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Effect of capsianoside, a diterpene glycoside, on tight-junctional permeability

Kei Hashimoto [a,*], Hirokazu Kawagishi [b], Tsutomu Nakayama [a], Makoto Shimizu [c]

[a] *School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, 52-1 Yada, Shizuoka 422, Japan*
[b] *Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422, Japan*
[c] *Department of Applied Biological Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*

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Abstract

Previous work (Hashimoto et al., (1994) Biosci. Biotech. Biochem. 58, 1345) revealed that a sweet pepper extract enhanced the tight-junctional (TJ) permeability of a human intestinal Caco-2 cell monolayer. In the present study, the substance which modulated the TJ permeability was chromatographically purified from the extract. The active substances were identified as capsianosides A–F, diterpene glycosides. Treatment of the cells with capsianoside F, the most active compound, decreased the cellular G-actin content by 40% and increased the F-actin content by 16%. The effect of capsianoside F was significantly suppressed by disturbing the cytoskeletal structure with cytochalasin D at a low dose (50 ng/ml). These results suggest that capsianosides affected the cytoskeletal function by modulating the reorganization of actin filaments, by which the TJ structure and permeability were changed. The possible involvement of a PKC inhibition in the mechanism of an increase in TJ permeability is also suggested.

Keywords: Caco-2 cell; Cultured epithelial monolayer; Tight junction; Capsianoside; Actin ring; Protein kinase C

1. Introduction

The intestinal epithelial cell layer functions as a barrier which separates the luminal and vascular fluid compartments. This barrier function is substantially dependent on the intercellular tight junction (TJ) that restricts the flow through the paracellular pathway. On the other hand, it is becoming evident that TJ does not act as an impermeable gasket but is physiologically regulated in its permeability by intracellular messengers [1] or such surfactants as bile salts [2]. The paracellular pathway is, therefore, thought to provide a highly dynamic transport route for certain

Abbreviations: AR, actin ring; CD, cytochalasin D; DMEM, Dulbecco's modified Eagle's medium; DT-3K, dextran-Texas red of M_r 3000; DDW, deionized and distilled water; EGTA, ethylenedioxybis(ethylamine)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FAB-MS, fast atom bombardment mass spectrum; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; LDH, lactic acid dehydrogenase; LY, Lucifer yellow; PB, phosphate buffer; PBS, phosphate-buffered saline; PKC, protein kinase C; SDS, sodium dodecyl sulfate; TEER, transepithelial electrical resistance; TFA, trifluoroacetic acid; TJ, tight junction; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ZOT, zonula occludens toxin

* Corresponding author. Fax: +81 (54) 2645522; e-mail: hashimot@fns1.u-shizuoka-ken.ac.jp.

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ions and macromolecules [3], contributing to the intestinal transport of various nutrients. Food components that are orally ingested and present in the intestinal lumen are also likely to participate in regulating TJ permeability. Pappenheimer [4] first demonstrated that the treatment of rat and hamster intestinal mucosa by glucose or alanine increased the paracellular permeability, resulting in rapid absorption of the nutrients. Alteration in macromolecular permeability via the paracellular route by L-tryptophan has also been reported by Madara and Carlson [5].

In addition to these nutrients, there may be other food-derived substances that modulate TJ. In the previous paper [6], using a human intestinal Caco-2 cell monolayer as a model of the intestinal epithelium, we demonstrated that milk whey proteins stabilized TJ, the active substances being identified as bovine serum albumin and β-lactoglobulin [7]. We also found that a sweet pepper (*Capsicum annuum* L. var. *grossum*) extract contained some substance(s) which reduced the transepithelial electrical resistance (TEER) and increased the TJ permeability of the Caco-2 monolayer. Caco-2 is a human colon carcinoma cell line which spontaneously exhibits various enterocytic characteristics; e.g., brush-border enzymes [8], nutrient transporters [9–11], and intercellular TJ [8]. The spontaneous formation of TJ in Caco-2 makes this cell a good in vitro model for investigating the regulation of intestinal TJ permeability.

The present study was undertaken to reveal the TJ-modulatory substances in the sweet pepper extract. The active substances were isolated, their molecular structures were determined, and the mechanism for TJ modulation by these substances was investigated.

