Short communication

Various non-digestible saccharides increase intracellular calcium ion concentration in rat small-intestinal enterocytes

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We have previously shown that non-digestible saccharides (NDS) stimulate intestinal Ca absorption via tight junctions. However, the cellular mechanisms activated by the NDS are not yet known. We investigated the effects of four NDS, difructose anhydride (DFA) III, DFAIV, fructo-oligosaccharides, and maltitol, on intracellular Ca signalling in isolated rat small-intestinal enterocytes. The changes in intracellular Ca^{2+} concentration were measured before and after the addition of capric acid (7·5 or 15 mmol/l, a positive control), glycerol, or each NDS (1 or 10 mmol/l) to fura-2-loaded enterocytes. Treatment with capric acid or each NDS caused an immediate and dose-dependent rise in intracellular Ca^{2+} concentration. Mechanical and osmotic stimulation achieved by adding glycerol had no effect on intracellular Ca^{2+} concentration. The intracellular Ca^{2+} concentration in enterocytes treated with DFAIII and fructo-oligosaccharides reached a peak level at about 30 s after stimulation, but those treated with DFAIV and maltitol showed further increases after the initial rapid rise. The maximum change in intracellular Ca^{2+} concentration obtained by the application of maltitol was higher than that of DFAIII at 10 mmol/l. These findings suggest that each of the four NDS directly stimulates rat enterocytes, and increases intracellular Ca^{2+} concentration. Thus, molecular structure may be more important than the size of the NDS in the induction of Ca signalling in the cells.

Non-digestible saccharides: Intracellular calcium ion: Rat enterocytes

Ca, in the form of hydroxyapatite, is a major constituent of bone, and an adequate intake is believed to be necessary for the maintenance of bone health. Bone Ca and intestinal Ca absorption are known to decrease with ageing, gradually in men and abruptly in women after the menopause (Bronner & Pansu, 1999). Therefore, increasing not only the dietary intake of Ca but also its bioavailability may provide an effective means of avoiding bone diseases. Intestinal Ca absorption proceeds by two pathways, the transcellular and the paracellular routes (Bronner, 1998). Transcellular absorption is an active-transport process regulated by 1, 25-dihydroxyvitamin D_3 and is mainly confined to the duodenum. Paracellular absorption is a passive-transport process that requires a gradient of Ca concentration between the luminal and the basolateral side and occurs throughout the intestine. The paracellular passage of solutes is regulated by tight junctions, which are located on the luminal side of adjacent epithelial cells (Mitic *et al.* 2000). The tight junctions are regulated by intracellular signalling (Karczewski & Groot, 2000). It is reported that increases in intracellular Ca^{2+} concentration lead to the opening of tight junctions following the

phosphorylation of myosin regulatory light chains (Lindmark *et al.* 1998; Ma *et al.* 2000). Some food-derived chemicals or drugs are reported to enhance paracellular permeability following increases in intracellular Ca^{2+} concentration; for example, lysophosphatidyl choline (Sawai *et al.* 2002) and sodium caprate (Tomita *et al.* 1996).

A high intake of non-digestible saccharides (NDS) is known to protect against bowel cancer as well as to alleviate other bowel problems such as constipation and diverticular disease (Geoffrey, 2002). Current average intakes of NDS are about 12 g/d in the UK, although the intake of 25–30 g/d is generally recommended (Geoffrey, 2002). Recent studies have shown that feeding NDS enhances intestinal Ca absorption in rats (Suzuki *et al.* 1998; Hara *et al.* 2000) and in human volunteers (van den Heuvel *et al.* 1999). One proposed mechanism for this effect is the solubilisation of Ca salts by acids produced through the microbial fermentation of the saccharides in the large-intestinal lumen (Suzuki *et al.* 1998; van den Heuvel *et al.* 1999; Hara *et al.* 2000). The large intestine has been shown to be a major site of increasing Ca absorption by NDS when acidic fermentation takes place (Younes

et al. 1996; van den Heuvel *et al.* 1999). However, we recently showed the importance of the small intestine to increase Ca absorption by NDS as another possible mechanism. That is, intact NDS enhance Ca absorption via a paracellular route by the direct stimulation of the small-intestinal epithelium (Mineo *et al.* 2001, 2002*a*). In *in vitro* studies using isolated intestinal epithelium of rats, difructose anhydride (DFA) III, DFAIV (both disaccharides), fructo-oligosaccharides (a mixture of 3, 4, and 5 saccharides), and maltitol (a disaccharide sugar alcohol) have been shown to enhance paracellular Ca absorption (Mineo *et al.* 2001, 2002*b*). However, the cellular mechanisms by which these saccharides enhance paracellular permeability have not been clarified.

