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The Nondigestible Disaccharide Epilactose Increases Paracellular Ca Absorption via Rho-Associated Kinase- and Myosin Light Chain Kinase-Dependent Mechanisms in Rat Small Intestines

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We previously showed that epilactose, a nondigestible disaccharide, increased calcium (Ca) absorption in the small intestines of rats. Here, we explored the mechanism(s) underlying the epilactose-mediated promotion of Ca absorption in a ligated intestinal segment of anesthetized rats. The addition of epilactose to the luminal solution increased Ca absorption and chromium (Cr)-EDTA permeability, a paracellular indicator, with a strong correlation ($R = 0.93$) between these changes. Epilactose induced the phosphorylation of myosin regulatory light chains (MLCs), which is known to activate the paracellular route, without any change in the association of tight junction proteins with the actin cytoskeleton. The epilactose-mediated promotion of the Ca absorption was suppressed by specific inhibitors of myosin light chain kinase (MLCK) and Rho-associated kinase (ROCK). These results indicate that epilactose increases paracellular Ca absorption in the small intestine of rats through the induction of MLC phosphorylation via MLCK- and ROCK-dependent mechanisms.

KEYWORDS: Epilactose; Ca absorption; tight junction; myosin light chain kinase; Rho-associated kinase

INTRODUCTION

Decreases in intestinal calcium (Ca) absorption and bone mineral density are characteristic of aging or menopause. However, together with lower calcium intake, these decreases can lead to bone diseases. Therefore, increasing not only the dietary intake of Ca but also its bioavailability may provide an effective means of avoiding bone diseases.

Epilactose (4-*O*-β-galactopyranosyl-D-mannose) is a rare nondigestible disaccharide. A considerable amount of epilactose can be produced from cow milk by heating and alkali treatments (*1*); however, the biological activity remains unknown. Recently, we developed a method for the preparation of epilactose using cellobiose 2-epimerase (EC 5.1.3.11) from the ruminal strain *Ruminococcus albus* NE1 (*2*), and we presented some biological activities of epilactose, including the promotion of intestinal Ca absorption (*3*, *4*). Our *in vivo* study, we demonstrated that epilactose increases Ca absorption and that the increase is greater than that induced by fructooligosaccharides (FOS) (*3*), which are also known to enhance Ca absorption. One mechanism responsible for this increase is the solubilization of Ca salts by acids produced through the microbial fermentation of the saccharide in the large intestine. Another mechanism is the promotion of Ca transport through the direct stimulation of the intestinal epithelium in the small intestine by the intact saccharide.

Intestinal Ca absorption occurs via both the transcellular and paracellular routes (*5*). The transcellular mechanism, predominant in the duodenum, is a saturable, carrier-mediated active transport process, whereas the paracellular mechanism is nonsaturable and diffusive, occurs through the intestines, and requires a gradient of Ca concentrations between the lumen and the basolateral side. The paracellular transport of ions and solutes is controlled by intercellular tight junctions (TJs) positioned around the apical end of the lateral cell membrane (*6*). The TJs are organized by specific interactions between a wide spectrum of proteins. Three integral transmembrane proteins, occludin (*7*), claudins (*8*), and junctional adhesion molecule (JAM) (*9*), have been identified and found to interact with other intracellular plaque proteins, such as zonula occludens (ZO)-1, ZO-2, ZO-3, cingulin, and 7H6, which in turn anchor the transmembrane proteins to the apical perijunctional actin cytoskeleton (*6*).

Stimuli, including nutrients and food factors, influence paracellular transport through the increase/decrease in the expression of TJ proteins and their association with the actin cytoskeleton. Murphy et al. (*10*) demonstrated that conjugated linoleic acid enhanced paracellular Ca transport through the alteration of TJ protein expression in human intestinal Caco-2 cells. We reported that quercetin, a phenolic compound, increased the association of ZO-2, occludin, and claudin-1 and claudin-4 expression in Caco-2