2. Materials and methods

2.1. Materials

The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui (Tokyo, Japan), and fetal calf serum (FCS) was from Bioserum (Victoria, Australia). Millicell-HA with 0.45-μm porosity cellulose membranes of 12 mm in diameter and a Millicell-ERS instrument with Ag/AgCl electrodes were purchased from Nihon Millipore (Tokyo, Japan). A type-I collagen solution was obtained from Nitta Gelatin (Osaka, Japan), and a Hanks' balanced salt solution (HBSS) was from Life Technologies (Grand Island, NY, USA). Lucifer yellow CH lithium salt (LY), dextran-Texas red of M_r 3000 (DT-3K), propidium iodide and rhodamine-labeled phalloidin were all from Molecular Probes (Eugene, OR, USA). DNase I (type II from bovine pancreas), DNA (calf thymus, type I) and pronase E were from Sigma Chemicals (St. Louis, MO, USA). G-Actin was from Funakoshi (Tokyo, Japan), the Bio-Rad Protein Assay and Protein DC Assay reagents were from Bio-Rad (Richmond, CA, USA). Lactic acid dehydrogenase (LDH) Test Wako was from Wako Chemicals (Osaka, Japan). Non-radioisotopic Protein Kinase Assay System (NRPK assay system) was from Upstate Biotechnology (Lake Placid, NY, USA), all other chemicals being of reagent grade.

2.2. Cells and method of culture

Caco-2 cells were maintained in DMEM supplemented with 10% FCS, 4 mM L-glutamine, penicillin at 50 IU/ml and streptomycin at 50 μg/ml. They were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were passaged at a split ratio of 4 to 8 every 3 or 4 days. For TEER measurements and transport studies, cells were seeded on Millicell-HA coated with type-I collagen at a density of $2 \cdot 10^5$ cells/cm^2. For measuring the F-actin content, cells were seeded on a collagen-coated cover glass in a 24-well plate at a density of $2 \cdot 10^5$ cells/cm^2.

2.3. TEER measurement

The TJ permeability of the Caco-2 cell monolayer was evaluated by measuring the TEER value. TEER is thought to be well correlated with the change in paracellular permeability of the cell monolayer [12]. After a 3-day culture of the cells on Millicell-HA, the monolayer cells were rinsed with HBSS and then set in a 24-well plate containing HBSS. HBSS was added to the apical side, and the TEER value was measured with a Millicell-ERS instrument. The TEER value of the Caco-2 monolayer was measured before

and after adding an assay sample, and the effect of each assay sample is expressed as the relative TEER value to the value at zero time.

2.4. Transport studies

The monolayer cells on Millicell-HA were set in a 24-well plate as just described. LY or DT-3K (100 μg/ml or 400 μg/ml in HBSS, respectively) was added to the apical side, and the plate was then incubated at 37°C. After 1 h of incubation, the medium was collected from the basal side, and the concentrations of LY and DT-3K were determined by measuring the fluorescence intensity of each with an F-4000 spectrophotofluorometer (Hitachi, Japan). The excitation–emission wavelengths were 430–540 nm for LY and 568–607 nm for DT-3K.

2.5. Isolation of the active compounds

Two ml of a sample solution (30 mg/ml) in a 50 mM formic acid–ammonium buffer at pH 8.0 was applied to a column of DEAE-Toyopearl 650M (10 × 110 mm; Tosoh, Japan) that had been equilibrated with the same buffer. The adsorbed materials were recovered by eluting with a 500 mM formic acid–ammonium buffer at pH 6.0, using a flow rate of 0.8 ml/min.

An aqueous sample solution was applied to a Sep-Pak-C_{18} cartridge (10 g; Millipore, MA, USA) that had been equilibrated with deionized and distilled water (DDW). The adsorbed materials were recovered by stepwise elution with 0–50% acetonitrile.

A lyophilized Sep-Pak fraction was dissolved in DDW and then applied to a Superose 12 column (8 × 300 mm; Pharmacia Biotech, Japan) that had been equilibrated with DDW. The flow rate was 15 ml/h, and the eluate (0.5-ml fractions) was monitored at 280 nm with a Bio mini UV monitor (Atto, Japan). For measuring the hexose content, 0.1 ml of each fraction was served for the phenol–sulfuric acid method [13]. The residue of each fraction was lyophilized and then used for the assay of TEER-decreasing activity.

The active fraction was further purified by HPLC. A sample solution was injected into HPLC equipment (Gulliver system, Jasco, Japan) that was equipped with a YMC-pack ODS-AM column (4.6 × 250 mm; YMC, Japan) that had been equilibrated with 0.1% trifluoroacetic acid (TFA). Elution was carried out with 0.1% TFA–acetonitrile as the mobile phase at a flow rate of 1.0 ml/min, the eluate being monitored at 210 nm. To prepare the samples for structural analyses, the mobile phase was replaced with DDW–acetonitrile, and for preparative-scale experiments, a YMC-pack ODS-AM column (10 × 250 mm) was also used.

