Permeation-Enhancing Effect of Aloe-emodin Anthrone on Water-Soluble and Poorly Permeable Compounds in Rat Colonic Mucosa

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The aims of this study were to examine the enhancing effects of aloe-emodin anthrone (AEA) on the colonic membrane permeability of water-soluble and poorly permeable compounds and to clarify the mechanism of the permeation-enhancing activity of AEA. The permeation-enhancing activity of AEA was estimated from changes in the permeability coefficient of 5(6)-carboxyfluorescein (CF) in rat colonic mucosa using a Ussing-type chamber. Various inhibitors were used to investigate the mechanism of action of AEA. The structural change in the membrane and the cytotoxicity of AEA in the intestinal mucosa were evaluated by measuring the electrical resistance of the membrane (R_m) and lactate dehydrogenase (LDH) activity, respectively. AEA significantly increased the permeation of CF in a dose-dependent manner. The enhanced permeability was significantly suppressed by a histamine H_1 receptor antagonist, pyrilamine, and a mast cell stabilizer, ketotifen, but not by a histamine H_2 receptor antagonist, cimetidine. The enhancing effect was also inhibited by an inhibitor of protein kinase C (PKC). Potential difference and short-circuit current values decreased, while R_m values remained constant throughout the experiment. The addition of AEA to the mucosal solution decreased R_m to 30%, but then remained constant. LDH activity with AEA was not significantly different from that of the control. In conclusion, AEA is a candidate for effective absorption enhancers without damage of the membrane and cytotoxicity. We propose that AEA stimulates mast cells within the colonic mucosa to release histamine, which probably bind to the H_1 receptor. The intracellular PKC route activated by H_1 receptor activation enhances the permeability of water-soluble and poorly permeable drugs *via* opening of tight junctions in rat colonic membrane.

Key words aloe-emodin anthrone; absorption enhancer; histamine; Ussing chamber; epithelial permeability; paracellular pathway

Aloe (Cape aloes from *Aloe ferox.* or *A. arborescens* MILL., Socotrine aloes from *Aloe perryi* BAK. and Curacao aloes from *Aloe vera* LINN.) is widely used in the manufacture of food products and beverages, pharmaceuticals, and cosmetics because of its aromatic properties, bitter taste, cathartic activity derived from anthraquinones, and other pharmacological activities (such as emolliency, reduction of inflammation, and acceleration of wound healing, although it is not yet well understood which activity is related to which component).[1] *R*- and *S*-barbaloins (10-glucopyranosyl-1,8-dihydroxy-3-(hydroxymethyl)-9(10*H*)-anthracenone: BAs), the main components of aloe, are two diastereoisomeric *C*-glucosides differing in the configuration of C-10 in the aloe-emodin anthrone (AEA) moiety.[2] It is generally accepted that BAs are the bitter and purgative principle of the aloe product.[1]

Ishii *et al.* reported that BAs were transformed into AEA (1,8-dihydroxy-3-(hydroxymethyl)-9(10*H*)-anthracenone) under anaerobic conditions and aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)-9,10-anthracenedione, AE) under aerobic conditions through an enzymatic redox reaction involving rat intestinal microflora.[3] Che *et al.* have shown the transformation of BA to AEA by human intestinal bacteria.[4]

Previously, we studied the antiinflammatory effects of BAs and related compounds and found that AEA induced the release of histamine from isolated rat mast cells, whereas AE neither induced nor inhibited it.[5] Histamine, an inflammatory mediator derived from mast cells, was reported to increase intestinal epithelial permeability.[6,7] Therefore we hypothesize that AEA enhances mucosal permeability, resulting in the release of histamine from mast cells in the colonic mucosa.

In this study, the effects of AEA on the permeability of water-soluble and poorly permeable compounds to the colonic epithelial cell membrane and the permeation-enhancing mechanism of AEA, particularly with regard to the role of histamine released from mast cells in the colonic mucosa, were investigated to elucidate the effectiveness of AEA as a new class of absorption enhancer for water-soluble and poorly permeable compounds.

