Permeability Characteristics of Tetragastrins Across Intestinal Membranes Using the Caco-2 Monolayer System: Comparison Between Acylation and Application of Protease Inhibitors

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Purpose. Three types of acyl tetragastrin (TG), acetyl-TG (C_2-TG), butyryl-TG (C_4-TG) and caproyl-TG (C_6-TG) were synthesized and their *in vitro* intestinal permeability characteristics were examined using Caco-2 monolayers.

Methods. The disappearance of acyl-TGs from the apical side of Caco-2 monolayers was estimated by analyzing degradation and permeation processes in terms of clearance.

Results. The amount of native TG transported to the basolateral side was very low due to its large degradation clearance (CLd) on the apical side. Degradation of TG was reduced by chemical modification with fatty acids, which resulted in an increase in the transport of TG across Caco-2 monolayers. In addition, the permeation clearance (CLp) value of carboxyfluorescein (CF), a paracellular transport and undegradable marker, was increased in the presence of acyl-TGs. Furthermore, we investigated the effects of the protease inhibitors bacitracin and gabexate on the transport of TG across Caco-2 monolayers. In the presence of a low concentration (0.1 mM) of protease inhibitor, the CLd value of TG was reduced, but they did not affect its CLp value. However, a higher concentration (1.0 mM) of bacitracin significantly reduced TG degradation on the apical side, and further increased its CLp value.

Conclusions. We demonstrated that acylation of TG made it resistant to intestinal proteases and caused it to enhance absorption of drugs, including itself, across Caco-2 monolayers. Further, bacitracin acted as both a protease inhibitor and an absorption enhancer.

KEY WORDS: Caco-2 cells; tetragastrin; chemical modification; membrane permeability.

INTRODUCTION

Tetragastrin (TG), the C-terminal tetrapeptide sequence of gastrin, Trp-Met-Asp-Phe-NH_2 (Fig. 1), possesses all the physiological properties (gastric output activity) as the intact 17-amino acid peptide (3,4). The intestinal absorption of TG is relatively poor because of extensive enzymatic degradation in the gastrointestinal tract (2,5,6). Therefore, it was difficult to assess its exact transport characteristics across the intestinal membrane. We have already synthesized acyl derivatives of TG which have retained their biological activities and we increased their lipophilicity by elongating the carbon chain of the acyl-modifiers (Fig. 1). In addition, the lipophilic modification of TG resulted in a significant increase in its enteral and transdermal absorption and also increased its bioavailability and stability (1,7,8).

In this study, we used Caco-2 cells, whose protease activity are reportedly lower than the small intestinal mucosal membrane, as an *in vitro* small intestinal membrane model, and determined the intestinal transport characteristics of TG. Furthermore, we assessed the contributions of the enzymatic and transport barriers on the intestinal transport of acyl-TGs. We also investigated the effects of protease inhibitors on the transport characteristics of TG across Caco-2 monolayers.

MATERIALS AND METHODS

Chemicals

TG was purchased from Peptide Institute, Inc. (Osaka, Japan). Three acyl-TG derivatives (Fig. 1), C_2-TG, C_4-TG and C_6-TG were synthesized according to the method described by Tenma *et al.* (1). Dimethylacetoamide (DMAc) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Trifluoroacetic acid (TFA) and acetonitrile were obtained from Nacalai Tesque, Inc (Kyoto, Japan). Caco-2 cells at passage number 74 were kindly donated by Dr. Kato (Eisai, Japan). Transwell polycarbonate inserts (3 μm pore size) were purchased from Costar (Cambridge, MA). Culture reagents were obtained from GIBCO BRL (Grand Island, NY). All other chemicals used in these experiments were of analytical grade.

Cell Culture

Caco-2 cells were maintained by serial passage in T-25 culture flasks. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL amphotericin B. The monolayer cultures were grown in an atmosphere of 5% CO_2–95% air at 37°C. The cells were subcultured every 7 days using 0.02% EDTA and 0.05% trypsin. Each the flask (25 cm^2) was inoculated with 5×10^5 cells in 10 mL of the culture medium. Caco-2 cells between passage number 80 and 85 were used in this study.