2.6. Structural analyses

NMR experiments were conducted with a JEOL α-500 spectrometer, while FAB-MS spectra were recorded with a JEOL DX-303HF mass spectrometer.

2.7. Surface activity

Surface activity was measured with a Wilhelmy plate type surface tensiometer (Kyowa CBVP surface tensiometer A3, Kyowa Kagaku, Tokyo, Japan) using a glass plate (24 × 24 mm) at 19°C.

2.8. Membrane permeability

The intercalating dye, propidium iodide, was used to evaluate the cell membrane permeability [14]. A Caco-2 cell monolayer grown on a collagen-coated cover glass for 3 days was treated for 15 min with or without each sample to be tested. After the incubation, the cells were rinsed and then stained with propidium iodide at 50 μg/ml. The stained material was observed by fluorescence microscopy.

2.9. Ca^{2+}-chelating activity of the capsianosides

The chelating activity of capsianoside F was evaluated as relative to the activity of EDTA. Several concentrations of capsianoside F or EDTA were added to a 1 nM quin-2 solution, in which quin-2 had been chelating Ca^{2+} in DDW, and the fluorescence intensity was measured with a F-4000 spectrophotofluorometer. The excitation–emission wavelengths were 334–490 nm. The relative chelating activity was determined by comparing the dose that was needed to reduce the fluorescence intensity by 0.5.

2.10. Determination of cellular actin

F-actin. The relative content of F-actin was determined by a fluorescent phalloidin-binding assay [15]. Caco-2 cell monolayers that had been incubated on a cover glass for 3 days were rinsed with HBSS and then incubated for 15 min more with or without a sample being tested. After the incubation, the cell monolayers were fixed with acetone/methanol (1:1, v:v), and then the actin ring (AR) was stained for 20 min with rhodamine-labeled phalloidin diluted 10-times with phosphate-buffered saline (PBS). Stained cells were extracted with 2 ml of methanol, and the fluorescence intensity of the extract was measured with a F-4000 spectrophotofluorometer. The excitation–emission wavelengths were 545–578 nm.

G-actin. The G-actin content was determined by a DNase I inhibition test [16]. Caco-2 cell monolayers that had been incubated on a 24-well plate for 3 days were rinsed with HBSS and then incubated for 30 min more with or without a sample being tested. After the incubation, the cells were lysed in a 50 mM Tris-HCl buffer at pH 7.5 (500 μl per well), containing 1% Triton X-100, 2 mM $MgCl_2$ and 1 mM ethylenedioxybis(ethylamine)-*N,N,N′,N′*-tetraacetic acid (EGTA). Twenty μl of a DNase I solution at 100 μg/ml in 50 mM Tris-HCl at pH 7.5, containing 0.1 mM $CaCl_2$ and 10 μM phenylmethylsulfonylfluoride was added to 100 μl of the cell lysate. Three ml of DNA at 80 μg/ml in 100 mM Tris-HCl (pH 7.5), containing 4 mM $MgSO_4$ and 1.8 mM $CaCl_2$, was also added to the mixture. The cellular G-actin content was determined by measuring the rate of absorbance increase at 260 nm, using purified rabbit muscle G-actin as a standard. The protein content was determined with a Bio-Rad protein assay reagent [17], the G-actin content being expressed as μg of G-actin per mg of protein.

2.11. In vitro PKC assay

Cell treatment and subcellular fractionation. Confluent Caco-2 monolayers on a 100-mm culture dish were rinsed twice with HBSS, and then incubated with or without capsianoside F (50 μg/ml) at 37°C for 30 min. After removing the medium, the cells were quickly rinsed with ice-cold HBSS and then scraped in 25 mM Tris buffer (pH 7.5), containing 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM 4-amidinophenylmethanesulfonyl fluoride and 0.2 mg/ml Triton X-100 (2 ml/dish). The cells were homogenized with Polytron (PT-MR3000, Kinematika AG, Littau, Switzerland) for 30 s, and the lysates were centrifuged at $100\,000 \times g$ for 30 min (Beckman TLA-100.3, fixed angle rotor), the supernatants being used as cytosolic fractions. The protein concentration of the fractions was determined with a Protein DC Assay reagent [18].

Measurement of PKC. The PKC content was measured by a NRPK assay system, which was based on an enzyme-linked immunosorbent assay (ELISA) [19]. Briefly, 15 μg of the cytosolic protein was incubated with a synthetic peptide corresponding to the residues 3–13 of porcine glial fibrillary acidic protein which had been coated on microwell plates. The phosphorylated peptides were detected by incubating with the monoclonal antibody, YC-10, followed by incubating with anti-mouse IgG conjugated to peroxidase. Peroxidase substrate was added to the microwells, and the color intensity was measured photometrically at 415 nm. Results were shown as the ELISA values.

