Melibiose, Difructose Anhydride III and Difructose Anhydride IV Enhance Net Calcium Absorption in Rat Small and Large Intestinal Epithelium by Increasing the Passage of Tight Junctions In Vitro

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ABSTRACT An Ussing chamber technique was used to determine the effects of three indigestible disaccharides on net Ca transport from the luminal side to the basolateral side of isolated preparations of jejunal, ileal, cecal and colonic epithelium in rats. Permeability of Lucifer Yellow (LY) and transepithelial electrical resistance (TEER), which are indicators of intercellular passage of the intestinal mucosa, were also determined. The concentrations of Ca in the serosal and mucosal media were 1.25 mmol/L and 10 mmol/L, respectively. After a 30-min incubation, the net Ca transport, LY passage and TEER were determined. In the control experiment, LY permeability was lowest, and TEER value was highest in the colon. The addition of 1–100 mmol/L melibiose, difructose anhydride (DFA)III, or DFAIV to the mucosal medium increased the net Ca absorption and LY permeability dose-dependently in the jejunum, ileum, cecum and colon preparations. Melibiose decreased TEER dose-dependently in the jejunum and cecum, but not in the ileum and colon. DFAIII decreased TEER dose-dependently in the jejunum, cecum and colon, but not in the ileum. DFAIV decreased TEER dose-dependently in all four intestinal portions. Positive linear relationships were found between net Ca transport and LY passage in all portions of the intestine, whereas negative linear relationships were found between net Ca absorption and TEER. We concluded that the three indigestible saccharides directly affect the epithelial tissue and activate the passage of tight junctions, thereby promoting Ca absorption in the small and large intestine in vitro. J. Nutr. 132: 3394–3399, 2002.

KEY WORDS: • *Ca absorption* • *indigestible saccharide* • *Ussing chamber* • *intestine* • *tight junction*

Ingestion of indigestible saccharides, including various types of sugar alcohols (1,2), oligosaccharides (3,4) and polysaccharides (5,6), increases Ca absorption in rats, as demonstrated by in vivo balance studies. These sugars increase the Ca content and the breaking force of bone (7,8) in rats. Sugar alcohols also retard bone resorption in rats (9). Thus, the ingestion of indigestible sugars might play a beneficial role in the absorption of Ca and the retention of Ca in the body.

Intraluminal infusion of sorbitol (a monosaccharide-type sugar alcohol) increases Ca absorption in the ileal loop of rats (10). Using a tracer technique with ^{45}Ca, maltitol (a disaccharide-type sugar alcohol) was shown to stimulate Ca absorption in the small intestine in rats (11). In in vitro experiments using everted sacs, maltitol (12), difructose anhydride (DFA)[2] III (13) and DFA IV (all disaccharides) (14), and polydextrose (PD, a polysaccharide) (15) have been found to stimulate Ca transport from the mucosal side to the serosal side of the rat small intestine. We have also shown using an Ussing chamber technique that various types of indigestible carbohydrates such as sugar alcohols, disaccharides and oligosaccharides or polysaccharides enhanced net Ca absorption in both the small and large intestine in vitro (16). The addition of indigestible sugars to the luminal side directly stimulated the intestinal epithelium and increased net Ca transport in a dose-dependent manner.

In contrast, improved fermentation in the large intestine is also thought to be involved in promoting resistant sugar-induced transepithelial Ca transport in the large intestine (17,18). Short-chain fatty acids (SCFA) and other organic acids produced through microbial fermentation decrease the pH of the cecal contents and thereby convert insoluble Ca to a soluble or ionic form (19). Thus, indigestible saccharides have the ability to promote Ca absorption directly (through the saccharide itself) and indirectly (through the organic acids produced from the saccharide) in the large intestine.

