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Abstract: A nondigestible disaccharide, diffructose anhydride (DFA) III, is known to activate calcium transport via tight junctions (TJs); however, the characteristics of and mechanisms for the increase in paracellular transport induced by DFAIII have not been clarified. We compared the effect of DFAIII with that of sodium caprate (C10), a well-known enhancer of TJ permeability, on the changes in TJ proteins, transport of paracellular markers, and effects of nine cellular signaling blockers using Caco-2 monolayers. The addition of DFAIII (0-100 mmol/L) and C10 (0-10 mmol/L) to the apical medium of the Caco-2 monolayers dose-dependently decreased transepithelial electrical resistance (TER), which is an indicator of TJ permeability. The reduction with C10 was much faster than that with DFAIII. Transport of the paracellular markers of various molecular weights (182-43,200) was elevated by the addition of 100 mmol/L DFAIII and 10 mmol/L C10. The transport rates were much in the presence of C10 than of DFAIII, while the reduction in

TER by two treatments was similar (from 1,000 to 300  $\Omega \cdot \text{cm}^2$ ). Treatment with DFAllI and C10 changed the distribution of actin filament and claudin-1, but not occludin, junctional adhesion molecule-1, or zonula occludens-1; however, alterations in the patterns of the TJ proteins differed according to treatment. An inhibitor of myosin light chain kinase and a chelator of intracellular calcium ion ( $[\text{Ca}^{2+}]_i$ ) attenuated the TER reduction by C10, but not by DFAllI. These data demonstrate that the increase in TJ permeability induced by DFAllI results from the alterations to actin and claudin-1 via  $[\text{Ca}^{2+}]_i$ -independent mechanisms.

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## Abstract

A nondigestible disaccharide, difructose anhydride (DFA) III, is known to activate calcium transport via tight junctions (TJs); however, the characteristics of and mechanisms for the increase in paracellular transport induced by DFAIII have not been clarified. We compared the effect of DFAIII with that of sodium caprate (C10), a well-known enhancer of TJ permeability, on the changes in TJ proteins, transport of paracellular markers, and effects of nine cellular signaling blockers using Caco-2 monolayers. The addition of DFAIII (0-100 mmol/L) and C10 (0-10 mmol/L) to the apical medium of the Caco-2 monolayers dose-dependently decreased transepithelial electrical resistance (TER), which is an indicator of TJ permeability. The reduction with C10 was much faster than that with DFAIII. Transport of the paracellular markers of various molecular weights (182-43,200) was elevated by the addition of 100 mmol/L DFAIII and 10 mmol/L C10. The transport rates were much in the presence of C10 than of DFAIII, while the reduction in TER by two treatments was similar (from 1,000 to 300  $\Omega\cdot\text{cm}^2$ ). Treatment with DFAIII and C10 changed the distribution of actin filament and claudin-1, but not occludin, junctional adhesion molecule-1, or zonula occludens-1; however, alterations in the patterns of the TJ proteins differed according to treatment. An inhibitor of myosin light chain kinase and a chelator of intracellular calcium ion ( $[\text{Ca}^{2+}]_i$ ) attenuated the TER reduction by C10, but not by DFAIII. These data demonstrate that the increase in TJ permeability induced by DFAIII results from the alterations to actin and claudin-1 via  $[\text{Ca}^{2+}]_i$ -independent mechanisms.

## Keywords

Difructose anhydride III, Sodium caprate, Paracellular transport, Tight junction, Caco-2 cells

## Abbreviations

Ca, calcium; C10, sodium caprate; DFA, difructose anhydride; FD, fluorescein isothiocyanate-conjugated dextran; FITC, fluorescein isothiocyanate; JAM, junctional adhesion molecule; LDH, lactate dehydrogenase; LY, lucifer yellow CH dipotassium salt; MLCK, myosin light chain kinase; PLC, phospholipase C; TJ, tight junction; ZO-1, zonula occludens 1

## Introduction

Difructose anhydride III (DFAIII; di-D-fructo-furanose 1,2':2,3 dianhydride) is a nondigestible disaccharide produced by inulin fructotransferase, an enzyme of *Arthrobacter* sp. H65-7 (Saito and Tomita, 2000). Our recent studies demonstrated that ingestion of DFAIII enhanced intestinal calcium (Ca) absorption in rats (Mineo et al., 2003; Mitamura et al., 2002; Suzuki et al., 1998) and humans (Shigematsu et al., 2004). One of the major mechanisms responsible for the increase in Ca absorption is the promotion of paracellular Ca transport through the direct stimulation of DFAIII in the small intestine (Mineo et al., 2003; Mitamura et al., 2002; Shigematsu et al., 2004; Suzuki et al., 1998). Some cellular events may be involved in the promotion of paracellular Ca transport induced by DFAIII. We also showed that DFAIII enhanced paracellular Ca transport in human intestinal Caco-2 cells (Suzuki and Hara, 2004), which indicates that the Caco-2 monolayer is a useful model of the small intestinal epithelium for examining the mechanisms underlying the promotion of paracellular Ca transport by DFAIII.

Ca absorption in the small intestine proceeds by two routes, an active transcellular transport and a passive paracellular transport (Bronner, 1998; Bronner and Pansu, 1999). Transcellular transport is saturable, regulated by 1, 25-dihydroxy vitamin D<sub>3</sub>, and mainly confined to the duodenum (Bronner, 1998; Bronner and Pansu, 1999). Paracellular transport is an unsaturable process that requires a gradient of Ca concentrations between the luminal and basolateral sides, and occurs throughout the intestines (Bronner, 1998; Bronner and Pansu, 1999). Paracellular passage of ions, water, and some water-soluble molecules is regulated by tight junctions (TJs) located on the uppermost portion of the lateral plasma membranes of adjacent cells (Mitic et al., 2000). The TJ is a multiprotein complex composed of both transmembrane and cytosolic proteins, although their functions are not completely understood. Three integral transmembrane proteins of TJs have been identified: occludin (Furuse et al., 1993), claudin (Furuse et al., 1998), and junctional adhesion molecule (JAM) (Martin-Padura et al., 1998). Occludin and claudin are tetra-spanning transmembrane proteins. Occludin plays an important role in the organization of the TJ, but does not appear to be essential for its assembly (Furuse et al.,

1 1998; Saitou et al., 2000). The claudin family has been expanded to 24 members, and they are known  
2 to play a crucial role in the gate-keeping function of TJs (Furuse et al., 2002). JAM is a single  
3 transmembrane protein that has been shown to participate in the sealing of the TJs (Liu et al., 2000).  
4 The carboxyl terminal ends of these three transmembrane proteins interact with zonula occludens 1  
5 (ZO-1), a cytosolic plaque protein (Bazzoni et al., 2000; Furuse et al., 1994; Itoh et al., 1999). Several  
6 TJ proteins, including occludin and ZO-1, associate with the actin cytoskeleton, which also regulates  
7 the function of TJs (Itoh et al., 1997; Wittchen et al., 1999).

8           Paracellular transport via TJs is physiologically regulated by various intracellular signalings  
9 (Karczewski and Groot, 2000). Extracellular stimuli, including food factors, drugs, and chemicals, are  
10 reported to affect the paracellular transport through signal transduction (Nusrat et al., 2000). Sodium  
11 caprate (C10), a medium-chain fatty acid, has been shown to increase paracellular transport via the  
12 contraction of actin filaments following the phosphorylation of myosin light chains (MLCs) by  
13  $Ca^{2+}$ /calmodulin-activated MLC kinase (MLCK) in Caco-2 cells (Lindmark et al., 1998).