We hypothesise that the mechanism for the saccharide-induced activation of the paracellular route may involve intracellular Ca signalling. The present study was designed to explore the possibility of the direct influence of four NDS (DFAIII, DFAIV, fructo-oligosaccharides, and maltitol) on Ca signalling in rat enterocytes.

Materials and methods

Chemicals

DFAIII and DFAIV were kindly provided by Nippon Beet Sugar MFG, Ltd (Obihiro, Japan). DFAIII and DFAIV are disaccharides consisting of two fructose residues and are isomers of one another. Fructo-oligosaccharides, a mixture of 34 % 1-kestose, 53 % nystose and 9 % 1F-β-fructofuranosylnystose, were kindly provided by Meiji Seika Kaisha, Ltd (Tokyo, Japan). Maltitol, obtained by the hydrogenation of maltose, was purchase from Wako Pure Chemical Industries, Ltd (Osaka, Japan). All other chemicals were obtained from Wako Pure Chemical Industries, Ltd.

Animals

Twenty-four male Sprague–Dawley rats (7 weeks old; Japan SLC, Shizuoka, Japan) were housed in individual stainless-steel cages. The cages were placed in a room with controlled temperature ($22 \pm 2°C$), relative humidity (40–60 %) and lighting (lights on from 08.00 to 20.00 hours). The rats had free access to water and a solid laboratory chow (CE-2; Japan CLEA, Tokyo, Japan) for at least 1 week before experimentation. They were used in the experiments at 8–10 weeks old (250–350 g). The present 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.

Enterocyte isolation

On the day of the experiments, the rats were anaesthetised with pentobarbital sodium (30 mg/kg body weight), and the jejunum (100 mm in length after the ligament of Treitz) was quickly removed and washed with saline (154 mmol NaCl/l). The intestinal segments were everted with a blunt plastic rod and both ends ligated, and the everted sacs were sufficiently filled with Ca- and Mg-free

Hank's balanced salt solution (HBSS) supplemented with D-glucose (10 mmol/l) and L-glutamine (6 mmol/l) (saturated with 100 % O_2, pH 7·3; HBSS (−)). The everted intestines were placed into solution A (HBSS (−) supplemented with sodium citrate (27 mmol/l), pH 7·3) for 10 min at 37°C with continuous shaking (125 oscillations/min). They were then placed into solution B (HBSS (−) supplemented with EDTA (1·5 mmol/l) and dithiothreitol (0·5 mmol/l), pH 7·3) for 5 min at 37°C with continuous shaking (550 oscillation/min). The isolated enterocytes were sedimented by centrifugation for 3 min at 700 g, washed twice with solution C (HBSS (−) supplemented with CaCl$_2$ (1·26 mmol/l), MgCl$_2$ (0·49 mmol/l) and MgSO$_4$ (0·41 mmol/l), pH 7·3), and then re-suspended in solution C. Cell viability was assessed by the trypan blue technique. Exclusion of the dye in more than 90 % of the cells was observed after the isolation process.

Measurements of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was measured according to a general protocol on the basis of changes in the excitation spectrum of the fluorescent probe, fura-2, when complexed with free Ca ions. Three or four measurements per rat were carried out. The isolated enterocytes were loaded with fura-2–acetoxymethyl ester (10 μmol/l) and cremophor EL (0·2 g/l) in solution C for 20 min at 37°C in the dark. The cell suspension was washed three times with solution C to eliminate unloaded dye. The fura-2-loaded cells were put into cuvettes at a density of 2×10^6 cells/ml, then introduced into the sample chamber of a thermostatically controlled (37°C) spectrofluorometer (CAF-110; JASCO International Co., Ltd, Tokyo, Japan) with constant stirring (400 rpm). Following the measurement of the steady state for at least 30 s, solutions containing the individual NDS (1 or 10 mmol/l), capric acid (7·5 or 15 mmol/l), or glycerol (1 or 10 mmol/l) were added into the cuvette. Fura-2 fluorescence intensity was monitored at an emission wavelength of 500 nm by alternating the excitation wavelength between 340 and 380 nm with a dual excitation monochromator. The ratio of signals at 340 and 380 nm was calculated, and maximal and minimal intracellular probe fluorescences were determined by the addition of Triton X-100 (2 g/l) and EGTA (10 mmol/l) in tri(hydroxymethyl)-aminomethane (18 mmol/l), respectively (final concentration). The transformation of fluorescence signals into intracellular Ca^{2+} concentration was performed by the method of Grynkiewicz *et al.* (1985).