Transepithelial Ca transport in the intestine occurs by two routes, a transcellular pathway and a paracellular pathway (20,21). Transcellular absorption is dependent on an active transport process driven by metabolic energy. The diffusion of Ca ions across the cytoplasm is the rate-limiting step because this process is dependent on a Ca-binding protein (21). Paracellular Ca absorption, involving passive transport (diffusion),

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[2] Abbreviations used: DFA, difructose anhydride; LY, Lucifer Yellow; TEER, transepithelial electrical resistance; TJ, tight junction; HBS, HEPES buffer solution; MLCK, myosin light-chain kinase.

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requires a chemical Ca concentration gradient between the lumen and the basolateral side of the intestinal mucosa. The activity of tight junctions (TJ), located on the luminal side of adjacent epithelial cells, is thought to regulate the passage of various nutrients, including Ca (20). To date, the route of Ca absorption in the intestinal epithelium has not been sufficiently clarified when net Ca transport is increased by the addition of luminal indigestible sugars.

Thus, the aim of the present study was to clarify the route, particularly focusing on the intercellular pathway of Ca absorption regulated with the activated TJ, when net Ca transport from luminal to basolateral side of intestine was increased by the addition of intraluminal indigestible saccharides. The permeability of Lucifer Yellow (LY), which is a paracellular passage marker, and the changes in transepithelial electric resistance (TEER) across the mucosal tissue were determined simultaneously with the velocity of net Ca transport in isolated rat intestinal epithelium using an Ussing chamber technique (22).

MATERIALS AND METHODS

Animals and diets. Six-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) were housed in individual stainless steel metabolic cages with wire-mesh bottoms. The cages were placed in a room with controlled temperature (22–24°C), relative humidity (40–60%) and lighting (light 0800–2000 h). The rats had free access to water and a solid nonpurified diet (CE-2, Japan Clea, Tokyo, Japan) for more than one week before the start of the experiments. The rats (body weight 220–290 g) were used in the experiments at 7 to 9 wk old. This study was approved by the Hokkaido University Animal Committee and the rats were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Tissue preparation. On the day of the experiment, the rats were anesthetized with pentobarbital sodium (30 mg/kg). The jejunum (12 cm in length after Trietz), ileum (12 cm before a point 2 cm from the ileocecal junction), cecum (whole sac) or colon (middle and distal parts, about 12 cm in length) were quickly removed. The outside surface of each specimen was washed with ice-cold (4°C) saline (154 mmol/L NaCl), then each specimen was cut open along the mesenteric border to produce a flat sheet, and rinsed with ice-cold HEPES buffer solution (HBS). The HBS used consisted of 125 mmol/L NaCl, 4 mmol/L KCl, 6 mmol/L L-glutamine, 30 mmol/L HEPES and 1.25 or 10 mmol/L $CaCl_2 \cdot 2H_2O$, and it was gassed with 100% O_2 to maintain a constant pH of 7.4. HBS containing 1.25 mmol/L Ca was used as the bathing solution for the mucosal component during the experiment and as a stabilizing solution for both the mucosal and the serosal sides between experiments. The serosa and muscle layers were removed, and stripped preparations, consisting of the mucosa and the submucosa, were mounted onto Ussing chambers (Diffusion chamber system, Corning Costar, Cambridge, UK) that exposed a circular area of epithelium of 0.64 cm^2. The serosal and mucosal sides of the segments were bathed in 1 mL of HBS containing 1.25 mmol/L Ca and continuously exposed to 100% O_2 gas. After a 30-min stabilization period, the medium on both sides of the tissue was removed by aspiration and 1 mL portions of the appropriate solutions were added to the mucosal and serosal sides.