14 Lysophosphatidic acid increases TJ permeability caused by actin contraction via the  
15 Rho-kinase-dependent pathway (Hirase et al., 2001). Hydrogen peroxide also induces alterations in  
16 occludin and ZO-1 via the activation of phosphatidylinositol 3-kinase and protein tyrosine kinase, and  
17 enhances TJ permeability in Caco-2 cells (Sheth et al., 2003). Protein tyrosine kinase participates in a  
18 number of cellular signal cascades, and the tyrosine phosphorylation of occludin affects its localization  
19 and interaction with ZO-1, -2, and -3 (Kale et al., 2003). Stimulation of protein kinase A or C is also  
20 known to regulate paracellular permeability in epithelial cells (Kottra and Vank, 2003; Stenson et al.,  
21 1993). We reported that DFAIII enhanced paracellular transport with the induction of  $Ca^{2+}$  signaling in  
22 Caco-2 cells (Suzuki and Hara, 2004). Therefore, we suggest that the promotion of paracellular  
23 transport by DFAIII results from alterations in the cytoskeleton or TJ proteins via an intracellular event  
24 after DFAIII recognition by a putative sensory system in Caco-2 cells.

25           Paracellular pathways are important for the transport of ions and water, while TJs form a  
26 physical barrier to the diffusion of allergens, toxins, and pathogens. Uncontrolled increases in intestinal

1 permeability or disruption of TJ function allow the entry into the blood stream of these undesirable  
2 molecules. Thus, it is important to evaluate the characteristics of the increase in paracellular transport  
3 by DFAIII.

4           The aims of the present study were to examine the effects of DFAIII on the alterations in TJ  
5 proteins and transport of paracellular markers of various molecular weights, and to examine the effects  
6 of the following cellular signaling blockers on the action of DFAIII: inhibitors of phospholipase C  
7 (PLC), MLCK, protein kinase C, alpha-subunit of Gi protein, adenylate cyclase, protein tyrosine kinase,  
8 phosphatidylinositol 3-kinase, and Rho-associated protein kinase and a chelator of intracellular  $Ca^{2+}$ .  
9 And we also compared the action of DFAIII with that of C10, as C10 is a well-known enhancer of TJ  
10 permeability and the mechanism underlying the action of C10 has been extensively investigated.

## Materials and methods

### Chemicals

Difructose anhydride III (DFAIII; di-D-fructo-furanose 1,2':2,3 dianhydride) was kindly provided by Nippon Beet Sugar MFG., Ltd. (Obihiro, Japan). Rhodamine phalloidin was purchased from Molecular Probe Inc. (Eugene, OR). Mouse anti-occludin-FITC conjugate, rabbit anti-claudin-1, rabbit anti-ZO-1, rabbit anti-JAM-1, and goat anti-rabbit IgG-FITC conjugate were purchased from Zymed laboratories Inc. (San Francisco, CA). U73122 (an inhibitor of PLC), BAPTA-AM (a cell-permeable chelator of  $\text{Ca}^{2+}$ ), ML-7 (an inhibitor of MLCK), H-7 (an inhibitor of protein kinase C), and pertussis toxin (an inhibitor of alpha-subunit of Gi protein) were purchased from Sigma Chemical (St. Louis, MO). SQ22536 (an inhibitor of adenylate cyclase), genistein (an inhibitor of protein tyrosin kinase), LY294002 (an inhibitor of phosphatidylinositol 3-kinase), and Y-27536 (an inhibitor of Rho-associated protein kinase) were purchased from Calbiochem (San Diego, CA). [ $^3\text{H}$ ]-mannitol (specific activity, 740 TBq/mol) was purchased from American Radiolabeled Inc. (St. Louis, MO). All other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

### Cell culture

Caco-2 cells (HTB-37; American Type Culture Collection, Rockville, MD) were propagated and maintained in high-glucose (4.5 g D-glucose/L) DMEM supplemented with 100 mL/L decomplemented fetal bovine serum, 44 mmol/L sodium bicarbonate, 1 mmol/L sodium pyruvate, 50,000 U/L penicillin, and 50 mg/L streptomycin, and adjusted to pH 7.4 (Suzuki and Hara, 2004). The cells were seeded into permeable polyester membrane filter supports (Transwell, 12 mm diameter, 0.4  $\mu\text{m}$  pore size, 1.0  $\text{cm}^2$  growth area; Corning Costar Co., Cambridge, MA) at a density of 63,000 cells/ $\text{cm}^2$ . Prior to all experiments, cultures were maintained for at least 24 d after seeding. The medium was refreshed every 3 d. Cultures were used for experiments between passage 35 and 60.

### Measurement of tight junction permeability

Transepithelial electrical resistance (TER) across the cell monolayers was measured using a commercial apparatus (Millicell-ERS; Millipore Co., Billerica, MA). The Caco-2 cells grown on the

1 permeable membrane filters were rinsed with pre-warmed HBSS [134 NaCl, 4.2 NaHCO<sub>3</sub>, 0.34  
2 Na<sub>2</sub>HPO<sub>4</sub>, 5.4 KCl, 0.44 KH<sub>2</sub>PO<sub>4</sub>, 1.25 CaCl<sub>2</sub>, 0.49 MgCl<sub>2</sub>, 0.41 MgSO<sub>4</sub>, 5.6 D-glucose, 4.0  
3 L-glutamine, and 10 HEPES (mmol/L), pH 7.4] at 37°C. The basolateral and apical chambers of the  
4 cells were bathed in 1.0 and 0.5 mL pre-warmed HBSS at 37°C, respectively. After a 60-min  
5 equilibration period, the experiments were initiated by adding DFAIII (0, 25, 50, 75, or 100 mmol/L) or  
6 C10 (0, 5, 7.5, or 10 mmol/L) to the apical chamber. TER was measured before (0 min) and at several  
7 points after the start of incubation (15-180 min). The experiments using C10 were carried out using  
8 standard HBSS in the basolateral chamber and Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS in the apical chamber to avoid  
9 precipitation of the fatty acid. These conditions had no effect on TER across the monolayers.

10 To examine the osmotic effect of DFAIII on TJ permeability, 100 mmol/L ethylene glycol or  
11 DFAIII was added to the apical or basolateral chambers in the following combinations: apical  
12 chamber/basolateral chamber; none/none; DFAIII/none; none/DFAIII; ethylene glycol/none;  
13 none/ethylene glycol; DFAIII/ethylene glycol. TER across the Caco-2 monolayers was measured  
14 before and after incubation for 3 h with each of the six treatments.

15 Transport of paracellular markers for 1 h was assessed from the apical to the basolateral  
16 chamber in the monolayers after 2-h exposure to 100 mmol/L DFAIII or 0.5-h exposure to 5 mmol/L  
17 C10. Monolayers incubated for 2 h without DFAIII or C10 were used as the control group. Mannitol  
18 (containing 370 MBq/L), lucifer yellow CH dipotassium salt (LY), and FITC-conjugated dextran 4  
19 (FD-4; average molecular weight 4,400), 10 (FD-10; 10,500), 20 (FD-20; 19,500), and 40 (FD-40;  
20 43,200) were used as paracellular markers, and were added into the apical chamber at a final  
21 concentration of 100 μmol/L. The concentrations of LY and FDs in the basolateral solution were  
22 determined by measuring fluorescence (CAF-110; JASCO International Co., Ltd., Tokyo, Japan). The  
23 radioactivity of [<sup>3</sup>H]-mannitol in the basolateral solution was measured by means of a liquid  
24 scintillation counting system (LSC-5100; Aloka, Tokyo, Japan) (Suzuki and Hara, 2004).

