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Transepithelial Transport of *p*-Coumaric Acid and Gallic Acid in Caco-2 Cell Monolayers

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The transepithelial transport of such common dietary phenolic acids as *p*-coumaric acid (CA) and gallic acid (GA) across Caco-2 cell monolayers was examined. CA transport was dependent on pH, and in a vectorial manner in the apical-basolateral direction. The permeation was concentration-dependent and saturable, the Michaelis constant and maximum velocity being 17.5 mM and 82.7 nmol min^{-1} (mg of protein)$^{-1}$, respectively. Benzoic acid and acetic acid inhibited the permeation of CA. These results indicate that the transepithelial transport of CA was *via* the monocarboxylic acid transporter (MCT). On the other hand, the permeation of GA was not in a polarized manner, was independent of pH and linearly increased with increasing concentration of GA. The transport rate of GA was about 100 times lower than that of CA, suggesting the transepithelial transport of GA to be *via* the paracellular pathway. Dietary phenolic acids thus showed diversified characteristics in their intestinal absorption.

Key words: *p*-coumaric acid; gallic acid; monocarboxylic acid transporter; intestinal absorption; Caco-2

Phenolic acids are derived from benzoic or cinnamic acids depending on the position of the carboxylic group on the benzene ring, and are natural constituents of many plants families; for instance, *p*-coumaric acid (CA), gallic acid (GA) and ferulic acid (FA). CA (4-hydroxycinnamic acid) is a ubiquitous plant phenolic acid that is typically esterified to arabinoxylan residues of hemicellulose or to lignin in graminaceous plants, including maize, oats,[1)] and wheat.[2)] It is also presented as an ester conjugate and as the free acid in fruits and vegetables such as apples,[3)] grapefruits, oranges, tomatoes, potatoes, and spinach.[4)] In respect of maize, CA has been reported to account for up to 4% of the dry weight including the stalk, root, and cob.[5)] Bryngelsson *et al.* have reported that common commercial processing such as steaming and autoclaving increased the levels of CA and ferulic acid in dehulled oat groats, while the levels of caffeic acid and tocotrienols were not increased.[6)] The daily consumption of cereals, vegetables, fruits, and common processed foods may therefore result in the ingestion of a large amount of CA. FA and caffeic acids are better inhibitors of peroxynitrite-mediated tyrosine nitration than CA.[7)] However, CA inhibited morpholine nitrosation,[8)] and reacted with peroxynitrite to reduce 3-nitrotyrosine formation *in vitro*,[9,10)] suggesting that CA would exhibit its physiological effects through regular intake.

Gallic acid (GA) is one of the main endogenous phenolic acids found in plants in the free or esterified form, and is distributed in a large amount in tea leaves, being present at about 5% of the weight of the tea leaf.[11)] GA is a potent natural antioxidant exhibiting antimutagenic and anticarcinogenic acitivity,[12–15)] and can be expected to reduce the risk of diseases and bring a health benefit through its daily intake.

The physiological importance of CA and GA depends upon its availability for intestinal absorption and subsequent interaction with target tissues. Estimating the bioavailavility of CA and GA is therefore important to evaluate its real physiological effects. Although only a few studies have been published about the availability of CA and GA from the diet, that of catechin and its gallic acid-esterified derivatives in tea polyphenols have been fully investigated.[16–18)] As to the intestinal absorption of polyphenols, the first limiting step for ingested polyphenols, only passive diffusion seems to be involved in permeation.[19)] Few attempts to discover other

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Abbreviations: HBSS, Hanks' balanced salt solution; TER, transepithelial electrical resistance; MCT, monocarboxylic acid transporter; CA, *p*-coumaric acid; GA, gallic acid; FA, ferulic acid; ECD, electrochemical detector

permeation mechanisms for the intestinal absorption of CA and GA have been made.

We have recently reported that FA was transported across human intestinal Caco-2 cells *via* the monocarboxylic acid transporter (MCT).[20,21] In addition, by measuring the effect of various types of phenolic acid on fluorescein transport, we have reported that CA would also be absorbed *via* MCT, although it had lower affinity for MCT than FA did.[22] On the other hand, GA had no inhibitory effect on fluorescein transport, suggesting no affinity for MCT.[22] This study was therefore designed to investigate and clarify the absorption mechanism for CA and GA by directly measuring their transepithelial transport across Caco-2 cell monolayers. This cell monolayer is a useful model system for evaluating the intestinal epithelial permeability,[23–25] because it expresses a number of solute transport systems for amino acids,[26] bile acids,[27] dipeptides,[28] and so on.

