data point represents the mean ± S.E. ( $n=4$ ). \* $p<0.05$  vs. Control.

gray value. The total junctional fluorescence intensity (mean gray value–background gray value) was calculated for each sample and was expressed as a percentage of the control.

**Immunoblot Analysis** Caco-2 cell monolayers were washed twice with HBSS and lysed in radio immunoprecipitation assay (RIPA) buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 1.0% NP-40, 1.0% sodium deoxycholate, 0.1% SDS, Protease inhibitor cocktail). Total cell lysates were prepared by sonication and centrifugation (15000×*g*) for 5 min at 4°C. The protein content in the supernatant was determined by the bicinchoninic acid (BCA) protein assay. The supernatants were mixed with equal volumes of Laemmli sample buffer (2×concentrated) and heated at 95°C for 5 min. Proteins (10 µg) were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in PBS–T with 3% skim milk. They were subsequently incubated in primary antibody in blocking buffer at 4°C overnight, followed by incubation for 1 h with the secondary antibodies.

The chemiluminescence was detected using an LAS-1000 instrument (GE Healthcare, U.K.). The pixel density of tight junction proteins was determined using the Image J software program.

**Statistical Analysis** The results are presented as the means ± S.E. The statistical analyses were performed using a one-way ANOVA, together with Dunnett's test, in order to compare the data from different groups. Comparisons of the  $P_{app}$  were done using Student's *t*-test. A value of  $p<0.05$  was considered to be significant.

## RESULTS

**PLA Induced FD-4 Permeability and Decreased the TEER with No Effect on the Cell Viability** The TEER of the Caco-2 cell monolayer was decreased in a PLA concentration-dependent manner, and the TEER rapidly reduced and reached a minimum at 45 min after treatment (Fig. 1A). The enhanced permeability to FD-4 induced by PLA was also

