Physiological concentrations of short-chain fatty acids immediately suppress colonic epithelial permeability

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Colonic fermentation products, SCFA, have various effects on colonic functions. Here, we found that physiological concentrations of SCFA immediately promote epithelial barrier function in the large intestine. Solutions of mixed and individual SCFA were applied to the caecal walls mounted on Ussing-type chambers. Transepithelial electrical resistance (TER) increased rapidly and reached a peak 35 % higher than that in the control specimen within 10 min post application of the SCFA mixture (80 acetate, 40 propionate, 20 butyrate (mmol/l)). The Lucifer yellow permeability, a paracellular transport marker, was dose-dependently reduced by the mixed SCFA, acetate and propionate solutions. Inhibition of monocarboxylate transporter-1 did not influence the increase in TER with acetate; however, lowering the pH (from 7·5 to 5·5) clearly enhanced the effect of acetate. Non-metabolizable, bromo and chloro derivatives of SCFA also increased TER. These results suggest that passive diffusion of SCFA is dominant and the metabolism of SCFA is not required for the promotive effect of SCFA on barrier function. We also observed that individual SCFA dose-dependently increased TER in T84 and Caco-2 cells, which indicates that SCFA directly stimulate epithelial cells. Depletion of membrane cholesterol and inhibitors of phosphatidylinositol-3 kinase and Gq protein attenuated the acetate-mediated promotive effect. Finally, we found that the mucosal application of the SCFA mixture dose-dependently suppressed [³H] mannitol transport from the caecal lumen to the mesenteric blood in the anaesthetized rats. We conclude that physiological concentrations of SCFA immediately enhance barrier function of the colonic epithelium through cholesterol-rich microdomain in the plasma membrane.

SCFA: Barrier function: Tight junction: Caecum: T84 cells: Caco-2 cells

The fermentation products of intestinal bacteria, SCFA, have various physiological effects on the large intestine. These organic acids promote colonic epithelial cell proliferation[1,2], mucosal blood flow[3] and colonic motility[4,5]. Moreover, butyrate is the principal energy source for the colonic epithelia[6,7] and excludes mutated mucosal cells through the induction of apoptosis[8,9]. These activities contribute to the maintenance of large intestinal function.

A number of commensal and possibly some pathogenic bacteria colonize the large intestinal lumen and produce harmful substances. To maintain beneficial relationships between the host and intestinal microbes, the barrier functions of the colonic mucosa are critical and the tight junction between the mucosal epithelial cells is the primary physical barrier in the intestines. The tight junction is composed of several transmembrane proteins, such as claudins[10] and occludin[11], and cytoplasmic plaque proteins, such as zonula occludens[12].

The permeability of the tight junction is closely regulated via intracellular signalling[13]; however, there is limited information on the effects of luminal factors apart from mid-chain fatty acids, which are known to induce a rapid increase in paracellular permeability via activation of myosin light chain kinase[14]. Non-digestible saccharides are known to enhance paracellular transport in the intestines[15,16]. It is also well known that SCFA are produced from non-digestible saccharides through intestinal bacterial fermentation. In an intestinal epithelial model consisting of cultured Caco-2 cell monolayers, SCFA, particularly butyrate, reduced monolayer permeability with increasing transepithelial electrical resistance (TER) during cell culture[17]. This effect of butyrate on TER reportedly depends on the promotion of cell differentiation, as butyrate is known as an inducer of cell differentiation[18,19] and the significant effects on TER appeared at a considerable time, more than 24 h, after the application of butyrate[17].

The present study was conducted to define details of the short-term effects of SCFA on mucosal barrier function and to examine the specificity of the organic acids and involvement of intracellular signalling molecules in the SCFA-mediated effect in rat isolated caecal mucosa, anaesthetized rats and cultured epithelial cell monolayers. We found that physiological concentrations of SCFA, particularly acetate, suppressed the permeability of the caecal mucosa within 10 min. This result differs from previous observations and represents a newly identified phenomenon.

