In Vitro and *In Vivo* Evaluation of the Enhancing Activity of Glycyrrhizin on the Intestinal Absorption of Drugs

Teruko Imai,[1,3] Michinori Sakai,[2] Hiroshi Ohtake,[2] Hidekazu Azuma,[2] and Masaki Otagiri[1]

Received July 7, 1998; accepted October 5, 1998

Purpose. The enhancing activity of dipotassium glycyrrhizinate (Grz) on the intestinal absorption of drugs has been demonstrated in an *in vitro* study using Caco-2 cell monolayers and in an *in vivo* absorption study in rats.

Methods. The hydrolysis of Grz by luminal content and mucosa of the rat colon was investigated. The absorption-enhancing activity of Grz and its hydrolysates was estimated by changes in transepithelial electrical resistance (TEER) and the permeation of sodium fluorescein (Flu-Na) in Caco-2 cell monolayers. It was further evaluated through the absorption of salmon calcitonin (sCT) in the rat colon.

Results. Grz was not hydrolyzed to glycyrrhetinylmonoglucuronide (GrMG) and glycyrrhetinic acid (GA) by colonic mucosa, but, rather by the β-glucuronidase in colonic flora. The hydrolysis of Grz to GrMG was extremely slow and the GrMG produced was rapidly regenerated to GA. Grz and GrMG had no effect on TEER nor on the permeability of Flu-Na across Caco-2 cell monolayers. On the other hand, GA decreased TEER and increased the permeability of Flu-Na in a dose-dependent manner. However, Grz and GrMG enhanced the plasma calcium-lowering effect of sCT after administration in the rat colon. The coadministration of sCT and GA in the rat colon induced the strongest plasma calcium-lowering effect and the highest plasma concentration of sCT.

Conclusions. The *in vivo* enhancing-activity of Grz in the absorption of drugs is dependent on GA, a hydrolysis product of Grz resulting from the action of β-glucuronidase in intestinal flora.

KEY WORDS: dipotassium glycyrrhizinate; glycyrrhetinylmonoglucuronide; glycyrrhetinic acid; salmon calcitonin; absorption enhancer; β-glucuronidase.

INTRODUCTION

Glycyrrhizin (Grz), a major constituent of licorice a steroid-like, anti-allergic, anti-viral, and interferon-inducing activity, has been used orally as a sweetener and a component of oriental medicines (1–4). In the field of pharmaceutics, Grz is used to enhance the transdermal absorption of drugs. Recently, Grz has been reported to possess *in vivo* enhancing activity with respect to the nasal and rectal absorptions of antibiotics, insulin, and calcitonin (5–7). We (8) have previously studied the mechanism of the absorption-enhancing activity of this compound using Caco-2 cell monolayers, an intestinal epithelial cell model (9–11). However, Grz showed negligible effects on TEER and the transport of several model fluorescent compounds in Caco-2 cell monolayers. Thus, the *in vivo* results were not consistent with the *in vitro* studies using Caco-2 cell monolayers. Grz contains two glucuronosyl moieties linked to a steroid. The compound is hydrolyzed in a stepwise manner to the monoglucuronide and then to glycyrrhetinic acid (GA, the aglycone) by the action of endogenous (biliary or enteric) and bacterial β-glucuronidases in the intestinal lumen (12–15). Therefore, it is highly possible the *in vivo* absorption enhancing activity of Grz could be attributed, not to Grz itself, but to its hydrolysis products.

In the present study, the hydrolysis of Grz was examined in the rat colon. The absorption-enhancing activity of Grz and its hydrolysates was evaluated in the Caco-2 cell monolayers and through the absorption of salmon calcitonin (sCT) in the rat colon.

MATERIALS AND METHODS

Materials

Caco-2 cells were purchased from the American Type-Culture Collection (Rockville, Maryland, USA). DMEM, non-essential amino acids (NEAA), benzylpenicillin G, streptomycin, 0.05% trypsin/0.53 mM EDTA solution, Flu-Na, kanamycin sulfate, and D-saccharic acid 1,4-lactone were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Fetal bovine serum (FBS) was purchased from Cytosystems PTY (Castle Hill, Australia). Grz and GA were purchased from Alps Pharm. Co. (Tokyo, Japan) and Tokyo Kasei Kogyo Co. (Tokyo, Japan), respectively. GrMG was purchased from Maruzen Co. (Hiroshima, Japan) and purified by high performance liquid chromatography (HPLC) prior to use. sCT was obtained from Calbiochem-Novabiochem corporation (San Diego, California, USA).

