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Absorption-enhancing effect of glycyrrhizin induced in the presence of capric acid

Teruko Imai [a,*], Michinori Sakai [b], Hiroshi Ohtake [b], Hidekazu Azuma [a], Masaki Otagiri [a]

[a] *Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan*
[b] *Fundamental Research Laboratories, Hisamitsu Pharmaceutical Co. Inc., 1-25-11 Kannondai, Tsukuba, Ibaraki 305-0856, Japan*

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Abstract

The absorption-enhancing effect of the simultaneous administration of sodium caprate (Cap-Na) and dipotassium glycyrrhizinate (Grz-K) was investigated to clarify an effect of Grz-K. A combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K had a rapid and long-lasting absorption-enhancing activity in Caco-2 cell monolayers under conditions where Cap-Na and Grz-K showed a weak and no activity, respectively. The simultaneous treatment of a Caco-2 cell monolayer with Cap-Na and Grz-K showed no change in intracellular calcium ion level, although a major mechanism of absorption-enhancing effect for Cap-Na was elevation of intracellular calcium ion level. On the other hand, the simultaneous enhancing effect of Cap-Na and Grz-K was inhibited by H7, a PKC inhibitor. Possibly, Grz-K showed an absorption-enhancing effect via PKC cellular signaling pathway after penetration into cell according to increasing membrane permeability by Cap-Na. The absorption of sCT by the rat colon was enhanced by a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K, and its effect continued even 9 h after the onset of the experiment. Furthermore, the simultaneous treatment of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K showed a negligible histological changes to the colon mucosal membrane and a negligible toxicity on Caco-2 cell monolayer. A combination of Cap-Na and Grz-K shows a synergistic absorption-enhancing effect with little mucosal injury, which is applicable to colon-specific delivery.

Keywords: Absorption enhancer; Sodium caprate; Glycyrrhizin; Caco-2 cell; Colon delivery

Abbreviations: Cap-Na, sodium caprate; Grz-K, dipotassium glycyrrhizinate; TEER, transepithelial electrical resistance; Flu-Na, sodium fluorescein; FD-4, fluorescein isothiocyanate-dextran 4000; EDTA, sodium ethylenediaminetetraacetate; sCT, salmon calcitonin; IP_3, inositol 1,3,4-triphosphate; PKC, protein kinase C; HBSS, Hanks' balanced salt solution; HBSS/CMF, Hanks' balanced salt solution/calcium–magnesium-free; H7, 1-(5-isoquinolinesulfonyl)-2-methylpirerazide dihydrochloride

* Corresponding author. Tel.: +81 96 371 4626; fax: +81 96 371 4639.
E-mail address: iteruko@gpo.kumamoto-u.ac.jp (T. Imai).

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1. Introduction

Oral drug delivery is still preferred by a majority of patients. Delivery systems in the colon are important in some cases, to minimize side effects and to maximize the therapeutic response of drugs. Colon delivery system can be improved in several ways. The timed-release system (Pulsinap®, Geomatrix®), pH-dependent coating (Hu et al., 1999), microbially degradable polymers (Prasad et al., 1998), intestinal pressure-controlled system (Jeong et al., 2001) and redox-sensitive polymer (Stubbe et al., 2001) are commonly used for this purpose. When drugs reach the colon via the delivery system, they should be effectively absorbed from the colon lumen. A successful way to improve drug absorption in the colon is the use of absorption enhancers. It is necessary to select a highly useful enhancer that induces potent and long-lasting absorption-enhancing activity without no or minimal mucosal injury.

We previously examined the absorption-enhancing activity of several representative absorption enhancers, i.e., sodium deoxycholate (Deo-Na), a bile acid, sodium caprate (Cap-Na), a fatty acid, and dipotassium glycyrrhizinate (Grz-K), a terpene (Sakai et al., 1997). In a study using an intestinal epithelial cell model, Caco-2 cell monolayer (Pinto et al., 1983; Hidalgo et al., 1989; Artursson, 1990), we found potent absorption-enhancing activity in the cases of Deo-Na and Cap-Na. Cap-Na induced a rapid response, while Deo-Na showed a relatively slow response. On the other hand, Grz-K that possessed low membrane permeability due to hydrophilicity rarely showed an effect on drug permeability through a Caco-2 cell monolayer, although it has been reported to enhance the in vivo transmucosal absorption of antibiotics and insulin (Tanaka et al., 1992; Mishima et al., 1989). Furthermore, we demonstrated that the discrepancy between the in vitro and in vivo study can be explained by the hydrolysis of Grz to its aglicon, glycyrrhetic acid, by bacterial β-glucuronidases in the intestinal lumen (Imai et al., 1999). Glycyrrhetic acid had a more potent enhancing activity than Cap-Na and Deo-Na at the same concentration. In addition, we examined the absorption-enhancing activity of simultaneous treatment with Deo-Na and Grz-K (Sakai et al., 1999). Consequently, the absorption-enhancing effect of a combination of Grz-K and Deo-Na was much greater than that of Deo-Na alone. However, the response of their simultaneous treatment was similar to Deo-Na. Moreover, we found that the simultaneous enhancing response was related to a cellular signaling system mediated by protein kinase C, the same mechanism as was found for Deo-Na (Qiao et al., 2000; Milovic et al., 2002). Therefore, two possible explanations for an effect of simultaneous treatment were considered. (1) The activity of Deo-Na was enhanced in the presence of Grz-K. (2) After transport into Caco-2 cell, Grz-K itself showed absorption-enhancing effect by a same mechanism as Deo-Na. If Grz-K itself possessed an absorption-enhancing response related to a cellular signaling system, in vivo activity of Grz-K was not induced by only a hydrolyzed product, glycyrrhetic acid, but also Grz itself. To demonstrate an absorption-enhancing effect of Grz-K itself is important for a pharmaceutical modification with Grz-K.