Calculations and statistics

All values are expressed as means with their standard errors. Intracellular Ca^{2+} concentration is expressed as nmol/l after subtraction from the concentration at the point when the solutions containing each NDS were added. Maximum changes in intracellular Ca^{2+} concentration were analysed by two-way ANOVA. Duncan's multiple-range test was used for comparisons among several groups (Duncan, 1955). A difference with $P < 0.05$ was considered significant. These statistical analyses were done by the general linear models procedure of the

Statistical Analysis Systems program (version 6.07; SAS Institute Inc., Cary, NC, USA).

Results

Changes in intracellular Ca^{2+} concentration in rat enterocytes were recorded before and after the addition of capric acid (7·5 or 15 mmol/l), glycerol (1 or 10 mmol/l), or each NDS (1 or 10 mmol/l) (Fig. 1). The addition of capric acid caused an immediate dose-dependent rise in intracellular Ca^{2+} concentration. Stimulation with each

NDS (1 or 10 mmol/l) caused an immediate dose-dependent rise in intracellular Ca^{2+} concentration. Treatment with DFAIII or fructo-oligosaccharides caused an initial rapid rise in intracellular Ca^{2+} concentration with the intracellular Ca^{2+} concentration reaching a peak level at about 30 s after stimulation, which was sustained for 90 s. Treatment with DFAIV or maltitol caused moderate, but continued, increases following the initial rapid rises in the intracellular Ca^{2+} concentration. The addition of a vehicle (solution C) and glycerol had no effect on intracellular Ca^{2+} concentration. Treatment with DFAIII and IV
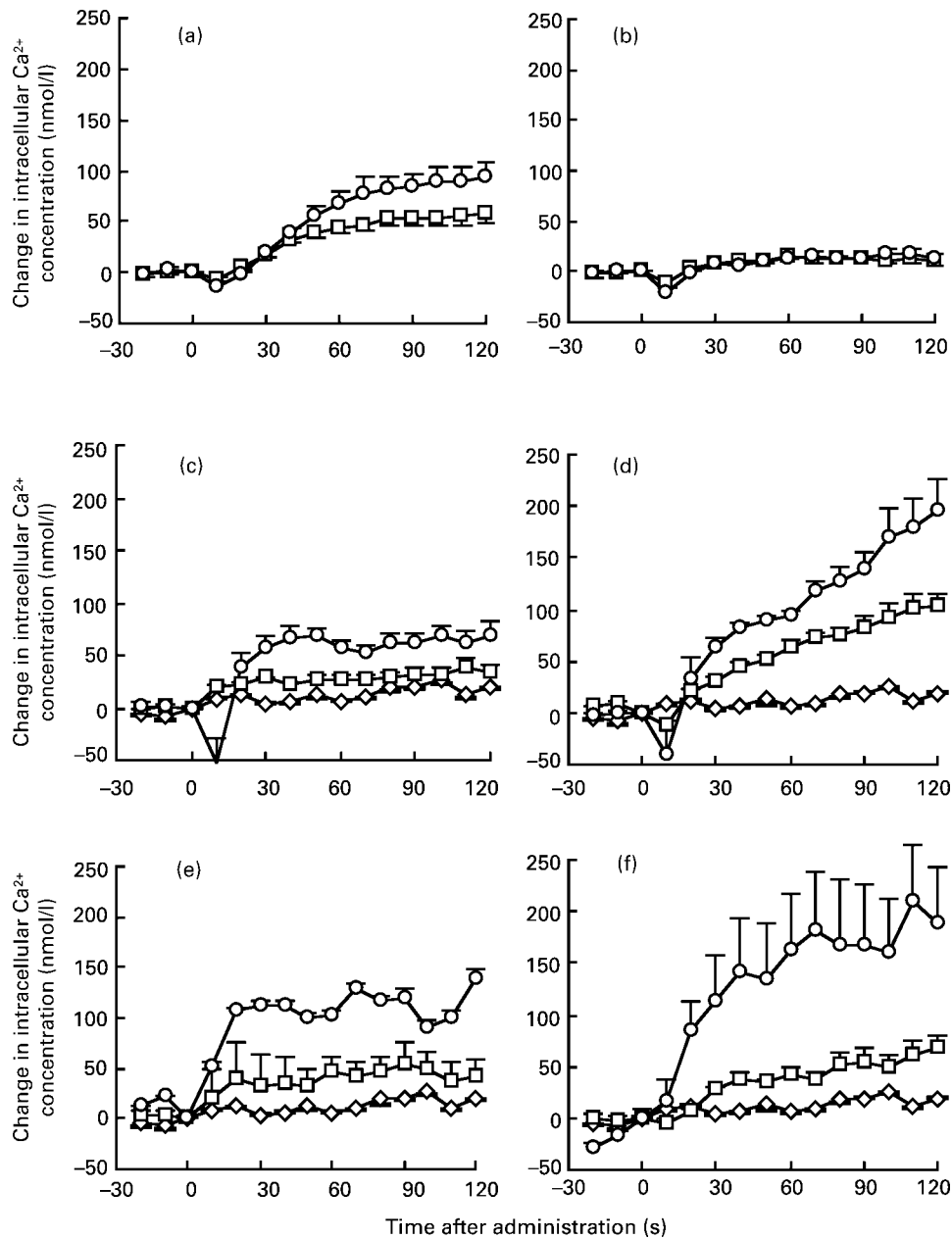
Fig. 1. Changes in intracellular calcium ion concentration in rat enterocytes after stimulation with (a) capric acid (□, 7·5 mmol/l; ○, 15 mmol/l), or with (b) glycerol, (c) difructose anhydride (DFA) III, (d) DFAIV, (e) fructo-oligosaccharides or (f) maltitol (◇, 0 mmol/l (vehicle); □, 1 mmol/l; ○, 10 mmol/l). Solutions containing each solute were added at 0 s. The data are mean values of four or five rats, with their standard errors represented by vertical bars.