Cell Cultures

Caco-2 cells between passages 75 and 90 were routinely cultured in DMEM (pH 7.4) which was supplemented with 1% NEAA solution, 10% (v/v) heat-inactivated FBS, benzylpenicillin G (100 U/ml), and streptomycin (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% trypsin/0.53 mM EDTA before reaching confluence and were then seeded at a density of approximately 1×10^4 cells/cm^2 on Transwell®-inserted filters (Costar, Cambridge, Massachusetts, 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 TEER ranging from 600–800 Ω·cm^2.

[1] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan.

[2] Hisamitsu Pharmaceutical Co., Inc., Central Research Center, Tsukuba Laboratories, 1-25-11 Kannondai, Tsukuba, Ibaraki 305-0856, Japan.

[3] To whom correspondence should be addressed. (e-mail: iteruko@gpo.kumamoto-u.ac.jp)

ABBREVIATIONS: Grz, dipotassium glycyrrhizinate; sCT, salmon calcitonin; GrMG, glycyrrhetinylmonoglucuronide; GA, glycyrrhetinic acid; TEER, transepithelial electrical resistance; DMEM, Dulbecco's Modified Eagle's Medium; HBSS, Hank's balanced salt solution; HBSS/CMF, calcium-and magnesium-free HBSS; Papp, apparent permeability coefficient; Flu-Na, sodium fluorescein; PBS, phosphate buffered saline; PBS/CMF, calcium- and magnesium-free PBS.

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Measurement of TEER

After the apical side of each Caco-2 cell monolayer was exposed to fresh HBSS/CMF for 1 hr, TEER of the monolayers was measured at t = 0 with a MILLICELL® electrical resistance system (Millipore Corporation, Bedford, Massachusetts, USA) and taken as the initial value. The HBSS/CMF in the apical side was then replaced with 0.5 ml of HBSS/CMF with or without Grz, GrMG, and GA. After incubation for 20 min, the apical and basal chambers were gently washed twice with fresh HBSS/CMF (0.5 ml) and DMEM (1 ml), respectively. The TEER of the cell monolayers was measured periodically, and expressed as a percentage of the initial (t = 0) value of the same monolayer.

Transport Experiment

After the cell monolayer was treated with an enhancer in the same manner as in the TEER measurement, 0.5 ml of Flu-Na solution (100 μg/ml in HBSS/CMF) 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 HPLC. Apparent permeability coefficient, Papp (cm/sec), was calculated using the following equation: $Papp = dQ/dt/A/C_0$, where dQ/dt (μg/sec) is the steady state rate of appearance of Flu-Na in the basal chamber after initial lagtime, C_0 (μg/ml) is the initial concentration in the apical chamber, and A (cm^2) is the area of the transwell.

Hydrolysis of Grz by Luminal Content and a Mucosal Homogenate in the Rat Colon

The SD male rats (ca. 300 g), fasted for 24 hr, were laparotomized under ether anesthesia. The colon, from ~2 cm distal to the cecum to ~2 cm proximal to the rectum was immediately removed and cut open. The luminal content was collected as quickly as possible, and suspended in ice-cold PBS (20 ml PBS/g wet weight). After washing the colon with ice-cold PBS, colonic mucosa was removed by scrapping with a glass slide under ice-cold conditions. The mucosa were homogenized with 10 mM phosphate buffer (pH 7.4) containing 1.15% KCl (3 ml buffer/g wet weight) using a Potter-Elvehjem glass homogenizer under ice-cold conditions. The supernatant (S10) fraction was obtained from the homogenate by centrifugation at 10,000g for 20 min at 0°C. The S10 fraction was stored at −80°C until required for use. The hydrolysis of Grz and GrMG (final concentration: 0.22 mM) was determined in 2.5 ml of luminal content suspension and/or the S10 fraction (final protein concentration: 0.1–1 mg/ml) previously preincubated for 5 min at 37°C. The reaction was stopped by the addition of 2 ml of MeOH, and the concentrations of Grz, GrMG, and GA in the supernatant obtained by centrifugation (1600g, 10 min) were determined by HPLC.