2.12. Statistical analyses

All results are expressed as mean $\pm$ S.D. Student's *t*-test was used to compare means and ranges.

3. Results and discussion

3.1. Isolation and partial characterization of the active substance from the sweet pepper extract

The crude sweet pepper extract, which had been prepared as described previously [20], was dialysed against DDW. The lyophilized material was dissolved in a 50 mM formic acid-ammonium buffer and applied to a DEAE-Toyopearl ion-exchange column. The passed-through fraction having TEER-decreasing activity was then applied to a Sep-Pak-C_{18} cartridge, and the adsorbed materials were recovered by stepwise elution with acetonitrile. The highest activity was detected in the Sep-Pak fraction eluted with between 30% and 50% acetonitrile (data not shown).

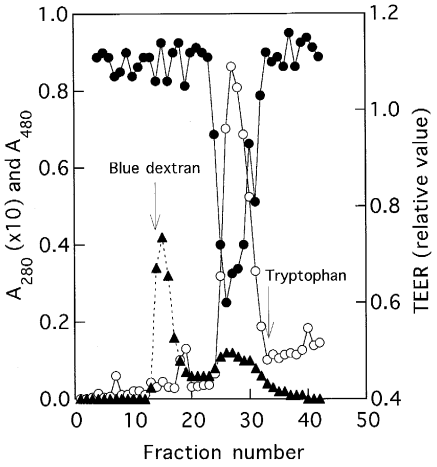

Fig. 1. Purification of the TEER-decreasing substances. The Sep-Pak fraction with TEER-decreasing activity was further fractionated in a Superose 12 column. The isolated fractions were each assayed for TEER-decreasing activity (●). The protein content was monitored by measuring the absorbance at 280 nm (▲). The hexose content was measured by the phenol–sulfuric acid method and is shown by the absorbance at 480 nm (○).

After a 30-min exposure of the Caco-2 cell monolayer to this fraction from the apical side at a concentration of 300 μg/ml, the TEER value was reduced to 60% of the value at zero time.

The active fraction was further fractionated by Superose 12 chromatography, and the TEER-decreasing activity of the fractions was measured. As shown in Fig. 1, the fractions from No. 25 to No. 29 (named SP-Fr) decreased the TEER value to less than 70% of the value at zero time. An analysis of all the fractions for hexoses by the phenol–sulfuric acid method showed SP-Fr to be rich in hexose, whereas the protein content of SP-Fr seemed to be small. No significant change was apparent in the TEER-decreasing activity of SP-Fr by heating at 100°C or by Pronase treatment (data not shown). These experimental results suggest that the active substance in SP-Fr was not a proteinaceous material, but rather a thermally stable complex carbohydrate or a related substance. The yield of SP-Fr was 27.5 ± 2.8 mg from 100 g (wet weight) of sweet pepper.

Various concentrations of SP-Fr were added to the apical side of the Caco-2 cell monolayer in HBSS and incubated at 37°C for 30 min. The TEER-decreasing activity of SP-Fr increased with increasing concentration and reached a plateau at about 200 μg/ml, at which the TEER value was decreased to 60% of the value at zero time. The half-maximal TEER-decreasing dose was 110 μg/ml.

Cytotoxic materials may damage the cell monolayer, resulting in a TEER decrease [20]. An optical microscopic observation, however, indicated that no detachment of cells from the monolayer was induced by the SP-Fr treatment (data not shown). No significant difference in the amount of LDH released from the cells was observed between the SP-Fr-treated (300 μg/ml; $2.9 \pm 0.7\%$, $n = 3$) and control ($2.1 \pm 0.2\%$, $n = 3$) cells. The decrease in TEER caused by SP-Fr was not an irreversible change, and when the medium containing SP-Fr was removed from the apical side of the cell layer and the cells were incubated in a culture medium containing 10% FCS, the TEER value of the monolayer had completely recovered after 5 h (the relative TEER value was 0.55 ± 0.05 for the cells after a 30-min treatment with SP-Fr (200 μg/ml), while the value was increased to 1.08 ± 0.04 in 5 h after removing the SP-Fr, $n = 4$). These experimental results suggest that the decrease in TEER value by the SP-Fr treatment was not due to any damage to the cell monolayer, but rather due to the reversible opening of a paracellular route.