In this study, we have investigated an absorption-enhancing effect of Grz-K itself by means of a simultaneous treatment with Cap-Na in Caco-2 cell. It has been reported that Cap-Na is capable of opening the paracellular route, when intracellular calcium concentrations are increased via inositol 1,3,4-triphosphate (Tomita et al., 1995). Therefore, the absorption-enhancing effect of Grz-K could be characterized by a comparison of the simultaneous effect of Cap-Na and Grz-K and the effect of Cap-Na itself. Furthermore, the in vivo efficacy of a combination of Cap-Na and Grz-K has been confirmed by the absorption of salmon calcitonin (sCT) in the rat colon. The safety of a combination of Cap-Na and Grz-K has been evaluated by examining a morphological changes in the rat colon mucus membrane.

2. Materials and methods

2.1. Materials

Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Sodium fluorescein (Flu-Na), fluorescein isothiocyanate dextran (FD-4), 1-(5-isoquinolinesulfonyl)-2 methylpiperazide dihydrochloride (H7), Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids, benzylpenicillin G, streptomycin and sodium ethylenediaminetetraacetate (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fura-2 acetoxymethyl ester (Fura-2 AM) and

Fura-2 were purchased from Molecular Probes Inc. (Eugene, OR, USA). Fetal bovine serum (FBS) was purchased from Cytosystems PTY (Castle Hill, Australia). Sodium caprate (Cap-Na) and dipotassium glycyrrhizinate (Grz-K) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and Alps Pharm. Co. (Tokyo, Japan), respectively. Ionomycin and salmon calcitonin were purchased from Calbiochem-Novobiochem Corporation (San Diego, CA, USA).

2.2. Cell culture

Caco-2 cells between passages 75 and 90 were routinely cultured in DMEM (pH 7.4) and were supplemented with a 1% (w/v) NEAA solution, 10% (v/v) heat-denatured FBS, benzylpenicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37 °C under an atmosphere of 95% air and 5% CO_2. The cells were harvested by treatment with 0.05% (w/v) trypsin/0.53 mM EDTA before reaching confluence and were then seeded at a density of approximately 1×10^4 cells/cm^2 on a Transwell-inserted filter (Costar, Cambridge, MA, USA) with an area of 1 cm^2 and a 3 μm pore size. Cells were allowed to reach confluence and to differentiate for 3 weeks prior to use. All cell monolayers in these studies exhibited the transepithelial electrical resistance (TEER) ranging from 700 to 900 Ω cm^2.

2.3. TEER measurement

Hank's balanced salt solution/calcium–magnesium-free (HBSS/CMF) was used as an apical side solvent, to prevent the formation of a chelate of Cap-Na with metal divalent ions such as calcium and magnesium. The results of a preliminary experiment indicated that the use of HBSS/CMF on the apical side of the Transwell filter reduced the TEER value by about 10–20% within 30 min after replacement and that the TEER values stabilized 45 min after replacement. Thus, all of the subsequent in vitro experiments were started 1 h after the addition of HBSS/CMF to the apical side. After a Caco-2 cell monolayer had equilibrated for 1 h in fresh HBSS/CMF on the apical side and DMEM on the basal side, the TEER of the monolayers was measured at an initial time ($t = 0$) with the MILLICELL® electrical resistance system (Millipore Corporation, Bedford, MA, USA) and taken as the initial value. For inhibition experiments, 100 μM H7 in HBSS/CMF was added to the apical side, instead of HBSS/CMF. HBSS/CMF on the apical side was replaced with 0.5 ml of HBSS/CMF with or without Cap-Na and/or Grz-K. The pH of HBSS/CMF was adjusted to 5.5 or 7.0 with HCl or NaOH. After incubation for 10 or 20 min, the apical and basal chambers were gently washed twice with fresh HBSS/CMF and DMEM, respectively, in order to wash out the enhancer. Fresh HBSS/CMF (0.5 ml) and DMEM (1 ml) were placed in the apical and basal chambers, respectively. The TEER values of the cell monolayers were measured periodically, and are expressed as a percentage of the initial ($t = 0$) value of the same monolayer.

2.4. Transport experiment

The test solutions containing Flu-Na and FD-4 were prepared in HBSS/CMF at final concentrations of 100 μg/ml and 1 mg/ml, respectively. After the monolayers were treated with an enhancer in the same manner as was used for the TEER measurement, 0.5 ml of the test solution and 1 ml of DMEM were added to the apical and basal chambers ($t = 0$), respectively. The samples (50 μl) from the basal chamber were analyzed by high performance liquid chromatography (HPLC). The apparent permeability coefficient (P_{app}, cm/s) was calculated using the following equation: $P_{app} = dQ/dt/A/C_0$, where dQ/dt is the permeability rate (steady state transport rate, μg/s) of appearance of the fluorescent model compound in the basal chamber after initial lag time, C_0 is the initial concentration in the apical chamber (μg/ml), and A is the surface area of the transwell (cm^2).