Materials and Methods

Materials. The human colon adenocarcinoma cell line Caco-2 was obtained from American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Fetal calf serum, glutamine, non-essential amino acids, penicillin and streptomycin (10,000 units/ml and 10 mg/ml in 0.9% sodium chloride, respectively), phosphate-buffered saline and Hank's balanced salt solution (HBSS) were all purchased from GIBCO (Gaithersburg, MD, USA). Type I collagen was purchased from Nitta Gelatin (Osaka, Japan). Plastic dishes, plates, and Transwell inserts with 0.4-μm polycarbonate membranes 12 mm in diameter were obtained from Corning (Corning, NY, USA). CA was from Sigma-Aldrich (St. Louis, MO, USA) and GA was from Kanto Chemical Co. (Tokyo, Japan), all other chemicals used in this study being of analytical grade.

Cell culture. Caco-2 cells were cultured in DMEM containing 10% fetal calf serum, 1% non-essential amino acids, 4 mM L-glutamine, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin, together with sodium bicarbonate to adjust the pH value to 7.4. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The monolayers became confluent 6 to 7 days after seeding at 1×10^5 cells per 100-mm dish, and the cells were passaged at a split ratio of 4 to 8 by a treatment with 0.1% trypsin and 0.02% EDTA acid in HBSS. All the cells used were between passages 55 and 75.

Measurement of the transepithelial electrical resistance (TER). Cells were grown for TER measurement in Transwell inserts with the semipermeable membrane first coated with type I collagen. The cells were seeded at a density of $1 \times 10^5/cm^2$, and the medium was changed every 1 or 2 days. Monolayers were found to have formed after 2 weeks of culture. The integrity of the cell layer was evaluated by measuring TER with Millicell-ERS equipment (Millipore, MA, USA). Monolayers with TER of more than 250 $\Omega \cdot cm^2$ were used for the experiments. The monolayer cells were gently rinsed three times with HBSS and left for equilibration in the same solution for 30 min at 37°C. The TER value of the monolayer was measured before and after an assay sample was added to the insert.

Transepithelial transport experiments. Monolayer cells cultured in a Transwell insert were set in a 12-well plate. To measure the apical-to-basolateral permeability, 1.5 ml of HBSS (pH 7.4, 37°C) was added to the basolateral side, and 0.5 ml of a test solution (at 37°C and of pH 6.0 or 7.4) containing CA (1 mM) or GA (5 mM) was added to the apical side of the insert. After the well had been incubated for an appropriate time at 37°C, samples were collected from the basolateral solution. The amount of CA or GA transported by the Caco-2 cells was evaluated by HPLC-electrochemical detector (ECD) with an ESA coulometric detection system (ESA, Boston, MA, USA). The results are expressed as the proportion of the original amount that permeated through the monolayer, this being calculated as the amount transported divided by the initial amount in the donor compartment. Unless otherwise indicated, transport experiments were done with a proton gradient (pH 6.0 for the apical side and pH 7.4 for the basolateral side).

To examine the basolateral-to-apical transport, HBSS (pH 6.0 or 7.4, 37°C) was added to the apical side, and 1.5 ml of a test solution (pH 7.4, 37°C) containing CA or GA was added to the basolateral side.

Chromatographic conditions. HPLC-ECD is well suited to the analysis of phenolic compounds like hydroxycinnamic acids in complex matrices. The coulometric detector was designed to provide high selectivity and sensitivity for these analytes, and the analytical subjects could be resolved in two dimensions, chromatographic and voltammetric, by arranging several coulometric detectors in series and set at different potentials, *i.e.* coulometric array detection.[29] Furthermore, with advanced data handling, including automatic gain ranging and baseline correction software, coulometric array detection permitted gradient chromatography without compromising on the sensitivity or dynamic range. A Shimadzu HPLC gradient pump (Shimadzu Corp., Kyoto, Japan) was coupled to an ESA coulometric array detection system (ESA, Boston, MA, USA), which