Stability of Acyl-TG Derivatives in Caco-2 Homogenates

Caco-2 homogenates were centrifuged at $1000 \times g$ in a refrigerated (4°C) centrifuge for 10 min to remove cellular and nuclear debris. The resulting supernatants were adjusted with Krebs-Ringer bicarbonate buffer (KRBB; pH 7.4) to a protein concentration of 1.5 mg/mL, as determined by the method of Lowry *et al.* (9) using bovine serum albumin fraction V as the standard. The degradation characteristics of acyl-TGs were studied by incubating 200 μL of the Caco-2 homogenate with 800 μL of acyl-TGs (50 μM). Fifty microliter samples were withdrawn from the incubating mixture at designated time intervals. The amount of acyl-TGs in each sample was determined by reversed phase HPLC.

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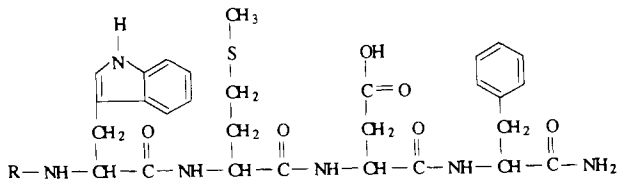

	R =	Lipophilic index	Biological activity (% of TG)
TG	H	3.13	100
C_2-TG	CH_3CO-	3.24	174
C_4-TG	CH_3-$(CH_2)_2$-CO-	3.62	n.d.
C_6-TG	CH_3-$(CH_2)_4$-CO-	4.33	173

n.d. : not determined.

Fig. 1. Chemical structures of acyl-TGs and their physicochemical and biological properties. Lipophilic indices (log k') were calculated by the following formula; $\log k' = \log (t_r - t_{zero})/t_{zero}$. Where t_r is the retention time of TG and acyl-TGs and t_{zero} is that of solvent (acetonitrile: acetic acid = 1:1). HPLC conditions were as follows; a linear gardient of acetonitrile (20–100%, 30 min) in 0.1% TFA at a flow rate of 0.7 mL/min, UV detection at a wavelength of 230 nm (1,7). The biological activities of acyl-TGs following intravenous injection to rats were examined by activity of gastric acid secretion previously reported (1).

Transport Studies Using tbe Transwell System

The transport of TG and its acyl-TG derivatives was examined in monolayer cultures grown in Transwell chambers. The confluent monolayers were washed three times with KRBB before KRBB containing 100 μM of drug and 10 μM of carboxyfluorescein (CF: a marker drug of paracellular transport) was added. After the addition of a solution containing acyl-TGs to the apical side, 100 μL aliquots were sampled from the basolateral side at designated time intervals, and these were immediately replaced with an equal volume of KRBB. In some transport experiments, either bacitracin or gabexate, protease inhibitors, was added to the drug solution.

Assessment of the Transport and Degradation Clearance of TG and Its Acyl-Derivatives

The disappearance of TG and its acyl-derivatives from the apical side of the Caco-2 monolayers can be explained by both permeation clearance (CLp) and degradation clearance (CLd). The CLp value is the actual transport parameter across the Caco-2 monolayer, while the CLd value represents the degradation by proteases on the apical membrane. These parameters were estimated by the following equations:

$$CL = \frac{\text{eliminated amount from apical side}}{AUC_{0-120}}$$

$$CLp = \frac{\text{transported amount to basolateral side}}{AUC_{0-120}}$$

$$CLd = CL - CLp$$

where CL is the elimination clearance of TG and acyl-derivatives from the apical side of Caco-2 monolayer and AUC_{0-120} is the area under the apical concentration-time curve from 0–120 min. This was calculated by assuming that the decrease of acyl-TGs on the apical side followed first-order kinetics. The analysis of acyl-TG derivatives was carried out by a reversed-phase HPLC.

HPLC Conditions

TG and acyl-TG were analyzed by a gradient HPLC system (Shimadzu LC-10A system, Kyoto, Japan) and a data processor (Chromatopac 6A, Shimadzu, Kyoto, Japan) using a C_{18}-column (4.6 × 150 mm, Cosmosil AR-300, Nacalai Tesque, Kyoto, Japan). The mobile phase was a mixture of 0.1% trifluoroacetic acid (mobile phase A) and acetonitrile (mobile phase B). The gradient system was programmed by linearly increasing the proportion of mobile phase B from 20–50% within 30 min. The gradient mobile phase was run at a flow rate 0.7 mL/min. The eluate was monitored with a UV detector at 230 nm.