2.5. HPLC analysis

The determination of Flu-Na and FD-4 were according to previously reported method (Sakai et al., 1997). The HPLC system consisted of a pump (L-6000, Hitachi Co., Tokyo, Japan), a fluorescence detector (F-1050, Hitachi Co., Tokyo, Japan), a chromato-integrator (D-2500, Hitachi Co., Tokyo, Japan), and a column (4.5 mm i.d. × 10 cm, LiChrospher® RP-18, Cica-MERCK, Darmstadt, Germany). The mobile phase used was 5 mM phosphate buffer (pH 7.4)/acetonitrile (88/12) at a flow rate of 1 ml/min. The excitation and emission wavelengths of the fluorescence detector were set at 494 and 518 nm, respectively.

2.6. Measurement of intracellular calcium ion levels

Intracellular calcium ion levels were measured using Fura-2-AM (Grynkiewicz et al., 1985). The cell monolayers, grown on filters, were incubated for 45 min at 37 °C after Fura-2-AM (final concentration, 30 μM) was added to 1.5 ml of HBSS/CMF on the apical side. After the Fura-2-AM solution was washed out, the cell monolayers were set in the calcium analyzer (CAF-100, Japan Spectroscopic Co. Ltd., Tokyo, Japan). Fresh HBSS/CMF (0.5 ml) and DMEM (2 ml) were placed in the apical and basal side, respectively. Since fluorescence of Fura-2 upon binding with calcium was immediately changed by addition of an absorption enhancer into apical side, fluorescent changes were recorded for 5 min and their maximum and/or minimal values were used for calculation of intracellular calcium ion level. The experimental system with cells was excited at wavelengths of 340 and 380 nm, and the intensity of the fluorescence was measured at an emission wavelength of 510 nm. The intracellular calcium concentration was calculated from the ratio of fluorescent intensity excited at 340 and 380 nm. Ionomycin was used as a positive control, which artificially increases intracellular calcium ion levels.

2.7. In vivo experiment

SD male rats (ca. 300 g) were subjected to fasting for 24 h, and their anus was occluded by a suture under ether anethesia. The animals were laparotomized to expose the colon at the site of the cecum, and a small opening was made at the root of the ascending colon. The sCT solution (160 units/head dissolved in 2 ml PBS/CMF with or without enhancer) was injected through the small hole into the ascending colon, and a point ∼0.5 cm distal to the small hole was ligated to prevent leakage of the solution. The exposed colon was then replaced into the peritoneal cavity and sutured. Sham operations were performed in the positive control group which received an intra-muscular injection (4 units of sCT/head) into the thigh. Blood (0.3 ml) was collected with a heparinized syringe from the jugular vein under ether anesthesia on a time-course basis (0, 0.5, 1, 2, 3, 6, and 9 h). The calcium concentration in the plasma sample obtained by centrifugation (1600 × *g*, 15 min) was measured by an autoanalyzer (AU-510, OLYMPUS, Tokyo, Japan). For histological examination, the colon (about 5 cm) was resected from the site of administration toward the rectum immediately after blood collection at 9 h and was then fixed in 10% (w/v) formaldehyde. The fixed colon was sliced, and then stained with hematoxylin and eosin.

2.8. Statistical analysis

Tukey's multiple rank test or Student's *t*-test, depending on the groups, were used to compare the data. *P*-values <0.05 were considered significant. Results are expressed as the mean ± standard deviation (S.D.).

3. Results

3.1. Tight junction opening in Caco-2 cell monolayer by Grz-K in the presence of Cap-Na

In the presence of Cap-Na, the effect of Grz-K on an opening of the tight junction was estimated by a change of TEER in a Caco-2 cell monolayer. Since Cap-Na leads to a decrease in TEER in a dose-dependent manner (Sakai et al., 1997), the concentration of Cap-Na was fixed at 0.1% (w/v) at which Cap-Na shows a slight TEER-lowering activity, in order to clearly observe the additive or synergic effect of Grz-K. Fig. 1 shows the dose-dependent effect of Grz-K on TEER values for Caco-2 cell monolayers in the presence of 0.1% (w/v) Cap-Na. Although Grz-K itself did not show any TEER-lowering effect, as previously reported (Sakai et al., 1997), a simultaneous treatment with 0.1% (w/v) Cap-Na induced a dose-dependent decreases in TEER. A rapid and long-lasting TEER-lowering activity was found when a combination of 0.1% (w/v) Cap-Na and more than 1% (w/v) Grz-K was used.

To examine whether or not the effects of this combination would occur under the co-existence of Cap-Na and Grz-K, each was allowed to exert its effects separately for 10 min. The results are shown in Table 1. A slight effect of the combination was found when each absorption enhancer was used separately. A marked decrease in TEER was found only when both Cap-Na and Grz-K were present. The TEER-lowering activity of the combination was sufficient even for a 10 min treatment period. These data suggest that the co-existence of Cap-Na and Grz-K is effective in opening the tight