25 To clarify the cellular signaling pathway involved in the promotive effect of DFAIII on TJ  
26 permeability, nine signaling blockers were used as follows: U73122, 5 μmol/L; BAPTA-AM, 20

1  $\mu\text{mol/L}$ ; ML-7, 30  $\mu\text{mol/L}$ ; H-7, 20  $\mu\text{mol/L}$ ; Y-27632, 10  $\mu\text{mol/L}$ ; SQ22536, 400  $\mu\text{mol/L}$ ; genistein,  
2 300  $\mu\text{mol/L}$ ; LY294002, 25  $\mu\text{mol/L}$ ; pertussis toxin, 200  $\mu\text{g/L}$ . Signaling blockers except for pertussis  
3 toxin were added to the apical side of the monolayers during the equilibration (1 h) and experimental (3  
4 h) periods. Pertussis toxin was added to the Caco-2 cell medium 24 h prior to the experiment. The TER  
5 across the monolayers was measured before and after incubation for 3 h with or without 100 mmol/L  
6 DFAIII. The effect of four signaling blockers, BAPTA-AM, ML-7, H-7, and Y-27632, on the  
7 C10-induced enhancement of TJ permeability was examined in the same manner. The TER across the  
8 monolayers was measured before and after a 0.5-h incubation with or without 5 mmol/L C10.

#### 9 Immunofluorescent localization of TJ proteins and F-actin

10 Caco-2 cell monolayers grown on the permeable membrane filters were used after 2-h  
11 exposure to 100 mmol/L DFAIII or 0.5-h exposure to 5 mmol/L C10. The monolayers incubated for 2 h  
12 without DFAIII and C10 were used as the control group. For F-actin staining, the cell monolayers were  
13 fixed in 3.7% formaldehyde for 20 min, permeabilized in ice-cold 0.5% Triton X-100, and incubated  
14 with 5,000 U/L rhodamine phalloidin for 30 min at room temperature. For immunostaining of TJ  
15 proteins, the cell monolayers were fixed and permeabilized with methanol for 5 min at  $-40^{\circ}\text{C}$  and were  
16 incubated with 10% normal goat serum for 30 min at room temperature. For claudin-1 and ZO-1, the  
17 cell monolayers were incubated with claudin-1 or ZO-1 antibody (1:50 dilution) for 1 h at room  
18 temperature. For JAM-1, the monolayers were incubated with JAM-1 antibody (1:50 dilution) for 24 h  
19 at  $4^{\circ}\text{C}$ . The cell monolayers were incubated with FITC-conjugated anti-rabbit IgG (1:100 dilution) for  
20 1 h at room temperature. For occludin, the cell monolayers were incubated with occludin  
21 antibody-conjugated FITC (1:50 dilution) for 24 h at  $4^{\circ}\text{C}$  after incubation with 10% normal mouse  
22 serum. For double staining of F-actin and claudin-1, the monolayers fixed with methanol were used. All  
23 monolayers were mounted in 1% n-propyl gallate in 90% glycerol and analyzed by confocal  
24 microscopy (Zeiss LSM 410; Zeiss, Thornwood, NY).

#### 25 Measurement of lactate dehydrogenase activity

26 Caco-2 cell viability was assessed by measuring the release of lactate dehydrogenase (LDH)

1 in the apical and basolateral solutions of the monolayers incubated with DFAIII (0, 25, 50, 75, or 100  
2 mmol/L) for 3 h or C10 (0, 5, 7.5, or 10 mmol/L) for 2 h. LDH activity was determined using a  
3 commercially available kit (LDH CII-Test Wako; Wako Pure Chemical Industries).

#### 4 Statistical analysis

5 All values are expressed as means  $\pm$  SEM. The TER is expressed as  $\Omega \cdot \text{cm}^2$  of surface area of  
6 the monolayer. The transport of paracellular markers across the monolayers are expressed as nmol of  
7 each marker transferred/ $\text{cm}^2$  of surface area or percentage increase against the values of the control  
8 group, and the two values were logarithmically transformed before statistical analyses, as  
9 heteroscedasticity was present within each data. Statistical analyses were performed by a repeated  
10 measure 2-way ANOVA or 1-way ANOVA followed by Duncun multiple range test (Duncan, 1955). A  
11 difference with  $P < 0.05$  was considered significant. These statistical analyses were done by the general  
12 linear models procedure of the Statistical Analysis Systems program (version 6.07; SAS Institute Inc.,  
13 Cary, NC).

14

15

## Results

### Effects of DFAIII and C10 on TER across the cell monolayers and LDH release

Incubation time, DFAIII concentration, and the interaction between them all had significant effects on TER ( $P < 0.001$ , 2-way ANOVA, **Fig. 1A**). DFAIII dose-dependently and gradually decreased TER across the monolayers from 30 min to 120 min after the start of incubation. There were no decreases with 25 mmol/L DFAIII and no differences in the TER at any dose of DFAIII at 30 min. At 60, 90, 120, and 180 min, values of TER with 50, 75, and 100 mmol/L DFAIII were lower than those without DFAIII or with 25 mmol/L DFAIII.

Incubation time, C10 concentration, and the interaction between them also had significant effects on TER ( $P < 0.001$ , 2-way ANOVA, **Fig. 1B**). C10 dose-dependently and rapidly decreased TER after the start of incubation. For all concentrations tested, TER reached its lowest value at 15 min. The TER values for 5, 7.5, and 10 mmol/L C10 were lower than the values without C10 and the values for 7.5 and 10 mmol/L C10 were lower than that for 5 mmol/L C10 at all time points except before incubation.

There were no differences in LDH activity, an indicator of cell membrane damage, in the apical and basolateral solutions after a 3-h incubation with 0, 25, 50, 75, and 100 mmol/L DFAIII (**Table 1**). The addition of C10 dose-dependently increased LDH release to the apical and basolateral solutions after a 2-h incubation. The LDH activity in the apical solution was higher in the monolayers incubated with 5, 7.5, and 10 mmol/L C10 than in those incubated without C10.

### The specificity of promotive effect of DFAIII on TJ permeability

Apical applications of DFAIII [DFAIII/none (apical chamber/ basolateral chamber) and DFAIII/ethylene glycol] clearly decreased TER values across the monolayers for 3 h (**Table 2**); however, the basolateral application of DFAIII had a smaller effect on the TER than did the apical application, and the value in the none/ DFAIII treatment was higher than those in the two apical DFAIII treatments. The basolateral application of ethylene glycol had no effect on TER. There were no differences in TER across the Caco-2 monolayers before the 3-h incubation among the six treatments.

## 1 Effects of DFAIII and C10 on transport of paracellular markers

2           The transport rates of LY and the four FDs with various molecular weights were higher in the  
3 monolayers incubated with 100 mmol/L DFAIII than in those incubated without DFAIII (**Fig. 2A**).  
4 The % increases against the control group were also significant for all markers except mannitol (**Fig.**  
5 **2B**). In the monolayers incubated with 5 mmol/L C10, the transport rates and the % increases of all  
6 markers were much higher than those in the control and the DFAIII groups. The transport rates of FDs  
7 in all groups decreased with increases in the molecular weight of the FDs. The rates of FD-40 in the  
8 control group and the values of FD-20 and -40 with DFAIII treatment were lower than those of FD-10  
9 with the same treatments. With increases in the molecular weight of the FDs, the % increase in FD  
10 transport tended to be larger with C10 treatment, but to be lower with DFAIII treatment. The %  
11 increases of FD-20 and -40 were significantly lower than those of FD-4 and -10 in the DFAIII group.  
12 The transport rates of mannitol were higher than those of other markers for each treatment, and the  
13 value was increased by C10, but not by DFAIII. The rate of LY was lower than that of FD-4 in the  
14 control group.

15           In this experiment, we also checked TER to confirm similar reductions in the TER between  
16 the monolayers after 2-h exposure to 100 mmol/L DFAIII and 0.5-h exposure to 5 mmol/L C10. The  
17 TER with DFAIII and C10 were lower than the control values for all markers, and the reductions in  
18 TER induced by DFAIII and C10 were very similar at around  $300 \Omega \cdot \text{cm}^2$  (**Table 3**). There were no  
19 differences in the TER before incubation with each marker (data not shown).