In Vivo sCT Absorption Experiment

The SD male rats (ca. 300 g) were subjected to fasting for 24 hr, and their anuses was occluded by suture under ether anesthesia. A small hole was made at the root of the ascending colon. The sCT solution (80 or 160 units/head dissolved in 2 ml of PBS/CMF with or without enhancer) was injected from the small hole into the ascending colon, and a point ~0.5 cm distal to the small hole was ligated to prevent the leakage of solution. Subsequently, the intestine was replaced in the peritoneal cavity and sutured. Sham operations were performed in the positive control group which received an intramuscular injection (4 units of sCT) into the thigh. Blood (0.3 ml) was collected with a heparinized syringe from the jugular vein under ether anesthesia. The plasma samples obtained from centrifugation (1600g, 15 min) were analyzed with an autoanalyzer (AU-510, OLYMPUS®, Tokyo, Japan) and a radioimmunoassay kit (Peninsula Laboratories, Inc., Belmont, California, USA), for calcium and sCT levels, respectively. For preparation of pseudo-germ-free rats, kanamycin sulfate dissolved in saline (200 mg/head) was administered orally twice daily for 2 days.

HPLC Analysis

The analysis conditions for Flu-Na were as follows: a column, C18 (4.5 mm × 10 cm, LiChrospher® RP-18, Cica-MERCK, Dermstadt, Germany); isocratic mobile phase, 5 mM phosphate buffer (pH 7.4)/acetonitrile (88:12 v/v); flow rate, 1 ml/min; the fluorescence detection, 494 nm for excitation and 518 nm for emission. The HPLC conditions for Grz, GrMG, and GA were as follows: a column, C18 (μbond pack C18, 3.9 mm × 30 cm, 10 μm particles, Waters Corporation, Massachusetts, USA); the gradient mobile phase, 2%(v/v) acetic acid (solvent A) and acetonitrile (solvent B) was accomplished using a gradient that consisted of 35%(v/v) B for 15 min, which was ramped to 35–70%(v/v) B over 10 min, an 8 min hold as 70%(v/v) B, and then returning to the starting composition during the final 5 min; the flow rate, 1 ml/min; UV detection, 254 nm.

Intracellular Calcium Ion Measurement

Intracellular levels of calcium ion were measured using Fura-2-AM. The cell monolayers were incubated with 0.5 ml of Fura-2-AM (final concentration, 30 μM) dissolved in HBSS/CMF on the apical side for 45 min at 37°C. After washing out the Fura-2-AM, the cell monolayers were placed in a Calcium Analyzer (CAF-100, Japan Spectroscopic Co. Ltd., Tokyo, Japan), and excited at wavelengths of 340 nm and 380 nm. The change in fluorescence (em. 510 nm) of Fura-2 upon binding with calcium was recorded.

Statistical Analysis

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

RESULTS

Effect of Grz on the Absorption of sCT in Rat Colon

The *in vivo* absorption-enhancing activity of Grz was estimated by the plasma calcium levels after administration of 160 units/head of sCT in the rat colon. As shown in Fig. 1, the group which received only sCT showed a transient decrease in calcium level by about 10–20% at 1 to 2 hr relative to the preadministration level. On the other hand, the group coadministered with 2.2 mM (=2% (w/v)) of Grz showed a gradual decrease in the calcium levels by about 30% at 3 hr, which

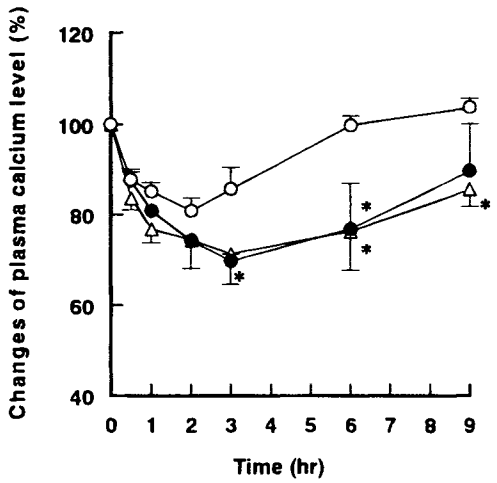

Fig. 1. Enhancing effect of 22.2 mM Grz on colonic absorption of sCT. ○: control buffer including 160 units sCT; ●: 160 units sCT with 22.2 mM Grz; and △: intramuscular injection of 4 units sCT. Data represent the mean ± SD (n = 4). *: $p < 0.05$; significant difference from control buffer (Tukey's multiple rank test).

was identical in terms of time profile to the intramuscularly administered group with 4 units/head of sCT as a positive control.