MATERIALS AND METHODS

Materials Histamine, cimetidine, ketotifen, pyrilamine, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine dihydrochloride (H7), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Compound 48/80 was an oligomeric mixture of condensation products of *N*-methyl-*p*-methoxyphenethylamine and formaldehyde (Purity: >98% by TLC)[8] and obtained from Sigma Chemical Co. 5(6)-Carboxyfluorescein (CF, MW 376.2) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). Other reagents were of analytical grade or higher.

Animals Male Wistar rats (250—300 g), at least 72 d old, were used in this study. The rats had free access to food and water prior to killing. All procedures involving experimental animals were carried out according to the guidelines of the Experimental Animal Care and Use Committee of Fukuoka University.

Preparation of AEA BA was prepared from commercial aloin (Sigma Chemical Co.) by recrystallization from water-methanol. The synthesis of AEA from BA was based

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on the method of Hay and Haynes.[9] An aqueous solution (100 ml) of purified BA (5 g), sodium borate (10 g), and phenylhydrazine hydrochloride (2 g) was refluxed for 3 h under an atmosphere of nitrogen. The dark red solution was acidified with diluted hydrochloric acid, and the precipitated yellow solid was extracted using ether (*ca.* 500 ml). Evaporation of the washed and dried (Na_2SO_4) ether extract gave a reddish solid, which on purification with a prepacked column (Lobor LiChroprep RP-18, 40—60 μm, 310×25 mm i.d., Merk Japan Ltd., Tokyo, Japan) gave a yellow crystal identified as AEA by FAB-MS (JEOL JMS-HX 110 controlled by a JEOL DA 7000 data system, Tokyo, Japan). Positive-ion FAB-MS: *m/z* 257 ($[M+H]^+$). Yield, 0.073 g, 2.4%, mp 199 °C. The purity of the synthesized AEA was determined by HPLC. The HPLC system (Shimadzu LC10AD, Shimadzu Co., Kyoto, Japan) consisted of a UV spectrometric detector (Shimadzu SPD-6AV), and chromatopac (Shimadzu C-R6A). The column used was a Wakosil II 5C 18HG (Wako, Osaka, Japan, 4.6 mm i.d.×25 cm) and the mobile phase was acetonitrile/water (2 : 1, v/v). The UV detector was set at 295 nm and the flow rate was 1.0 ml/min at 40 °C. The retention time of AEA was 21.6 min. The purity of AEA was found to be more than 98%.

Histamine Release from Rat Peritoneal Mast Cells The composition of the Tyrode HEPES solution (THS, pH 7.4) was as follows (g/l); NaCl (8.01), KCl (0.20), $NaH_2PO_4 \cdot 2H_2O$ (0.06), HEPES (4.76), $CaCl_4 \cdot 2H_2O$ (0.26), $MgCl_2 \cdot 6H_2O$ (0.20), glucose (1.01), and gelatin (1.0). The preparation of peritoneal mast cells and the assay of histamine release from mast cells were carried out by a modified version of the method described by Nakagomi *et al.*[10] The mast cells were collected from Wistar rat peritoneal cavities after injection of 20 ml of THS, and centrifuged in a 38% solution of BSA (d=1.068) for purification. The purity of the mast cells was about 88—92% as observed under an Olympus phase-contrast microscope. Various concentrations of sample solution (AEA or compound 48/80) was prepared with 0.1% BSA in THS. AEA was first dissolved in dimethyl sulfoxide (DMSO) and diluted by the solution. The final concentration of DMSO in the assay media was less than 1% (v/v), at which DMSO did not affect the cell viability and the subsequent histamine assay. An aliquot (20 μl) of the cell suspension (1—2×10^6 cells/ml, 0.1% BSA in THS) was warmed at 37 °C for 10 min, and then 20 μl of sample solution was added and incubated at 37 °C for 10 min. After the incubation, the cells were cooled on ice, then centrifuged at 1500 ***g*** for 15 min. Next, 0.5 ml of 0.8 M perchloric acid was added to 50 μl of the supernatant and centrifuged at 1500 ***g*** for 15 min. The supernatant (150 μl) was then used for the measurement of histamine release from the cells by fluorometric analysis with *o*-phthalaldehyde. After the addition of 2 ml of 0.1 M hydrochloric acid, 0.4 ml of 1 M sodium hydroxide, and 0.1 ml of 1% *o*-phthalaldehyde in ethanol to the supernatant, the solution was allowed to stand for 4 min. The reaction was stopped by adding 0.2 ml of 3 M hydrochloric acid. The concentration of histamine was determined fluorometrically using a Shimadzu model RF-500 spectrofluorometer (Shimadzu) at 355-nm excitation and 450-nm emission. The amount of histamine released was expressed relative to that from the 0.05% Triton X-100-treated cell supernatant. Thus the percentage of release was defined as 100 $[(S-B)/(C-B)]$, where B is the value of the control without sample, and S and C are the amount of histamine released by the sample and 0.05% Triton X-100, respectively.