Indigestible saccharides and permeable marker. Melibiose is a dicarbohydrate composed of galactose and glucose. DFAIII and DFAIV are disaccharides consisting of two fructose residues and are isomers of one another. These compounds are not hydrolyzed by the digestive enzymes present in the small intestine, but they are metabolized by microorganisms in the large intestine. The three sugars were kindly provided by Nippon Beet Sugar MFG., Ltd (Obihiro, Japan). These indigestible saccharides are soluble in water at concentrations in the range of 0–100 mmol/L. Lucifer Yellow CH dilithium salt (LY: FW 457.2; Sigma-Aldrich, St. Louis, MO) was used as the paracellular permeable marker at a concentration of 21.8 μmol/L in the 10 mmol/L Ca HBS mucosal medium. The pH of 10 mmol/L Ca plus LY-HBS containing any of these sugars at 0, 1, 10 or 100 mmol/L remained constant at 7.4.

Experimental procedure. The prepared segments of the intestine, including segments of the jejunum, ileum, cecum and colon, were used for experiments with each of the three indigestible saccharides tested. An experiment without indigestible saccharides added to the mucosal medium was performed as a control. Fresh HBS containing 1.25 mmol/L Ca was applied to the serosal bath and 10 mmol/L Ca-HBS was applied to the mucosal bath in the experiments using the jejunum, ileum, cecum and colon preparations. After a 30-min incubation period, the serosal solution was transferred to a polyethylene test tube. The transepithelial electric resistance (TEER) of the epithelial preparation in the jejunum, ileum, cecum and colon was measured before and after the 30-min incubation period with 1.25mmol/L Ca-HBS added to both mucosal and serosal chambers. The TEER was calculated from the load of a small external current (0.1mA) according to Ohm's law using a short-circuit current amplifier (CZE-9100; Nihon Cohden, Tokyo, Japan).

Analyses. The Ca^{2+} concentrations in the serosal medium were measured by a colorimetric method using a commercial kit (Calcium C-Test; Wako Chemical, Osaka, Japan). Inter- and intraassay CV for the Ca^{2+} determination were less than 9% (stock standard solution) and 2% (same sample at 5 assays). The net transepithelial passage of Ca was expressed as nmol Ca transferred per min per square cm of surface area. The LY in the serosal solution was determined fluorometrically at 430nm for excitation and 540nm for emission (FP-550; Jasco, Tokyo, Japan) after the appropriate dilution of the solution with purified water. The TEER was expressed as the percentage decrease in TEER relative to the control value (before incubation).

Statistical analyses. All results are expressed as mean ± SEM. Statistical analyses were performed by one-way or two-way ANOVA followed by Duncan's or Dunnett's multiple range tests. A difference with $P < 0.05$ was considered significant.

RESULTS

The basal values (0 mmol/L saccharides in the serosal medium) of net Ca absorption and LY permeability in the jejunum, ileum, cecum and colon are shown in **Table 1**. There was no difference in the net Ca transport from the mucosal to the serosal medium in the jejunum and ileum. However, the net Ca transport in the cecum was greater than that in the colon ($P < 0.05$). The LY permeability in the colon was significantly lower than that in the other three intestinal portions ($P < 0.05$). The basal TEER in the four intestinal portions before and after 30min-incubation are shown in **Table 2**. The basal TEER in the colon before incubation was the highest ($P < 0.05$) among the four intestinal portions. The 30-min incubation in the absence of saccharides did not affect the TEER for any portions of the intestine.

The effects of the intraluminal application of DFAIII on

TABLE 1

Net Ca transport and LY permeability from the luminal to basolateral side of the rat intestinal epithelium in the absence of saccharide application (control)

Portion	Net Ca absorption	LY permeability
	$nmol \cdot min^{-1} \cdot cm^{-2}$	
Jejunum	10.1 ± 1.4^{ab}	0.275 ± 0.038^{a}
Ileum	10.5 ± 1.7^{ab}	0.320 ± 0.039^{a}
Cecum	13.4 ± 1.5^{a}	0.401 ± 0.039^{a}
Colon	5.3 ± 0.7^{b}	0.082 ± 0.012^{b}
ANOVA *P*-value	=0.0327	<0.001

Values are means ± SEM, $n = 8$. Means in a column not sharing a superscript letter differ, $P < 0.05$ (Duncan's test).