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cells (*11*). On the other hand, circumferential contraction and subsequent tension in the apical perijunctional actin-myosin ring regulates solute transport via the paracellular route (*12*). Modification of the actin-myosin ring is achieved by the phosphorylation of the myosin regulatory light chain (MLC) of myosin II (*13*). MLC is phosphorylated at Thr 18 and Ser 19 by myosin light chain kinase (MLCK) in a Ca/calmodulin-dependent manner, and the phosphorylation triggers the contraction of the actin-myosin ring, resulting in the activation of paracellular transport (*13*). Rho-associated kinase (ROCK) is another kinase responsible for MLC phosphorylation (*14*), and it also phosphorylates the myosin phosphatase target subunit at its inhibitory sites, leading to an increase in MLC phosphorylation (*15*). MLCK and ROCK activities have been reported to be mediated by various signaling molecules, including phospholipase C and tyrosine kinase (*16*, *17*).

In the present study, we examined the promotive effect of epilactose on intestinal Ca absorption in a ligated small intestinal segment in anesthetized rats. Our results show that (1) epilactose enhances paracellular Ca absorption in the small intestines of rats, (2) epilactose increases the phosphorylation of MLC in the intestinal epithelial cells, and (3) the activities of MLCK and ROCK are responsible for the epilactose-mediated phosphorylation of MLC as well as the increase in intestinal Ca absorption.

MATERIALS AND METHODS

Chemicals. Epilactose was synthesized from lactose by using a recombinant cellobiose 2-epimerase from *Ruminococcus albus*, as described by Ito et al. (*2*). Rabbit anti-myosin regulatory light chain (MLC) and phospho-MLC were purchased from Cell Signaling Technology (Danvers, MA). Goat anti-claudin-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-claudin-3, rabbit anti-JAM-1, and horseradish peroxidase (HRP)-conjugated mouse anti-occludin were purchased from Zymed Laboratories (San Francisco, CA). Mouse anti-β-actin, HRP-conjugated antigoat, -mouse, and -rabbit IgG, and fluorescein isothiocyanate−dextran (FD20S; average molecular weight 20,000) were purchased from Sigma (St Louis, MO). ML-7 [a myosin light chain kinase (MLCK) inhibitor], Y27632 [a Rho-associated kinase (ROCK) inhibitor], and U73122 [a phospholipase C (PLC) inhibitor] were purchased from BIOMOL International (Plymouth Meeting, PA). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Animals. Male Sprague−Dawley rats (Japan SLC, Hamamatsu, Japan), weighing about 200 g, were acclimatized with a standard diet (CE-2; Clea Japan, Tokyo, Japan) for 7 days. Rats were housed in individual cages in a room with controlled temperature (23 ± 1 °C), relative humidity (55 ± 5%), and lighting (lights on from 8:00 to 20:00). 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 (Approval number: 08-0137).

Experimental Procedure Using Small Intestinal Loops. A small intestinal segment was prepared in each rat in all experiments. All experiments were repeated at least 2 times. In the first experiment, we examined the effect of epilactose on Ca absorption at two different doses (50 and 100 mM) and compared its potency with that of fructooligosaccharides (FOS) at 100 mM. The number of rats in each treatment was six. An abdominal midline incision (about 4 cm) was made under pentobarbital anesthesia (sodium pentobarbital, 40 mg/kg body weight; Abbott Laboratories, Tokyo, Japan). After ligations at about 3 and 30 cm distal from the ligament of Treitz, two small cuts were made just inside the two ligations. The lumen was gently washed out with saline, and a ligation was made at 15 cm distal from the proximal cut. 1.5 mL of each test solution [30 mM MOPS, 110 mM NaCl, 4 mM KCl, 10 mM $CaCl_2$, 6 mM L-glutamine, 0.9 μM Cr-EDTA (as a paracellular indicator) (*18*), 100 μg/mL FD20S (as a nonabsorbable marker, average molecular weight 20,000), pH 6.5] without or with epilactose (50 or 100 mM) or FOS (100 mM) was injected into the segment through the proximal cut, and another ligation was made just distal to the cut (producing a 15-cm segment of the small intestine). Epilactose and FOS were substituted with NaCl (50%) in the test solutions so as not to impair the isotonicity of the solutions. Cr-EDTA was prepared as described previously (*19*). The luminal solutions were collected 20 min after the start of the experiments for the measurements of Ca, Cr, and FD-20S. The small intestinal mucosa was immediately collected by scraping with glass slides and subjected to detergent-insoluble fraction preparation as described below. Rats were kept under anesthesia on the heater (37 °C) in a room with controlled temperature (23 ± 1 °C) throughout the experiments.