Statistical Analysis

Results are expressed as the mean ± S.D. and statistical significance was assessed by the Student's *t* test.

RESULTS

Stability of Acyl-TGs in Caco-2 Homogenates

The degradation of acyl-TGs in Caco-2 homogenates followed first-order kinetics (Fig. 2). Native TG was rapidly degraded in Caco-2 homogenates. The half-life of TG was significantly increased by acylation, indicating that these acyl-derivatives are more stable than native TG. In the case of acyl-TGs, no native TG was observed in their metabolites. No definite relationship was observed between the length of acyl-chain and the stability of the acyl-TGs.

Transport of Acyl-TGs Across the Caco-2 Monolayers

Next we determined the permeation of acyl-TGs across the Caco-2 monolayers as shown in Fig. 3. Only 0.5% of the total amount of TG was transported to the basolateral side. In contrast, acyl-modification enhanced the permeability of TG. The amounts of C_2-TG, C_4-TG and C_6-TG transported were 13-, 19- and 18-times larger than the amount of the native TG transported, respectively (Fig. 3a). The amount of TG remaining on the apical side at the end of the experiment was about 10%,

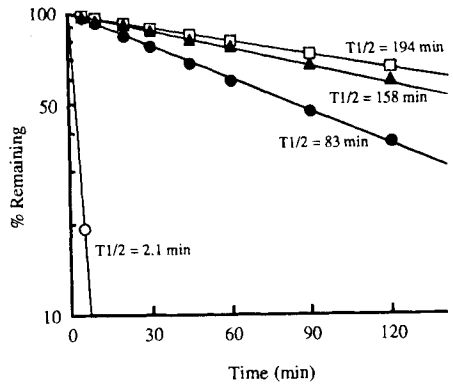

Fig. 2. Degradation profiles of acyl-TGs in Caco-2 homogenates. Keys: TG (○); C_2-TG (▲); C_4-TG (□); C_6-TG (●).

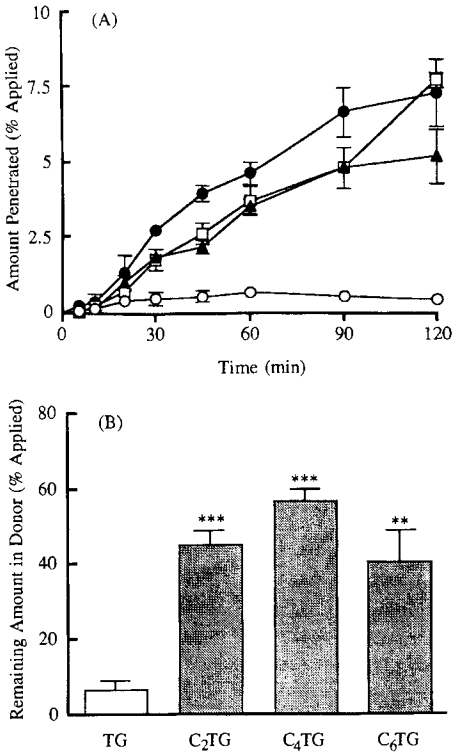

Fig. 3. Permeation profiles of acyl-tetragastrins across Caco-2 monolayers (A) and the amounts remaining on the apical side after 120 min (B). Results are expressed as the mean ± S.D. of three experiments. Keys: TG (○); C_2-TG (▲); C_4-TG (□); C_6-TG (●). (***) $p < 0.001$, (**) $p < 0.01$, compared with TG.