TABLE 2

TEER in the rat intestinal epithelium before and after incubation for 30 min in the absence of saccharide application (control)

Portion	TEER Value ($\Omega \cdot cm^{-2}$) Pre-incubation	Post-incubation
Jejunum	123.9 ± 3.5[a]	120.2 ± 3.6[a]
Ileum	117.4 ± 4.2[a]	115.7 ± 3.5[a]
Cecum	110.8 ± 4.1[a]	112.0 ± 3.8[a]
Colon	230.8 ± 8.3[b]	224.2 ± 6.3[b]
ANOVA *P*-values		
Portions (P)	<0.001	
Pre- or post-incubation (I)	0.5718	
P × I	0.9481	

Values are means ± SEM, $n = 8$. Values in two column not sharing a superscript letter are significantly different ($P < 0.05$) according to Duncan's test. TEER, transepithelial electrical release.

net Ca absorption, LY permeability and relative TEER in rat intestinal mucosa are shown in **Figure 1**. Net Ca transport from the luminal side to basolateral side in the jejunum, cecum and colon was increased dose-dependently with the application of DFAIII ($P < 0.05$). LY permeability was also increased in a dose-dependent manner in the four intestinal portions ($P < 0.05$). TEER was decreased by the luminal DFAIII ($P < 0.05$) in the jejunum, cecum and colon, but not in the ileum. The application of melibiose or DFAIV in the mucosal medium increased net Ca transport and LY permeability dose-dependently in all four intestinal preparations ($P < 0.05$, data not shown). The addition of melibiose to the luminal side decreased TEER dose-dependently in the jejunum and cecum ($P < 0.05$), but not in the ileum or colon (data not shown). The luminal application of DFAIV decreased TEER dose-dependently in all four intestinal portions ($P < 0.05$, data not shown). The maximal effects of three sugars on net Ca absorption, LY passage and TEER were obtained at a dose of 100 mmol/L of each sugar. The comparative data for the effects of the three saccharides applied at 100 mmol/L are shown in **Figure 2**. For net Ca absorption and LY permeability, the saccharides tested ($P < 0.001$ and $P < 0.001$) and the portions of intestine ($P = 0.0068$ and $P < 0.001$) differed but did not interact significantly. TEER was affected by the portion of the intestine ($P < 0.001$), but not by the sugars tested, and the interaction was not significant.

The relationship between the mean TEER (after a 30-min incubation) and LY permeability obtained by the application of various doses of the three sugars was analyzed (**Fig. 3**). The plots formed two large clusters. The colon cluster consisted of plots with high TEER (above 150 $\Omega \cdot cm^{-2}$) and low LY permeability (below 0.25 nmol $\cdot min^{-1} \cdot cm^{-2}$), whereas the other cluster (jejunum, ileum and cecum) consists of plots with low TEER (below 150 $\Omega \cdot cm^{-2}$) and high LY permeability (above about 0.25 nmol $\cdot min^{-1} \cdot cm^{-2}$). The characteristics of the permeation parameters, therefore, differed among the portions of the intestine. Regression analysis of net Ca transport and LY permeation, or of Ca transport and TEER was conducted for the small (**Fig. 4**) and large (**Fig. 5**) intestines. Positive linear relationships ($P < 0.001$) were found between net Ca transport and LY permeability and negative linear relationships ($P < 0.001$) between net Ca absorption and TEER in all four intestinal portions.