consisted of two cell packs in series, each pack containing four porous graphite working electrodes with associated palladium reference electrodes and platinum counter-electrodes. Chromatographic separation was done in an ODS column (C18; 150 mm × 4.6 mm i.d.; 5-μm particle size) from MC Medical (Tokyo, Japan). The column and detector array were housed in a thermostatic chamber at 40°C. Mobile phase A was 50 mM sodium acetate containing 5% methanol (pH 3.0 adjusted with phosphoric acid), while mobile phase B was 50 mM sodium acetate containing 40% acetonitrile and 20% methanol (pH 3.5 adjusted with phosphoric acid). To measure the amount of CA, the elution profile (0.6 ml/min) was as follows: 0–28.5 min, 85% solvent A/15% solvent B→20% solvent A/80% solvent B; 28.5–31 min, 0% solvent A/100% solvent B; 31–35 min, return to initial mobile phase conditions and then equilibration. The eight electrode detector potentials were from 0 to 700 mV in increments of 100 mV. To measure the amount of GA, the elution profile (0.6 ml/min) was as follows: 0–5 min, 100% solvent A/0% solvent B; 5–28.5 min, 100% solvent A/0% solvent B→20% solvent A/80% solvent B; 28.5–31 min, 0% solvent A/100% solvent B; 31–35 min, return to initial mobile phase conditions and then equilibration. The five electrode detector potentials were from 0 to 400 mV in increments of 100 mV.

Distribution of CA and GA after the transport experiments. CA and GA in both the apical and basolateral solutions were measured after the transport experiments. The monolayer cells were rinsed with HBSS (pH 6.0 or 7.4) and then extracted with methanol/solvent A above-mentioned (10:1, v/v) for 30 min. CA and GA in the extract were measured and used as an index of the intracellular fractions taken up by the Caco-2 cells.

Data analysis. The permeation rate [nmol min^{-1} (mg protein)$^{-1}$], J, was evaluated from the slope of the initial linear part of a plot of the amount transported [nmol min^{-1} (mg protein)$^{-1}$] against time (in minutes), this being calculated by a linear regression analysis. The kinetic parameters for saturable transport across the Caco-2 cells were evaluated by fitting equation 1 by the MULTI nonlinear least-squares regression analysis program:[30]

$$J = J_{max}\,[C]/(K_t + [C]) \quad (1)$$

where C is the initial concentration of FA, J_{max} is the maximum permeation rate, and K_t is the Michaelis-Menten constant. Each result is expressed as the mean ± SD. A statistical analysis was done with Student's two-tailed t-test, and differences with $P<0.01$ are considered significant.

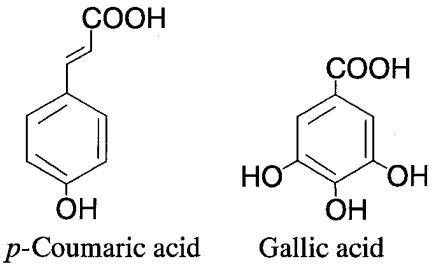

Fig. 1. Chemical Structures of CA and GA.

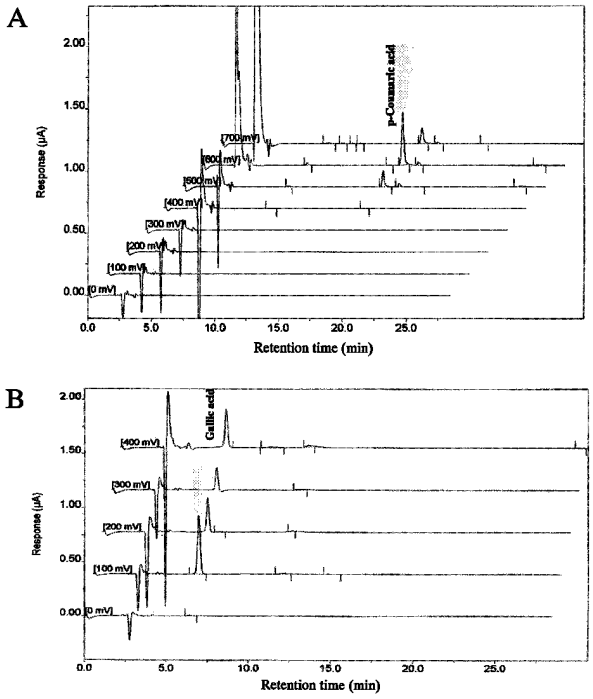

Fig. 2. Chromatograms from the Coulometric Array Detection of CA (A) and GA (B) Transported Across Caco-2 Cell Monolayers.