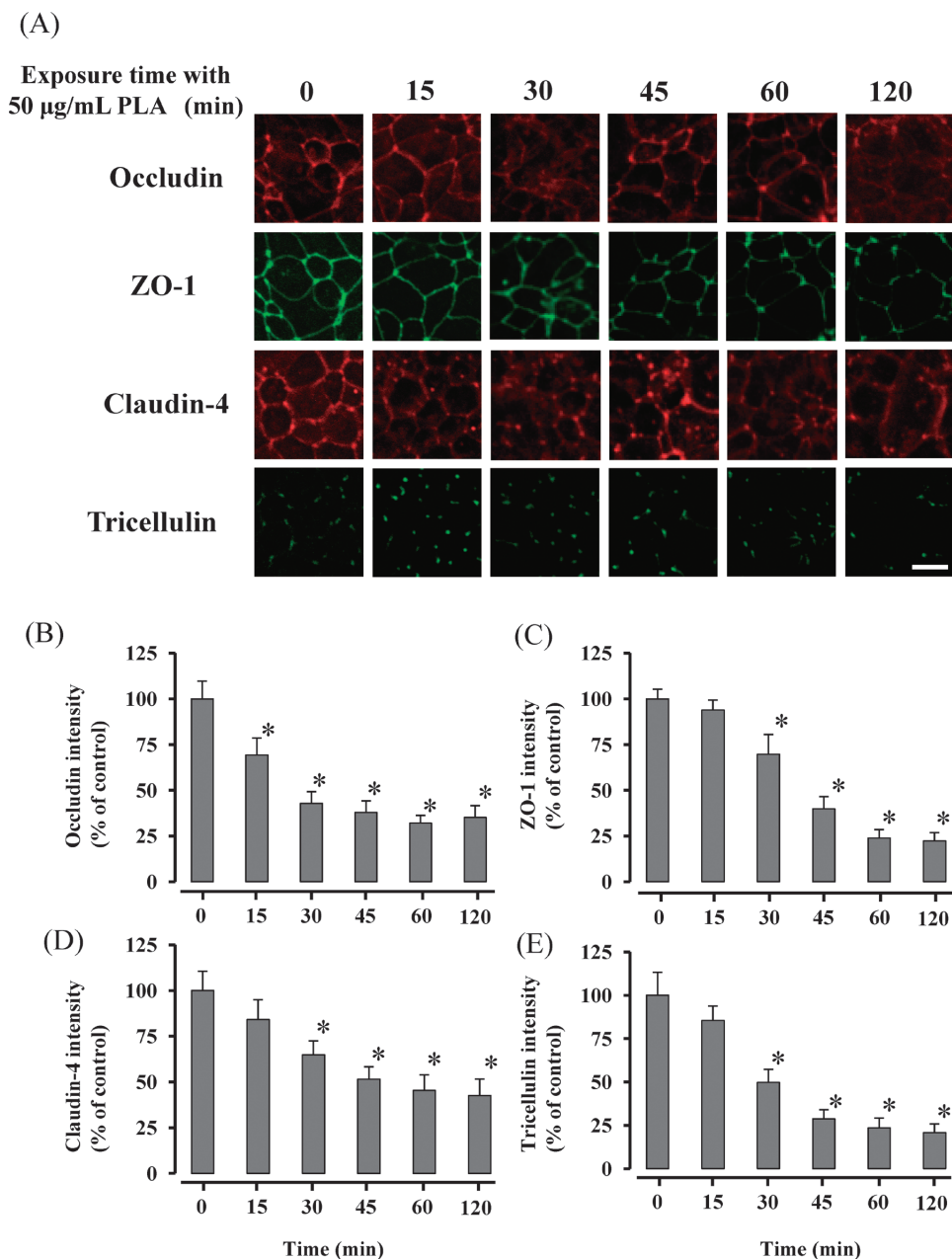


Fig. 2. Time-Dependent Alterations in the Distribution of Tight Junction Proteins after the Application of 50  $\mu\text{g/mL}$  PLA to Caco-2 Cell Monolayers  
 The tight junction proteins disappeared from cell–cell contacts in a time-dependent manner following the treatment with PLA (A). The intensities in all junction proteins were decreased with increasing application times (B–E). Occludin and tricellulin were decreased more than the other proteins (B and E). Each data point represents the mean  $\pm$  S.E. ( $n=4$ ). \* $p<0.05$  vs. Control.

dependent on the PLA concentration (Fig. 1B). The apparent permeability coefficients,  $P_{\text{app}}$ , calculated from steady-state fluxes of FD-4 between 45 and 120min were increased 1.2-, 4-, 16-, and 35-fold by treatment with PLA of 10, 25, 50, and 100  $\mu\text{g/mL}$ , respectively, and application of more than 25  $\mu\text{g/mL}$  PLA led to a significant increase in the  $P_{\text{app}}$  (Fig. 1C). A good linear relationship between the  $1/\text{TEER}$  and  $P_{\text{app}}$  was found (Fig. 1D). The fluorescence of FD-4 120 min after exposure to 100  $\mu\text{g/mL}$  PLA was observed mainly in the intercellular space (Fig. 1E). As shown in Fig. 1F, the Caco-2 cell viability was not affected by PLA in the concentration range used in this study. These results suggest that PLA enhanced the paracellular permeability of FD-4 in Caco-2 monolayers while maintaining the cell viability.

**The Effect of PLA on the Distribution of TJ Proteins**  
 The distribution of TJ proteins (occludin, ZO-1, claudin-4, and tricellulin) was visualized by immunostaining 15, 30, 45, 60, and 120min after exposure to 50  $\mu\text{g/mL}$  PLA (Fig. 2A), and the time course of each protein band intensity at the cell–cell junctions was calculated (Figs. 2B–E). The levels of the TJ proteins localized in the cell–cell junctions were decreased by treatment with PLA in a time-dependent manner. In particular, occludin decreased significantly to about 70% of the initial value after 15 min, even though the levels of other TJ proteins were not significantly altered at that time point. Tricellulin also showed a statistically significant decrease of about 50% compared with the initial value 30min after PLA exposure. The times required to reduce the protein intensity by 50%, as