Materials and methods

Chemicals

Genistein (an inhibitor of protein tyrosine kinase), LY294002 (an inhibitor of phosphatidylinositol-3 kinase), YM-254890 (an inhibitor of Gq protein), Calphostin C (a inhibitor of protein kinase C) and Y-27 536 (an inhibitor of Rho-associated protein kinase) were purchased from Calbiochem (San Diego, CA). [³H] mannitol (specific activity, 740 TBq/mol) was purchased from American Radiolabeled Inc. (St Louis, MO, USA). All other chemicals were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Animals and diets

Male Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan), weighing about 200 g, were acclimatized with a solid unpurified diet (CE-2; Clea Japan, Tokyo, Japan) for at least 3 d and fasted for 1 d before experiments. The diet contained 50·5 % carbohydrate, 25 % protein, 4·7 % fat, 4·0 % fibre, 6·8 % ash and 9 % moisture.

The present study was approved by the Hokkaido University Animal Committee and animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Paracellular permeability in the caecal wall using Ussing chambers

A Ussing chamber technique was used to investigate the suppressive effect of SCFA on paracellular permeability in the caecum as described previously[15,20]. Briefly, the caecum was quickly removed from rats under pentobarbital anaesthesia (sodium pentobarbital, 40 mg/kg). Each specimen was cut open along the mesenteric border to produce a flat sheet and then rinsed with ice-cold Hanks' balanced salt solution (HBSS) (117 NaCl, 5·4 KCl, 0·4 KH_2PO_4, 4·2 $NaHCO_3$, 0·3 Na_2HPO_4, 1·3 $CaCl_2$, 0·5 $MgCl_2$, 0·4 $MgSO_4$, 10 piperazine-1,4-bis(2-ethanesulfonic acid) dipotassium salt, 10 D-glucose, 4 L-glutamine (mmol/l), pH 7·4). Appropriately prepared caecal tissues were mounted on Ussing chambers (Costar, Cambridge, MA, USA) that exposed a circular area of epithelium of 0·64 cm^2. The serosal and mucosal sides of the specimens were bathed in 1 ml HBSS at pH 7·4 and 6·5, respectively, at 37°C to mimic physiological conditions and the medium was continuously exposed to 100 % O_2 gas. After a 10 min stabilization period, the mucosal medium was replaced with SCFA-containing HBSS (acetate–propionate–butyrate 80:40:20, 40:20:10 and 20:10:5 (mmol/l), pH 6·5). The composition of SCFA was based on that in the rat caecum[20,21]. Acetate, propionate and butyrate were each added to the mucosal medium to examine the potency of individual SCFA. The effect of acetate (40 mmol/l) was examined in the mucosal medium at different pH values (5·5, 6·5 and 7·5). The pH in rat caecum and human faeces is reportedly between 5 and 8[22,23]. Non-metabolizable bromoacetate, chloroacetate (80 mmol/l) and bromopropionate (40 mmol/l) were added to the mucosal medium to examine whether metabolism of the SCFA is essential for their effect. The SCFA and their derivatives were substituted with NaCl so as not to impair the isotonicity of medium. To investigate

whether monocarboxylic acid transporter (MCT)-1 is involved in the acetate effect (80 mmol/l), inhibitors of MCT-1[24,25], 2-cyano-4-hydroxycinnamate (CHC, 10 mmol/l) and phloretin (1 mmol/l) were added to the mucosal and serosal compartments during experiments.

Paracellular permeability was evaluated by measurement of TER and unidirectional Lucifer yellow flux as described previously[15,16]. Lucifer yellow (50 µmol/l) was added to the mucosal medium at the beginning of incubation. After experiments, the medium was removed and fluorescence was measured using a fluorescence reader (CAF-110; JASCO International Co. Ltd, Tokyo, Japan). The mucosal:serosal flux was calculated as nmol/h per cm^2 surface area. TER was measured at various time points during the experiments using a Millicell-ERS system (Millipore, Bedford, MA, USA) as described previously[15,20]. TER was expressed as Ω per cm^2 surface area or % of initial values.

Transport rates of acetate from the mucosal to serosal side were evaluated by measurement of acetate concentration transferred into the serosal medium. The concentration was measured by the previously described method using HPLC (LC-10ADpv; Shimadzu, Kyoto, Japan) equipped with two Shim-pack SCR-102H columns (8 mm internal diameter, 30 cm long; Shimadzu) and an electroconductibility detector (CDD-6A, Shimadzu)[26].