3.2. Effect of SP-Fr on the permeability of the cell monolayer

The effect of SP-Fr on the permeability of the cell monolayer was examined by using such leakage markers as LY and DT-3K. After 1 h of incubation with LY or DT-3K in the presence of SP-Fr (200 μg/ml), the medium on the basal side was collected, and the leakage-marker concentration was determined by measuring the fluorescence intensity. The SP-Fr treatment increased the permeability for LY (M_r 457.24) and DT-3K (average M_r of about 3000) by 120% and 30%, respectively (Fig. 2), suggesting that the opening of the paracellular route was not due to TJ destruction, but due to a subtle modulation of the TJ structure.

3.3. Purification and identification of the active substances in SP-Fr

An HPLC analysis of SP-Fr, using an ODS column, indicated at least five major components (SP-

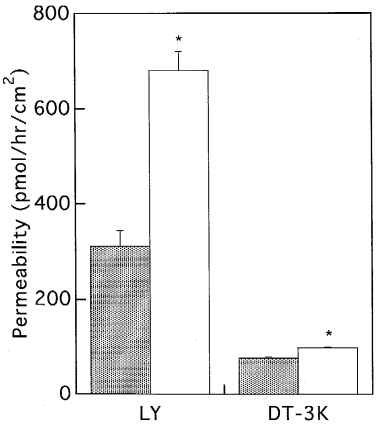

Fig. 2. Effect of SP-Fr on the permeability of the Caco-2 monolayer. The permeability of the Caco-2 cell monolayer was examined with LY and DT-3K as leakage markers. Data bars represent the means ± S.D. for four samples. Shaded bars, control; open bars, treated with SP-Fr (200 μg/ml). * Significantly different ($P < 0.001$) from the control sample.

1–5; Fig. 3). SP-1–5 corresponding to the five major peaks by HPLC were purified by rechromatography, and the structure of SP-1–5 was then analyzed by ^{1}H- and ^{13}C-NMR and FAB-MS. SP-1–5 had a similar structure and were identified as capsianosides, dimeric esters of monomeric diterpene glycosides.

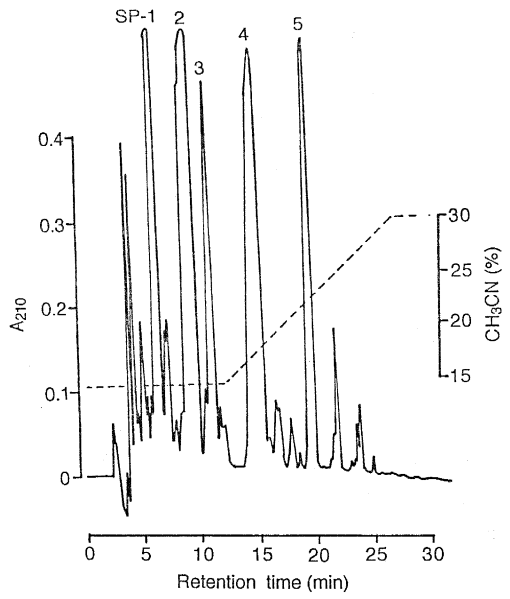

Fig. 3. Reverse-phase HPLC of SP-Fr in a YMC-Pack ODS-AM column. 800 μg of SP-Fr was applied to the column. The elution profile (acetonitrile concentration) is shown by the broken line.

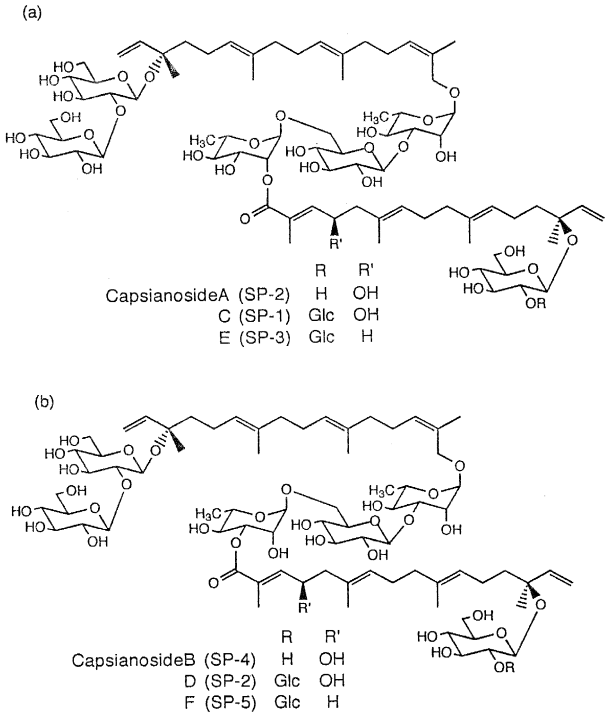

Fig. 4. Structures of capsianosides A–F. (a) Capsianosides A, C and E. (b) Capsianosides B, D and F.