## 20 Alterations in F-actin and TJ proteins induced by DFAIII and C10

21           Stronger staining of the F-actin was observed with C10 treatment than with the control and  
22 DFAIII treatments on the adjacent borders of Caco-2 cells in the perijunctional images (**Fig. 3**). In the  
23 monolayers treated with DFAIII, F-actin was strongly stained only at the points where 3 or 4 cells were  
24 in contact. The four TJ proteins, occludin, claudin-1, JAM-1, and ZO-1, were observed as continuous  
25 pericellular bands in the perijunctional images of the control monolayers (**Fig. 3**). In the monolayers  
26 treated with DFAIII, strong fluorescent intensity of immunostained claudin-1 was observed at the

1 points where 3 or 4 cells were in contact, which was similar to F-actin staining and was distinguishable  
2 from the control monolayers. In the monolayers treated with C10, a dispersed and fragmented staining  
3 pattern of claudin-1 was observed. The distributions of occludin, JAM-1, and ZO-1 in the monolayers  
4 were not affected by treatment with DFAIII or C10.

5 The perijunctional images of double immunostained F-actin and claudin-1 showed their  
6 co-localization in the control monolayers (**Fig. 4A-C**). In the monolayers treated with DFAIII, the  
7 co-localized redistributions of F-actin and claudin-1 were observed at the cell corners (**Fig. 4D-F**).  
8 Strong staining of the F-actin was observed on the borders of the cells treated with C10; however, the  
9 staining pattern of F-actin was not identical to the dispersed location of claudin-1, which showed as red  
10 (**Fig. 4G-I**).

#### 11 Effect of signaling blockers on DFAIII- and C10-induced reductions in TER

12 In all sets of experiments without inhibitors, TER across the monolayers treated with DFAIII  
13 was much lower than that in those without DFAIII (**Fig. 5A-I**). The DFAIII-induced reduction in TER  
14 was not affected by treatment with any of the signaling blockers tested except for an inhibitor of  
15 adenylate cyclase (SQ22536), by which TER in the presence of the inhibitor was slightly, but  
16 significantly, higher than TER without the inhibitor in the DFAIII-treated monolayers. In control  
17 monolayers, no blockers had any effects on the TER across the monolayers.

18 TER with the C10 treatment was reduced to less than a half of the value of the control  
19 monolayers, and the reduced TER was partly reversed by the inhibitors MLCK (ML-7) and a  
20 cell-permeable Ca<sup>2+</sup> chelator (BAPTA-AM) (**Fig. 6**). The inhibitors of Rho-associated protein kinase  
21 (Y-27632) and protein kinase C (H-7) had no effect on the C10-induced reduction in TER.

22

## Discussion

1  
2 DFAIII and C10 dose-dependently decreased TER across Caco-2 cell monolayers (**Fig. 1**),  
3 which is consistent with results of previous reports (Lindmark et al., 1998; Suzuki and Hara, 2004).  
4 However, the patterns of the decreases in TER were distinct from each other. Specifically, the treatment  
5 with DFAIII lowered the TER much more slowly than did C10. This finding implies that DFAIII and  
6 C10 enhance paracellular transport via different mechanisms. Treatment with C10, but not DFAIII,  
7 increased LDH release, an indicator of membrane damage, in both the apical and basolateral solutions  
8 (**Table 1**). DFAIII activates paracellular routes without cell membrane damage and is not harmful to  
9 intestinal cells even at high concentrations.

10 Paracellular passage of solutes is regulated by TJs, showing that the TJs function not only as  
11 a barrier to large molecules but also as a transport pathway for ions and water (Tsukita and Furuse,  
12 2000; Tsukita et al., 2001). The enhanced transport rates of paracellular markers were much higher in  
13 the C10-treated monolayer than in the DFAIII-treated one (**Fig. 2A**), although the electrolyte transport  
14 indicated by TER is identical in the two treatments (**Table. 2**). The % increases in FD transport were  
15 enhanced by C10, but were reduced by DFAIII with increases in the molecular weight of FDs (**Fig. 2B**).  
16 The enhancement of paracellular transport is known to result from increasing both the radius and  
17 number of the pathways constituted by TJs (Watson et al., 2001). These results indicate that pore size of  
18 the paracellular pathways activated by DFAIII is smaller than that of the pathways activated by C10. It  
19 has been reported that C10 treatment increased the pore radius in the TJ pathway from 8 to 20 angstrom  
20 in the rat colon (Hayashi et al., 1997). The excess increase in the pore size of TJ pathways may allow  
21 transport of undesirable molecules; e.g., toxic compounds or allergens. Conversely, DFAIII appears to  
22 be safe as a food additive for the promotion of absorption via TJs.

23 The transport rates of mannitol and FDs, which are electrically neutral, depended on their  
24 molecular weight in all the monolayers (**Fig. 2A**). However, the rates of LY, having negative charge,  
25 were lower than the values of FD-4 for each treatment though the molecular weight of LY (522 Da)  
26 was much smaller than that of FD-4 (4,400 Da). The transport of solutes via TJs depends on the charge

1 as well as the molecular size (Knipp et al., 1997). Our result agrees with that of a previous report in  
2 which the TJ permeability of Caco-2 monolayers was higher for positive and neutral molecules than for  
3 negative ones (Knipp et al., 1997).

4 The activation of paracellular pathways by both DFAIII and C10 appears to result from  
5 alterations to F-actin and claudin-1, not other TJ proteins (occludin, JAM-1, and ZO-1) (**Fig. 3 and 4**).  
6 Immunohistochemical analysis demonstrates that the alteration of the patterns of F-actin and claudin-1  
7 were different between DFAIII and C10 groups. Treatment with DFAIII caused increases in the  
8 fluorescent signals of F-actin and claudin-1 at the cell corners, and the both signals were co-localized  
9 (**Fig. 4**). In contrast, the contraction of F-actin after treatment with C10 was much stronger than that  
10 with DFAIII, and the claudin-1 staining after the C10 treatment showed a discontinuous feature on the  
11 adjacent border of the cells (**Fig. 4**). Claudins are known to be essential for the sealing of TJs (Furuse et  
12 al., 2002) and the actin cytoskeleton also regulates TJ functions (Itoh et al., 1997; Wittchen et al., 1999).  
13 Therefore, the different changes in the two TJ proteins may reflect different increases in the  
14 paracellular transport of markers between the two treatments. TJ proteins, especially claudins, need to  
15 be continuously distributed on the adjacent borders of the cells, as shown in the control and DFAIII  
16 groups, to regulate paracellular transport. The discontinuous feature of claudin-1 induced by C10 may  
17 result in the formation of the larger pores and allowing the permeation of larger markers. Further  
18 investigations may define the relationship between alterations to TJ proteins and characteristics of  
19 paracellular transport.

20 The contraction of actin filaments is reported to be caused by phosphorylation of MLCs via  
21  $Ca^{2+}$ /calmodulin-activated MLCK and Rho-kinase pathways in the epithelial cells (Hirase et al., 2001;  
22 Ma et al., 2000). The C10-induced reduction in TER was partially, but clearly, attenuated by a  
23 cell-permeable chelator of  $Ca^{2+}$  (BAPTA-AM) and an inhibitor of MLCK (ML-7) (**Fig. 6**). This result  
24 shows that one of the major mechanisms underlying the action of C10 is actin contraction following the  
25 activation of MLCK, which is consistent with the results of a previous report (Lindmark et al., 1998).  
26 Our recent study suggests that  $Ca^{2+}$  signaling may be involved in the promotive effect of DFAIII on

1 paracellular transport (Suzuki and Hara, 2004). However, BAPTA-AM and ML-7 did not attenuate the  
2 reduction in TER induced by DFAIII (**Fig. 5**). This finding reveals that the  $\text{Ca}^{2+}$  signaling induced by  
3 DFAIII is not involved in the mechanisms underlying the action of DFAIII. Also, an inhibitor of  
4 Rho-associated protein kinase (Y-27536) did not affect the action of DFAIII (**Fig. 5A, C, and E**). The  
5 cellular mechanisms leading to the alterations to TJ proteins including the claudin family are not  
6 completely understood. The inhibitor of adenylate cyclase (SQ22536) slightly, but significantly,  
7 attenuated the effect of DFAIII (**Fig. 5G**). However, treatment with SQ22536 similarly increased TER  
8 in the control group (but not significantly), suggesting that the inhibition of adenylate cyclase increased  
9 TER irrespective of DFAIII action. The other signaling blockers used did not affect the action of  
10 DFAIII. Therefore, other signaling mechanisms may possibly be involved in the alteration to F-actin,  
11 claudin-1, and TER by DFAIII, and we should consider also mechanisms not involving intracellular  
12 signal transduction.