Hydrolysis of Grz in Colonic Preparations

The hydrolysis of Grz (0.22 mM) in the colon was determined by both a luminal content suspension and a mucosal homogenate. The disappearance of Grz and the appearance of GrMG and GA were simultaneously determined by HPLC. Grz gradually decreased while approximately 1% of GrMG and 13% of GA were formed in 3 hr (Fig. 2-A). Mass balance (>97%) was achieved up to 1 hr, but the total amount of Grz and its hydrolysis products was 95% at 3 hr after incubation. As shown in Fig. 2-B, GrMG was rapidly hydrolyzed to GA. Mass balance was not achieved even at 3 min after incubation (90%) and the total amount of Grz and its hydrolysis products decreased with incubation time, indicating the presence of further metabolism of GA. The rate constant of each step was calculated by simultaneously fitting each time-course data set for the hydrolyses of Grz and GrMG in Fig. 2-A and -B. The calculation was performed based on the following assumptions: 1) Grz is hydrolyzed to GA via GrMG; 2) GA is further metabolized; 3) all transformation processes follow first-order kinetics. The rate constants obtained were 0.054 ± 0.011 hr^{-1} for the hydrolysis of Grz to GrMG, and 4.91 ± 1.05 hr^{-1} for the hydrolysis of GrMG to GA. Nearly the same parameters were obtained at five times higher concentration (1.1 mM) of substrates, suggesting that the hydrolysis of Grz to GrMG is the rate-limiting step for the hydrolysis of Grz.

In addition, the hydrolysis of Grz was completely inhibited by 1 mM of D-saccharic acid 1,4-lactone, a specific β-glucuronidase inhibitor, as shown in Table I. The selective inhibitor for carboxylesterase, bis-nitrophenyl phosphate, failed to inhibit the hydrolysis of Grz. The decomposition of Grz was also studied in the S10 fraction of the colonic mucosa. However, no degradation of Grz and GrMG was observed for periods of

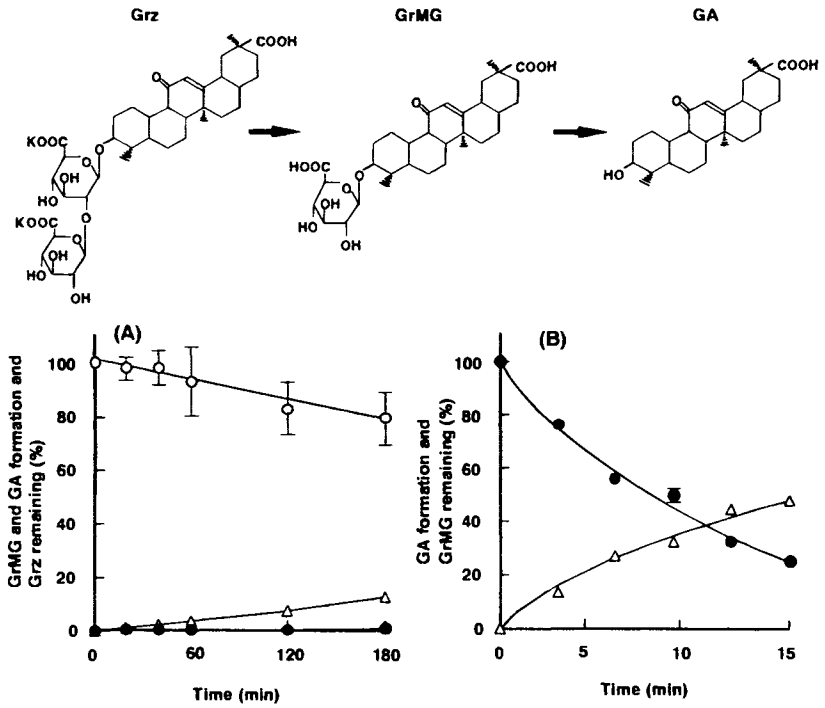

Fig. 2. Scheme for the hydrolysis of Grz and a time course of Grz (A) and GrMG (B) hydrolysis by luminal contents in rat colon. 0.22 mM of Grz and GrMG were incubated in luminal content (50 mg wet weight/ml) at 37°C. Data represent the mean ± SD (n = 3). ○: Grz; ●: GrMG; and △: GA.