SP-1, 3, 4 and 5 corresponded to capsianosides C, E, B and F, respectively, while SP-2 was a mixture of capsianosides C and D (Fig. 4). SP solutions in HBSS were centrifuged at 16 000 × g and the SP-content in the supernatants was determined by HPLC analysis. SP-1–5 were all soluble in HBSS within the concentrations used in this experiment (up to 200 μg/ml).

The TEER-decreasing activity of SP-1–5 varied according to each, suggesting a relationship between the structure and activity of the capsianosides (Fig. 3 and Fig. 5). For example, capsianosides C and E, whose TEER-decreasing activity was lower than that of the other capsianosides, were less hydrophobic (retention times by reversed-phase HPLC were shorter), whereas capsianoside B and F were more hydrophobic than the other capsianosides. This difference in hydrophobicity seem to have been dependent on the different mode of ester bonding between the inner rhamnosyl moiety and the terpene glycoside (Fig. 4a for capsianosides C and E, and Fig. 4b for B and F). The co-incubation of capsianoside C (100 μg/ml) with Pluronic F-127, a reagent which in-

creases the permeability of the cell membrane for hydrophilic substances [21], rendered decrease in the TEER value (Fig. 6), indicating penetration through the cell membrane to be an important step for the TEER-decreasing activity of capsianosides.

Capsianosides A–F had already been isolated from various *Capsicum annuum* species and their structures determined by spectroscopic and chemical means [22]. However, the physiological functions of capsianosides are hardly known, except for the inhibitory activity toward the angiotensin-converting enzyme of capsianosides C and D [21]. A new function of capsianosides, i.e., the regulation of paracellular absorption in the intestine, has therefore been revealed by the present study.

3.4. Chemical and physicochemical properties of capsianosides

Uvomorulin, a Ca^{2+}-dependent adhesion molecule, mediates cell–cell adhesion that is a prerequisite for the assembly of the intracellular junctional complex involving TJ [23]. Ca^{2+} removal would affect these Ca^{2+}-dependent adhesion molecules, inducing the disassembly of cytoskeletal and junctional components [14]. The Ca^{2+}-chelating activity of capsianoside F was therefore investigated using quin-2 as a Ca^{2+}-specific indicator. Capsianoside F was observed to have a Ca^{2+}-chelating activity, about one-tenth that of EDTA (data not shown). Previous studies using certain epithelial cell lines demonstrated that Ca^{2+} removal by chelating agents did not initiate the disintegration of TJ when the agents were added from the apical side [24–27]. Capsianosides, however, decreased the TEER value from any side of the monolayer (the relative TEER value was 0.67 ± 0.07 from the apical side, and 0.71 ± 0.04 from the basal side, $n = 3$). Further addition of Ca^{2+} (up to 4 mM) to the incubation medium did not affect the activity of SP-Fr (the relative TEER values were 0.63 ± 0.06 in the presence of 4 mM Ca^{2+} and 0.63 ± 0.02 for the control, $n = 3$). Consequently, it is unlikely that the increased monolayer permeability by capsianosides was due to its Ca^{2+}-chelating activity.

Capsianosides A–F were shown to be surface active (Fig. 7). Anderberg and Artursson [28] have reported that the treatment of Caco-2 monolayers with such surface active substances as SDS increased the intracellular Ca^{2+} level ($[Ca^{2+}]_i$), resulting in

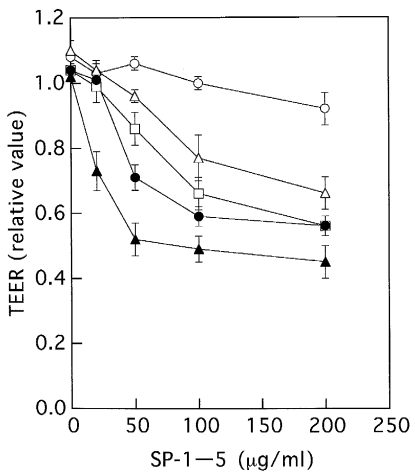

Fig. 5. Dose dependency of SP-1–5 for TEER-decreasing activity. The Caco-2 cell monolayer was treated with the indicated amounts of SP-1–5 for 30 min at 37°C. The TEER values are presented as relative to the values at zero time. The values shown are the mean ± S.D. bars for four samples. (○) SP-1; (△) SP-2; (□) SP-3; (●) SP-4; (▲) SP-5.