Permeability Experiments Using Ussing-type Chambers The composition of Krebs-Hensleit bicarbonate Ringer's solution (KBR solution, pH 7.4) was as follows (g/l): NaCl (6.9), KCl (0.354), $CaCl_2$ (0.282), $NaHCO_3$ (2.1), KH_2PO_4 (0.162), $MgSO_4 \cdot 7H_2O$ (0.294), and glucose (1.8). CF was used as a model of water-soluble and poorly permeable compounds.

The colon was enucleated from male Wistar rats under ether anesthesia. The colon mucosa stripped of the underlying muscle was mounted as a flat sheet in an Ussing-type chamber (WPI, Inc., U.S.A., 0.636 cm^2 exposed surface area). Briefly, KBR solution was added to both the mucosal and serosal sides of the Ussing chamber, and the specimen was incubated for 20—30 min at 37 °C under bubbling with 95% O_2 and 5% CO_2. Then the mucosal solution was replaced with 10 ml of KBR solution containing CF (100 μg/ml) together with AEA (0.5 mM), histamine (0.01 mM), and/or compound 48/80 (0.1 mM) and incubated for 1 h at 37 °C. An aliquot of 0.2 ml was taken from the serosal side and 3 ml of 1 M sodium hydroxide was added. The concentration of CF was determined fluorometrically at 490-nm excitation and 520-nm emission. The apparent permeability coefficient (P_{app}) was calculated using the following equation: $P_{app}=dQ/dt\times 1/(AC_o)$, where dQ/dt represents the steady-state flux of CF on the serosal side, C_o the initial concentration of CF on the mucosal side, and A the gross surface area of the membrane.[11] The short-circuit current (I_{sc}) and transmucosal potential difference (PD) were measured and transmucosal electrical resistance (R_m) was obtained according to Ohm's law as the electrophysiological parameters.

Treatment with Various Inhibitors: Pyrilamine (20 mg/kg) or cimetidine (100 mg/kg) was intraperitoneally administered to the rats 10 min prior to excision of the colonic segment as described by Murakami *et al.*[12] Ketotifen (3.125 mg/kg) was intraperitoneally given 30 min prior to the preparation of the colon.[12] The colonic segment pretreated with various inhibitors was subjected to permeability experiments. Compound 48/80 (2.5 μg/ml), W7 (50 μM), or H7 (50 μM) was applied on the mucosal side of the chamber and incubated for 20 min at 37 °C as described previously.[13]

Measurment of Lactate Dehydrogenase Activity Male Wistar rats (250—300 g) were intraperitoneally anesthetized with sodium pentobarbital. The intestine was exposed through a midline incision, and a closed loop was prepared using approximately 5 cm of the colon by ligation. One milliliter of KBR solution containing 0.5 mM AEA was introduced into the loop *via* a vinyl catheter immediately after ligation. After 1 h, the loop was removed to measure lactate dehydrogenase (LDH) release in the loop using a commercial enzymatic assay kit (LDH-10; Shimadzu Chemical Co.).

Statistical Analysis Statistical analysis was performed using the unpaired Student's *t*-test or ANOVA followed by Scheffe's *F* test. A value of $p<0.05$ was considered significant. The intraobserver or interobserver variation was <5% in each experiment.