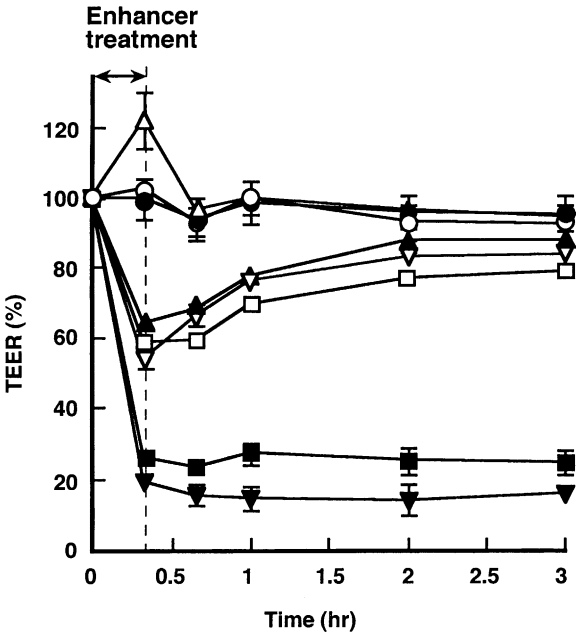

Fig. 1. Effect of Grz-K on TEER values of Caco-2 cell monolayers in the presence of 0.1% (w/v) Cap-Na: (●) control, (○) 0.1% (w/v) Grz-K, (△) 2.0% (w/v) Grz-K, (▽) 0.1% (w/v) Cap-Na, (▲) 0.1% (w/v) Cap-Na/0.1% (w/v) Grz-K, (□) 0.1% (w/v) Cap-Na/0.2% (w/v) Grz-K, (■) 0.1% (w/v) Cap-Na/1.0% (w/v) Grz-K and (▼) 0.1%Cap-Na/2.0%Grz-K. The treatment period of the absorption enhancers was 20 min. Data are expressed as the mean ± S.D. of TEER ($n = 4$).

junction. The uptake of Trypan blue and neutral red, a respective marker of damage of a cellular membrane and a lysosomal membrane, from the apical membrane was same under the both condition of a control and a treatment with combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K (data not shown). Furthermore, the lowering TEER by combination of 0.1% Cap-Na and 2% Grz-K was recovered after change of HBSS to DMEM in both apical and basolateral sides, although 0.5% Cap-Na that showed nearly same TEER-lowering activity did not show any recovery of TEER (Sakai et al., 1998). These observations indicated that the enhanced permeability of Caco-2 cell monolayer by treatment of Cap-Na and Grz-K was not related to a damage of cell membrane.

3.2. Effect of pH on the simultaneous treatment of Cap-Na and Grz-K

The TEER-lowering effects by combination of Grz-K and Cap-Na might be due to a lowering of the pH by Grz-K, because the pH of a 2% (w/v) solution of Grz-K is 5.2. Possibly, Cap-Na could easily permeate into a Caco-2 cell at pH 5.2, and this might show a significant tight junction opening effect. A permeability of water-soluble model compounds, Flu-Na and FD-4 across Caco-2 cell monolayers was measured at pH 5.5 and 7.0. The treatment method for Cap-Na and Grz-K was identical to the TEER measurement. The apparent permeation coefficients (P_{app}) are shown in Table 2. The P_{app} for Flu-Na in the presence of Cap-Na was nearly same at pH 5.5 and 7.0, indicating that a lowering pH of medium was not significant factor in enhancing permeability of Flu-Na under simultaneous treatment of Grz-K and Cap-Na. Interestingly, a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K enhanced the permeability of Flu-Na across Caco-2 cell monolayer at both pH conditions. Especially, at pH 5.5, the P_{app} was 2.5-fold greater under the treatment with combination

Table 1
Interdependency and combination effects of Cap-Na and Grz-K on the TEER in Caco-2 cell monolayers

The first treatment for 10 min	TEER value (%) at 10 min	The second treatment for 10 min	TEER value (%) at 20 min
Control buffer	100.8 ± 1.3	Control buffer	97.5 ± 1.1
0.1% Cap-Na	69.4 ± 1.5*	Control buffer	84.3 ± 2.5
2% Grz-K	118.3 ± 4.5*	Control buffer	103.3 ± 4.7
0.1% Cap-Na/2% Grz-K	31.0 ± 4.9*	Control buffer	17.9 ± 3.2*
Control buffer	96.4 ± 3.2	0.1% Cap-Na	68.2 ± 0.2*
2% Grz-K	118.9 ± 3.9*	0.1% Cap-Na	76.1 ± 5.6*
Control buffer	93.8 ± 4.7	2% Grz-K	104.4 ± 9.4
0.1% Cap-Na	70.1 ± 8.9*	2% Grz-K	102.5 ± 10.6

The first treatment of the enhancer to the monolayers was for 10 min, and the second treatment was for 10 min after washing out the retained enhancer with HBSS/CMF. Control buffer: HBSS/CMF not including enhancer. Data represent the mean ± S.D. of TEER ($n = 4$).

* $P < 0.05$: significant difference from control buffer (Tukey's multiple rank test).