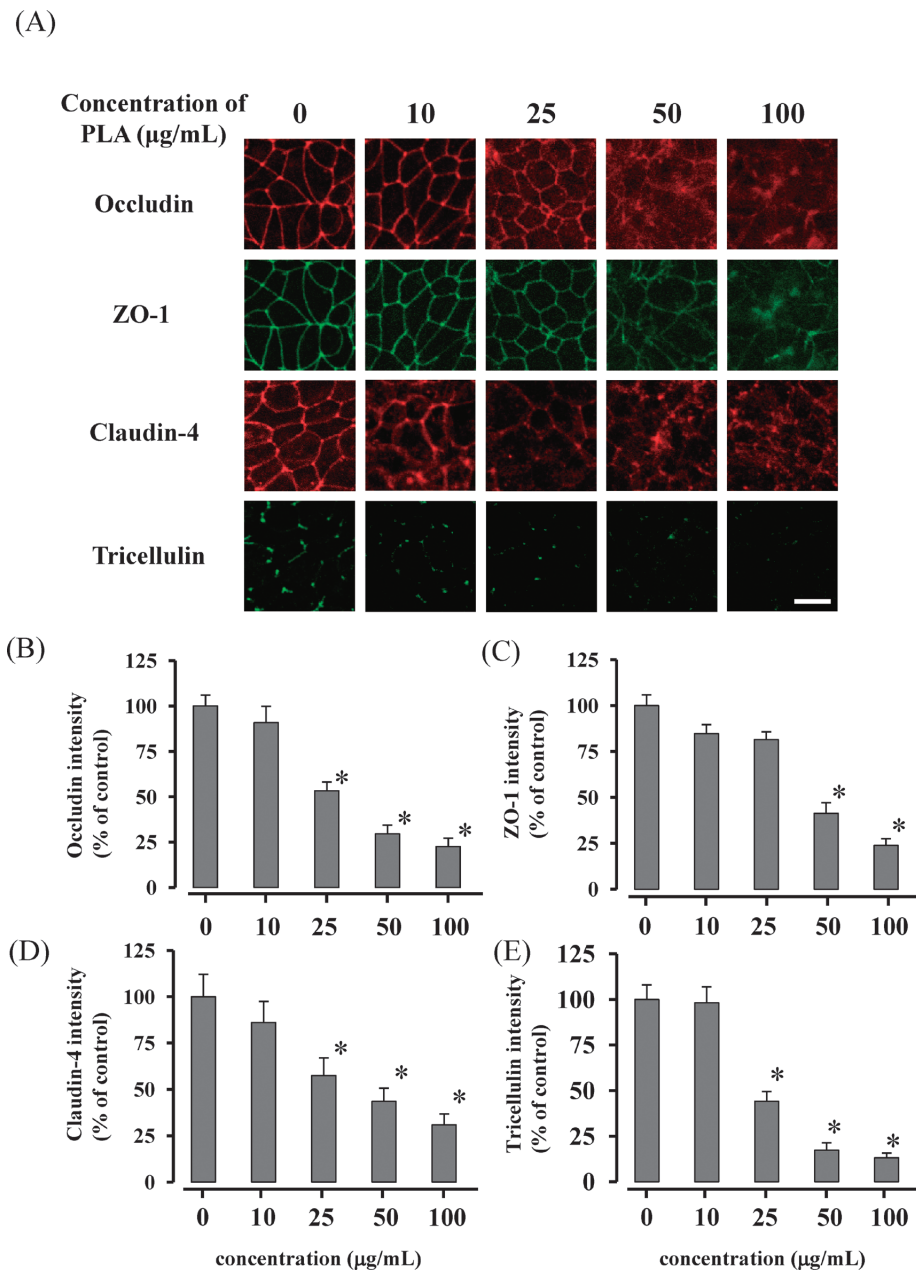


Fig. 3. Effects of the PLA Concentration on the Distribution of Tight Junction Proteins in Caco-2 cell Monolayers 120 min after Treatment with PLA

The tight junction proteins disappeared from the cell-cell contacts in a PLA dose-dependent manner (A). The intensities in all junction proteins were decreased with increasing PLA concentrations (B–E). Each data point represents the mean  $\pm$  S.E. ( $n=4$ ). \* $p<0.05$  vs. Control.

calculated by graphs, were 51 and 41 min for claudin-4 and ZO-1, respectively, whereas they were 27 and 31 min for occludin and tricellulin, respectively.