DISCUSSION

In our preliminary study using the isolated intestinal epithelium in rats in vitro, we show that various types of indigestible sugars enhanced net Ca absorption (16). However, the route of Ca transport activated by luminal indigestible saccharides was not clear. In the present study, we determined the route of Ca transport enhanced by the application of luminal indigestible sugars in the isolated rat intestinal mucosa using an Ussing chamber technique. Nonabsorptive permeable dye (LY) and TEER were used as indicators of the activation of paracellular passage. Permeability of the leakage marker and

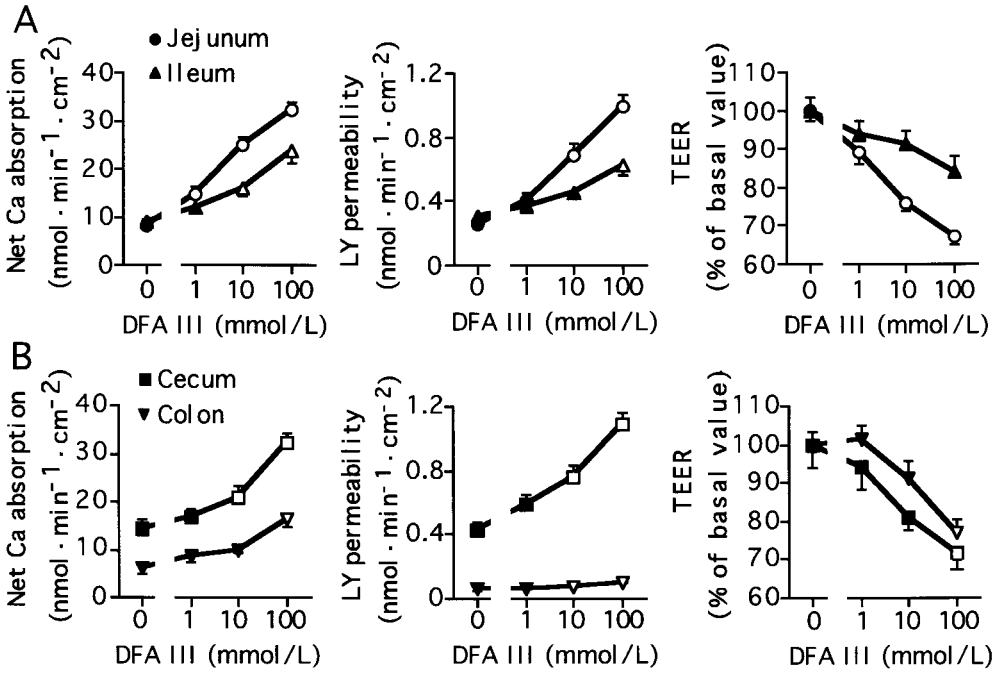

FIGURE 1 Effects of difructose anhydride (DFA)III on net Ca absorption, Lucifer Yellow (LY) permeability and transepithelial electrical resistance (TEER) in isolated rat intestinal epithelium. Values are mean ± SEM, $n = 8$. Open symbols indicate significantly different from the control value (0 mmol/L DFAIII), $P < 0.05$ (Dunnett's test).

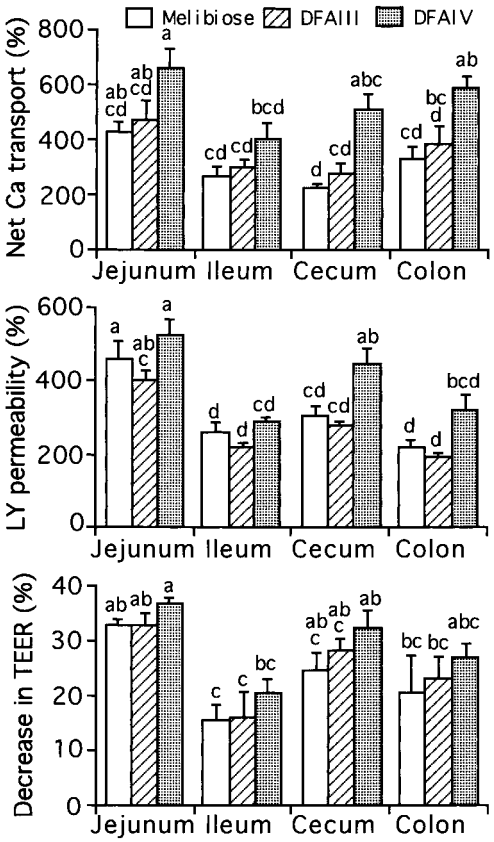

FIGURE 2 Effects of individual saccharides at 100 mmol/L on net Ca absorption, Lucifer Yellow (LY) permeability and transepithelial electrical resistance (TEER) in isolated rat intestinal epithelium. Values are means ± SEM, $n = 8$. Means without a common letter differ, $P < 0.05$ (Duncan's test).