Table 2
Comparison of apparent permeability coefficients (P_{app}) on the transports of Flu-Na and FD-4 in Caco-2 cell monolayers

Enhancer	P_{app} ($\times 10^{-7}$ cm/s)		
	Flu-Na (pH 5.5)	Flu-Na (pH 7.0)	FD-4 (pH 5.5)
Control buffer	8.32 ± 0.43	6.65 ± 1.71	0.36 ± 0.10
0.1% Cap-Na	9.35 ± 1.24	9.24 ± 0.80	0.34 ± 0.07
2% Grz-K	7.58 ± 0.48	7.58 ± 0.51	0.29 ± 0.09
0.1% Cap-Na/2% Grz-K	23.36 ± 2.21*,#	14.06 ± 2.16*,#	3.56 ± 0.01*,#

Data are expressed as the mean ± S.D.
* $P < 0.05$: significant difference from control (Tukey's multiple rank test).
# $P < 0.05$: significant difference from 0.1% Cap-Na (Tukey's multiple rank test).

of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K than the treatment with 0.1% (w/v) Cap-Na. Regarding the permeability of FD-4 at pH 5.5, the combination showed a significant increase about 10.5-fold greater than that of the treatment with 0.1% (w/v) Cap-Na. Furthermore, the effects of a combination of Cap-Na and Grz-K on TEER in Caco-2 cell monolayers at pH 5.5 and 7.0 are shown in Fig. 2. The control buffers had no affect on TEER at pH 5.5 and 7.0. A simultaneous treatment with 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K showed a remarkable TEER-lowering activity at pH 5.5 in comparison with that at pH 7.0.

These results suggest that a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K effectively opens tight junction and enhances the absorption of water-soluble compounds in the pH range of 5.5–7.0 where the physiological in vivo conditions are assumed (Evans et al., 1988; Kararli, 1995).

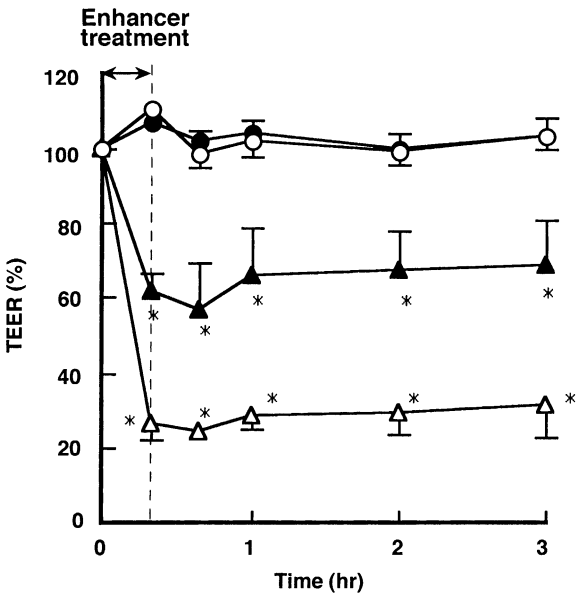

Fig. 2. Influence of apical pH on the simultaneous effect of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K on the TEER in Caco-2 cell monolayers. The treatment period for the combination was 20 min for a control buffer of pH 5.5 (○), pH 7 (●), the combination at pH 5.5 (△), and the combination at pH 7 (▲). Data are expressed as the mean ± S.D. of TEER ($n = 4$). * $p < 0.05$: significant difference from each control buffer (Student's t-test).

3.3. Mechanism of a tight junction opening effect by simultaneous treatment of Cap-Na and Grz-K

To elucidate the mechanism of TEER lowering by the combination of Cap-Na and Grz-K, intracellular calcium ion levels were measured. The results are listed in Table 3. However, a significant increase in intracellular calcium levels was found with 0.5% (w/v) Cap-Na and 0.0015% (w/v) ionomycin, a calcium ionophore, used as a positive control. Intracellular calcium ion levels were very slightly increased by treatment with 0.1% (w/v) Cap-Na alone, and slightly decreased for

Table 3
Effects of Cap-Na, Grz-K and the combination of Cap-Na and Grz-K on intracellular calcium ion levels in Caco-2 cell monolayers

Enhancer	Intracellular calcium ion levels (nM)
Control buffer	55.8 ± 3.4
0.1% Cap-Na	69.7 ± 4.4
2% Grz-K	23.7 ± 7.4
0.1% Cap-Na/2% Grz-K	52.4 ± 20.7
0.5% Cap-Na	320.2 ± 46.7*
0.0015% ionomycin	142.5 ± 38.2*

Data are expressed as the mean ± S.D. for intracellular calcium ion level ($n = 3$–5).
* $P < 0.05$: significant difference from control buffer (Tukey's multiple rank test).

2% (w/v) Grz-K. In addition, a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K had no affect on intracellular calcium ion levels at all, possibly due to opposite effect of Cap-Na and Grz-K on intracellular calcium level.

Furthermore, the effect of H7, a PKC inhibitor, was examined. After each monolayer was exposed to HBSS/CMF containing 100 μM H7 only from apical side for 1 h, the TEER of Caco-2 cell monolayers was measured. The concentration of H7 used, i.e., 100 μM, was selected on the basis of the result of a preliminary study by Tomita et al. (1996). Namely, 100 μM of H7 was loaded to a sufficient level, and had no effect on TEER values. Interestingly, the TEER-lowering activity by a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K was significantly reduced when the cells were pretreated with H7, as shown in Fig. 3. In contrast, the TEER lowering by 0.1% Cap-Na was not affected by treatment with H7. When 0.3% (w/v) EDTA, a compound previously reported to have PKC-mediated absorption-enhancing activity (Tomita et al., 1996), was used as a positive control, approximately a 30% decrease in TEER was found with the use of EDTA alone; however, this TEER-lowering activity was inhibited completely by pretreatment with H7.