Cell culture

T84 cells (CCL-248; American Type Culture Collection, Rockville, MD, USA) and Caco-2 cells (HTB-37; American Type Culture Collection) were propagated and maintained in high-glucose (4·5 g/l D-glucose) Dulbecco's modified Eagle's medium supplemented with 100 ml/l decomplemented fetal bovine serum, 44 mmol/l sodium bicarbonate, 1 mmol/l sodium pyruvate, 50 000 U/l penicillin and 50 mg/l streptomycin and adjusted to pH 7·4. Cells were grown on polyester membranes in Transwell inserts (12 mm; Costar) and experiments were conducted on days 25–27 post seeding. T84 cells between passages 66 and 88 and Caco-2 cells between passages 40–60 were used for experiments.

Paracellular permeability in the T84 and Caco-2 cell monolayer

T84 and Caco-2 cell monolayers were washed with HBSS and the basal and apical chambers of the cells were bathed in 1·5 ml HBSS at pH 7·4 and 0·8 ml HBSS at pH 6·5, respectively. After a 60 min equilibration period, the experiments were initiated by application of SCFA-containing HBSS to the apical wells (0–80 acetate, 0–40 propionate and 0–20 butyrate (mmol/l), pH 6·5). To examine the specificity of the suppressive effect of SCFA on paracellular permeability, three other organic acids (formate, lactate and succinate; 50 mmol/l) were used in T84 cells. Paracellular permeability was demonstrated by measurement of TER and Lucifer yellow transport as described earlier.

Treatment of cells with methyl-β-cyclodextrin and sphingomyelinase

To examine the involvement of cholesterol-rich membrane microdomain, such as raft or caveolae, in the promotive

effect of acetate on barrier function, methyl-β-cyclodextrin (MCD, a cholesterol-depleting agent) and sphingomyelinase (SMase) were used. Caco-2 cell monolayers were pre-treated with MCD (0, 0·2 and 0·4 %) or SMase (0, 1, 2 U/ml) for 1 h prior to the experiment and incubated with or without 80 mmol/l acetate. Paracellular permeability was evaluated by measurement of TER as described earlier.

Experiments with intracellular signalling inhibitors

Caco-2 cell monolayers were pre-treated with the following six intracellular signalling inhibitors to investigate whether their activities were required for the acetate-mediated effect on barrier function: Y27632, 10 μmol/l; genistein, 200 μmol/l; Calphostin C, 1 μmol/l; LY294002, 25 μmol/l; YM-254890, 5 μmol/l; ML-7, 30 μmol/l. Cells were incubated with or without 80 mmol/l acetate and paracellular permeability was evaluated by measurement of TER as described earlier.

Paracellular permeability in the caecum using in situ loops

A perfusion segment of the caecum was prepared in each rat through an abdominal midline incision (about 3 cm) under pentobarbital anaesthesia. Briefly, the proximal and distal ends of the caecum were tied and a small cut was made at the distal end of the caecum. The lumen was gently washed out with saline and the experiment was initiated by the injection of 3 ml HBSS containing [^{3}H] mannitol (7·4 MBq) at pH 6·5. To examine the effect of SCFA on paracellular permeability, HBSS containing SCFA (acetate–propionate–butyrate 80:40:20 and 40:20:10 (mmol/l)) was applied. The paracellular permeability was evaluated by measurement of mannitol transport from the caecal lumen into the mesenteric blood. Blood samples were collected via the caecal vein 30 min after injection and the radioactivity of [^{3}H] mannitol was measured by means of a liquid scintillation counting system (LSC-5100; Aloka, Tokyo, Japan)[16]. The permeability of mannitol was calculated as pmol/h per g caecal tissue.

Statistical analysis

All experiments were conducted at least three times. All values are expressed as means with their standard errors of the mean. Statistical analyses was performed by a one-way ANOVA, two-way ANOVA or repeated measure two-way ANOVA followed by Duncan's multiple range test[27] (one-way ANOVA, Tables 1, 3, 4, 5 and 7; two-way ANOVA, Tables 2 and 3 and Figs. 3 and 4; repeated measure two-way ANOVA, Figs. 1 and 2). A difference with $P < 0·05$ was considered significant. These statistical analyses were performed using the general linear models procedure of the Statistical Analysis Systems program (version 6.07; SAS Institute Inc., Cary, NC, USA).