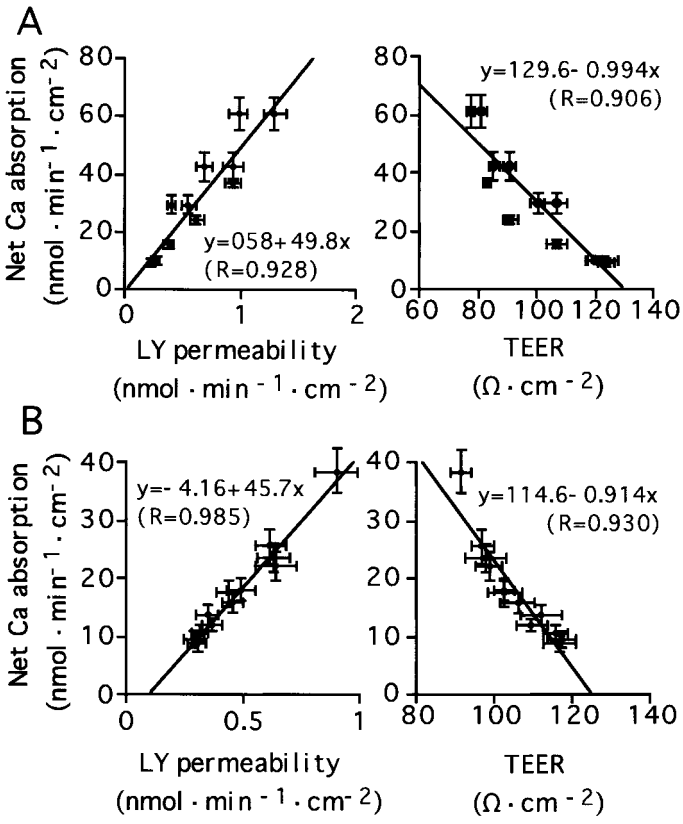

FIGURE 4 Relationships between net Ca transport and permeability parameters in the jejunum (*A*) and ileum (*B*) in rats. Regression for net Ca absorption and Lucifer Yellow (LY) permeability (*left panel*), and net Ca absorption and transepithelial electrical resistance (TEER) (*right panel*) after 30-min incubations are shown. Values are means ± SEM, $n = 8$. Plots in each panel include the data obtained for melibiose, difructose anhydride (DFA)III and DFAVI applications.

TEER were reported to change according to the degree of cell-to-cell connection (mainly the function of TJ) and reflect paracellular passage in cell lines (23) or isolated intestinal mucosa (24). The slope of the regression between LY flux and

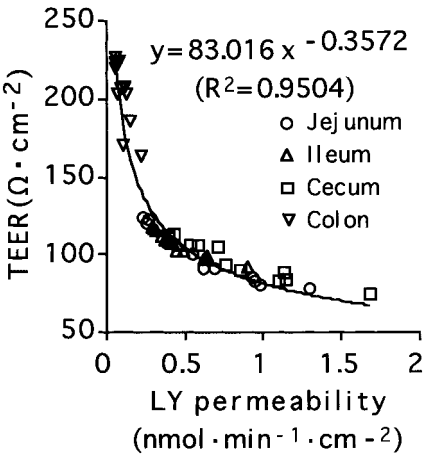

FIGURE 3 Relationships between Lucifer Yellow (LY) permeability and transepithelial electrical resistance (TEER) (after a 30-min incubation) in isolated rat intestinal epithelium. Means ($n = 8$) are plotted for melibiose, difructose anhydride (DFA)III and DFAVI applications in the isolated rat jejunal, ileal, cecal and colonic epithelia.