The effect of the PLA concentration on the distribution of TJ proteins was also assessed (Fig. 3). Immunostaining of TJ proteins showed that all of them disappeared from the cell-cell junctions in a PLA concentration-dependent manner (Fig. 3A). In particular, the intensities of occludin and tricellulin were largely reduced even after exposure to lower concentrations of PLA (Figs. 3B–E). The concentrations required to reduce the protein intensity by 50%, as calculated using Hill's equation, were 46.6, 45.5, 38.7, and 38.6  $\mu\text{g/mL}$  for claudin-4, ZO-1, occludin, and tricellulin, respectively. These results suggested that the disappearance of TJ proteins was induced by PLA treatment in a concentration- and time-dependent

manner, and that occludin and tricellulin were especially sensitive.

#### The Effect of PLA on the Distribution of AJ Proteins

The effects of PLA on the distribution of E-cadherin and  $\beta$ -catenin, components of AJs, were also examined. Most of the E-cadherin and  $\beta$ -catenin were located in the cell-cell junctions prior to PLA treatment (Fig. 4A). In contrast, the expression levels of E-cadherin decreased to about 71% and 55% of the initial value 120 min after treatment with PLA at concentrations of 50 and 100  $\mu\text{g/mL}$ , respectively (Figs. 4A, B). Similarly, the expression levels of  $\beta$ -catenin decreased to about 64% and 52% after treatment with PLA at concentrations of 50 and 100  $\mu\text{g/mL}$ , respectively (Figs. 4A, C). However, the disappearance of AJ proteins was less extensive than that of TJ proteins.

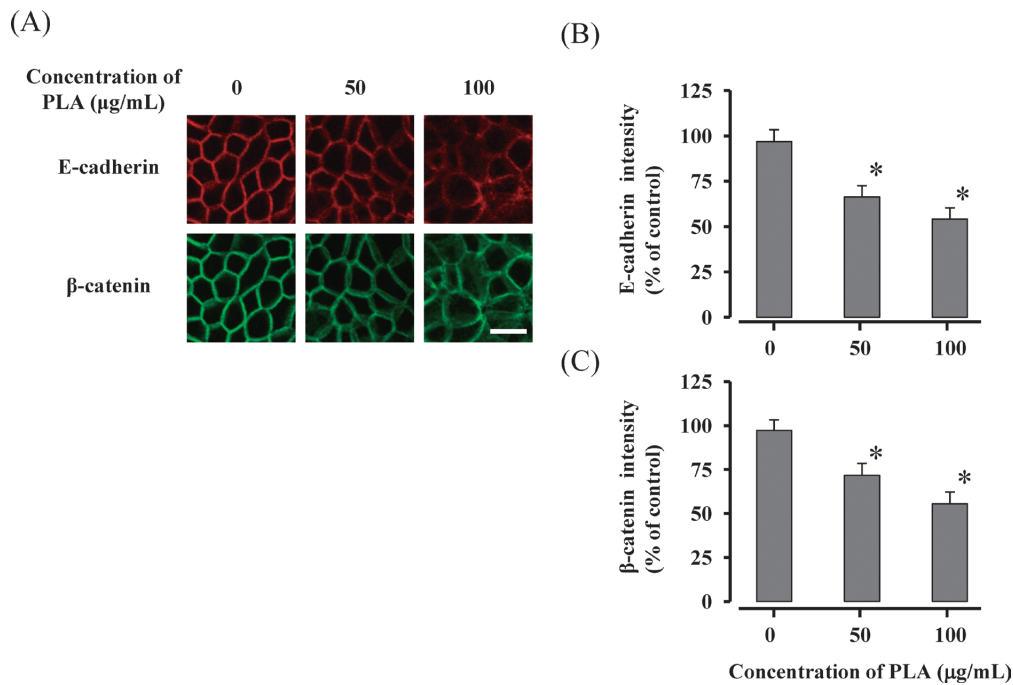


Fig. 4. Effects of the PLA Concentration on the Distribution of Adherens Junction Proteins 120 min after Treatment of Caco-2 Cell Monolayers with PLA

Adherens junction proteins disappeared from the cell–cell contacts in a PLA dose-dependent manner (A). The intensities of the expression of all junction proteins were decreased with increasing PLA concentrations (B,C). Each data point represents the mean±S.E. (n=4). \*p<0.05 vs. Control.