RESULTS

AEA-Induced Histamine Release Figure 1 shows the histamine release induced from rat peritoneal mast cells by AEA or compound 48/80. The release was rapid and reached a maximum within 1 min of the initiation of incubation. Ten minutes of incubation was sufficiently long to measure the release. Although AEA was less effective than compound 48/80, AEA induced the release of histamine in a dose-dependent manner at concentrations of 2×10^{-4}—10^{-2} M. The threshold concentration was around 1×10^{-4} M. At concentrations above 1×10^{-2} M, the percentage of histamine released by AEA reached a plateau (55—60%).

Effect of AEA on the Permeation of CF in Rat Colonic Mucosa The permeation of CF in rat colonic mucosa was studied using the Ussing chamber method. Table 1 lists the permeability coefficient (P_{app}) values for CF when various concentrations of AEA were added to the mucosal solution. Since CF is water soluble, CF could not readily permeate through the isolated colonic mucosa in the absence of AEA. The P_{app} values increased from the AEA concentration of 0.3 mM, but became saturated as the concentration increased over 0.5 mM. Thus AEA caused the release of histamine from mast cells (≧0.4 mM) and enhanced the permeability of CF in the colon mucosa (>0.1 mM) as shown in Fig. 1 and Table 1, respectively. Therefore the concentration of AEA was fixed at 0.5 mM in all the following experiments.

Role of Histamine in the Permeation-Enhancing Effect of AEA To clarify whether the permeation-enhancing activity of AEA was related to the histamine stored in mucosal mast cells, the influence of exogenous and endogenous histamine on the CF permeability was studied in the presence of 0.01 mM of histamine and 0.1 mM of compound 48/80, respectively. The results are shown in Fig. 2. The CF permeability was increased approximately two-fold compared with the control by the addition of exogenous histamine and compound 48/80. Compound 48/80 at 0.1 mM released about 57% of the histamine stored in mucosal mast cells (Fig. 1) and 0.01 mM of histamine added to the mucosal solution was sufficiently in excess. However, P_{app} values following both additions were almost equal to that upon the addition of 0.5 mM AEA, where only 13% of histamine was released from mast cells (Fig. 1). The permeability of CF significantly decreased in the colonic mucosa of rats pretreated with pyrilamine as an antagonist of the H_1 histamine receptor, with a significant difference of $p<0.001$, even if histamine existed in excess.

Figure 3 shows the enhancing effect of AEA on the CF permeability in the isolated colonic mucosa of rats pretreated with pyrilamine as an H_1 receptor antagonist, cimetidine as an H_2 receptor antagonist, or ketotifen as a mast cell stabilizer. These histamine inhibitors were administered intraperitoneally to the rats before excision of the colonic segment and had no effect on the permeation of CF. In the colonic mucosa of rats pretreated with pyrilamine and ketotifen, P_{app} values in the presence of 0.5 mM AEA were significantly reduced to the levels of the control without AEA ($p<0.001$), indicating that the permeation-enhancing activity of AEA was inhibited by pyrilamine and ketotifen. On the other hand, the enhancing activity of AEA was not influenced by cimetidine, resulting in a much higher P_{app} in the presence *vs.* the absence of 0.5 mM AEA ($p<0.001$). Thus the histamine released by AEA and bound to the H_1 receptor plays an important role in the permeation-enhancing actions of AEA.

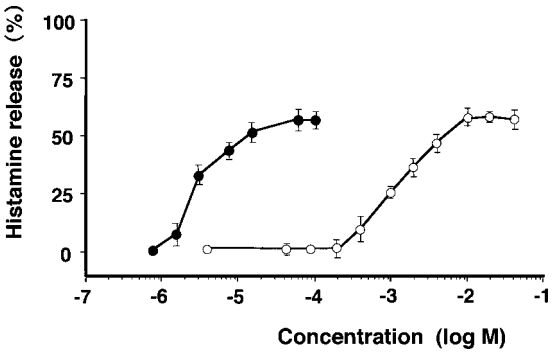

Fig. 1. Histamine Release from Rat Peritoneal Mast Cells Induced by AEA or Compound 48/80

Histamine release was expressed as a percentage of the release induced by 0.05% Triton X-100. Each point represents the mean±S.D. of five experiments. ○, AEA; ●, compound 48/80 (comp 48/80).