TEER value obtained in our study (**Fig. 2**) was almost the same as that from a previous report using Caco-2 cell monolayers (23). There was the inverse relationship between LY permeability and TEER. This result supports that the increase in Ca transport induced by luminal application of three sugars occurs via paracellular route in the isolated epithelial tissue of the intestine in vitro.

Intraluminal application of the three indigestible saccharides increased net transepithelial transport of Ca and permeability of LY from the luminal to basolateral side of the intestinal epithelium, and decreased TEER dose-dependently. There was a positive linear correlation between LY and net Ca transport ($P < 0.001$), whereas a negative linear relationship was observed between TEER and net Ca absorption ($P < 0.001$) in mucosal tissue preparations of the rat intestine (**Fig. 4 and Fig. 5**). These results suggest that an increase in net Ca transport stimulated by the application of indigestible disaccharides to the luminal side occurred via the paracellular route in the epithelial tissue.

Transepithelial Ca transport in the intestine occurs by two routes, a transcellular pathway and a paracellular pathway (20,21). Transcellular absorption is dependent on an active transport process driven by metabolic energy. The transport of Ca ions across the cytoplasm is the rate-limiting step, because this process depends upon a Ca-binding protein (21). It is essentially localized in the upper duodenum and is totally dependent on vitamin D (21,25). Paracellular Ca absorption,

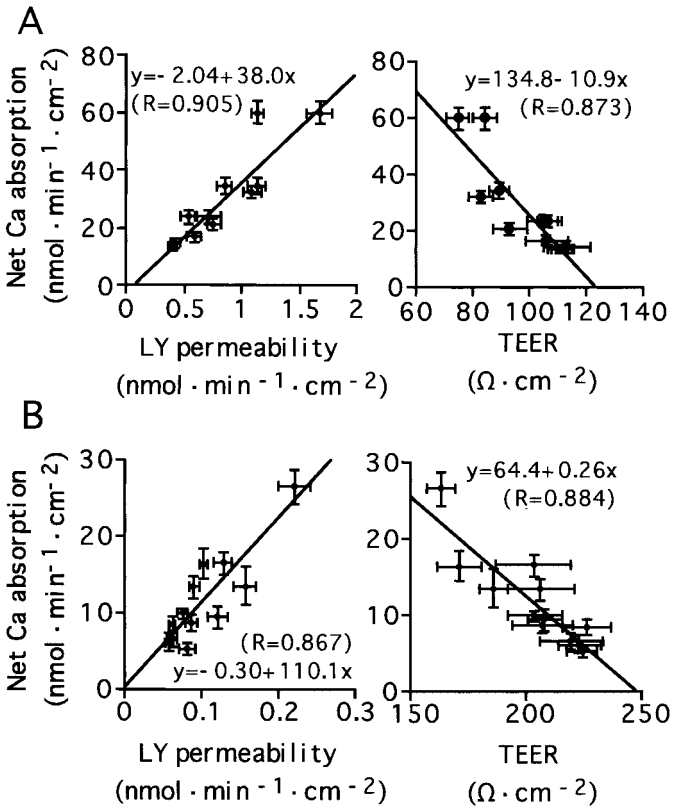

FIGURE 5 Relationships between net Ca transport and permeability parameters in the cecum (A) and colon (B) in rats. Regression for net Ca absorption and Lucifer Yellow (LY) permeability (*left panel*), and net Ca absorption and transepithelial electrical resistance (TEER) (*right panel*) after 30-min incubations are shown. Values are means ± SEM, $n = 8$. Plots in each panel include the data obtained for melibiose, difructose anhydride (DFA)III and DFAVI applications.

involving passive transport (diffusion), requires a chemical gradient of Ca concentrations between the lumen and the basolateral side of the intestinal mucosa. Evidence has accumulated indicating that tight junctions (TJ), located on the luminal side of adjacent epithelial cells, regulate the absorption of various nutrients including Ca (20).