**The Effect of PLA on the Expression of TJ Proteins**

Figure 5 shows the effects of PLA on the expression levels of TJ proteins. Although the expression levels of ZO-1, claudin-4 and tricellulin were not affected by 100 µg/mL PLA treatment, the expression of occludin was decreased significantly to about 75% of the initial value 30 min after treatment (Figs. 5A,B).

**The TEER, TJ Protein Distribution, and Occludin Expression after PLA Depletion** Following depletion after treatment with 50 µg/mL PLA, the decreased TEER recovered to the initial level after 24h. On the other hand, it took 48h for the TEER to completely recover after depletion of the 100 µg/mL PLA (Fig. 6A). In the cells exposed to 100 µg/mL PLA, ZO-1 was observed to be localized around TJs 24h after PLA depletion, and its expression recovered to the initial level (Fig. 6C). Claudin-4 was also re-localized to the cell–cell junctions, and its expression had fully recovered at 48h after PLA depletion (Figs. 6B,C). In contrast, the re-localization and recovery of occludin and tricellulin were incomplete even at 48h after PLA depletion (Figs. 6B,C). However, the total cellular expression levels of occludin at 24 and 48h after PLA depletion were almost identical to the initial value (Fig. 6D).

**DISCUSSION**

In the present study, we investigated whether PLA promotes the paracellular permeability of Caco-2 cell monolayers, as well as excised rabbit nasal epithelium, to FD-4. We also characterized the involvement of the redistribution of TJ proteins (ZO-1, claudin-4, occludin, and tricellulin) in the enhanced paracellular permeability by monitoring the alterations in the TEER, distribution of TJ proteins, and occludin expression following PLA depletion. As a result, PLA increased the

paracellular permeability of Caco-2 monolayers to FD-4 due to the disassembly of TJ proteins (Fig. 1). In previous study, we have reported that the interaction between PLA and ion channels as a calcium channel and chloride channel has no relation to an increase paracellular permeability.<sup>22)</sup> Therefore, as a possibility, the ionic interaction between a cationic PLA and anionic proteins (but not identified) may trigger the enhanced absorption. After that, dispersion of occludin and ZO-1 via activation of Serine/Threonine kinases and protein tyrosine phosphatases in intracellular signaling induced TJ disassembly.<sup>11)</sup> In consequence, disappearance of TJ proteins in intercellular enhanced an absorption of macromolecules as a FD-4.

Interestingly, occludin and tricellulin in the paracellular space disappeared more rapidly than ZO-1 and claudin-4 when the permeability to FD-4 was increased by PLA treatment (Fig. 2). In addition, following PLA exposure at a lower concentration, the occludin and tricellulin intensities around the cell–cell junctions were found to be reduced more remarkably than those of claudin-4 and ZO-1 (Fig. 3). Hence, it is likely that the internalization of occludin as well as claudins, known as a TJ protein related to macromolecules permeability, play an important role in the regulation of paracellular permeability to hydrophilic macromolecules induced by PLA.<sup>14)</sup> Occludin was the first TJ transmembrane protein identified.<sup>12)</sup> In previous studies, occludin was not a crucial component protein required for the TJ barrier function with regard to TJ formation.<sup>23,24)</sup> In contrast, recent studies using short interfering RNA (siRNA)- or micro RNA (miRNA)-mediated loss of occludin in Caco-2 cells demonstrated that occludin regulates the penetration of macromolecular substances.<sup>14,24)</sup> This finding suggests that occludin plays a crucial role in the regulation of hydrophilic macromolecule penetration, thus supporting the results of the present study. Our study indicates that the