Table I. Percentage of Presence of Grz and Its Metabolits at 3 hr After Hydrolysis of Grz in Colonic Content Suspension in the Absence and Presence of D-Saccharic Acid 1,4-Lactone and Bis-Nitrophenyl Phosphate

Component	Without inhibitor	D-saccharic acid 1,4-lactone	Bis-nitrophenyl phosphate
Grz	79.63 ± 9.91	107.12 ± 2.63*	79.72 ± 7.16
GrMG	0.98 ± 0.46	0.46 ± 0.29*	0.98 ± 0.46
GA	12.99 ± 0.34	0.87 ± 0.67*	12.34 ± 0.26

Note: Grz(0.22 mM) was incubated in luminal content (50 mg wet weight/ml) for 3 hr at 37°C in the absence and presence of 1 mM of D-saccharic acid 1,4-lactone and bis-nitrophenyl phosphate.
* $p < 0.05$ significant difference from data without inhibitors (Student's *t*-test).

up to 3 hr in 0.1–1 mg/ml protein content of S10 fraction (data not shown). These data suggest that Grz was hydrolyzed to GA via GrMG by β-glucuronidase in the colonic flora.

TEER of Caco-2 Cell Monolayers After Treatment with Grz and Its Hydrolysates

Figure 3 shows the effects of Grz and its metabolites on TEER, a parameter for the opening and closing of the paracellular route of cells, in the Caco-2 cell monolayers. Grz had little effect on TEER which slightly increased in a time-course basis (8). GrMG had a negligible effect on TEER irrespective of its concentration, while GA showed a progressive TEER-lowering effect with increasing concentration. GA, at concentrations exceeding 1.11 mM induced almost a complete decrease of TEER within 20 min of treatment.

Transport of Flu-Na in Caco-2 Cell Monolayers

The permeability of Flu-Na, a water-soluble model compound, across Caco-2 cell monolayers is shown in Fig. 4 and the transport parameters are listed in Table II. The Papp of the control monolayer was $6.23 \pm 0.43 \times 10^{-7}$ cm/sec, which is identical to the previously reported value ($5.86 \pm 0.48 \times 10^{-7}$ cm/sec) (8). The permeation of Flu-Na was not affected by treatment with ~5.55 mM Grz and GrMG. However, GA at concentrations of 0.55 and 1.11 mM markedly enhanced the permeability of Flu-Na, and the corresponding Papps were significantly higher than that of the control by about 2- and 4-fold, respectively. These data are in agreement with the results, based on TEER (see Fig. 3).

Intracellular Calcium Concentration

The intracellular calcium concentration was 23.72 ± 7.38 nM by treatment with 2.22 mM of Grz, in contrast to 68.36 ± 1.52 nM of control Caco-2 cell monolayers. In addition, the treatment by GA gives values of 117.29 ± 5.13 nM and 147.51 ± 15.75 nM for 0.55 mM and 1.1 mM, respectively. GrMG shows no different calcium level from control monolayers in a range of 1.11 to 5.55 mM.

Effects of GrMG and GA on the Colonic Absorption of sCT in Rats

The enhancing activity of 1.11 and 5.55 mM of GrMG and GA on *in vivo* absorption in the colon was examined. As shown in Fig. 5-A, the control group showed a transient decrease in calcium levels up to about 10% from the preadministration level in 0.5 to 1 hr after administration. The group treated with GrMG showed a gradual decrease in calcium level up to about 20% in 1 hr. GA induced a significant change in the calcium level, in a dose-dependent manner. Furthermore, only GA enhanced plasma sCT levels (Fig. 5-B). The higher dosing of GA (5.5 mM) induced the same plasma peak level of sCT as did the lower dose (1.11 mM) but retained the high level of sCT up to 2 hr.

We assumed the effect of Grz is dependent on GA produced by β-glucuronidase in colonic flora. In order to confirm the assumption, the enhancing effect of Grz was examined using the rats which had been orally administered kanamycin sulfate