In the second and third experiments, the involvement of MLCK, ROCK, phospholipase C (PLC), and tyrosine kinase activity in the epilactose-mediated promotive effect on Ca absorption was examined using their specific inhibitors. The number of rats in each treatment was six. After making the abdominal midline incision, ML-7 (a MLCK inhibitor), Y-27632 (a ROCK inhibitor), U73122 (a phospholipase C inhibitor), and genistein (a tyrosine kinase inhibitor) were intra-abdominally administrated to the rats (ML-7; 1 μmol/rat, Y-27632; 2 μmol/rat, genistein; 2 mmol/rat, U73122; 1 μmol/rat). These inhibitors were dissolved in 1 mL of 15% dimethyl sulfoxide and 50% polyethylene glycol 400, and the solution without inhibitors was used for mock treatment. After preparation of the small intestinal segments, 1.5 mL of the test solutions described above without or with 100 mM epilactose in the absence or presence of 5 μM ML-7, 10 μM Y27632, 200 μM genistein, or 5 μM U73122 (final concentrations) was injected into the segments. The luminal solutions were collected after 20 min for the measurements of Ca, Cr, and FD-20S.

Measurements of Ca, Cr, and FD20S. Concentrations of Ca and Cr in the luminal solutions were measured by atomic absorption spectrophotometry (Z-5310; Hitachi, Tokyo, Japan) in the presence of 1000 ppm lanthanum chloride and 0.2 g/L ammonium chloride after appropriate dilutions. The concentration of FD20S was determined by fluorescence measurement (FP-5500; JASCO International, Tokyo, Japan).

Ca absorption and Cr-EDTA permeate rates were calculated using the following equation; 100 − 100[Ca (Cr) concentration/FD20S concentration in the collected solution]/[Ca (Cr) concentration/FD20S concentration in the injected solution].

Preparation of the Detergent-Insoluble Fraction. The detergent-insoluble fraction, corresponding to the proteins associated with the actin cytoskeleton, was prepared as described previously (*11*, *20*). Fifty milligrams of mucosa was suspended in 500 μL of lysis buffer-CS [1% TritonX-100, 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid in 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol containing protease inhibitors (5 μg/mL aprotinin, 3 μg/mL leupeptin hemisulfate, 5 mM benzamidine hydrochloride, and 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (2 mM sodium orthovanadate and 10 mM sodium fluoride), pH 7.5] and incubated for 15 min on ice. Cell lysates were centrifuged at 15,600*g* for 10 min at 4 °C to sediment the high density actin-rich fraction. The pellet was resuspended in 200 μL of RIPA buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 150 mM NaCl, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and 1 mM EDTA in 25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol containing the protease and phosphatase inhibitors described above, pH 7.5]. Protein concentrations in the lysates were measured using the BCA method (Pierce Biotechnology, Inc., Rockford, IL). The lysates were mixed with a half volume of Laemmli sample buffer [3× concentrated; 6% (w/v) SDS, 30% (v/v) glycerol, 15% (v/v) 2-β-mercaptoethanol, and 0.02% (w/v) bromophenol blue in 188 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol, pH 6.8] (*21*) and heated at 100 °C for 5 min.

Immunoblot Analysis. Proteins (50 μg) were separated by SDS-PAGE (12%) and transferred to polyvinylidene difluoride membranes. Membranes were blotted for pMLC, total MLC, occludin, JAM-1, claudin-1, claudin-3, and β-actin using specific antibodies in combination with HRP-conjugated anti-mouse IgG or anti-rabbit IgG antibodies. The blots were developed using the ECL chemiluminescence method (GE Healthcare, Buckinghamshire, U.K.). Quantification was performed by densitometric analysis of specific bands on the immunoblots using Image J software.