Table 1. Effect of AEA on the Permeability of CF

AEA Concentration (mM)	P_{app} ($\times10^{-6}$ cm/s)
0 (control)	3.691±0.992
0.05	3.922±3.501
0.1	5.100±2.561
0.3	7.925±2.035[a)]
0.5	11.593±3.308[a)]
0.7	11.950±2.503[a)]
1.0	12.073±3.020[a)]

Each value represents the mean±S.D. of five to seven rats. *a)* $p<0.001$ (*vs.* control).

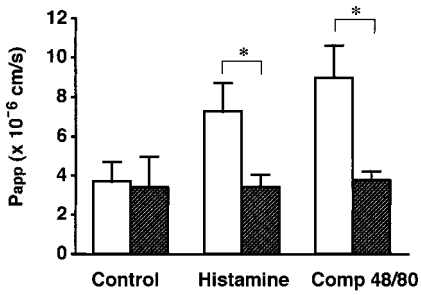

Fig. 2. Effects of Exogenous and Endogenous Histamine on CF Permeability in Isolated Rat Colonic Mucosa with and without *in Vivo* Pretreatment with Pyrilamine

Pyrilamine (20 mg/kg) was administered intraperitoneally to the rats 10 min prior to excision of the colonic segment. Black or white column represents CF permeability coefficients in colonic mucosa with or without pretreatment by pyrilamine, respectively. $*p<0.001$, P_{app} with *vs.* without pretreatment with pyrilamine.

Inhibition of Permeation-Enhancing Activity of AEA To clarify the mechanism of action of AEA, the effects of several inhibitors such as W7, H7, and compound 48/80 on CF permeation were investigated. W7 is a calmodulin antagonist and H7 a protein kinase C (PKC) inhibitor. Compound 48/80 was used as a specific inhibitor of phospholipase C (PLC) at low concentrations.[8] Figure 4 shows the permeation coefficients for CF when inhibitors were added to the mucosal solution in the absence and presence of 0.5 mM AEA.

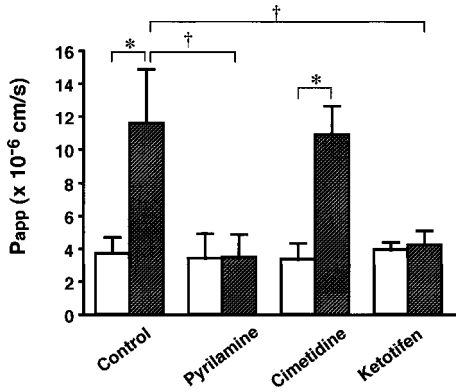

Fig. 3. Enhancing Effect of AEA on CF Permeability through Colonic Mucosa of Rats Pretreated with Pyrilamine, Cimetidine, or Ketotifen

Pyrilamine (20 mg/kg) or cimetidine (100 mg/kg) was administered intraperitoneally to the rats 10 min prior to excision of the colonic segment. Ketotifen (3.124 mg/kg) was given intraperitoneally to the rats 30 min prior to the preparation of the colon. AEA (0.5 mM) was added to the colonic mucosal solution. Black or white column represents CF permeability coefficients in the presence or absence of AEA, respectively. Results are expressed as the mean±S.D. of 5 to 7 experiments. $*p<0.001$, P_{app} in the presence *vs.* the absence of 0.5 mM AEA; $\dagger p<0.001$, P_{app} *vs.* the corresponding control in the presence of 0.5 mM AEA.

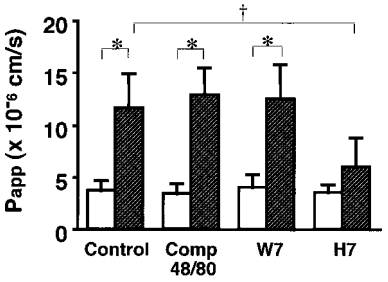

Fig. 4. Inhibitory Effects of W7, H7, and Compound 48/80 on Permeation-enhancing Activity of AEA

AEA (0.5 mM) was added to the colonic mucosal solutin. Compound 48/80 (comp 48/80, 2.5 μg/ml), W7 (50 μM), or H7 (50 μM) was applied to the mucosal side of the chamber, and the preparation was incubated for 20 min at 37 °C. Each column expresses the mean±S.D. of 5 to 7 experiments. $*p<0.001$, P_{app} in the presence *vs.* the absence of 0.5 mM AEA; and $\dagger p<0.01$, P_{app} *vs.* the corresponding control without inhibitor.