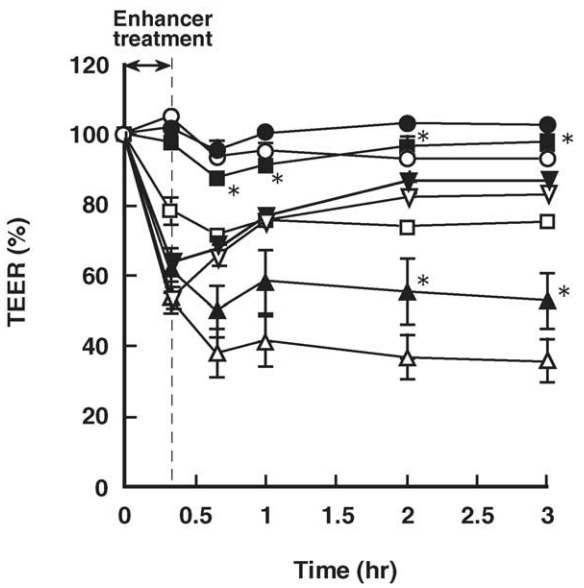

Fig. 3. Effect of H7 on the TEER in Caco-2 cell monolayers treated with 0.1% (w/v) Cap-Na, a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K, and 0.3% (w/v) EDTA. Caco-2 cell monolayer was treated with 0.1% (w/v) Cap-Na (▼, ▽), a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K (▲, △), 0.3% (w/v) EDTA (■, □), and control buffer (●, ○) for 20 min. Closed and open symbols show TEER values for a Caco-2 cell monolayer treated with and without H7, respectively. Data are expressed as the mean ± S.D. of TEER ($n = 4$). * $p < 0.05$: significant difference between with H7 and without H7 (Student's *t*-test).

To elucidate the differences of the mechanism of TEER-lowering by the combination of Cap-Na and Grz-K between pH 5.5 and 7.0, an intracellular calcium level and an effect of H7 on TEER-lowering activity were also measured. An inhibition effect of H7 and no change of intracellular calcium level were observed at pH 5.5 similar to pH 7.4. These data suggested that the mechanism of tight junction opening activity by combination of 0.1% Cap-Na and 2% Grz-K in Caco-2 cell monolayer was quite different from Cap-Na itself that increased intracellular calcium level.

3.4. In vivo experiment

To examine the effects of the simultaneous treatment of the two absorption enhancers on in vivo absorption, sCT was selected as a model peptide drug. Blood calcium levels were measured at appropriate times after the dosing of sCT alone or in combination with the absorption enhancers in the colon. The results are shown in Fig. 4. A transient decrease in blood calcium levels was found up to 2 h after the dosing (160 units of sCT) of a control buffer solution. Thereafter the calcium levels returned to the preadministration level within 6 h. When sCT was administered with 0.1% (w/v) Cap-Na, a gradual decrease in blood calcium levels was found for period of up to 3 h after dosing. A maximum decrease of about 30% in calcium level was observed and then returned to the preadministration level within 9 h. Furthermore, the decrease was nearly identical to the case of the buffer solution containing 2% (w/v) Grz-K alone when 4 units of sCT was dosed intra-muscularly as a positive control. Treatment with 2% Grz-K and intra-muscular dosing maintained an about a 20% decrease against the preadministration value, even 9 h after dosing. On the other hand, a gradual decrease in blood calcium levels occurred after the dosing of a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K, showing about a 40% decrease; the effect persisted up to 9 h after dosing.

Regarding in vivo safety, the colon was stained with hematoxylin and eosin after the termination of the in

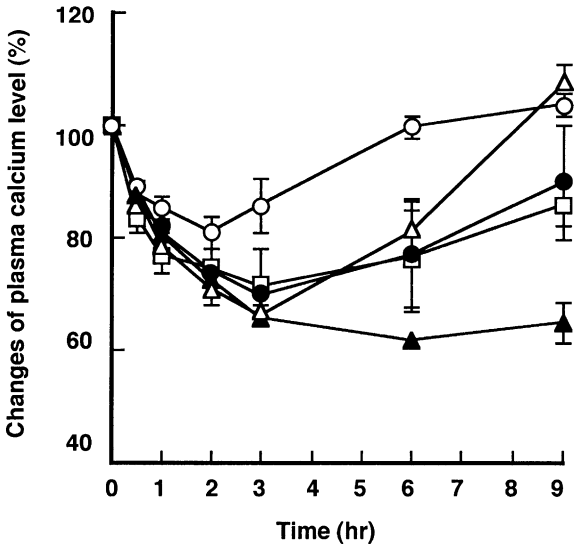

Fig. 4. Changes in plasma calcium levels after the administration of sCT into the rat colon. A control (sCT itself) was administered into the colon (◯) at 160 unit/head and muscle (□) at 4 unit/head. The simultaneously administered absorption enhancer was 0.1% (w/v) Cap-Na (△), 2% (w/v) Grz-K (●), and the combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K (▲), respectively. Data are expressed as the mean ± S.D. of plasma calcium level ($n = 4$).

vivo sCT absorption experiment; the tissue was examined histologically, and a typical photograph of the stained material is shown in Fig. 5. No histological effect was observed at all as compared with a control even when a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K was used.