Statistical Analysis. All values are expressed as means ± SEM. Statistical analyses were performed by 1- or 2-way ANOVA followed by

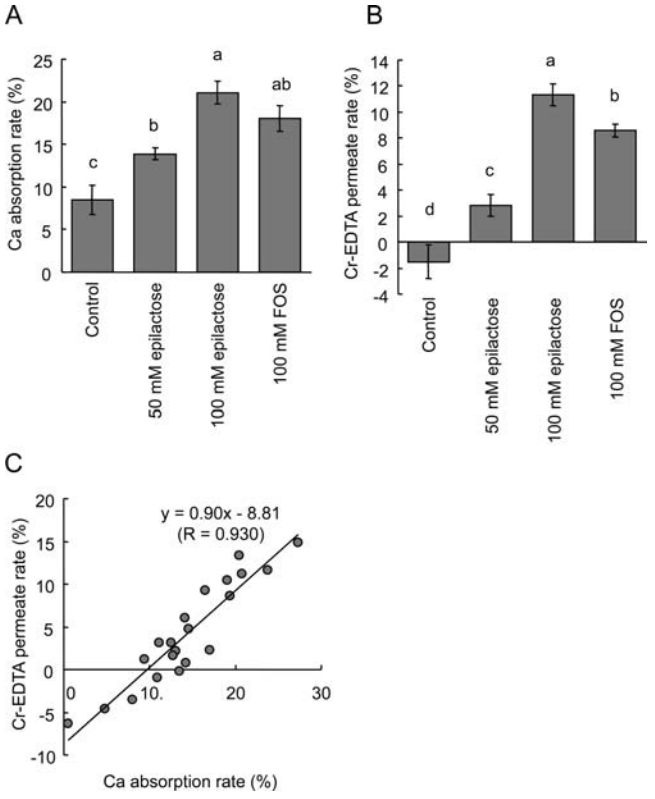

Figure 1. Ca absorption rate (A), Cr-EDTA permeate rate (B), and their correlation (C) in small intestinal loops infused with solutions containing epilactose (50 and 100 mM) or fructooligosaccharides (100 mM). Values are means with their standard errors for 6 rats. Mean values for groups not sharing a common letter are significantly different ($P < 0.05$). For details of the animals and procedures, see the Materials and Methods.

Duncan's multiple range test. The correlation between Ca absorption and Cr-EDTA permeate rates was evaluated by Pearson's correlation. A difference with $P < 0.05$ was considered significant. Statistical analyses were performed using the general linear models procedure of the SAS program (version 6.07; SAS Institute Inc., Cary, NC).

RESULTS

Intestinal Ca Absorption and Cr-EDTA Permeate Rates. The addition of epilactose to the luminal solution increased the Ca absorption rate and Cr-EDTA permeate rate, an indicator of paracellular transport, in a dose-dependent manner (**Figure 1**). The Ca absorption and Cr-EDTA permeate rates in the 100 mM epilactose group were higher than those in the 0 and 50 mM epilactose groups. The addition of FOS also increased the Ca absorption and Cr-EDTA permeate rates; however, the potency was lower than that of epilactose in the Cr-EDTA permeate. The Ca absorption rate in the 100 mM FOS group was higher than the control value, but not that of the 50 mM epilactose group. The Cr-EDTA permeate rate in the 100 mM FOS group was higher than those in the 0 and 50 mM epilactose groups but lower than that in the 100 mM epilactose group. A positive correlation was found between Ca absorption and Cr-EDTA permeate rates in the luminal solutions containing 0, 50, and 100 mM epilactose, indicating that epilactose promotes Ca absorption through the paracellular route.

MLC Phosphorylation and TJ Proteins in the Detergent-Insoluble Fraction. MLC phosphorylation and TJ proteins associated with the actin cytoskeleton were evaluated by immunoblot analysis using the detergent-insoluble fraction of the mucosa collected from the intestinal segments perfused with epilactose (**Figure 2**). The ratio of phosphorylated to total MLC was increased by the addition of 50 and 100 mM epilactose in a dose-dependent manner (**Figure 2**A and B). The ratio in the 100 mM epilactose group was higher than the control value.