Results and Discussion

HPLC analysis of CA and GA transported across the Caco-2 cell monolayers

A standard chromatogram showing the resolution of CA and GA transported (Fig. 1) to the basolateral solution is presented in Figs. 2A and B. This method had a detection limit of less than 0.5 pmol for CA or GA in the column, and its reproducibility was good without requiring any sample pre-treatment. The dominant oxidation potential of CA and GA was 600 mV and 100 mV, respectively. The peak purity was assessed by using the peak area ratio accuracy for the adjacent oxidation channels (lower or upper) to the dominant oxidation channel, because the voltammetric response of the analyte across these channels had its own natural characteristics.[29]

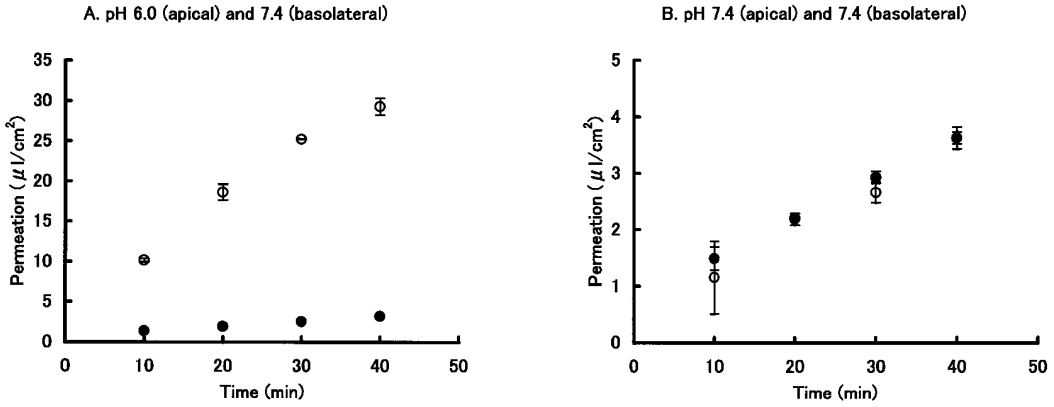

Fig. 3. Characteristics of the Transepithelial Transport of CA Across Caco-2 Cell Monolayers.
Permeation of CA (1 mM) from the apical side to the basolateral side (○) and from the basolateral side to the apical side (●) was measured at 37°C both in the presence (A) and absence (B) of a proton gradient (apical side, pH 6.0 or 7.4; basolateral side, pH 7.4). Each point is the mean ± SD of three experiments.

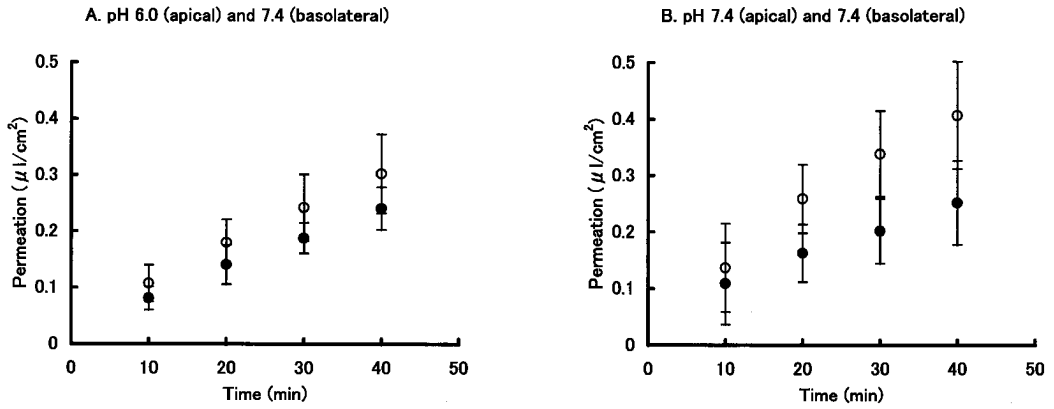

Fig. 4. Characteristics of the Transepithelial Transport of GA Across Caco-2 Cell Monolayers.
Permeation of GA (5 mM) from the apical side to the basolateral side (○) and from the basolateral side to the apical side (●) was measured at 37°C both in the presence (A) and absence (B) of a proton gradient (apical side, pH 6.0 or 7.4; basolateral side, pH 7.4). Each point is the mean ± SD of three experiments.