In the absence of AEA, no effect of any inhibitor on CF permeability was observed. The value of P_{app} increased threefold compared with the control upon the addition of 0.5 mM AEA, but was significantly reduced by H7 ($p<0.01$), but not by W7 and compound 48/80. Accordingly, it was suggested that PKC contributed to the permeation-enhancing effect of AEA in the intestinal mucosa and that the AEA activity was not influenced by the calmodulin antagonist or PLC inhibitor.

Effect of AEA on Electrophysiological Parameters of Colonic Mucosa CF permeability increased in the rat colonic mucosa as the AEA concentration increased. However, it is apparent that many, if not most, of the compounds examined as membrane permeation enhancers *in vitro* often cause cytotoxicity or membrane damage. Both the permeability enhancement and cytotoxicity depended on the length of exposure, and thus we assessed the changes in colonic mucosa caused by AEA using the electrophysiological parameters PD, I_{sc}, and R_m. Under control conditions, PD and I_{sc} reached maximum values at 10 min after mounting the colonic mucosa; this was followed by a slow decrease to 30 min and constant values of 3.5 mV and 38 $\mu A \cdot cm^{-2}$, respectively. When 0.5 mM AEA was added to the mucosal solution at 30 min after the mounting of the tissue, PD and I_{sc} decreased slowly: PD, 3.5 mV to 2.0 mV; and I_{sc}, 44 $\mu A \cdot cm^{-2}$ to 28 $\mu A \cdot cm^{-2}$ in 40 min. Figure 5 shows the time course of R_m after mounting the colonic mucosa. R_m also remained nearly constant under the control conditions. After the addition of 0.5 mM AEA to the mucosal solution, R_m was immediately lowered by about 20—25 ohm $\cdot cm^{-2}$ and then remained at about 70 ohm $\cdot cm^{-2}$.

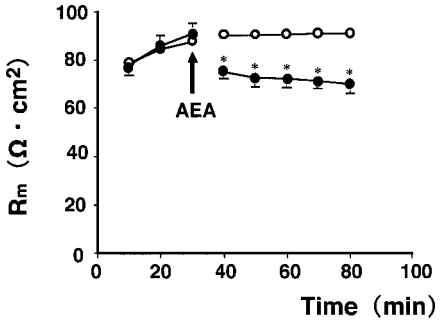

Fig. 5. Effect of AEA on R_m of the Rat Colonic Mucosa at pH 7.4 and 37 °C

AEA (0.5 mM) was added to the mucosal solution 30 min after the mounting of the colonic mucoa (indicated by an arrow). Results are expressed as the mean±S.D. of 11 experiments. Opened or closed circles represent the control or the sample in the presence of 0.5 mM AEA, respectively.

The cytotoxicity of AEA in the colonic mucosa was evaluated using the *in situ* loop method by measuring the LDH crossing the rat colonic epithelium into the lumen for 1 h. This method could measure LDH leakage with much higher sensitivity than the Ussing chamber method,[14] and the leakage of LDH was shown to be a useful marker of local toxicity.[15] LDH activity with 0.5 mM AEA (49.4±18.0 U/l/cm) was not significantly different from that of the control (43.2±7.7 U/l/cm). Since AEA had little potential to cause toxicity at this concentration, the decrease in R_m after the addition of AEA shown in Fig. 5 might indicate a reduction in paracellular resistance. Thus a transient opening of tight junctions would seem less damaging than a disruption of the cell membrane structure.