Table 1. Maximum changes in intracellular calcium ion concentration (nmol/l) in rat enterocytes*

(Mean values with their standard errors)

Treatment	Mean	SE
1 mmol/l		
DFAIII	58·7[d]	10·4
DFAIV	117·9[cd]	23·9
Fructo-oligosaccharides	65·1[d]	19·2
Maltitol	87·2[d]	28·2
10 mmol/l		
DFAIII	136·7[bcd]	15·1
DFAIV	252·2[ab]	72·5
Fructo-oligosaccharides	238·3[abc]	53·6
Maltitol	270·7[a]	88·4
Two-way ANOVA (*P* values)		
Type of saccharides	0·128	
Dose of saccharides	< 0·001	
Type × dose	0·533	

DFA, difructose anhydride.

[a,b,c,d] Mean values with unlike superscript letters were significantly different according to Duncan's multiple range test ($P < 0.05$).

* The data were obtained after the application of each non-digestible saccharide (1 or 10 mmol/l).

caused an initial decrease in intracellular Ca^{2+} concentration at the higher concentration.

The maximum changes in intracellular Ca^{2+} concentration for 120 s after the application of the four NDS are presented in Table 1. The maximum changes in intracellular Ca^{2+} concentration induced by each saccharide (10 mmol/l) were, except for DFAIII, higher than the values for treatment with the lower concentration of each saccharide (1 mmol/l). The maltitol-induced increase was higher than that produced by DFAIII at 10 mmol/l. There were no significant differences in the values among the saccharides tested at 1 mmol/l.

Discussion

We demonstrated that each NDS (at 1 mmol/l) obviously increased intracellular Ca^{2+} concentration, but the NDS did so more strongly at 10 mmol/l in rat enterocytes. This result is consistent with previous reports, in which the NDS dose-dependently enhanced paracellular permeability in stripped rat jejunal mucosa (Mineo *et al.* 2001, 2002*a,b*). The NDS concentrations tested in the present study were chosen in consideration of following two rationales: first, the NDS concentrations were estimated to be in the range of 1·5–22 mmol/l in jejunal contents when rats were fed diets containing 30 g NDS/kg (T Suzuki, unpublished results). Second, the NDS of the concentrations are shown to promote Ca transport via a paracellular route in the rat jejunum (Mineo *et al.* 2001, 2002*a,b*). Also, the two rationales reveal that the concentrations of 1 and 10 mmol/l were in the physiological range.