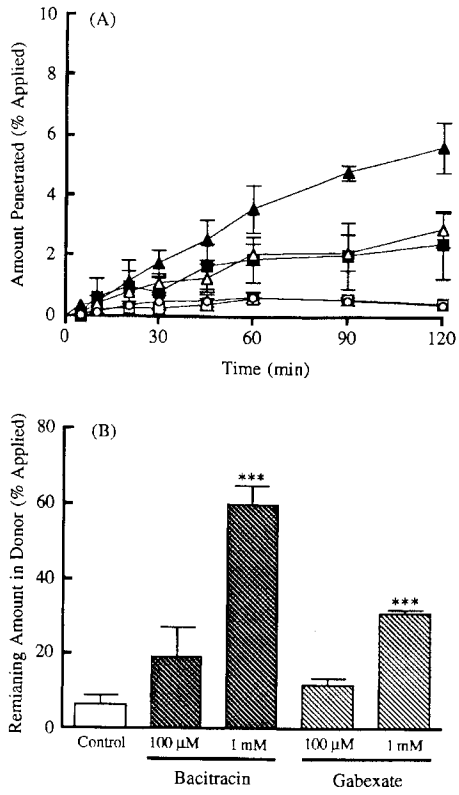

Fig. 4. Permeation profiles of tetragastrins across Caco-2 monolayers in the presence or absence of protease inhibitors (A) and the amounts remaining on the apical side after 120 min (B). Results are expressed as the mean ± S.D. of three experiments. Keys: TG (○); TG + 100 μM bacitracin (△); TG + 1 mM bacitracin (▲); TG + 100 μM gabexate (□); TG + 1 mM gabexate (■). (***) $p < 0.001$, compared with TG.

while about 40% of the acyl-TGs still remained (Fig. 3b). Interestingly, the transport of carboxyfluorescein (CF), which was coadministered with acyl-TGs as a paracellular transport marker, was significantly enhanced in the presence of acyl-TGs (Fig. 5).

Effects of Protease Inhibitors on the Transport of TG Across the Caco-2 Monolayers

The permeability of TG in the presence or absence of protease inhibitors was determined as shown in Fig. 4. By adding bacitracin (0.1 and 1 mM) and gabexate (1 mM) to the apical side, the amount of TG that permeated the Caco-2 cell monolayers was significantly increased as compared with in the absence of protease inhibitors. The amount of TG remaining on the apical side was also increased by the addition of protease inhibitors dose-dependently. Moreover, bacitracin enhanced the permeability of CF as well as permeability of the acyl-TGs (Fig. 5).

Assessment of the Transport Characteristics of Acyl-TGs Across Caco-2 Monolayers in the Presence or Absence of Protease Inhibitors

The disappearance of TG and acyl-TGs from the apical side of Caco-2 monolayers was divided in permeation clearance (CLp) and degradation clearance (CLd). Table 1 summarizes the permeation, degradation and elimination (permeation plus degradation) clearances of TG and its acyl-derivatives in the presence or absence of protease inhibitors. Figure 6a shows the relationship between the lipophilicity of acyl-TGs and their CLp values. The permeation clearance of TG was increased by acylation, however, no significant differences in the transport of C_4-TG and C_6-TG across the Caco-2 monolayers were observed. Conversely, the CLd values of TG on the apical membrane were remarkably reduced by acylation. A strong correlation was observed between the CLd values of acyl-TGs and their half-lives in Caco-2 homogenates ($r = -0.901$, Fig. 6b).

Both protease inhibitors dose-dependently decreased the CLd values of TG (Table 1). The CLp value of TG was also enhanced by 1 mM bacitracin (Table 1). In addition, bacitracin enhanced the CLp value of CF, a paracellular marker (data not shown). These results suggest that 1 mM of bacitracin possesses both a protease inhibitory activity and an absorption enhancing activity.

DISCUSSION

TG is rapidly degraded by proteolytic enzymes such as aminopeptidase and amidase in the gastrointestinal tract (1), and this proteolytic degradation lowers its bioavailability after oral administration. Therefore, it may be possible to improve the intestinal absorption of TG by suppressing its degradation. We previously showed that the chemical modification of TG with fatty acids significantly improved its absorption in the