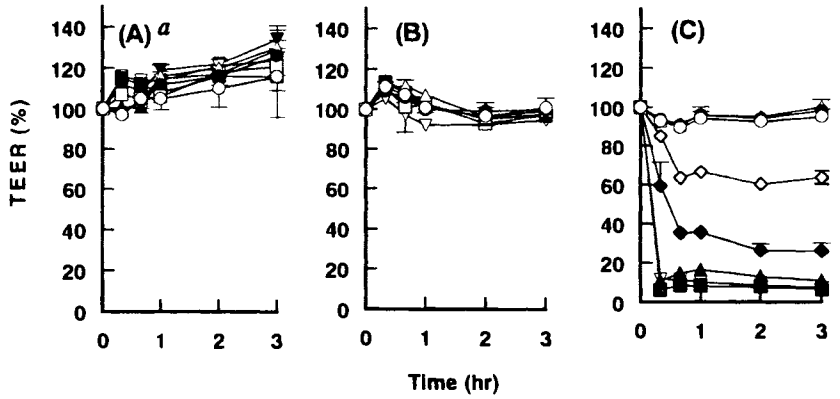

Fig. 3. Effects of various concentration of Grz (A), GrMG (B), and GA (C) on TEER in Caco-2 cell monolayers. The treatment period for the absorption enhancers was 0 to 20 min. The used medium are HBSS/CMF and DMEM in apical and basal chambers, respectively. ○: control; ●: 0.11 mM; △: 0.22 mM; ◇: 0.27 mM; ◆: 0.55 mM; ▲: 1.11 mM; □: 2.22 mM; ■: 5.55 mM; ▽: 11.1 mM and ▼: 22.2 mM. Data represent the mean ± SD of 3 TEER measurements of each monolayer. [a]Reference (8).

Table II. Comparison of Apparent Permeability Coefficients (Papp) on the Transport of Flu-Na

Enhancer	Papp (10^{-7} cm/sec)
Control[a]	5.86 ± 0.48
Control	6.23 ± 0.43
1.11 mM GrMG	6.30 ± 0.32
5.55 mM GrMG	6.57 ± 0.33
0.55 mM GA	11.78 ± 1.11*
1.11 mM GA	25.56 ± 1.37*
2.22 mM Grz[a]	5.77 ± 0.17
5.55 mM Grz[a]	5.98 ± 0.38

Note: Data were calculated from the results of transport experiments (Figure 5) and present the mean ± SD.
[a] Reference 8.
* $p < 0.05$; significant difference from control (Tukey's multiple rank test).

(200 mg/head twice daily) for 2 days. As shown in Fig. 6, the enhancing effect of Grz on the absorption of sCT was significantly decreased by the treatment of kanamycin sulfate. Interestingly, the treatment with kanamycin sulfate enhanced the colonic absorption of sCT in the control group. The enhancing mechanism of kanamycin sulfate is unclear, but these data suggest that kanamycin sulfate might affect the colonic membrane. In contrast, the effect of kanamycin sulfate on the absorbability of sCT, caused a reduction in enhancement by Grz of about 50%, suggesting that GA formation by colonic flora is very important to the enhancing activity of Grz.

DISCUSSION

The development of biotechnologies in recent years has allowed commercialization of numerous peptides and proteins as drugs. However, the majority of these are intended for injection due to poor permeability through mucosal membrane and to their instability in the gastro-intestinal lumen. To improve the poor intestinal absorption of these drugs, several techniques have been designed, e.g., the derivatization of the peptide to give, for example, a prodrug (16) and colon delivery systems. The colon delivery system mainly takes advantage of the specific disintegration of coated materials in the colon based on issues such as intestinal flora, luminal pH, and the long transit time after oral administration (17). Furthermore, absorption enhancers are available to improve the absorption of these proteins and peptides in colon delivery systems. In recent years, absorption enhancers have been actively investigated and some of their activity and enhancing mechanisms in transepithelial membranes (18–20) have been clarified. Grz is now in use as an absorption enhancer for transdermal formulations, and is reported to have *in vivo* mucosal enhancing properties with regard to peptides and shows no local toxicity problems (6,7). However, Grz showed no enhancing effect when examined in Caco-2 cell monolayers in a previous report (8) as shown in Fig. 3-A and Table II. The present study was designed to elucidate this observed discrepancy between *in vivo* and *in vitro* activity.

From the hydrolysis study, it is clear that Grz slowly hydrolyses to GrMG followed by rapid hydrolysis to GA by β-glucuronidase in colonic flora (Fig. 2 and Table I). The following organisms are thought to be responsible for the hydrolysis of Grz in rats: *E. coli*, *C. perfringens*, *Streptococci*, *Lactobacilli*, yeasts, and bacteroides (21). This represents the first evidence that the hydrolysis of Grz to GrMG is the rate-limiting step for the hydrolysis of Grz to GA by β-glucuronidase, although the hydrolysis of Grz by colonic flora has been reported (14). Furthermore, the mass balance was not obtained for the hydrolysis, especially from GrMG. The possibility for the formation of other metabolites, e.g., 3-oxo-GA as reported from Akao (12). These should be studied further in detail.