Results

TER of the caecal wall was slightly but significantly higher after a mucosal application of medium containing a low level of SCFA (70 mmol/l) compared with that after the application of a SCFA-free medium and the TER values after treatment with a high but physiological level of SCFA (140 mmol/l) was much higher than those after treatment with low levels of SCFA at all time points (Fig. 1). The TER of the caecal tissues reached a maximum 10 min after the application of 70 and 140 mmol/l SCFA and maintained a high value for 60 min. After an application of 40 or 80 mmol/l acetate and 20 or 40 mmol/l propionate, TER was dose-dependently higher than that in the absence of SCFA. Butyrate did not significantly affect caecal wall TER. We also observed that a serosal application of SCFA did not increase TER (data not shown).

Lucifer yellow transport rates, an indicator of the paracellular pathway, were dose-dependently reduced by a mucosal application of SCFA, acetate and propionate, but not by butyrate, within physiological concentrations (Table 1). The reduction in the transport rates by each application was 40–50 % of the rate in the absence of SCFA.

Inhibitors of MCT-1, CHC and phloretin, which have a role in SCFA absorption in the intestinal epithelial cells, did not attenuate the increases in the TER of the caecal wall induced by the application of 80 mmol/l acetate (Table 2). The addition of CHC itself increased TER, whereas the addition of phloretin reduced TER in the absence of acetate. However, the application of 80 mmol/l acetate recovered the TER to the same level as that induced by acetate in the absence of the inhibitors.

TER and Lucifer yellow transport across the caecal wall changed in a pH-dependent manner (Table 3). Decreasing the pH of the mucosal medium to 5·5 amplified the enhancement of TER induced by 80 mmol/l acetate, although the change in pH did not have any effect on TER by itself. Paracellular transport, as indicated by Lucifer yellow, after application of acetate was higher in the pH 7·5 medium with acetate than in the pH 6·5 or 5·5 medium. Lucifer yellow permeability was higher in the pH 7·5 in the absence of acetate. Acetate transport from the mucosal to serosal sides increased as the pH of the mucosal medium was decreased.

The caecal wall TER after an application (80 mmol/l) of acetate derivatives, bromoacetate or chloroacetate, was higher than that in the absence of SCFA (Table 4). The increased levels of TER after the application of these derivatives were similar to the level after an application of acetate. The TER after bromopropionate (40 mmol/l) treatment was also greater than that in the absence of SCFA, with the effect of bromopropionate being comparable to that of propionate.