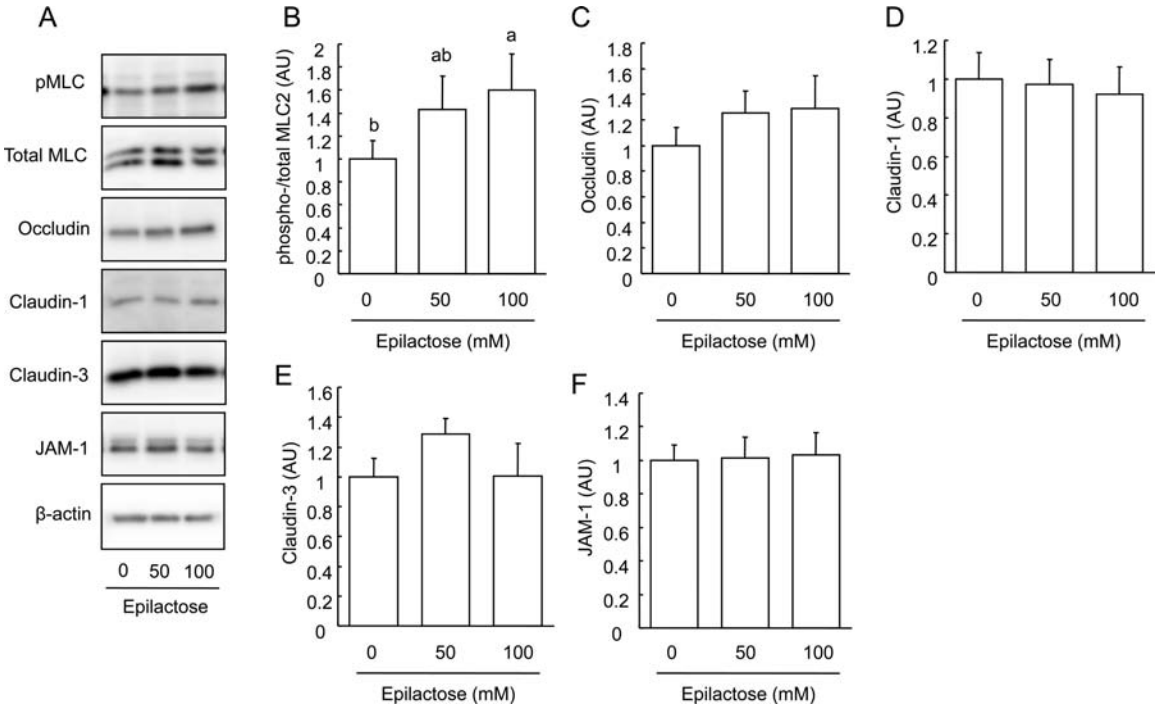

Figure 2. Immunoblot analysis of phospho-MLC (pMLC), total MLC, occludin, claudin-1, claudin-3, junctional adhesion molecule (JAM)-1, and β-actin in the detergent-insoluble fractions prepared from the mucosa of small intestinal loops infused with solutions containing epilactose (50 and 100 mM). Each immunoblot is representative of six rats (A). Each specific band of proteins was quantified by densitometric analysis (B, the ratio of pMLC/total MLC; C, occludin; D, claudin-1; E, claudin-3; F, JAM-1). Values are means with their standard errors for six rats. Mean values for groups not sharing a common letter are significantly different ($P < 0.05$). For details of the animals and procedures, see the Materials and Methods.