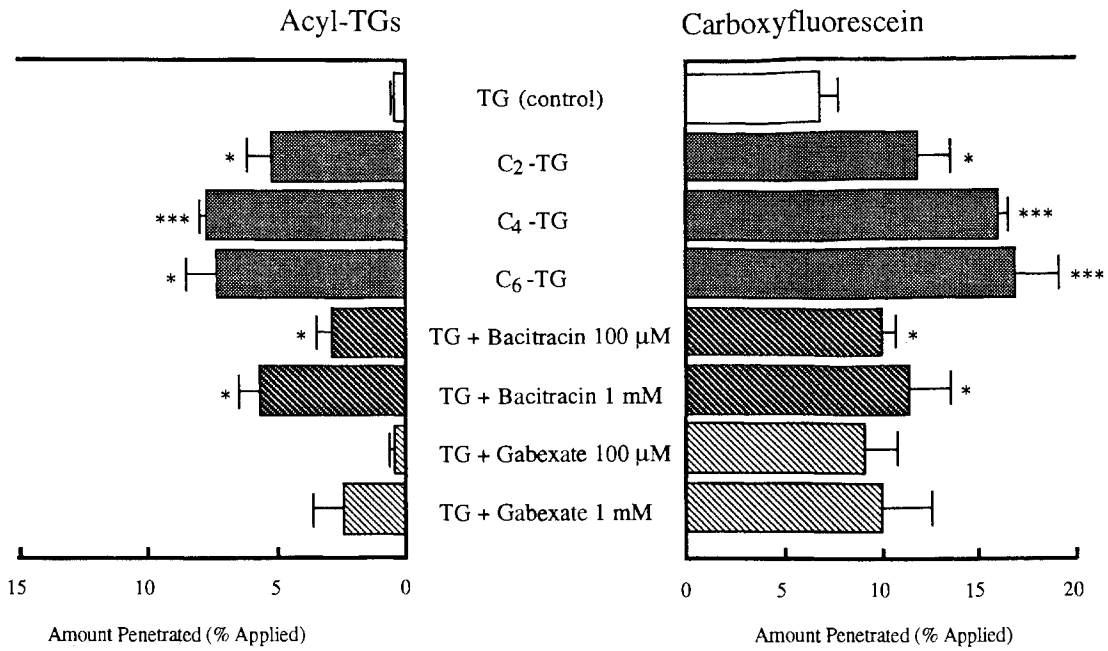

Fig. 5. The permeability of carboxyfluorescein coadministered with acyl-tetragastrins across the Caco-2 monolayers in the presence or absence of protease inhibitors. Results are expressed as the mean ± S.D. of three experiments. (***) $p < 0.005$, (*) $p < 0.05$, compared with tetragastrin (control).

large intestine (2,7). In our previous study, however, we could not analyze the relationship between the intestinal permeability characteristics of TG and its stability in intestinal homogenates because it was rapidly degraded on the intestinal mucosal surface.

Caco-2 cell monolayers have been extensively used as a model intestinal epithelium system. Caco-2 monolayers share morphological and functional similarities with the human intestinal mucosa, such as the development of cell polarity, the presence of tight junctions and the presence of cell-surface peptidases (10–12). For example, aminopeptidases N, P and W, dipeptidyl peptidase IV, endopeptidase-24.11, and γ-glutamyl transpeptidase have been found in Caco-2 cells (13).

This study has demonstrated that native TG is rapidly degraded in Caco-2 homogenates as well as in the intestinal mucosal homogenates (Fig. 2 and Fig 6b). In transport experiments, TG was also markedly degraded on the apical side (Fig. 3).

Therefore, TG is thought to be mainly degraded by aminopeptidases on the brush border membrane of Caco-2 monolayers. However, acyl-TGs were more stable than native TG in Caco-2 homogenate. This result concurs with our previous findings (7). Therefore, N-terminal acylation of TG with fatty acids improves its stability in the gastrointestinal tract. Interestingly, C6-TG, which had the highest lipophilicity, is the most unstable, although the instability is slight, among acyl-TGs in Caco-2 homogenates and on the apical side of Caco-2 monolayers (Fig. 2 and Fig. 3b). The reason for its unstability is not clear. However, chemical modification of TG with a highly lipophilic moiety might increase the affinity of TG to proteases, resulting in a decrease in stability of acyl-TG.

The transport of TG across Caco-2 monolayers was improved by acylation with fatty acids (Fig. 3a). C4- and C6-TG had higher CLp values than C2-TG, but no significant difference in CLp values was observed between C4-TG and C6-TG. However, about 40% of the acyl-TGs still remained

Table 1. The Elimination, Degradation and Permeation Clearance Values of Acyl-TGs Under Various Conditions