In fully differentiated cultured T84 cell monolayers, TER increased dose- and time-dependently with increases in the physiological concentration of acetate and propionate, but not butyrate (Fig. 2). The TER of the colonic epithelium model rapidly increased after the application of 40 and 80 mmol/l acetate and reached maximum levels 30 min post application. The TER values in the presence of 40 and 80 mmol/l acetate and 20 and 40 mmol/l propionate were significantly higher than those in the absence of SCFA 15–120 min post application. The increases in TER by acetate and propionate were also observed in another human intestinal Caco-2 cell (Table 5). The TER values in Caco-2 cells incubated with 40 and 80 mmol/l acetate and 20 and 40 mmol/l propionate were significantly higher than each control value. The changes in TER in T84 and Caco-2 cells after the application of each SCFA were very similar to those in the rat caecal wall.
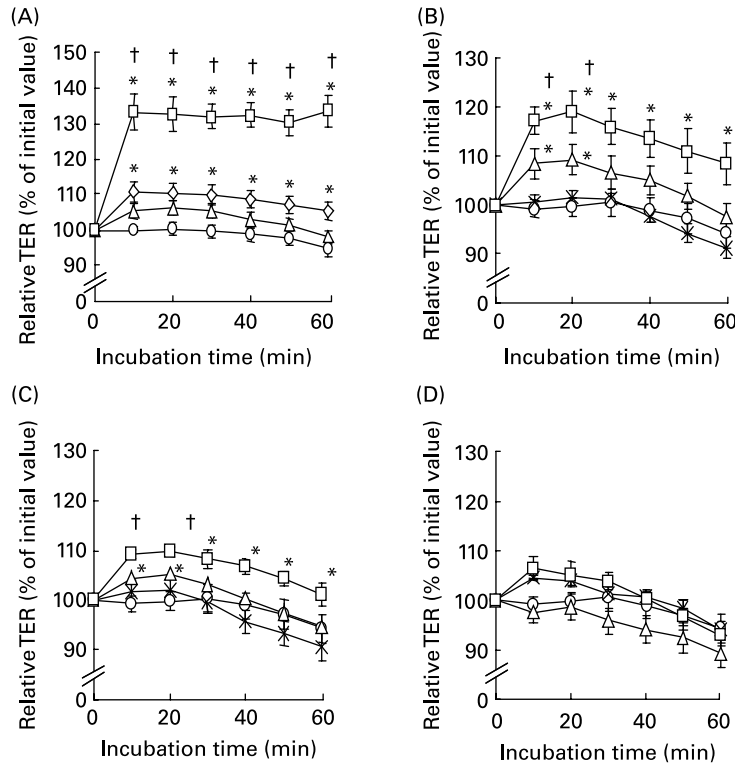
Fig. 1. Transepithelial electrical resistance (TER) in the rat caecal wall mounted on Ussing chambers incubated in the absence or presence of mixed SCFA (○, 0 mmol/l; △, 35 mmol/l; ◇, 70 mmol/l; □, 140 mmol/l) (A), acetate (○, 0 mmol/l; ×, 20 mmol/l; △, 40 mmol/l; □, 80 mmol/l) (B), propionate (○, 0 mmol/l; ×, 10 mmol/l; △, 20 mmol/l; □, 40 mmol/l) (C) and butyrate (○, 0 mmol/l; ×, 5 mmol/l; △, 10 mmol/l; □, 20 mmol/l) (D) in the mucosal chambers. Values are means with their standard errors for five rats. *$P < 0.05$ v. 0 mmol/l at each time point. †$P < 0.05$ v. 70 mmol/l SCFA (A), 40 mmol/l acetate (B) and 20 mmol/l propionate (C) at each time point. For details of animals and procedures, see Materials and methods.

The TER in T84 cell monolayers was higher 30 min after the application of the same concentrations (50 mmol/l) of propionate, acetate and butyrate and lactate, which increased TER in that order, compared with TER of the control group (Table 6). Formate did not change TER and succinate markedly reduced the TER of the monolayer.

Depletion of membrane cholesterol by MCD attenuated the acetate-mediated promotive effect on barrier function in a MCD dose-dependent manner (Fig. 3 (A)). The TER values in Caco-2 cells pre-treated with 0.2 and 0.4 % MCD were significantly lower than those without pre-treatment in the presence of acetate. No differences were observed in TER values between control and acetate groups in 0.4 % MCD treatment. The MCD treatment did not affect TER values by itself (control 1413 (SEM 18); 0.2 % MCD 1430 (SEM 73); 0.4 % MCD 1406 (SEM 29), after each treatment). Digestion of sphingomyelin by SMase also impaired the acetate-mediated effect on barrier function just like the MCD treatment (Fig. 3(B)). The TER value in the cells pre-treated with low-dose SMase (1 U/ml) was slightly, but not significantly, lower than that without pre-treatment in the presence of acetate. The TER values were significantly lower in the 2 U/ml SMase treatment than those in the 0 and 1 U/ml SMase treatments in the presence of acetate. There was no difference in TER values between the control and acetate groups in the 2 U/ml SMase treatment. The SMase treatment did not significantly change the TER values by itself

Table 1. Lucifer yellow transport (pmol/h per cm²) in the rat caecal wall mounted on Ussing chambers incubated in the absence or presence of mixed SCFA, acetate, propionate and butyrate in the mucosal chambers†

(Values are means with their standard errors of the mean for five rats)

Treatment	Mean	SEM
Mixed SCFA		
0 mmol/l	195	7
35 mmol/l	161	16
70 mmol/l	160	17
140 mmol/l	114*	5
Acetate		
0 mmol/l	122	14
8 mmol/l	113	9
40 mmol/l	79*	5
80 mmol/l	76*	14
Propionate		
0 mmol/l	122	14
4 mmol/l	101	15
20 mmol/l	86*	5
40 mmol/l	68*	4
Butyrate		
0 mmol/l	122	14
2 mmol/l	103	11
10 mmol/l	108	12
20 mmol/l	90	7

*$P < 0.05$ v. 0 mmol/l of each set, Duncan's multiple range test. One-way ANOVA was performed for each set (mixed SCFA, acetate, propionate and butyrate).
† For details of animals and procedures, see Materials and methods.