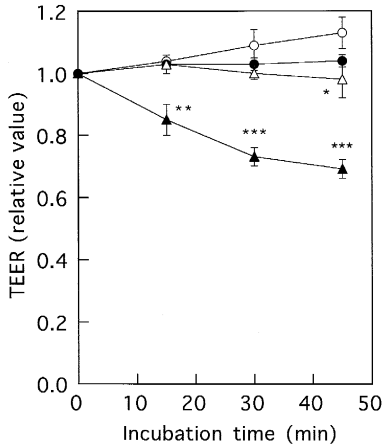

Fig. 6. Effect of Pluronic F-127 on the TEER-decreasing activity of capsianoside F. Cells were pretreated with Pluronic F-127 (200 μg/ml) for 15 min, and then treated with capsianoside C (100 μg/ml) for up to 45 min. The TEER values are presented as relative to the values at zero time. The values shown are the means ± S.D. ($n = 3$) bars, unless the error bar is smaller than the symbol. (○) control; (●) capsianoside C; (△) Pluronic F-127; (▲) Pluronic F-127 and capsianoside C. * Significantly different ($P < 0.025$) from the control sample. ** Significantly different ($P < 0.005$) from the Pluronic F-127-treated cells. *** Significantly different ($P < 0.001$) from the Pluronic F-127-treated cells.

increased TJ permeability. This increase in $[Ca^{2+}]_i$ was probably due to the stimulation of Ca^{2+} release from the intracellular store and/or the influx of extracellular Ca^{2+} caused by the increased cell membrane permeability. Treatment with capsianoside F (50 μg/ml), however, neither increased the cell membrane permeability nor induced increased $[Ca^{2+}]_i$ (data not shown). Although the TEER-decreasing activity of capsianosides A–F (Fig. 5) seems to be correlated with the surface activity (Fig. 7), the mechanism for the increase in TJ permeability by capsianosides was thought to be different from that by SDS. Probably, the surface active properties of capsianosides relate to their penetrability to cell membranes, thus affecting the TEER-decreasing activity.

3.5. Effect of capsianosides on the cytoskeletal structure of Caco-2

A linkage between TJ components and the actin ring (AR) has been shown [29]. Recently, Stuart and Nigam [30] reported that the TJ-associated protein complex, involving ZO-1 [31], ZO-2 [32] and occludin [33], associated with cytoskeletal elements. The functional association of AR and TJ was suggested by the experiments using cytochalasin D (CD) which was known to perturb the cytoskeletal structure [34,35]. The TEER value of the Caco-2 cell monolayer after a 30-min exposure to CD (10 μg/ml) was also reduced to 60% of the value at zero time. Perturbing the cytoskeletal structure, therefore, seemed to be the major mechanism for the TEER-decreasing activity of capsianosides. In order to reveal the effect of capsianosides on the cytoskeletal structure, the cellular G- and F-actin contents were determined (Fig. 8). The intracellular G-actin content was decreased to 60% by treating with capsianoside F at 50 μg/ml for 30 min. In contrast, the relative F-actin content was increased by 16% by the capsianoside F treatment at 50 μg/ml for 15 min. Pretreatment of the cells for 30 min with a low concentration (50 ng/ml) of CD, which had been reported to perturb the cytoskeletal structure but not to reduce the TEER value at this concentration [36], significantly reduced the activity of capsianoside F (the relative TEER values were 0.42 ± 0.06 for the cells without CD-pretreatment and 0.64 ± 0.07 with pretreatment, $P < 0.01$, $n = 3$). These findings strongly suggest that capsianoside F stimulated the reorganization of the actin filaments, by which the TJ permeability was increased.

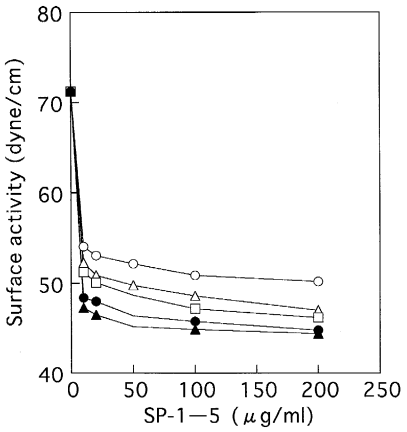

Fig. 7. Surface activity of capsianosides. SP-1–5 were dissolved in DDW at various concentrations. The values shown are the means of three replications. The S.D. bars were smaller than the symbol. (○) SP-1; (△) SP-2; (□) SP-3; (●) SP-4; (▲) SP-5.