13           The promotive effect of DFAIII on paracellular transport is not caused merely by increasing  
14 solutes, which was demonstrated by the result that the apical application of 100 mmol/L ethylene  
15 glycol had no effect on TER (**Table 2**). Our recent study showed that some nondigestible saccharides  
16 similarly decreased TER (Suzuki and Hara, 2004). In addition, the action of DFAIII was not influenced  
17 by the basolateral application of ethylene glycol, which counteracted the osmotic gradient between the  
18 apical and basolateral sides, and the basolateral application of DFAIII had no substantial effect on TER.  
19 These findings indicate that specific common structures in the saccharides are recognized by a putative  
20 sensory system in the brush border membrane of Caco-2 cells and activate paracellular routes. However,  
21 the molecular weight of ethylene glycol used as a negative control is smaller than those of the  
22 saccharides and its physiochemical properties may be different from the saccharides. We are  
23 conducting further studies to clarify the specificity of the sensory system for the nondigestible  
24 saccharides.

25           In summary, DFAIII and C10 reduced TER and enhanced the transport of paracellular  
26 markers with alterations to claudin-1 and F-actin in Caco-2 cell monolayers; however, the alterations

1 induced by DFAIII were distinguishable from those by C10, and the pore size of the paracellular routes  
2 activated by DFAIII may be smaller than that of those activated by C10. Further, C10 partially  
3 enhanced paracellular routes by activation of MLCK, while the DFAIII-induced reduction in TER was  
4 not attenuated by any signaling blockers. The results of the present study demonstrate that the cellular  
5 mechanisms underlying the enhancement of paracellular transport by DFAIII are distinct from those of  
6 C10. Further information on the mechanisms is necessary to fully establish the safety of DFAIII for  
7 human consumption.

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11

12

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24  
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## Figure legends

### Fig. 1

Transepithelial electrical resistance (TER) across the Caco-2 cell monolayers incubated in the presence of up to 100 mmol/L difructose anhydride (DFA) III (A) or 10 mmol/L sodium caprate (B) in the apical chambers. Values are means  $\pm$  SEM of 4 monolayers. Means not sharing a common letter are significantly different at each time point ( $P < 0.05$ ).

### Fig.2

Transport rates of paracellular markers across the Caco-2 cell monolayers incubated for 1 h after 2-h exposure to 100 mmol/L DFAIII or 0.5-h exposure to 5 mmol/L sodium caprate (A) and the % increases against the control value (B). Caco-2 monolayers incubated for 2 h without DFAIII or sodium caprate were used as the control group. Values are means  $\pm$  SEM of 5-7 monolayers. Means not sharing a common letter in the same treatment differ ( $P < 0.05$ ). \*Significantly different from the value of the control group for the same marker ( $P < 0.05$ ). #Significantly different from the value of the DFAIII group for the same marker ( $P < 0.05$ ).

### Fig. 3

Confocal images of actin filaments stained with rhodamine phalloidin (A, F, and K) and immunofluorescent staining of occludin (B, G, and L), claudin-1 (C, H, and M), JAM-1 (D, I, and N), and ZO-1 (E, J, and O) in Caco-2 cell monolayers. A-E, Caco-2 monolayers incubated for 2 h without DFAIII or sodium caprate; F-J, Caco-2 monolayers incubated for 2 h with 100 mmol/L DFAIII; E-O, Caco-2 monolayers incubated for 0.5 h with 5 mmol/L sodium caprate, respectively. The arrows in panel F indicate areas of high intensity of fluorescence-labeled F-actin in the monolayers incubated with DFAIII. The arrowheads indicate areas of high intensity of fluorescence-labeled claudin-1 in the monolayers incubated with DFAIII. The images are representatives of at least 4 monolayers, respectively. The scale bar represents 10  $\mu\text{m}$ .

### Fig.4

Confocal images of double immunostained F-actin (A, D, and G) and claudin-1 (B, E, and H)

1 in Caco-2 cell monolayers (C, F, and I, overlay images). A-C, Caco-2 monolayers incubated without  
2 DFIII or sodium caprate for 2 h; D-F, Caco-2 monolayers incubated with 100 mmol/L DFIII for 2 h;  
3 G-I, Caco-2 monolayers incubated with 5 mmol/L sodium caprate for 0.5 h, respectively. The  
4 arrowheads indicate areas of high intensity of fluorescence-labeled claudin-1 in the monolayers  
5 incubated with DFIII. The images are representatives of at least 4 monolayers, respectively. The scale  
6 bar represents 10  $\mu\text{m}$ .

7 Fig.5

8 Transepithelial electrical resistance (TER) across the Caco-2 monolayers incubated for 3 h  
9 with or without 100 mmol/L DFIII in the presence or absence of each signaling blocker. A, an  
10 inhibitor of phospholipase C (U73122); B, a cell-permeable chelaor of calcium ion (BAPTA-AM); C,  
11 an inhibitor of myosin light chain kinase (ML-7); D, an inhibitor of protein kinase C; E, an inhibitor of  
12 Rho-associated protein kinase (Y-27632); F, an inhibitor of phosphatidylinositol 3-kinase (LY294002);  
13 G, an inhibitor of adenylate cyclase (SQ22536); H, an inhibitor of tyrosine kinase (genistein); I, an  
14 inhibitor of alpha-subunit of Gi protein (pertussis toxin). Values are means  $\pm$  SEM of 4-5 monolayers.  
15 Means not sharing a common letter in each panel differ ( $P < 0.05$ ).

16 Fig.6

17 Transepithelial electrical resistance (TER) across the Caco-2 monolayers incubated for 0.5 h with  
18 or without 5 mmol/L C10 in the presence or absence of inhibitors of myosin light chain kinase (ML-7),  
19 protein kinase C, and Rho-associated protein kinase (Y-27632) and a cell-permeable chelaor of calcium  
20 ion (BAPTA-AM). Values are means  $\pm$  SEM of 4 monolayers. Means not sharing a common letter  
21 differ ( $P < 0.05$ ).