Table 2. Transepithelial electrical resistance (% of initial values) at 30 min in the rat caecal wall mounted on Ussing chambers incubated in the absence or presence of acetate with or without monocarboxylic acid transporter-1 inhibitors, 2-cyano-4-hydroxycinnamate and phloretin†

(Mean values with their standard errors for five rats)

Treatment	Mean	SEM
No inhibitor		
Control	102^c	2
Acetate	118^b	3
2-Cyano-4-hydroxycinnamate		
Control	111^b	3
Acetate	130^a	2
Phloretin		
Control	91^b	2
Acetate	117^d	2
Two-way ANOVA		
Inhibitor (I)		<0·01
Acetate (A)		<0·01
I × A		<0·01

a,b,c,d Mean values without a common superscript letter among treatments were significantly different ($P<0.05$, Duncan's multiple range test).
† For details of animals and procedures, see Materials and methods.

(0 U/ml, 1628 (SEM 57); 1 U/ml, 1467 (SEM 47); 2 U/ml, 1574 (SEM 73), after each treatment).

Inhibitors of phosphatidylinositol-3 kinase and Gq protein attenuated the promotive effect of acetate on barrier function in Caco-2 cells (Fig. 4). The TER in cells with LY294002 and YM254690 was lower than that without inhibitors in the presence of acetate. The other inhibitors, Y27632, genistein, Calphostin C and ML-7, did not influence the acetate-effect. The TER value in LY294002 treatment was lower and those in YM254690 and ML-7 were higher than the control value in the absence of acetate.

Transport of [^{3}H] mannitol, a paracellular route marker, from the caecal lumen to the mesenteric blood in anaesthetized rats after an injection of 140 mmol/l SCFA into the caecal lumen fell by more than 50 % compared with that after the injection of a SCFA-free medium, as shown in Table 7. The suppression of paracellular transport was dose-

dependent for both the SCFA mixture and acetate alone, but the changes for latter were not statistically significant.

Discussion

The present study demonstrates that higher SCFA concentrations, within the physiological range, enhance barrier function in the caecum, as indicated by increases in TER and decreases in the transport rates of a paracellular marker. The caecum is the segment of the rat alimentary tract in which the largest numbers of microbes exist. The tight junction represents a physical barrier to the intestinal epithelium[28,29]. This physical barrier is important in man and animals to prevent the permeation of toxic compounds produced by pathogenic bacteria and is also essential for them to co-exist with commensal bacteria.

We used sections of the whole caecal wall mounted on Ussing chambers to maintain the intact structure of the caecal epithelium and began experiments immediately after the removal of the tissues with confirmation of parallel changes in the paracellular transport between the whole caecal wall and the stripped caecal mucosa (unpublished results). The present study also shows rapid increases in TER in the presence of SCFA in T84 and Caco-2 cultured cell monolayers, a well-known model of the intestinal epithelium. The dose-dependent and time-dependent changes in TER induced by the apical application of acetate, propionate and butyrate were very similar to those observed in the rat caecal wall. These results reveal that the effects of SCFA in the caecum are evoked through direct action on the epithelial cells of the caecum, not on the vascular or nervous system of the caecal wall, even though SCFA are known to affect intestinal blood flow[3] and to induce contraction of smooth muscle in the colon via the enteric nervous system[5,30]. We also found that SCFA had a promotive effect on the caecal barrier function using anaesthetized rats. Permeation of [^{3}H] mannitol, which is a paracellular transport marker, into the blood was markedly and dose-dependently reduced by a luminal application of SCFA at the same concentrations as those in

Table 3. Transepithelial electrical resistance, Lucifer yellow transport and acetate transport in the rat caecal wall mounted on Ussing chambers incubated in the absence or presence of acetate at various pH levels (5·5, 6·5 and 7·5) in the mucosal chambers*