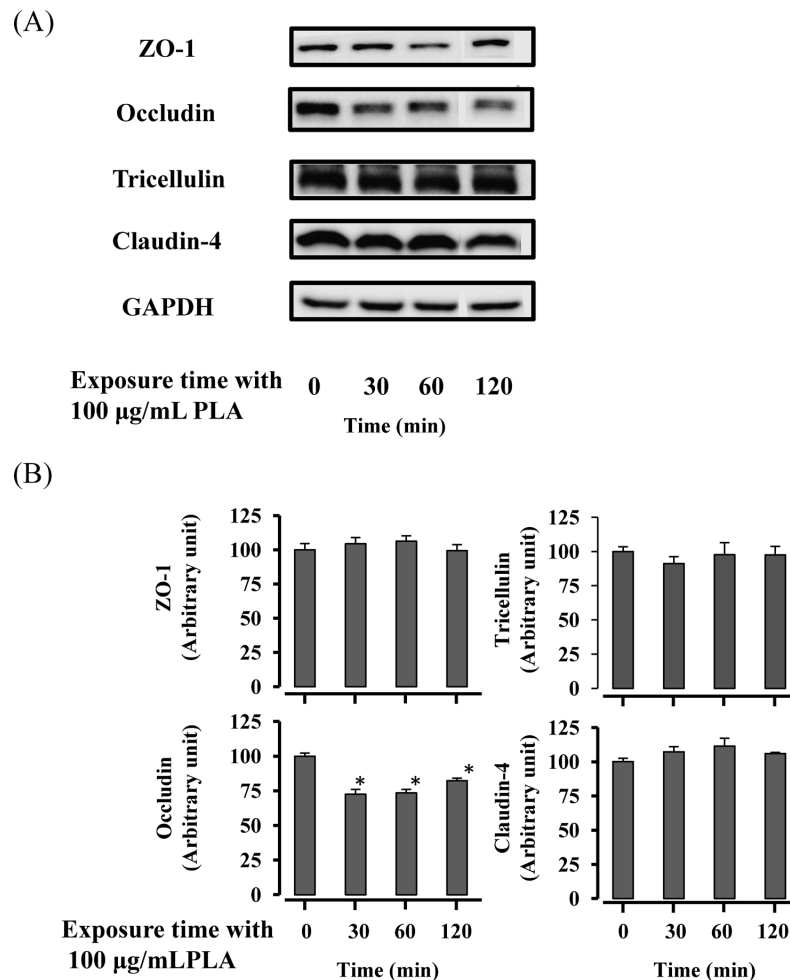


Fig. 5. Changes in the Tight Junction Protein Levels after Treatment with PLA

An immunoblot analysis of the tight junction proteins in Caco-2 cell monolayers was assessed after treatment of the cells with PLA for various times (A), and the density of the protein spots was also analyzed (B–D). Each data point represents the mean  $\pm$  S.E. ( $n=4$ ). \* $p<0.05$  vs. Control.

internalization of occludin mainly contributes to hydrophilic macromolecule penetration in bicellular TJ.

Claudin, also a four-transmembrane-domain protein, was discovered by Tsukita and colleagues,<sup>13</sup> and 24 claudin family members have been reported so far. It has been reported that claudin is the main protein involved in forming TJ strands, and, in addition, claudin functions as the major component regulating the permeability to small molecules, such as certain ions, through the paracellular pathway.<sup>26,27</sup> Furthermore, claudins form homo- and/or hetero-strands in TJs.<sup>28</sup> Combination of claudin members determines the properties of TJs-seal in various tissues. Claudin-1, 3, 4, and 7, *etc.* are expressed in Caco-2 cells,<sup>29</sup> and claudin-4 is closely involved in the paracellular barrier function of TJs and has been investigated as a target for increasing drug delivery.<sup>30</sup> In this study, the rate of disappearance of claudin-4 from the paracellular region caused by PLA exposure was slower than those of occludin and tricellulin. These results suggest that PLA has an effect on both occludin that regulates the permeability of macromolecules and claudin-4 that plays an important role in the paracellular barrier function of TJs. Therefore, claudin-4 is partially contributed the paracellular permeability of macromolecules by PLA exposure.