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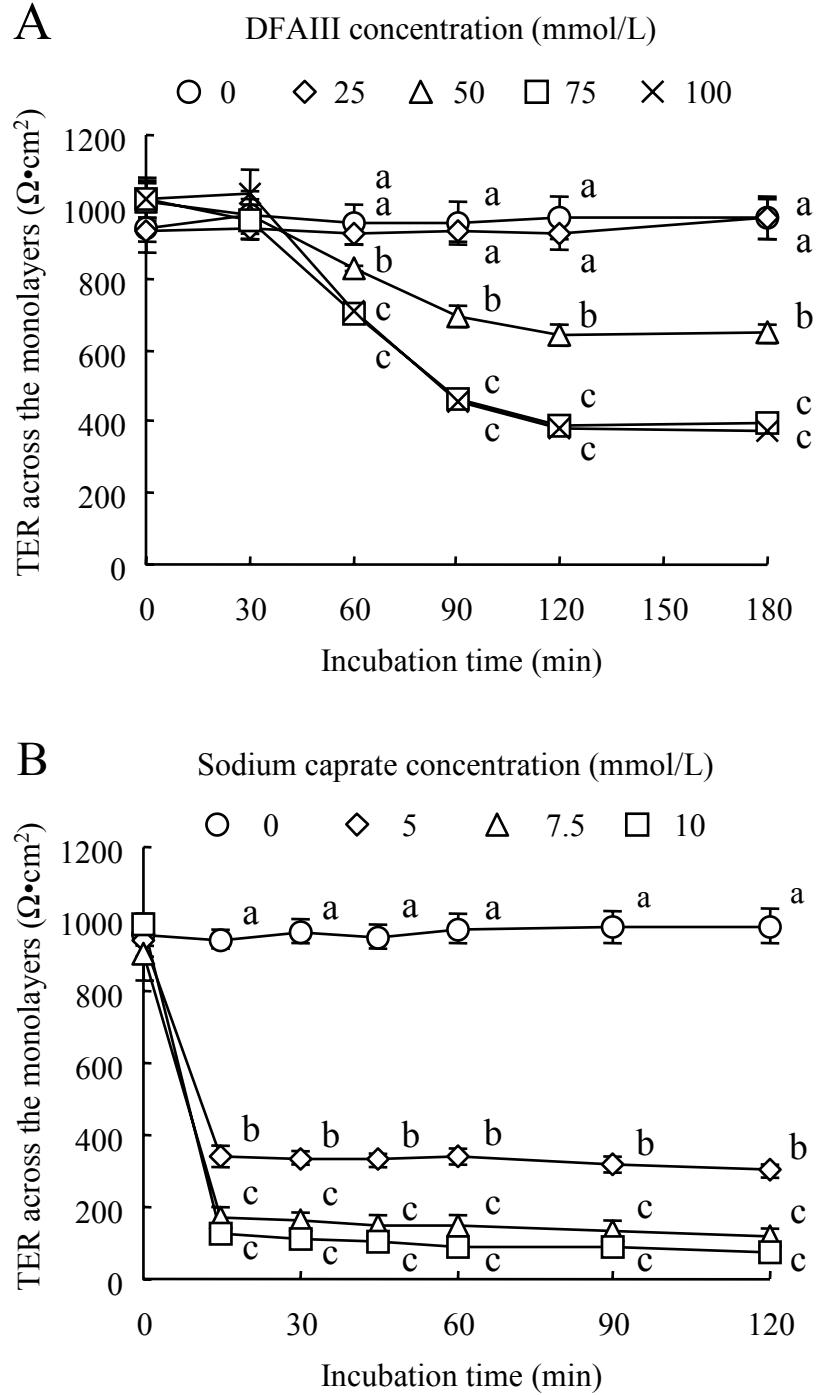
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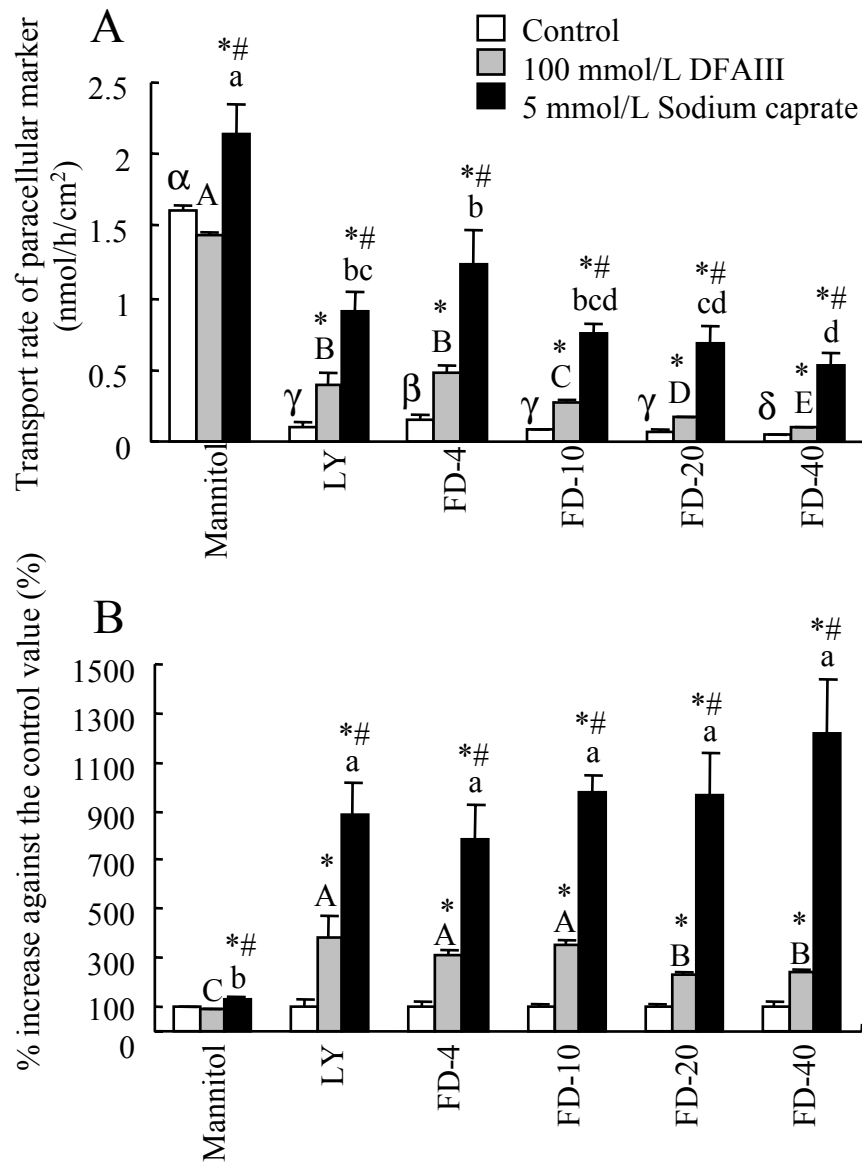
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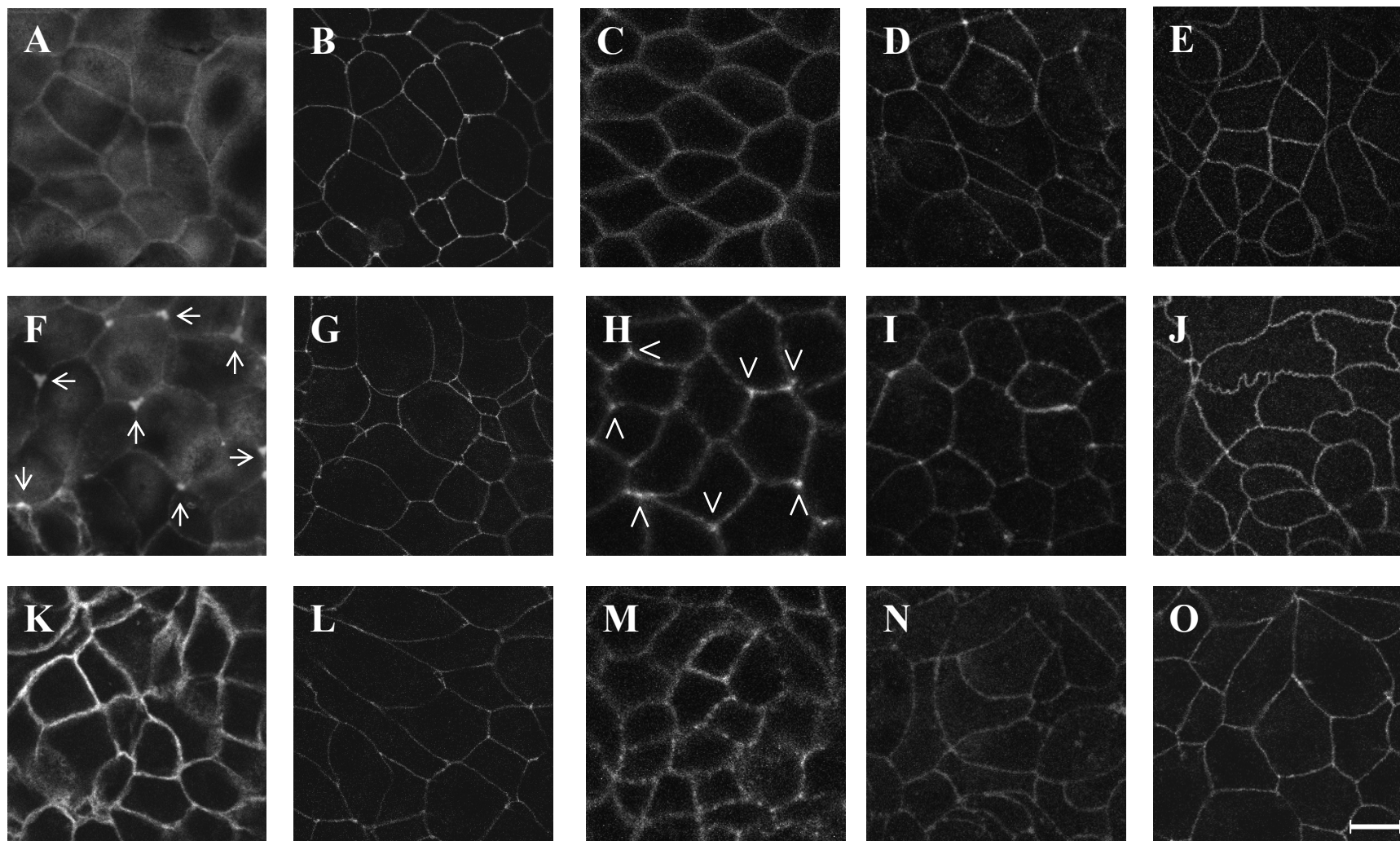