4. Discussion

We previously reported that Grz-K showed an absorption-enhancing activity after hydrolysis of Grz to glycyrrhetic acid by bacterial β-glucuronidase in the intestinal lumen (Imai et al., 1999), although Grz-K did not show any in vitro absorption-enhancing effect in Caco-2 cell monolayer due to low permeability depended on its hydrophilicity. In addition, we also found that the absorption-enhancing activity obtained from the simultaneous treatment of Deo-Na and Grz-K was much greater than Deo-Na alone in Caco-2 cell monolayers, where Grz-K showed no absorption-enhancing effect (Sakai et al., 1999), and this action showed the same slower response as Deo-Na. This result expected that Deo-Na would affect cell membrane permeability followed by a modification of a tight junction opening activity by Grz-K. However, the enhancing activity of Grz itself could not be identified, because a mechanism of synergistic enhancing effect of Grz-K and Deo-Na was similar to Deo-Na. Possibly, Grz penetrated in Caco-2 cell monolayer under certain condition when membrane permeability increased by Deo-Na, and then Grz itself showed absorption-enhancing effect. In this study, Cap-Na, an absorption enhancer with a different mechanism from Deo-Na, was selected as a coadministered absorption enhancer with Grz-K and an absorption-enhancing activity of Grz-K itself was identified.

The effect of the combination of Cap-Na and Grz-K was observed only when both individual enhancers were present (Table 1), similar to the combination of Deo-Na and Grz-K. Concerns were elicited by the following factors: (1) an increase in the penetration of Cap-Na into the cell due to the lowering of the medium pH by Grz-K; (2) a direct effect of potassium salt contained in 2% (w/v) Grz-K; and (3) facilitated penetration of Grz-K that might affect the tight junction. Since the concentration of Grz-K used is relatively high, the effects of lowering pH and potassium concentration should be considered. Although the medium pH was lowered to 5.2 for the addition of 2% (w/v) Grz-K, the permeability of Flu-Na was equally increased by 0.1% (w/v) Cap-Na at both pH 5.5 and 7.0, which excluded the first possibility. In general, potassium concentrations in the extracellular fluid of culture cells are relatively low. Furthermore, to elucidate an effect of a high level of extracellular potassium ion, disodium glycyrrhizinate (Grz-Na) and potassium chloride was used at same molar concentration as 2% (w/v) Grz-K in a combination with 0.1% (w/v) Cap-Na. Consequently, Grz-Na showed a similar potent effect, and potassium chloride did not show any additional effect on the response of 0.1% (w/v) Cap-Na. These facts provide evidence to show that the effect of a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K was not due lowering pH and potassium ion level.

The final possibility that is the cooperative effect of Cap-Na and Grz-K penetrated into cell in the presence of Cap-Na may be an appropriate explanation. Regarding the mechanism, as already reported by Tomita et al. (1995), Cap-Na promotes the release of calcium ions from the intracellular calcium pool via IP_3 and

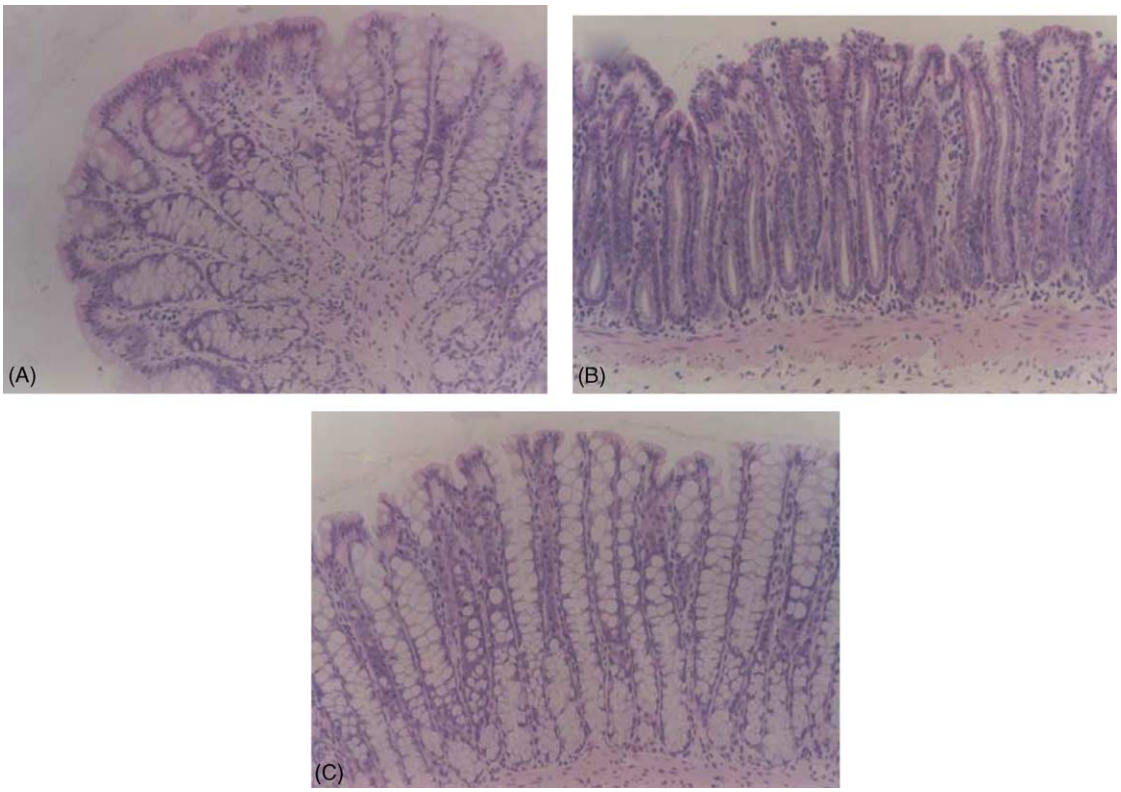

Fig. 5. Hematoxylin and eosin staining of the colonic mucosa 9 h after administration of buffer (A), the combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K (B), and 0.1% (w/v) SDS (C); magnification 50×.