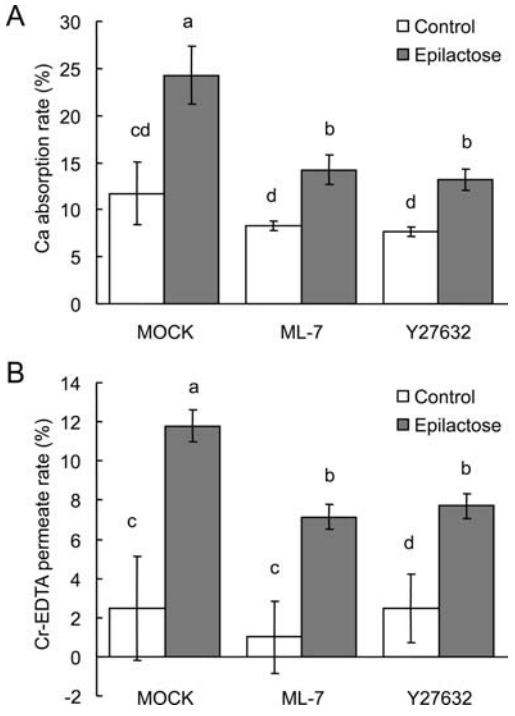

Figure 3. Ca absorption rate (A) and Cr-EDTA permeate rate (B) in small intestinal loops infused with solutions containing epilactose (0 and 100 mM) in the absence and presence of two signaling inhibitors (ML-7 and Y27632). Values are means with their standard errors for six rats. Mean values for groups not sharing a common letter are significantly different ($P < 0.05$). For details of the animals and procedures, see the Materials and Methods.

There was no differences in the amount of TJ proteins, occludin, claudin-1, claudin-3, JAM-1, in the detergent-insoluble fractions among the groups (**Figure 2**A, C, D, E, and F).

Involvement of MLCK and ROCK in the Epilactose-Mediated Promotion of Ca Absorption. Specific inhibitors of MLCK (ML-7) and ROCK (Y27632) were used to examine their involvement in the epilactose-mediated promotive effect on intestinal Ca absorption (**Figure 3**). Among the 100 mM epilactose groups, the Ca absorption and Cr-EDTA permeate rates in the presence of ML-7 and Y27632 were lower than those after mock treatment, with the rates in the presence of ML-7 and Y27632 both being higher than the respective control values but lower than those of the 100 mM epilactose group in the absence of any inhibitors. Both rates in the presence of ML-7 and Y27632 were approximately 60% of the respective control values without inhibitors in the epilactose groups. ML-7 and Y27632 had no effect on the Ca absorption or Cr-EDTA permeate rates in the absence of epilactose.

U73122 (a PLC inhibitor) and genistein (a tyrosine kinase inhibitor) did not affect the epilactose-mediated increase in Ca absorption or Cr-EDTA permeate rates (**Figure 4**). The two rates in the epilactose groups were similar to and higher than those in the control groups in the absence and presence of inhibitors, respectively. Among the control groups, the Ca absorption rate after genistein treatment was slightly, but not significantly, lower than that after MOCK treatment.

DISCUSSION

This study demonstrates that epilactose, a nondigestible disaccharide, enhances paracellular Ca absorption in the small intestine of rats. Our results show that epilactose directly stimulates the small intestinal epithelium and induces MLC phosphorylation via MLCK- and ROCK-dependent mechanisms, resulting in the activation of paracellular Ca transport (Figure **5**).

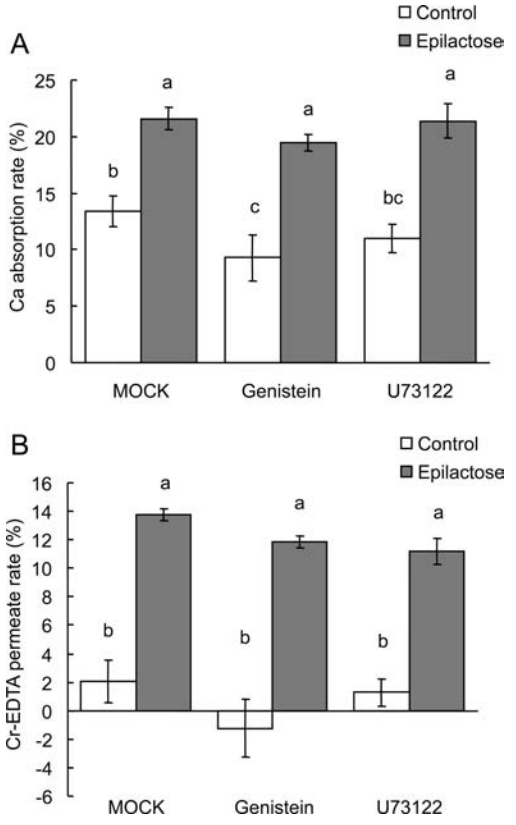

Figure 4. Ca absorption rate (A) and Cr-EDTA permeate rate (B) in small intestinal loops infused with solutions containing epilactose (0 and 100 mM) in the absence and presence of two signaling inhibitors (U73122 and genistein). Values are means with their standard errors for six rats. Mean values for groups not sharing a common letter are significantly different ($P < 0.05$). For details of the animals and procedures, see Materials and Methods.