To date, there is no evidence to suggest that indigestible sugars directly affect the mucosal cells of the intestine to enhance intracellular active Ca transport. On the basis of balance tests in rats, increased Ca absorption induced by the ingestion of xylitol was shown to be independent of vitamin D action (26). However, some reports have suggested that indigestible saccharides enhance Ca absorption via activation of paracellular passage in the rat intestinal epithelium in vitro. The amount of Ca in the serosal medium was directly proportional to the Ca concentrations in the mucosal medium at 1–20 mmol/L using everted preparations of rat ileum in vitro (27). In previous experiments using isolated mucosal tissue in rats (28) or cell monolayers (29), Ca concentration in the serosal medium increased linearly with the incubation time. Under the experimental conditions used in our study, the Ca concentration in the mucosal medium was 10 mmol/L, higher than the concentration in the serosal medium (1.25 mmol/L). In our preliminary trial, net Ca absorption was not observed when same Ca concentration (1.25 mmol/L) was applied to media on both the luminal and basolateral sides (data not shown). These results indicate that the Ca concentration in the serosal medium increased proportional to the incubation time in epithelial preparations of rat intestine. We consider the transcellular Ca passage to be very low in ileum, cecum and colon but not jejunum under the conditions used in this study (21). Therefore, further experiments are needed to clarify the contribution of the transcellular Ca transport in the small intestine close to the duodenum, which is the main portion of the active Ca transport, in this experimental condition.

A variety of food-derived chemicals or drugs have been reported to change the intercellular permeability of the intestinal epithelium via the activation of TJ in cell lines (30,31) and isolated intestinal epithelium (24). It has been proposed that condensation of actin microfilaments induced by myosin light-chain kinase (MLCK) participates in the opening of TJ (32). It was shown that maltitol-induced Ca transport in everted ileal sacs is completely inhibited by the application of a calmodulin-dependent MLCK antagonist (27). Maltitol is an indigestible sugar alcohol reported to enhance Ca absorption in rats in vivo (1,7) and isolated rat intestinal tissue in vitro (12,16). A common mechanism via the paracellular pathway may be involved in saccharide-induced Ca absorption in the epithelium of the gastrointestinal tract.

The degree of tightness in the TJ varies with the physiological requirements of the various portions of the body (33). The basal permeability of LY and TEER varied among the portions of the intestine tested (Table 1). The LY permeability of the colon is lower than that of the other three portions ($P < 0.05$). Basal net Ca absorption in the colon was lower than that in the cecum ($P < 0.05$). TEER was highest in the colon among the four intestinal portions ($P < 0.05$). The main functions of the colon, especially the middle and distal portions used in this study, are the formation of feces and its excretion. Jejunum, ileum and cecum are important portions for absorption of nutrients. Differences in the dye permeability and TEER between the colon and other three portions might reflect their different functions. In addition, differences in Ca absorption and the response of permeable parameters occurred among intestinal portions and saccharides tested (Fig. 2). In previous studies using everted sacs (27) or epithelial tissues of the intestine (28), Ca transport was also found to vary according to the type of saccharide and portion of the intestine tested. These results suggest that differences in recognition of saccharides and/or characteristics of TJ may exist between portions of the intestine, and may affect Ca transport in the small and large intestine.

In conclusion, three indigestible disaccharides, melibiose, DFAIII and DFAIV increased the permeability of intercellular passage in which TJ function may be an important factor. The three sugars may open the passages of TJ, thereby promoting Ca absorption in the small and large intestine in rats. Further study is required to clarify the recognition of resistant sugars by the epithelial tissue of the intestine, and the postulated mechanism of enhancement of TJ-regulated Ca absorption via the paracellular route.

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