pH dependence and vectorial characteristics of the transepithelial transport

Figure 3 shows the time-dependent change in the apical-to-basolateral permeation of CA across the Caco-2 cell monolayers in the presence and absence of an inwardly directed proton gradient. The permeation of CA increased linearly with time, and was also higher with lower pH of the apical solution, as was the case with the transepithelial transport of FA (Figs. 3A and B).[21] The initial permeation rate, J, was 3.73 ± 0.43 (at pH 6.0) and 0.39 ± 0.03 (at pH 7.4) nmol min^{-1} (mg of protein)$^{-1}$. The apical-to-basolateral flux of CA ($J_{ap \to bl} = 3.73 \pm 0.43$ nmol min^{-1} (mg of protein)$^{-1}$) was approximately tenfold that of the basolateral-to-apical flux ($J_{bl \to ap} = 0.30 \pm 0.04$ nmol min^{-1} (mg of protein)$^{-1}$) with the inwardly directed proton gradient, showing vectorial transepithelial transport (Fig. 3A). The unidirectional transport of CA probably occurred because of a mechanism such as carrier-mediated transport; the transport of acetamide (passive diffusion) and mannitol or Lucifer Yellow (paracellular transport) is never polarized.[31,32] On the other hand, in the absence of an inward proton gradient, the apical-to-basolateral flux of CA ($J_{ap \to bl} = 0.39 \pm 0.03$ nmol min^{-1} (mg of protein)$^{-1}$) was approximately the same as the basolateral-to-apical flux ($J_{bl \to ap} = 0.35 \pm 0.04$ nmol min^{-1} (mg of protein)$^{-1}$), showing the passive diffusion of CA (Fig. 3B). The results obtained here match the case of FA transport previously reported.[21]

Figure 4 shows the change with time in the apical-to-basolateral permeation of GA across the Caco-2 cell monolayers in the presence and absence of an inwardly directed proton gradient. Unlike the case of CA, the permeation of GA was in no way polarized, and was little affected by the pH of the apical solution, as was the case with Lucifer Yellow (Figs. 4A and B).[20] The initial permeation rates, $J_{ap \to bl}$, were 0.16 ± 0.03 (at pH 6.0) and 0.22 ± 0.05 (at pH 7.4)

Table 1. Distribution of CA after Transepithelial Transport Experiments in the Presence and Absence of a Proton Gradient

pH gradient	Proportion of compound (%) recovered		
	Apical side	Basolateral side	Cells
6.0/7.4	88.93 ± 0.36	10.87 ± 0.36	0.20 ± 0.01
7.4/7.4	98.33 ± 0.18	1.55 ± 0.19	0.12 ± 0.01

Transepithelial transport experiments were done at 37°C for 40 min as described in the Materials and Methods section both in the presence and absence of a proton gradient (apical side, pH 6.0 or 7.4; basolateral side, pH 7.4). Each value is the mean ± SD of three experiments.

nmol min^{-1} (mg of protein)$^{-1}$, and, $J_{bl \to ap}$, were 0.13 ± 0.03 (pH 6.0), and 0.12 ± 0.06 (pH 7.4) nmol min^{-1} (mg of protein)$^{-1}$.

Distribution of CA and GA after the transport experiments

After the transport experiments in the absence of a proton gradient (apical side, pH 7.4; basolateral side, pH 7.4), most of CA added to the apical side of the cell monolayers remained on that side; only a small percentage had been transported to the basolateral side (Table 1), so the transepithelial transport of CA in the absence of a proton gradient seems to have been restricted by the tight junctions. In the presence of a proton gradient (apical side, pH 6.0; basolateral side, pH 7.4), a considerable amount of CA was detected on the basolateral side; it seems that CA on the apical side had been taken up by the cells and transported to the basolateral side in the presence of a proton gradient. These observations suggest that CA was transported across the Caco-2 cell monolayers *via* a transcellular pathway identical to the one for FA transport. The proton-coupled specific transport mechanism seems to have been involved in the transport of CA. Considering that a larger amount of CA on the basolateral side was observed than that of the intracellular fraction, it is possible that CA was transported through the cells quite efficiently. The transported samples on the basolateral side were treated by the deconjugating enzyme, sulfatase H-5 (Sigma-Aldrich, St. Louis, MO, USA) containing both sulfatase and glucuronidase. Since no change in the CA content was apparent after the enzyme treatment (Table 2), CA is not likely to have been conjugated during the process of transport across the Caco-2 cells.