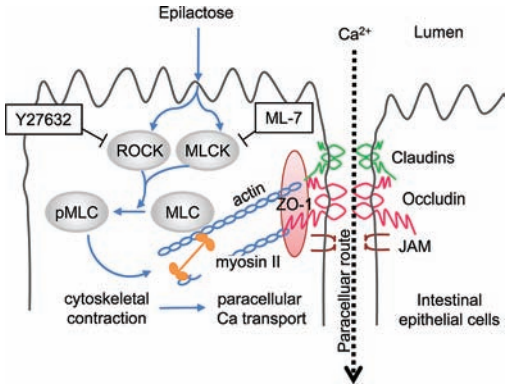

Figure 5. Diagrammatic representation of the molecular mechanismunderlying the epilactose-mediated activation of paracellular Ca transport.

Epilactose increases both Ca absorption and Cr-EDTA permeability, a paracellular indicator, in the small intestinal segments of rats in a dose-dependent manner. The strong correlation ($R = 0.93$) between the two rates clearly shows that epilactose promotes paracellular Ca absorption, although transcellular Ca absorption also occurs. Intestinal Ca absorption takes place via two routes: the transcellular and paracellular routes (*5*). It is reported that transcellular Ca absorption predominantly occurs in the duodenum and is saturated at luminal Ca concentrations < 5 mM (*22*, *23*), which is much lower than in the luminal solutions used in our study (10 mM). In our experiments, paracellular Ca absorption seemed to be more efficiently driven

than transcellular absorption, as the midpart of the small intestines was used and the Ca concentration of the solutions was high. Furthermore, our previous study showed that another nondigestible disaccharide, difructose anhydride III, enhanced paracellular Ca transport in intestinal Caco-2 cells without any concomitant change in transcellular Ca transport (*24*). We found that some control rats gave the negative values in the Cr-EDTA permeability according to the calculation. The negative values may have been given because Cr-EDTA permeability was nearly 0% and because of the trace amount of Cr-EDTA adhered to the epithelial mucosa in these rats.

The promotive effects of epilactose on Ca absorption and Cr-EDTA permeability are more potent than those of FOS at 100 mM, although we only found statistically significant differences between the two groups in regard to Cr-EDTA permeability. This result corresponds to our *in vivo* study showing more efficient increases in intestinal Ca absorption in rats fed epilactose than in those fed FOS (*3*). We have previously shown that the increases in Ca absorption induced by various nondigestible oligosaccharides in the isolated small intestines of rats vary (*25*). Importantly, the osmolarity in the test solutions was identical among treatments in the present study. This evidence indicates that the epilactose-mediated increases in paracellular Ca absorption are not merely the result of increases in the solute concentration in the luminal solution and suggest that the number, type, and/or configuration of the monosaccharides in the nondigestible saccharide molecules determine the potency of the increase in paracellular Ca absorption. Epilactose is a nondigestible disaccharide that does not permeate the plasma membrane of the intestinal cells. This means that extracellular epilactose directly stimulates the intestinal cells, resulting in the activation of paracellular Ca transport; however, further investigations are required to clarify how the intestinal cells sense the extracellular epilactose.

Our result suggests that epilactose activates paracellular Ca absorption through the induction of MLC phosphorylation and, therefore, the contraction of the perijunctional actin-myosin ring in the intestinal cells. Columnar intestinal epithelial cells have a prominent perijunctional actin-myosin ring that encircles the apical pole of polarized cells, and paracellular transport is regulated by the lateral tension within this actin-myosin ring (*12*, *26*). In contrast, epilactose did not have any effect on the association of TJ proteins (occludin, claudin-1, claudin-3, and JAM-1) with the actin-myosin ring, which is another determinant of paracellular transport (*12*,*27*). We did not examine the changes in expression of any TJ proteins, because the epilactose-mediated promotive effect on paracellular Ca absorption emerged within 20 min, and it is unlikely that their expression would significantly change within such a short period.