A significant increase in intracellular Ca^{2+} concentration was observed after the application of capric acid (7·5 and 15 mmol/l), which was used as a positive control for the present experiment as it is known to enhance paracellular permeability subsequent to increases in intracellular Ca^{2+} concentration in the intestines (Tomita *et al.* 1996). The paracellular transport of solutes is regulated by tight junctions (Mitic *et al.* 2000). The opening of tight junctions is known to follow the condensation of actin microfilaments induced by myosin light-chain kinase (Lindmark *et al.* 1998; Ma *et al.* 2000), and the calmodulin-regulated protein, myosin light-chain kinase, is activated by an increase in intracellular Ca^{2+} concentration (Vetter & Leclerc, 2003). Furthermore, it has been reported that Ca absorption in the ileal sacs induced by maltitol was completely inhibited by a calmodulin antagonist (Kishi *et al.* 1996). These results indicate that the increases in intracellular Ca^{2+} concentration stimulated by the NDS may lead to the enhancement of paracellular Ca transport observed in previous studies (Mineo *et al.* 2001, 2002*a,b*).

Ca transport via the paracellular pathway, which is activated by NDS, occurs throughout the intestine and is non-saturable, and this transport mechanism is more important when Ca concentrations in the intestinal lumen are sufficiently high with adequate or high Ca intake (Bronner, 1998). In contrast, 1, 25-dihydroxy vitamin D_3-dependent active Ca transport is localised to the upper duodenum and is the dominant mechanism when Ca intake is low (Bronner, 1998). This suggests that feeding NDS enhances Ca absorption throughout the intestine, and feeding NDS combined with Ca is effective to prevent postmenopausal or age-related osteoporosis.

We found that treatment with a vehicle and glycerol had no effects on intracellular Ca^{2+} concentration. This finding suggests that the effect of the NDS is not merely mechanical or osmotic stimulation, and that common structures in these saccharides are recognised by the enterocytes and induce Ca signalling. However, the profiles of the intracellular Ca^{2+} concentration increases vary between the four saccharide treatments. Increases in intracellular Ca^{2+} concentration induced by DFAIII or fructo-oligosaccharides reached a plateau level after initial rapid rises. In contrast, the application of DFAIV or maltitol led to continued moderate increases throughout the whole 120 s. The maximum changes obtained by treatment with maltitol (10 mmol/l) was higher than that with DFAIII (10 mmol/l), though the two saccharides similarly increased Ca absorption in stripped rat intestinal mucosa (Mineo *et al.* 2001). Both DFA and maltitol are disaccharides, and fructo-oligosaccharide is a mixture of 3, 4, and 5 saccharides. This suggests that a specific molecular structure rather than the molecular size of the saccharides is important in inducing Ca signalling. The reason for the initial decreases in DFAIII and IV is not known; however, these saccharides may influence fluorescence themselves. Having accounted for this initial decrease, the increments of intracellular Ca^{2+} concentration after the decrease by DFAIII and IV is comparable with those of fructo-oligosaccharides and maltitol, respectively.

The intracellular Ca^{2+} concentration is determined by the balance between the passive influx of Ca^{2+} from both extracellular and intracellular compartments and their active extrusion from the cytosol (Berridge *et al.* 2003). Ca^{2+} entry from outside occurs through various channels located on the cellular membrane, whereas the release of Ca^{2+} from internal stores takes place through either inositol 1, 4, 5-trisphosphate- or ryanodine-sensitive Ca^{2+} channels (Berridge *et al.* 2003). Capric acid, used as a

positive control in the present study, has been shown to increase intracellular Ca^{2+} concentration independent of extracellular Ca^{2+} in Caco-2 cells (Tomita *et al.* 1994). Further investigations are required to determine which routes are responsible for the increases in enterocyte intracellular Ca^{2+} concentration induced by NDS.

The present study was performed with non-polarised isolated intestinal cells that do not possess functional tight junctions. The tight junction has roles not only in regulating paracellular transport of solutes but also in distinguishing between apical and basolateral molecules. Capric acid similarly enhances paracellular permeability from the apical and basolateral side, while decanoylcarnitine has a stronger effect when applied to the apical than to the basolateral side of Caco-2 cell monolayers (Tomita *et al.* 1996). It is important to examine whether the putative sensory system for the NDS leading to the increases in intracellular Ca^{2+} concentration is located on the apical membrane because the basolateral concentration of saccharides is much less than the apical concentration under physiological conditions. It is necessary to examine this mechanism in cultured cell monolayers bearing tight junctions in a future study, while the results of the present study performed in normal intestinal cells are important even though the cells do not have the polarity.

In summary, four NDS, DFAIII, DFAIV, fructo-oligosaccharides, and maltitol, directly stimulated rat enterocytes, and induced intracellular Ca signalling. The saccharide-induced Ca signalling may lead to the activation of the paracellular route.

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