DISCUSSION

There are two permeation pathways for colorectal drug absorption: the transcellular pathway across the intestinal epithelial cell membrane and paracellular pathway to the lateral intercellular space *via* tight junctions between epithelial cells.[16] The transcellular pathway is generally the principal route for drugs with some degree of lipophilicity. On the other hand, for water-soluble compounds or poorly permeable macromolecules such as polypeptides and proteins, the paracellular pathway is considered an important absorption route. The opening of the paracellular route is effective for the absorption enhancement of such compounds. In recent years, absorption enhancers have been actively investigated and some of their actions and enhancing mechanisms in transepithelial membranes have been clarified.[17—19] Some absorption enhancers have also been found in folk medicines and health foods. It was reported that Glycyrrhizin (Grz), a major constituent of licorice with steroid-like activity, possesses *in vivo* enhancing activity with respect to the nasal and

rectal absorption of antibiotics, insulin, and calcitonin.[20—22] Imai *et al.* showed the mechanism of action of Grz *in vivo* and *in vitro.*[23]

Previously, we studied the antiinflammatory effects of BA, a component of aloes and related compounds, and found AEA of the anaerobic product from BA to have histamine-releasing activity in isolated rat mast cells.[5] It has been known that mast cell-derived inflammatory mediators such as histamine, platelet-activating factor, and nitric oxide increase the intestinal epithelial permeability of drugs and disrupt the barrier function.[6,7,24] It is also reported that histamine contributes to the chemically induced hypermeability in intestinal mucosa caused by absorption enhancers.[25—27]

First, to confirm the activity of AEA as an absorption enhancer, we investigated the effect of AEA on the permeability of CF as a water-soluble and poorly permeable model compound. AEA clearly enhanced the permeability of CF at the low concentrations, as shown in Table 1, indicating that AEA has an enhancing effect.

Next, we investigated the role of histamine in the permeability of CF produced by AEA. The enhanced permeability of CF as a result of the treatment with AEA was inhibited by an H_1 receptor antagonist of pyrilamine and a mast cell stabilizer of ketotifen, but not by an H_2 receptor antagonist of cimetidine (Figs. 2, 3). The result suggests that small amounts of histamine released from mast cells play an important role in the permeation-enhancing effect of AEA. In other words, AEA stimulated mast cells within the colonic mucosa membrane to release histamine, which probably bound to the H_1 receptor, and resulted in the response.

To elucidate the mechanism of action of AEA further, we investigated the effects of several inhibitors on the permeability of CF. As shown in Fig. 4, the enhanced permeability of CF caused by AEA was inhibited by H7, but not by W7 or compound 48/80. H7, W7, and compound 48/80 used in the study of the inhibition of AEA permeation enhancement were a PKC inhibitor, a myosin light chain kinase (MLCK) inhibitor of a calmodulin antagonist, and a PLC inhibitor, respectively. The following mechanisms to open the tight junction are well known: PLC in plasma membranes cleaves phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol 1,3,4-triphosphate (IP_3); DAG activates PKC, which induces the phosphorylation of the membrane protein or lipid to open the tight junction; IP_3 releases calcium from intracellular stores[28,29]; a calcium–calmodulin complex formed due to calcium binding with calmodulin activates MLCK; the condensation of actin microfilaments is then induced by the activated MLCK[30]; and microfilaments and microtubules participate in the opening of the tight junctions.[31] Since the increment in P_{app} by AEA was inhibited by only the PKC inhibitor, it was suggested that AEA extends the tight junction *via* activation of PKC to improve the permeability. Furthermore, the decrease in R_m caused by AEA also indicates the opening of the paracellular route (Fig. 5). Groot *et al.* reported that the activation of the intracellular PKC route by histamine receptor activation could induce a decrease in the intestinal barrier function.[32] And according to Kachintorn *et al.*, histamine could stimulate PLC-mediated phosphatidylinositol turnover, theoretically yielding the endogenous PKC activator DAG.[33] In this study, it was suggested that the histamine released from mast cells treated with AEA might interact with the H_1 receptor to activate PKC, and further with cell membrane protein to widen tight junctions, resulting in improved permeability. Further work is in progress to measure DAG and the intracellular level of calcium ions to elucidate the detailed intracellular mechanism induced by AEA.

In conclusion, AEA is of potential use as an absorption enhancer in colonic mucosa. The permeation-enhancing mechanism is based on the widening of tight junctions partially mediated *via* histamine, which binds to the H_1 receptor and activates PKC, without cytotoxicity in the colonic epithelium.

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