Tricellulin has been reported to be localized in the corners

at regions where three cells are in close contact. The disruption of the paracellular barrier function of TJs was demonstrated to be caused by tricellulin knockdown using siRNA in mouse cell lines, Eph4, CSG1, and MTD1A cells, and a dog cell line (Madin Darby canine kidney (MDCK) cells). Also, depletion of tricellulin leads to disruption of bicellular TJs.<sup>16</sup> In addition, it was reported that the penetration of hydrophilic macromolecules was reduced by tricellulin overexpression.<sup>31</sup> These findings also indicate that tricellulin might play an important role in regulating the penetration of hydrophilic macromolecules induced by PLA.

AJs are the structures situated underneath TJs, and they play a role in the adhesion between cells. The constituent structural molecules that assemble to form AJs are E-cadherin and  $\beta$ -catenin. Although PLA also affected these proteins in the present study, the effects of PLA on these proteins were relatively weak compared with those on TJ proteins (Fig. 4).

We examined whether the degradation of TJ proteins was regulated by the altered subcellular distribution of the proteins by performing a Western blot analysis with whole cell lysates. The results suggested that ZO-1, claudin-4, and tricellulin were not degraded following their internalization induced by PLA. In contrast, PLA led to an approximately 25% decrease in the intensity of occludin, thus indicating that the occludin