The specific inhibitors of MLCK (ML-7) and ROCK (Y27632), but not the inhibitors of PLC (U73122) and tyrosine kinase (genistein), diminished the epilactose-induced increases in Ca absorption and Cr-EDTA permeability. This result indicates that both MLCK and ROCK participate in the induction of MLC phosphorylation and activation of paracellular transport by epilactose. It is known that these two kinases directly phosphorylate MLC in the intestinal cells and that some food factors, such as glucose (*26*) and lysophosphatidic acid (*28*), induce the activation of these kinases, leading to the promotion of paracellular transport. On the other hand, PLC and tyrosine kinase were found not to participate in the epilactose-mediated effect, even though they have been reported to be involved in the regulation of MLCK and ROCK activities: capric acid activates MLCK through the induction of intracellular Ca signaling in a PLC-dependent mechanism in the intestinal cells (*17*), and ROCK-dependent MLC phosphorylation by thromboxane A2 is reported to be inhibited by genistein in the vascular endothelium (*16*). Further investigations are required to understand the upstream signaling pathway(s) leading to the activation of MLCK and ROCK by epilactose.

Ca deficiency is known to increase the risk of osteopenia; however, it can be efficiently restored by increasing Ca availability, because the intestinal Ca absorption rate is typically low. In particular, the promotion of paracellular Ca absorption seems to be an effective means to increase Ca bioavailability. It is known that the paracellular route contributes the greater portion of absorbed Ca at luminal Ca concentrations above 5 mM, which are typical Ca concentrations at feeding times (*29*). In addition, Slepchenko and Bronner (*22*) have recently modeled transcelluar Ca transport in rats using their extensive experimental data. They have shown that active transcellular transport is saturated at $K_m = 0.35$ mM and accounts for only a minor portion of the Ca absorbed at feeding times because of the short sojourn time in the duodenum (~3 min). We have previously demonstrated that epilactose promotes Ca absorption in both the jejunum and ileum (*3*), which is most likely driven through the paracellular route based on the results of the present study. These results suggest that the supplemental feeding of epilactose can efficiently restore Ca deficiency and reduce the risk of osteopenia in humans.

In the view of safety for human consumption, it should be emphasized that epilactose promotes paracellular Ca transport without any concomitant changes in the distribution of TJ proteins, including occludin and claudins. The TJs have a crucial role in the intestinal barrier function as well as the paracellular transport of nutrients (*30*). It is reported that some inflammatory cytokines such as TNFα and interferon γ disrupt the barrier function through the disassembly of TJ proteins from the actin cytoskeleton concomitantly with MLC phosphorylation (*31*,*32*), and TJ disruption is believed to be involved in the pathogenesis of some intestinal diseases such as inflammatory bowel disease (*33*). Additionally, we showed that rats fed epilactose for over 2 weeks did not exhibit any signs of physical or physiological problems (*3*, *4*).

In conclusion, epilactose stimulates paracellular Ca absorption in the small intestine through the direct interaction of epilactose and the intestinal epithelium. The epilactose-mediated promotive effect occurs via MLC phosphorylation through MLCK- and ROCK-dependent mechanisms.

ABBREVIATIONS USED

ANOVA, analysis of variance; Ca, calcium; Cr; chromium; EDTA, ethylenediaminetetraacetic acid; FOS, fructooligosaccharides; HRP, horseradish peroxidase; JAM, junctional adhesion molecule; MLC, myosin regulatory light chain; MLCK; myosin regulatory light chain kinase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PLC, phospholipase C; ROCK, Rho-associated kinase; SDS, sodium dodecyl sulfate; TJ, tight junction; ZO, zonula occludens.

SAFETY

The authors designed and performed all experiments with careful attention to safety.

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Science and Technology of Japan. All authors declare no conflict of interest and contributed to the research.

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