In Caco-2 cell monolayers, the GrMG produced has no effect on the TEER and the permeation of Flu-Na (see Fig. 3-B and Fig. 4-B). In contrast to the increase in TEER by Grz, GrMG might have a different effect on the cells from that of Grz. Although both Grz and GrMG contain attached glucuronic acid, the number of sugar moieties might influence the action on the cell. In addition, GA showed a strong paracellular opening effect, which might be related to ready permeation across

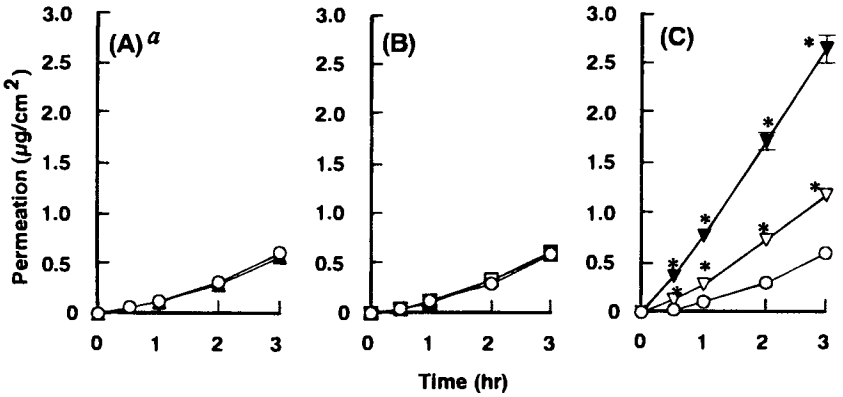

Fig. 4. Effects of Grz (A), GrMG (B), and GA (C) on the transport of Flu-Na in Caco-2 cell monolayers. Flu-Na (50 μg/0.5 ml) was applied to the apical side following the treatment of absorption enhancers for 20 min. ○: control buffer; △: 2.22 mM Grz; ▲: 5.55 mM Grz; □: 1.11 mM GrMG; ■: 5.55 mM GrMG; ▽: 0.55 mM GA; and ▼: 1.11 mM GA. Data represent the mean ± SD of the cumulative permeation per area (n = 3–4). *: $p < 0.05$; significant difference from control buffer (Tukey's multiple rank test). [a]Reference (8).

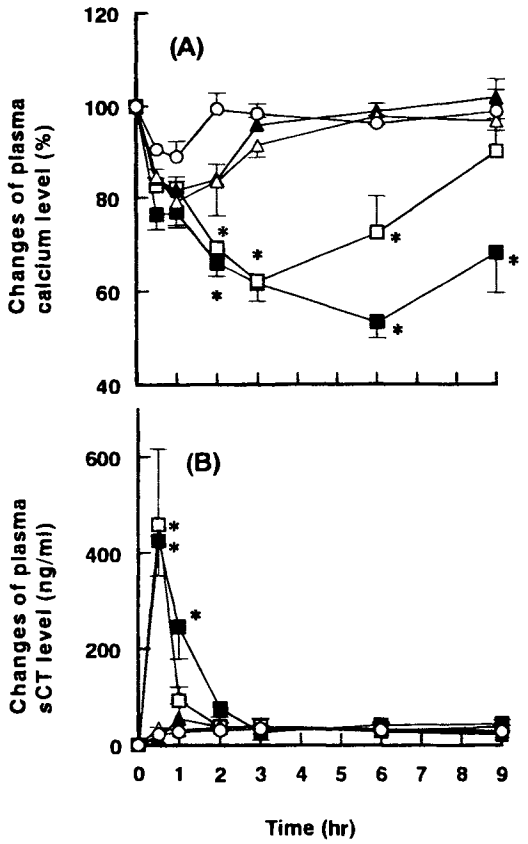

Fig. 5. Enhancing effects of GrMG and GA on colonic absorption of sCT. A and B show the changes of plasma calcium and sCT levels, respectively. ○: control buffer including 80 units sCT; △: with 1.11 mM GrMG; ▲: with 5.55 mM GrMG; □: 1.11 mM GA; ■: 5.55 mM GA. Data represent the mean ± SD (n = 4). *: $p < 0.05$; significant difference from control buffer (Tukey's multiple rank test).