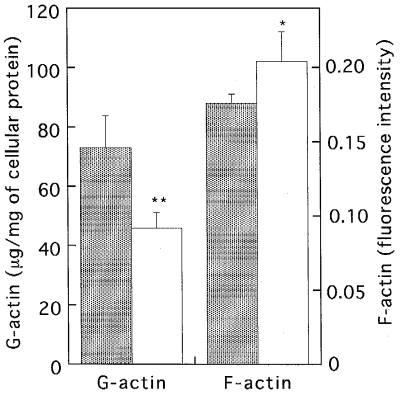

Fig. 8. Effect of capsianoside F on the cellular G- and F-actin contents. Data bars represent the means ± S.D. for four samples (G-actin) and for three samples (F-actin). Shaded bars, control; open bars, treated with SP-Fr (50 μg/ml). * Significantly different ($P < 0.05$) from the control sample. ** Significantly different ($P < 0.005$) from the control sample.

3.6. Effect of capsianosides on the activity of PKC

The protein kinase inhibitor has been shown to block junctional dissociation caused by Ca^{2+}-chelator [37,38], CD [39] or zonula occludens toxin [40] treatment. However, the pretreatment with a PKC in-

Table 1
Effect of TPA on the TEER-decreasing activity of capsianoside F

TPA (ng/ml)	relative TEER
0	0.45 ± 0.02
0.5	0.50 ± 0.06
1	0.52 ± 0.03 **
5	0.54 ± 0.04 **
10	0.57 ± 0.07 *

Cells were pretreated with various concentrations of TPA for 15 min from both the apical and basal sides, and then treated with capsianoside F (50 μg/ml) from the apical side for 15 min. The TEER values are presented as relative to the values at zero time. Values present the mean $\pm$ S.D. for three samples.
* Significantly different ($P < 0.05$) from the cells without TPA-treatment; ** significantly different ($P < 0.025$) from the cells without TPA-treatment.

hibitor, H-7 (20 μM), had no effect on the TEER-decreasing activity of capsianoside F (data not shown), suggesting that phosphorylation by PKC did not play a part in the disassembly of TJ by capsianoside F. On the other hand, pretreatment of Caco-2 cells with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), a PKC activator which was reported to cause immediate activation of PKC of Caco-2 cells [41], significantly reduced the TEER-decreasing activity of capsianoside F (Table 1). PKC-activation therefore seems to have negative effects on the disassembly of TJ by capsianoside F. Balda et al. [48] reported that the PKC activator stimulated the assembly of TJ. It has been reported that PKC activation was accompanied with the translocation of this enzyme from the cytosol to the plasma membrane [42–47]. In our preliminary experiment, the amount of PKC in the cytoplasmic fraction significantly increased ($P < 0.05$) after a capsianoside F-treatment (50 μg/ml) for 30 min (the ELISA values, which reflect the amount of PKC, were 0.037 ± 0.009 for the control and 0.070 ± 0.011 for the capsianoside F-treated cells, $n = 3$). Therefore, capsianosides seemed to reduce the cellular PKC, that might interfere with the maintenance of barrier functions and increase the TJ permeability. The mechanism for the PKC inhibition by capsianosides must be further investigated.

3.7. Concluding remarks

The results presented here lead to our postulated mechanism for the TEER-decreasing activity of capsianosides. Capsianosides pass through or are inserted into the cell membrane, after which they stimulate AR reorganization. Participation of PKC in the reorganization of actin filaments was also suggested. These effects would modulate the TJ structure, resulting in increased TJ permeability.

The present study has demonstrated that capsianosides would be useful to enhance the permeability for drugs or other biologically important hydrophilic substances across the intestinal mucosa. As capsianosides maintain their activity in the presence of a high concentration of Ca^{2+}, they would, for example, be able to enhance Ca^{2+} permeability. Since the majority of Ca^{2+} in the intestinal tract is transported by the paracellular pathway [49], capsianosides may lead to increased Ca^{2+} intake from the lumen into the vascular fluids.

Although capsianosides increased TJ permeability, this does not mean that the paracellular route would be wide-open without any restrictions. The enhancement of DT-3K permeability by capsianoside was much smaller than that of LY permeability (Fig. 2), suggesting that the permeability for large molecules, which could become allergens, would be restricted to a higher degree than that for drugs and Ca^{2+}.

Capsicum species have long been used as a food, and for spices and external medicines. The physiological functions of *Capsicum* substances in the human body are therefore of great interest. The present study demonstrated that capsianosides, diterpene glycosides, contained in *Capsicum* plants could modulate the intestinal absorptive functions. The in vivo effect of capsianosides on the absorption of certain nutrients including Ca^{2+} will be the subject of a further study.

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