	CL (μl/min)	CLp (μl/min)	CLd (μl/min)
TG	35.4 ± 5.5	0.15 ± 0.0	35.2 ± 5.5
C2-TG	10.1 ± 1.0*	0.95 ± 0.1***	9.2 ± 1.1*
C4-TG	7.2 ± 0.6*	1.28 ± 0.0***	5.9 ± 0.6*
C6-TG	11.7 ± 3.3*	1.43 ± 0.4**	10.3 ± 2.9**
TG + Bacitracin 100 μM	21.5 ± 4.4	0.75 ± 0.1***	20.7 ± 4.5
TG + Bacitracin 1 mM	6.5 ± 1.0*	0.91 ± 0.2*	5.6 ± 0.9*
TG + Gabexate 100 μM	27.0 ± 1.4	0.13 ± 0.0	26.8 ± 1.4
TG + Gabexate 1 mM	14.7 ± 0.3*	0.51 ± 0.2	14.2 ± 0.4*

Note: Results are expressed as the mean ± S.D. of three experiments.
(***) $p < 0.005$, (**) $p < 0.01$, (*) $p < 0.05$, compared with TG.

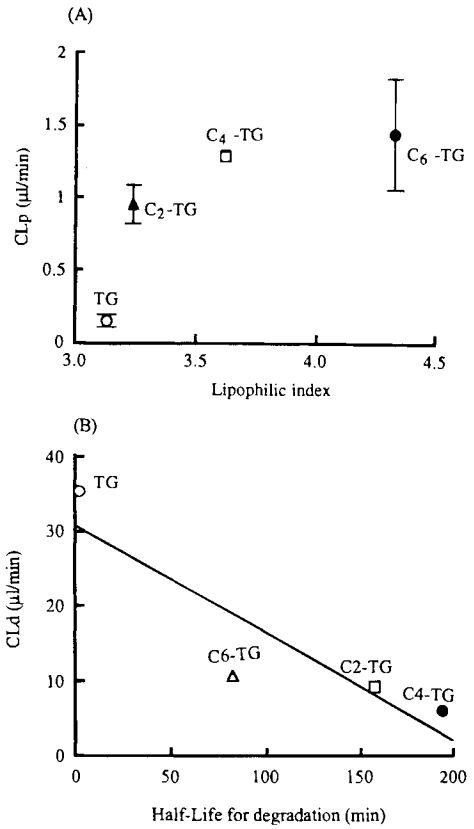

Fig. 6. Relationships between the lipophilic indices of acyl-tetragastrins and their permeation clearance values across Caco-2 monolayers (A) and between the degradation half lives of acyl-tetragastrins in Caco-2 homogenate and their degradation clearance values (B). Results are expressed as the mean ± S.D. of three experiments.

on the apical side at the end of the experiment, whereas only 10% of the native TG still remained (Fig. 3b). Among acyl-TGs, the remaining amount of C4-TG on the apical side was the largest (Fig. 3b). This finding is consistent with the other observation that C4-TG has the longest half-life in Caco-2 homogenates (Fig. 2). In our previous study (7), the permeability of peptides across the intestinal membrane was estimated by the following equation;

$$P_{app} \cdot A = dX_R/dt/C_0.$$

where $P_{app} \cdot A$ is the apparent permeation clearance, X_R is the amount of drug on the basolateral side and C_0 is the initial concentration of the drug on the apical side. In this equation, C_0 is assumed to be constant. Thus, in the case of a rapidly degraded drug such as TG, the $P_{app} \cdot A$ value might be underestimated compared to its actual permeation clearance. In this study, we separated the elimination clearance of TG from the Caco-2 monolayers into permeation and degradation clearances during the transport process. By this method, we could assess the contributions of the enzymatic and transport barriers to the intestinal transport of TG, which were expressed as clearance values. The present suggested that the degradation of TG on the apical surface of the Caco-2 monolayers account for most of the TG eliminated from the apical side of Caco-2 cell monolayers (Table 1).

On the other hand, the CLp value of TG was increased by chemical modification with fatty acids and by elongating the acyl-carbon chain. In addition, the transport of CF across the Caco-2 monolayers was simultaneously increased by the coadministration of acyl-TGs (Fig. 5). The enhancement mechanism of CF due to acyl-TGs is not clear. We speculate that acyl-TGs have some extent surfactant activity and absorption enhancement activity. Therefore acyl-TGs increased the transport of CF across the Caco-2 monolayers. Further studies are needed to clarify the absorption enhancement mechanism of acyl-TGs.