increases intracellular calcium concentrations, and the contraction of actin-myosin due to the calcium calmodulin complex results in opening the paracellular route. Increases in intracellular calcium ion levels, as evidenced in this experiment, were consistent with their report in this regard when only 0.5% (w/v) Cap-Na was used (Table 3). Intracellular calcium ion levels were slightly affected by either 0.1% (w/v) Cap-Na itself or a combination of 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K. It may be concluded that the combination did not activate the IP_3 pathway similar to an action of a high concentration of Cap-Na itself (0.5% (w/v)).

Furthermore, we attempted to elucidate the mechanism of a combination of Grz-K and Cap-Na via an activation of PKC signaling pathway. As shown in Fig. 3, the effect of the combination was reduced to 60% by the pretreatment with H7, a PKC inhibitor. This effect was considered to be similar to the action of a positive control as represented by EDTA (Tomita et al., 1996). It has been reported that Grz affects cellular signaling pathways, such as PKC, casein kinase II and transcription factors such as activator protein I and nuclear factor κB (Dai et al., 2001; Tanigawa et al., 2001). Probably, Grz-K might penetrate into cells in the presence of Cap-Na, and then might act on the tight junction through a PKC cellular signaling pathway.

In addition, the response of combination with Grz-K and Cap-Na was observed only when they were present. Since the absorption-enhancing effect of Cap-Na maintained after washing out it in Caco-2 cell monolayer, Grz-K might penetrate into cell. However, the separated treatment of Cap-Na and Grz-K hardly showed a synergistic response (Table 1). It can be assumed that a hydrophobic Cap-Na preferentially permeates into cell in the presence of both Cap-Na and Grz-K, and then limited amount of Grz-K penetrates in the cell. Then Grz-K might show an absorption-enhancing activity via PKC signaling pathway, then the permeability

of Cap-Na might be also enhanced. Thus cooperative response of Cap-Na and Grz-K is important for the activity of combination. Furthermore, the response for combination was greater in pH 5.5 than 7.0 (Table 2 and Fig. 2), because the membrane permeability of both acidic compounds, Cap-Na and Grz-K, might be higher in acidic condition. The Cap-Na mediated increase of membrane permeability of Grz-K is supported by the report that the colonic absorption of Grz is enhanced by a surfactant such as polysorbate 80 (Shibata et al., 2001). Consequently, the improved permeability of Grz-K into cell under certain condition might play an important role in the absorption-enhancing activity of simultaneous treatment of Cap-Na and Grz-K, although further experiments will be required to clarify the details of the enhancing activity by combination of Cap-Na and Grz-K.

We have already reported that Grz-K shows in vivo absorption-enhancing activity. Grz-K is partially metabolized to glycyrrhetinic acid, an aglycone, by β-glucuronidase, an enzyme present in the intestinal flora in the colon. Since glycyrrhetinic acid has shown considerably potent TEER-lowering activity in an in vitro study, we estimated that the decrease in TEER was probably provoked by the absorption-enhancing activity of glycyrrhetinic acid formed in the colon (Imai et al., 1999). In fact, the in vivo absorption of sCT was enhanced by 2% (w/v) Grz-K (Fig. 4) that showed no effect in Caco-2 cell monolayers. The in vivo effect of 2% (w/v) Grz-K was prolonged in compared with that for 0.1% (w/v) Cap-Na. The in vivo effect of Grz-K might be related to glycyrrhetinic acid and intact Grz that might be facilitated the penetration into mucosal membrane via the action of glycyrrhetinic acid. Therefore, it would be expected that the combination of Cap-Na and Grz-K would show a severe in vivo response in comparison with the results obtained for a Caco-2 cell monolayer. When 0.1% (w/v) Cap-Na and 2% (w/v) Grz-K were simultaneously administered, the initial response was almost the same as their individual response and the subsequent response was maintained for 9 h after dosing. Thus an initial response after treatment with a combination of Cap-Na and Grz-K showed neither an additive nor a synergistic effect, and the subsequent response was significantly potent in comparison with their individual activity. The initial rapid response might be related to the action of Cap-Na and the subsequent long-lasting effect might be explained by both the activity by glycyrrhetinic acid produced from Grz in the colon and the direct activity of Grz-K as shown in Caco-2 cell monolayers.

From the viewpoint of safety, the histological effects of the combination were examined after termination of the in vivo study. No histological differences were found against the control at all, which led us to consider that it constitutes a safe combination of these absorption enhancers.

The present study demonstrated that the intact Grz showed an absorption-enhancing response via PKC cell signaling pathway, and its effect was increased by the presence of the other absorption enhancer. The absorbability in the colon would be still poor when a water-soluble or protein drug is dosed orally as the active ingredient to be disintegrated in the colon; therefore, the use of a combination of absorption enhancers would improve the bioavailability of an active drug without intestinal injury. The results obtained in the present study provide useful information for applications to oral colon delivery preparations.

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