The distribution of GA was obviously different from that of CA. Regardless of the presence or absence of a proton gradient (apical side, pH 6.0 or 7.4; basolateral side, pH 7.4), most GA added to the apical side of the cell monolayers remained on that side, suggesting that the permeation of GA would have been restricted by the tight junctions (Table 3). Considering that no conjugated form of GA permeated across the Caco-2 cells to the basolateral side (Table 4), GA would have been transported *via* the paracellular pathway.

Table 2. Sulfatase Treatment of Transported CA after Transepithelial Transport Experiments in the Presence and Absence of a Proton Gradient

	Transported CA (nmol)	
pH (apical/basolateral)	6.0/7.4	7.4/7.4
Control	54.00 ± 7.00	7.90 ± 0.67
Sulfatase-treated	51.15 ± 6.36	7.22 ± 0.72

One mM CA was added to the apical chamber, and the transepithelial transport experiments were done at 37°C for 40 min both in the presence and absence of a proton gradient (apical side, pH 6.0 or 7.4; basolateral side, pH 7.4). Transported CA on the basolateral side was treated with or without 2 units of a sulfatase H-5 solution in a 0.1 M acetate buffer (pH 5.0) and incubated at 37°C for 45 min. Each value is the mean ± SD of three experiments.

Table 3. Distribution of GA after Transepithelial Transport Experiments in the Presence and Absence of a Proton Gradient

pH gradient	Proportion of compound (%) recovered		
	Apical side	Basolateral side	Cells
6.0/7.4	99.50 ± 0.07	0.13 ± 0.01	0.37 ± 0.07
7.4/7.4	99.60 ± 0.04	0.32 ± 0.06	0.08 ± 0.02

Transepithelial transport experiments were done at 37°C for 40 min as described in the Materials and Methods section both in the presence and absence of a proton gradient (apical side, pH 6.0 or 7.4; basolateral side, pH 7.4). Each value is the mean ± SD of three experiments.

Table 4. Sulfatase Treatment of Transported GA after Transepithelial Transport Experiments in the Presence and Absence of a Proton Gradient

	Transported GA (nmol)	
pH (apical/basolateral)	6.0/7.4	7.4/7.4
Control	2.16 ± 0.15	4.20 ± 1.25
Sulfatase-treated	2.13 ± 0.11	4.01 ± 1.18

5 mM GA was added to the apical chamber and the transepithelial transport experiments were done at 37°C for 40 min both in the presence and absence of a proton gradient (apical side, pH 6.0 or 7.4; basolateral side, pH 7.4). Transported GA on the basolateral side was treated with or without 2 units of a sulfatase H-5 solution in a 0.1 M acetate buffer (pH 5.0) and incubated at 37°C for 45 min. Each value is the mean ± SD of three experiments.

Concentration dependence of CA and GA transport

Figure 5A shows the relationship between the initial permeation rate of CA and its concentration in the presence or absence of a proton gradient. The permeation rate was saturable in the presence of a proton gradient. With the use of equation 1, the maximum permeation rate, J_{max}, was 82.7 ± 4.0 nmol min^{-1} (mg of protein)$^{-1}$, and the Michaelis-Menten constant, K_t, was 17.5 ± 1.5 mM. Non-saturable transport of CA seemed to have occurred in the