We also examined the effects of the protease inhibitors bacitracin and gabexate on the transport of native TG across Caco-2 monolayers. By adding bacitracin, an aminopeptidase inhibitor, large amounts of TG were retained on the apical side, and the amount transported was consequently improved (Fig. 4 and Table 1). Aminopeptidase activity is high in the mucosa, especially on the brush border membranes. We believe that aminopeptidases contribute significantly to the proteolytic degradation of TG on the apical side and the reduced CLd values of TG in the presence of protease inhibitors accounts for the increased amount of TG that permeated through the Caco-2 monolayers. In addition, gabexate, a guanidinobenzoate derivative, has an inhibitory effect on various proteolytic enzymes (14). Because camostat, a structurally related compound, has both aminopeptidase inhibitory and serine protease inhibitory action (15), gabexate might also inhibit aminopeptidase. However, the CLd values in the presence of gabexate were relatively larger than in the presence of bacitracin. This result suggests that gabexate has less aminopeptidase inhibitory activity than bacitracin.

In this study, the CLp values of TG transported across Caco-2 monolayers were increased in the presence of bacitracin (Table 1). Our laboratory has reported that bacitracin has an absorption enhancing effect on the intestinal absorption of phenol red (Mw 354) and FITC-dextran (Mw 4,000), two hydrophilic compounds (16,17). Furthermore, we found that bacitracin enhances the paracellular transport of FITC-dextran with average molecular weights of 4,000, using electrophysiological techniques (submitted for publication). Taken together, we conclude that bacitracin is both a protease inhibitor and an absorption enhancer. Taki et al. (18) investigated the effect of puromycin, an aminopeptidase inhibitor, on the transport of metkephamid (MKA), a stable analog of Met-enkephalin, by means of vascular perfusion of the small intestine of rats. They reported that the ability of puromycin to increase the CLp value reflects the inhibition of the enzymatic degradation of MKA that usually occurs during the permeation process; i.e. the CLp value was a hybrid parameter including a permeation process and an enzymatic inhibition process during its passage through Caco-2 monolayers. Thus, increased amounts of TG on the apical side as a results of the use of protease inhibitors might be caused by the saturation of metabolic enzymes involved in the Caco-2 transport process, and result in increased CLp values.

We analyzed the contributions of the transport barrier and the enzymatic barrier on the disappearance of acyl-TGs from the apical side of Caco-2 monolayers (Fig. 7). Figure 7 indicates that acylation of TG improves its stability (mainly by decreasing the CL value), which resulted in enhanced permeation (refer to CLp/CL) of TG across Caco-2 monolayers. The same results were observed when it was coadministred with protease inhibi-

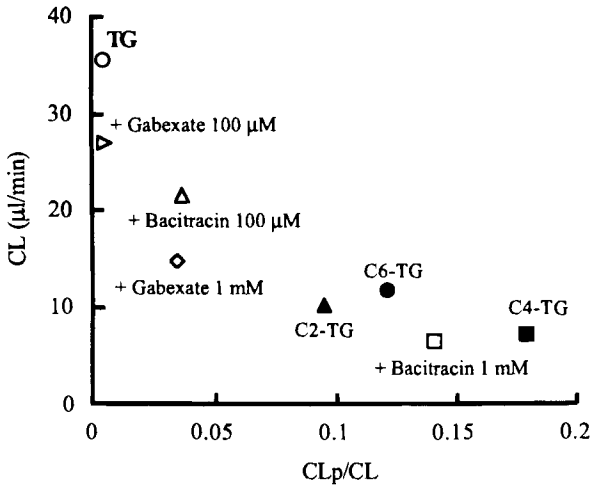

Fig. 7. Contribution of permeation and degradation clearances on the disappearance of acyl-tetragastrins from the apical side of Caco-2 monolayers. Results are expressed as the mean ± S.D. of three experiments.

tors. In particular, the CLp/CL value of TG were increased by coadministration of 1 mM bacitracin to values similar to those obtained with acyl-TGs.

In conclusion, we have demonstrated that the permeability of TG across Caco-2 monolayers can be improved by chemical modification with fatty acids and/or by the addition of various protease inhibitors. To improve the intestinal absorption of low-molecular weight peptides such as TG, which are susceptible to proteolytic degradation, it is necessary to reduce enzymatic degradation by chemical modification and the addition of protease inhibitors, in addition to increasing their lipophilicity. These results may illustrate a useful method for enhancing intestinal permeability and inhibiting degradation of peptide drugs after oral administration.

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