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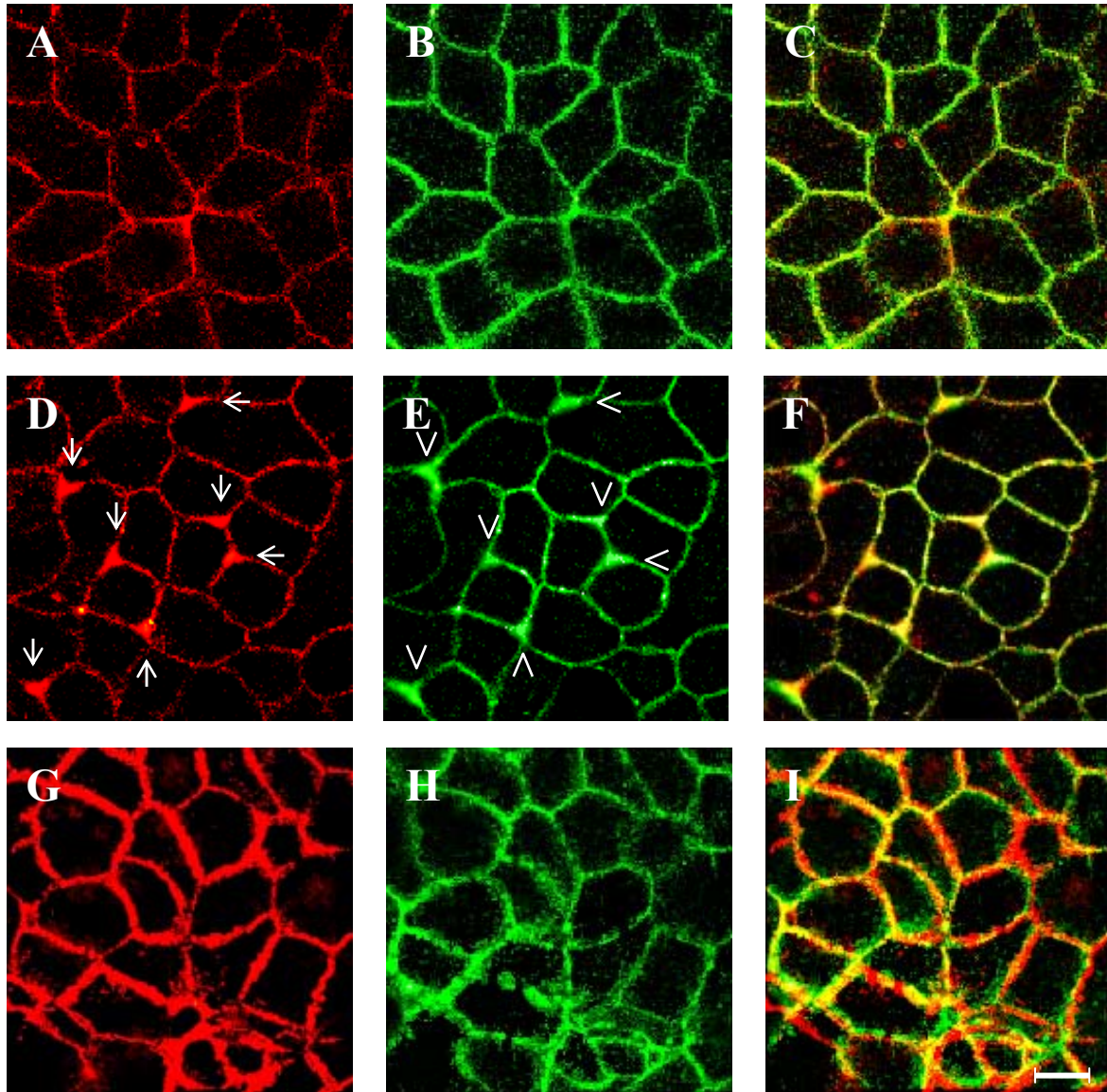
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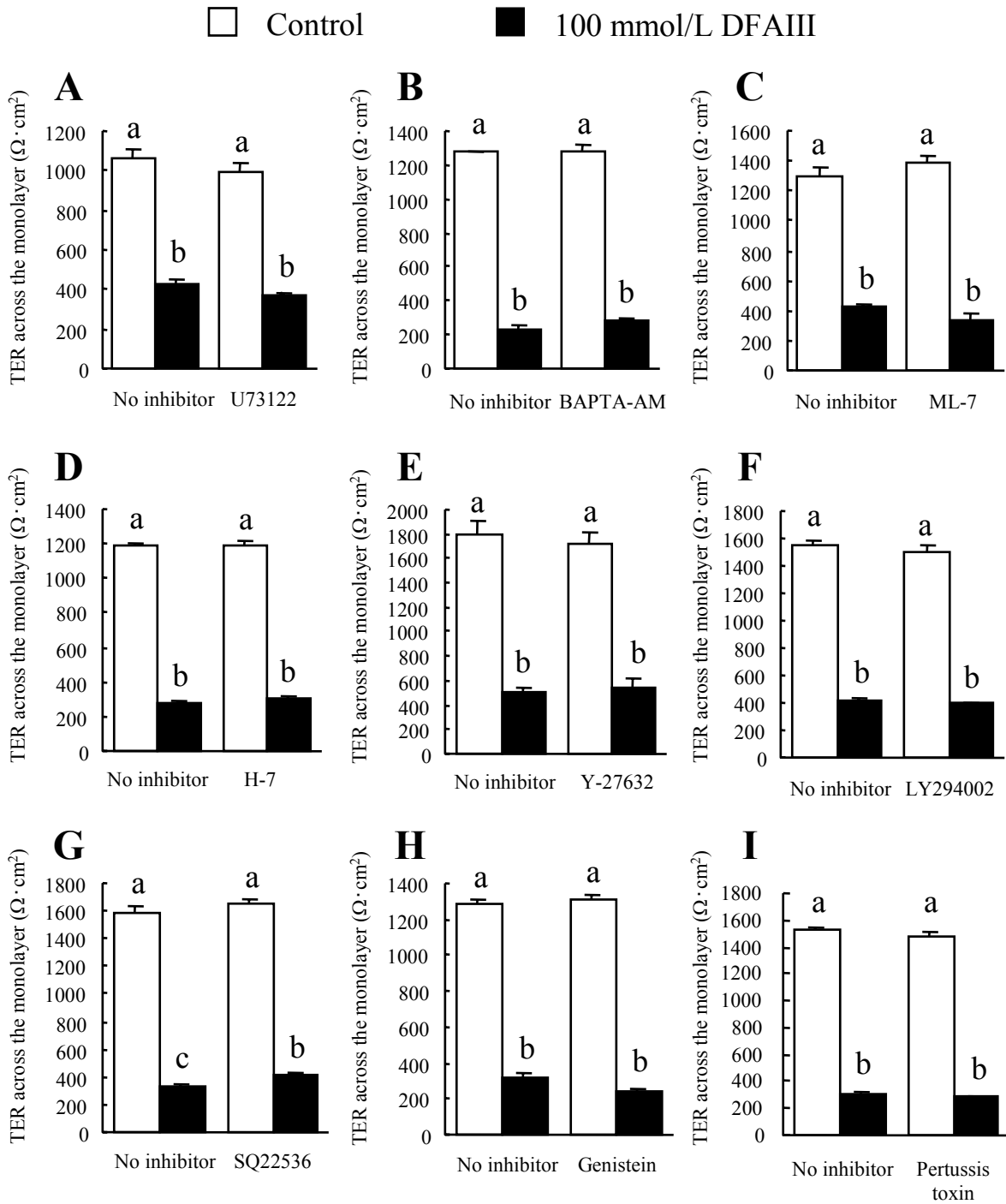
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 T. Suzuki et al.