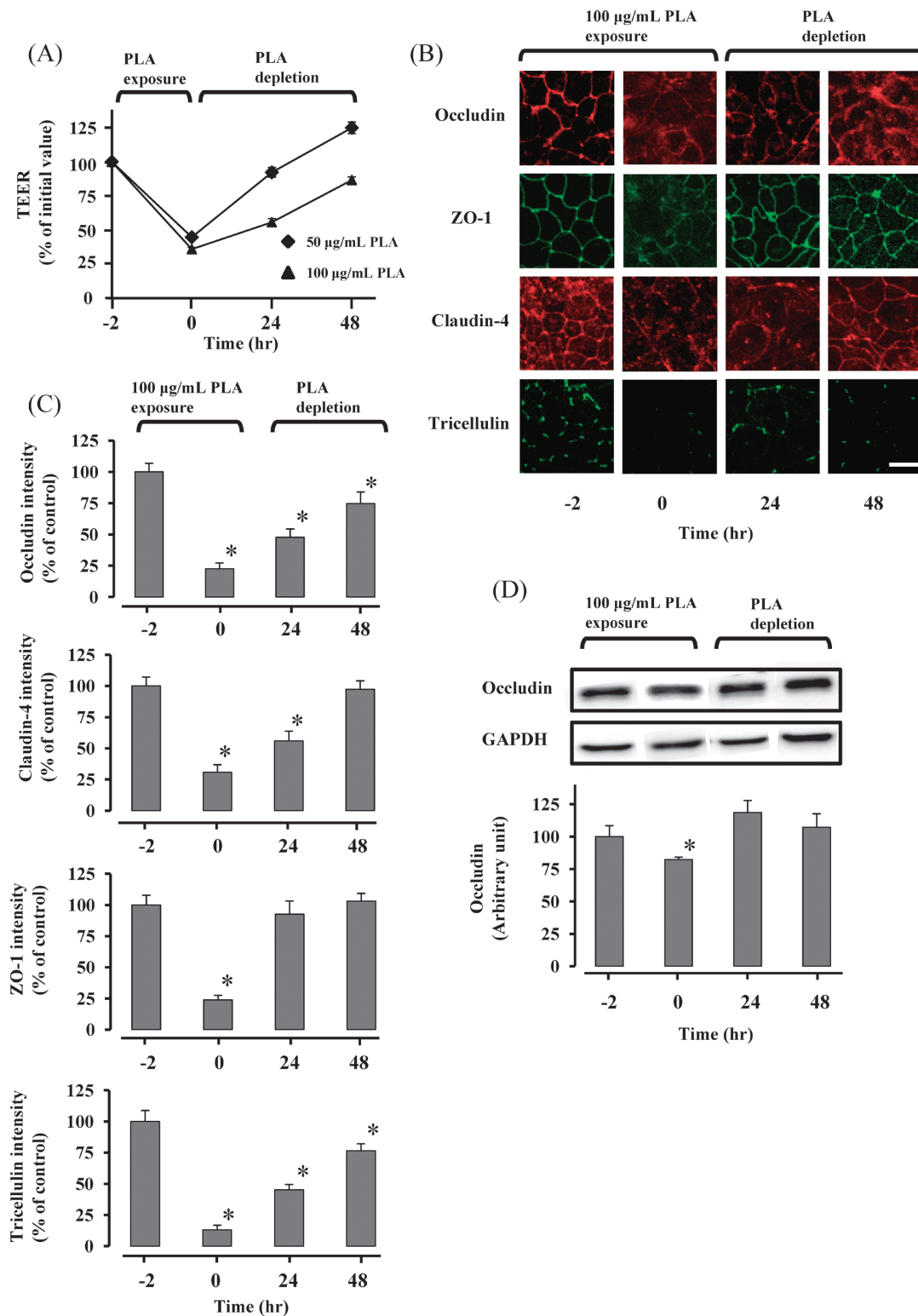


Fig. 6. Recovery of the TEER and Redistribution of Tight Junction Proteins after Removal of PLA from Caco-2 Cell Monolayers

After removal of PLA from the Caco-2 cell monolayers, the TEER values increased (A), tight junction proteins were redistributed to the cell-cell contact (B), and the expression levels of tight junction proteins recovered (C). An increased occludin level was detected by the immunoblot and densitometric analyses after the removal of PLA (D). Each data point represents the mean±S.E. (n=4). \*p<0.05 vs. Control.

protein appears to be partially degraded (Figs. 5A,B). The degradation of occludin was reported to occur when retinal endothelial cells and Caco-2 cell monolayers were treated with vascular endothelial growth factor (VEGF), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-1 $\beta$  (IL-1 $\beta$ ).<sup>32-34</sup> In addition,

ubiquitin-proteasome and matrix metalloprotease are known to be involved in a mechanism of occludin degradation. Further investigation will be needed to clarify the process of degradation of occludin following PLA treatment.

Following PLA depletion, the reductions in the TEER and



the intensities of TJ proteins gradually recovered, suggesting that there was a reconstitution of the TJ strand structures, *i.e.*, recovery of the barrier function (Fig. 6A). This also suggests that the effect of PLA is reversible. However, the decreased TEER had not recovered fully even 48h after withdrawal of the 100  $\mu\text{g}/\text{mL}$  PLA. The alteration in the TEER might have coincided with the behavior of TJ proteins. Therefore, we investigated the relocalization and disposition of the TJ proteins around cell–cell junctions after withdrawal of the 100  $\mu\text{g}/\text{mL}$  PLA. As shown in Figs. 6B and C, all of the TJ proteins were observed to have relocalized around cell–cell junctions, but their appearance rates were different. For example, the intensity of ZO-1 staining rapidly and completely recovered within 24h after PLA depletion. Claudin-4 was also fully relocalized by 48h after PLA depletion, although the localization of occludin and tricellulin had still not been fully restored. A decrease in the expression level of occludin was not responsible for this insufficient relocalization, because the occludin that degraded following PLA treatment was almost completely re-synthesized within the first 24h after PLA depletion, thus restoring it to its original expression level (Fig. 6D). Therefore, it was most likely that occludin was still partially internalized in the subcellular space. Thus, the incomplete reconstitution of the TJ strand structures, *i.e.*, incomplete reassembly of TJ proteins, might be resulted in an insufficient recovery of the TEER.

In the early stage of TJ formation, it is known that ZO-1 is formed first, and then transmembrane proteins, such as occludin and claudin, are recruited to TJs to form the mature and intact TJ structure.<sup>35</sup> Our data suggest that ZO-1 also plays an important role in the complete reformation of the cell sheet after disruption of TJ strand structures by PLA. This finding may be of interest in the fields of medical science and physiology, and may be useful for the further development of novel drug delivery strategies.

In conclusion, the mechanism underlying the enhancement of Caco-2 permeability to FD-4 permeation by PLA was concluded to be as follows: 1) the TJ proteins were internalized from the cell–cell junctions and then were translocated to the subcellular space; 2) the paracellular permeability to FD-4 mainly increased by means of a reduction in the barrier function due to the disruption of the TJ strand structures.

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