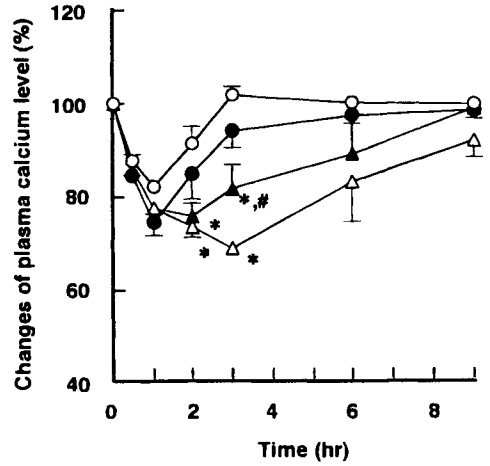

Fig. 6. Enhancing effects of Grz on colonic absorption of sCT in rats pretreated with kanamycin sulfate. Kanamycin sulfate dissolved in saline (200 mg/head) or saline alone was administered orally twice daily for 2 days prior to the sCT absorption experiment. ○: control buffer including 80 units sCT in rats pretreated with saline alone; ●: control buffer including sCT in rats pretreated with kanamycin sulfate; △: sCT with 22.2 mM Grz in rats pretreated with saline alone; ▲: sCT with 22.2 mM Grz in rats pretreated with kanamycin sulfate. Data represent the mean ± SD (n = 4). *: $p < 0.05$; significant difference from control buffer (Tukey's multiple rank test). #: $p < 0.05$; significant difference from the group with 22.2 mM Grz in rats pretreated with saline alone (Student's *t*-test).

the apical membrane. Furthermore, GA showed the strongest enhancing-activity on absorption of sCT in the rat colon among Grz and its hydrolysates (see Fig. 5). These results lead us to hypothesize that the *in vivo* enhancing-activity of Grz involves the hydrolysis of Grz to GrMG and GA by β-glucuronidase in colonic flora, and that GA is responsible for the observed enhancement. GA was produced at levels of 13% within 3 hr in a colonic luminal content suspension (see Fig. 2-A). Although the data for *in vitro* hydrolysis has not been extrapolated to *in vivo* hydrolysis after colonic administration, a certain amount of GA is produced from Grz in the colonic lumen. Furthermore, dosing with kanamycin sulfate (400 mg/head/day) for 2 days in rat reduced the enhancing effect of Grz, in spite of the enhancing effect of kanamycin sulfate itself on the absorption of sCT (see Fig. 6). Therefore, it is entirely possible that the result obtained in the *in vivo* study is due to the produced GA. However, GrMG revealed a weak enhancing effect in an *in vivo* experiment (see Fig. 5). If GrMG is rapidly hydrolyzed to GA, as in the *in vitro* study in the colonic luminal content suspension (see Fig. 2-B), GrMG should show an enhancing effect similar to GA. This may be due to the different conditions between *in vivo* and *in vitro* studies, i.e., amount of β-glucuronidase, stirring condition, etc.

In order to further investigate the enhancing action of GA, we measured intracellular calcium ion concentrations. Interestingly, the intracellular calcium ion levels were increased by treatment with GA. In contrast, they were decreased and unchanged by Grz and GrMG, respectively. Phospholipase C which is activated on the cell membrane cleaves inositol diphosphate to form inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases calcium ion from the intracellular stores, and DAG activates protein kinase C in the cell. It has been reported that these mediators regulate the paracellular permeability in Caco-2 cell monolayers (22). Tomita *et al.* (23) demonstrated that sodium caprate acts on cell membranes and causes an increase in intracellular calcium concentration via IP_3 and the calcium-calmodulin complex then provokes the contraction of actin-myosin filaments resulting in the opening of the paracellular route. This suggests that the increase in intracellular calcium ion concentration represents one of the enhancing mechanisms in drug absorption. Moreover, the intracellular calcium levels resulting from treatment with Grz and GrMG serve to explain their effects on TEER and transport of Flu-Na on Caco-2 cell monolayers. It is clear further studies are necessary for a full understanding of the enhancing mechanisms of Grz and GA.

The above results clearly show the essence of the absorption-enhancing activity of Grz, which has been observed *in vivo*, is due to GA, the aglycone, which is produced by the hydrolysis of Grz in the organism.

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