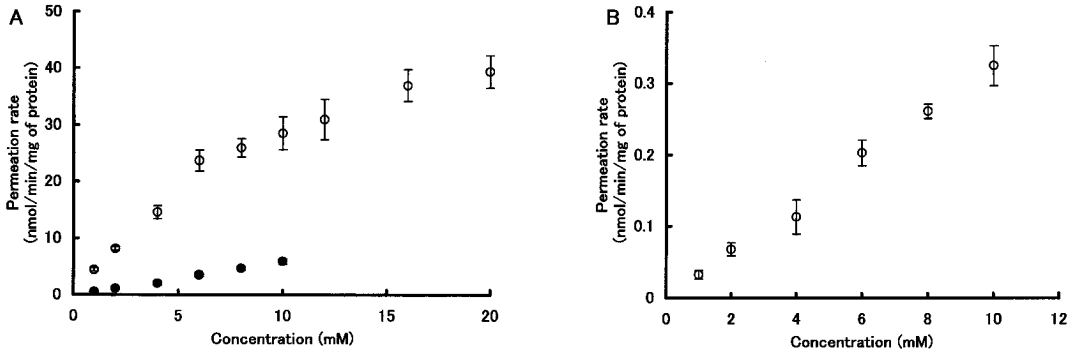

Fig. 5. Concentration Dependence of CA (A) and GA (B) Transport Across Caco-2 Cell Monolayers in the Presence (○) and Absence (●) of a Proton Gradient.

The initial permeation rate is shown. Each Value is the mean ± SEM of three or more experiments.

Table 5. Effects of Various Compounds on CA Transport Across Caco-2 Cell Monolayers

	Concentration (mM)	Relative permeation (% of control)
NaN_3	10	14.7 ± 3.8*
Benzoic acid	10	15.3 ± 2.5*
Lactic acid	10	117.3 ± 9.6
Acetic acid	10	70.8 ± 6.4*

The amount of CA transported was measured at 37°C for 40 min by incubating Caco-2 cells in the absence or presence of each compound at the concentration indicated (apical side, pH 6.0; basolateral side, pH 7.4). Each value represents the mean ± SD of three or more experiments. *$P < 0.01$.

absence of a proton gradient, indicating that passive diffusion was involved in the permeation of CA. This finding is in agreement with the case of FA previously reported, which was presumed to have been transported by the MCT in Caco-2 cell monolayers.[21] These results strongly suggest that the transcellular transport of CA involved a carrier-mediated mechanism in the presence of a proton gradient. As shown in Fig. 5B, the permeation of GA was independent of its concentration in the presence of a proton gradient (apical side, pH 6.0; basolateral side, pH 7.4), exhibiting passive diffusion characteristics, which is similar to that of Lucifer Yellow (paracellular transport).

Inhibition of CA transport by chemical compounds

To discover the properties of the transporter responsible for CA transport, we added 0.1 mM CA to the apical chamber and studied the effects of various compounds on the permeation rate of CA (Table 5). The metabolic inhibitor, NaN_3 (10 mM), strongly reduced the permeation of CA. The substrates for MCTs such as benzoic acid and acetic acid significantly inhibited the permeation of CA, although lactic acid, a good substrate for MCT1-MCT4 characterized by their structural specificity,[33] had no effect on CA transport, suggesting that the transporter responsible for CA transport might have been different from MCT1-MCT4. The inhibitory effect of the MCT substrates on CA transport was in the order of benzoic acid > acetic acid > lactic acid. This order is the same as the case of FA transport, indicating that CA shared the transporter carrier with FA. At pH 6.0, the carboxylic acid group of CA would dissociate and be present as a monoanion like FA and fluorescein, which would fulfil the conditions necessary for substrate recognition.[34]

Spencer *et al.*, by an analysis of the results from perfusion studies, investigated the absorption and metabolism of flavonoids and hydroxycinnamates in the rat gastrointestinal tract, and reported that large amounts of CA and FA were absorbed intact, although the absorption of intact caffeic acid and its quinic ester, chlorogenic acid, were low at all times.[35] They suggested that these critical differences among those phenolic compounds could be ascribed to the higher susceptibility to oxidation of the latter two compounds than the former one in the intestinal epithelium. However, considering that phenolic acids would have their own affinity for MCT by means of their chemical structure, the results reported by Spencer *et al.*[35] could be explained by the different transepithelial transport characteristics of these compounds; *i.e.*, transport *via* MCT-mediated and non-mediated systems.

The results of the present study provide feasible evidence for the involvement of MCT-mediated transport in the intestinal absorption of phenolic acids and the diversity of phenolic acids in their intestinal absorption characteristics. Further investigation on the combination or interaction among various phenolic acids in their availability and biological effects would be necessary to clarify their benefits to human health through their daily intake.

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