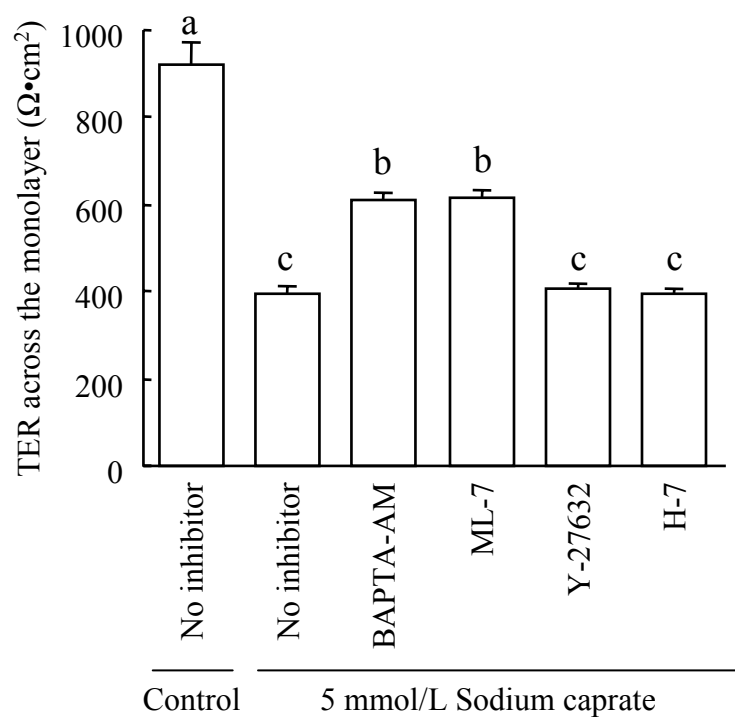
**Fig.3**  
**T. Suzuki et al.**



**Fig. 4**  
**T. Suzuki et al.**



**Fig.5**  
T. Suzuki et al.



**Fig. 6**  
**T. Suzuki et al.**

1 **Table 1.** Lactate dehydrogenase activity in the apical and basolateral medium of Caco-2 monolayers  
 2 incubated in the presence of up to 100 mmol/L DFAIII or 10 mmol/L sodium caprate in the apical  
 3 chambers

	Apical side (mU/0.5mL)	Basolateral side (mU/1.0mL)
DFAIII		
0 mmol/L	115 ± 14	14.9 ± 2.1
25 mmol/L	111 ± 5	14.5 ± 1.8
50 mmol/L	119 ± 4	14.2 ± 1.6
75 mmol/L	112 ± 4	14.8 ± 1.5
100 mmol/L	114 ± 6	13.9 ± 1.4
Sodium caprate		
0 mmol/L	144 ± 12 <sup>c</sup>	11.0 ± 0.6 <sup>b</sup>
5 mmol/L	236 ± 6 <sup>b</sup>	17.6 ± 0.6 <sup>b</sup>
7.5 mmol/L	269 ± 5 <sup>b</sup>	20.9 ± 2.3 <sup>b</sup>
10 mmol/L	449 ± 21 <sup>a</sup>	45.9 ± 5.8 <sup>a</sup>

17 Values are means ± SEM, n = 4. Means in a column not sharing a common letter are significantly  
 18 different ( $P < 0.05$ ). Apical and basolateral chambers of the cells were bathed in 1.0 and 0.5 mL  
 19 HBSS, respectively. Abbreviations used: DFA, difructose anhydride; FD, Fluorescein  
 20 isothiocyanate-conjugated dextran; TER, transepithelial electrical resistance.

1 **Table 2.** Transepithelial electrical resistance (TER) across the Caco-2 monolayers before and after  
 2 a 3-h incubation in the absence or presence of DFAIII or ethylene glycol in the apical or  
 3 basolateral chambers.

Administration site		TER across the monolayers ( $\Omega \cdot \text{cm}^2$ )	
Apical site	Basolateral site	0 h	3 h
None	None	1311 $\pm$ 82	1281 $\pm$ 81 <sup>a</sup>
DFAIII	None	1308 $\pm$ 62	276 $\pm$ 24 <sup>c</sup>
None	DFAIII	1329 $\pm$ 31	1166 $\pm$ 30 <sup>b</sup>
Ethylene glycol	None	1279 $\pm$ 81	1339 $\pm$ 67 <sup>a</sup>
None	Ethylene glycol	1239 $\pm$ 86	1308 $\pm$ 42 <sup>a</sup>
DFAIII	Ethylene glycol	1417 $\pm$ 66	312 $\pm$ 26 <sup>c</sup>

12 Values are means  $\pm$  SEM, n = 4-6. Values in a column not sharing a common letter are  
 13 significantly different ( $P < 0.05$ ). Abbreviations used: DFA, difructose anhydride; TER,  
 14 transepithelial electrical resistance.

1 **Table 3** Transepithelial electrical resistance (TER) of Caco-2 monolayer used in the transport  
 2 experiment after incubation

	MW	TER after incubation ( $\Omega \cdot \text{cm}^2$ )		
		Control	DFAIII	Sodium caprate
5 Mannitol	182	1153 $\pm$ 40 <sup>a</sup>	312 $\pm$ 9 <sup>b</sup>	281 $\pm$ 49 <sup>b</sup>
6 Lucifer Yellow	522	1002 $\pm$ 22 <sup>a</sup>	278 $\pm$ 13 <sup>b</sup>	265 $\pm$ 23 <sup>b</sup>
7 FD-4	4,400	1069 $\pm$ 41 <sup>a</sup>	313 $\pm$ 10 <sup>b</sup>	313 $\pm$ 57 <sup>b</sup>
8 FD-10	10,500	1141 $\pm$ 26 <sup>a</sup>	307 $\pm$ 8 <sup>b</sup>	272 $\pm$ 9 <sup>b</sup>
9 FD-20	19,500	1115 $\pm$ 20 <sup>a</sup>	305 $\pm$ 12 <sup>b</sup>	293 $\pm$ 25 <sup>b</sup>
10 FD-40	43,200	1153 $\pm$ 40 <sup>a</sup>	312 $\pm$ 9 <sup>b</sup>	281 $\pm$ 21 <sup>b</sup>

11 Values are means  $\pm$  SEM, n = 4-5. Values in a row not sharing a common letter are significantly  
 12 different ( $P < 0.05$ ). Abbreviations used: DFA, difructose anhydride; FD, Fluorescein  
 13 isothiocyanate-conjugated dextran; MW, molecular weight; TER, transepithelial electrical  
 14 resistance.

15