(Mean values with their standard errors for five rats)

		Transepithelial electrical resistance						Lucifer yellow permeability (pmol/h per cm^2)		Acetate transport (nmol/h per cm^2)	
		Ω per cm^2				% of initial values					
		0 min		20 min		20 min					
pH in mucosal medium	Treatment	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
pH 5·5	Control	228	4	240	4	104^c	1	92^{bc}	7	–	–
	Acetate	252	6	365	17	155^a	6	51^c	4	1·9^a	0·2
pH 6·5	Control	231	5	231	6	103^c	3	103^b	16	–	–
	Acetate	252	10	298	17	121^b	5	77^{bc}	6	1·6^a	0·0
pH 7·5	Control	241	8	235	8	100^c	2	176^a	25	–	–
	Acetate	244	7	265	7	115^b	2	121^b	17	1·2^b	0·1
One- or two-way ANOVA	pH	NS		<0·01		<0·01		<0·01		<0·01	
	Acetate	NS		<0·01		<0·01		<0·01		–	
	pH × Acetate	NS		<0·01		<0·01		<0·01		–	

a,b,c Mean values without a common letter among treatments were significantly different ($P<0.05$, Duncan's multiple range test).
* For details of animals and procedures, see Materials and methods.

Table 4. Transepithelial electrical resistance (% of initial values) at 30 min in the rat caecal wall mounted on Ussing chambers incubated in the absence or presence of derivatives of acetate and propionate in the mucosal chambers†

(Mean values with their standard errors for five rats)

Treatment	Mean	SEM
Derivatives of acetate (80 mmol/l)		
Control	102	3
Acetate	120*	4
Bromoacetate	122*	4
Chloroacetate	123*	5
Derivatives of propionate (40 mmol/l)		
Control	97	1
Propionate	113*	3
Bromopropionate	111*	3

*$P < 0.05$ *v.* control value of each set, Duncan's multiple range test. One-way ANOVA was performed for each set (derivatives of acetate and propionate).

† For details of animals and procedures, see Materials and methods.

the experiments using caecal tissue and cultured epithelial monolayers. The results indicate that the effects of SCFA on TER and paracellular permeation across the epithelium have physiological implications for the large intestinal barrier function.

The promotive effects of SCFA on barrier function indicated by TER appeared rapidly, just 10 min after mucosal application. There are a few previous reports showing that SCFA, particularly butyrate, increased the TER of Caco-2 monolayers[17]. However, that effect only emerged clearly more than 24 h after the addition of butyrate to the culture medium. In the present study, a physiological concentration of butyrate, which is higher than the concentration used in the previous study, did not significantly influence paracellular permeability in T84 and Caco-2 cell monolayers or caecal tissue. Together with the much faster effects of SCFA on TER and the different specificity among each SCFA, the findings on the effects of SCFA on colonic barrier functions shown in the present study represent a distinct phenomenon and mechanism from previous observations. The long-term action of butyrate in Caco-2 monolayers shown in the previous reports is associated with the promotive effect of butyrate on cell differentiation[18,19]. On the other hand, Mariadason *et al.* showed that butyrate at 10 mmol/l increased colonic mucosal permeability and decreased TER[31]. The present findings seem to disagree with those of this previous study; however, this inconsistency is possibly caused by differences between the caecum and colon. The caecum is the predominant segment for bacterial fermentation and the production of SCFA in the rat intestines and the physiological concentration of butyrate is much higher in the caecal lumen than in the colonic lumen[32], which may affect the mucosal tissue response to butyrate.

We found that MCT-1 inhibitors, CHC and phloretin, did not attenuate the effect of acetate, although MCT is one of the major transporters of SCFA in intestinal cells[24]. On the other hand, lower pH levels in the mucosal medium clearly amplified the promotive effect of acetate on TER with concomitant increases in acetate transport. Simple diffusion of SCFA, which is another major transport pathway[33], is accelerated at lower pH values, as protonated acetate more easily penetrates the cell membrane. These results suggest that the

permeation of SCFA into the cell membrane, but not the uptake of SCFA by MCT-1 in the epithelial cells, is involved in the promotion of barrier function by SCFA. The reason why the media in pH 7·5 and phloretin themselves increased
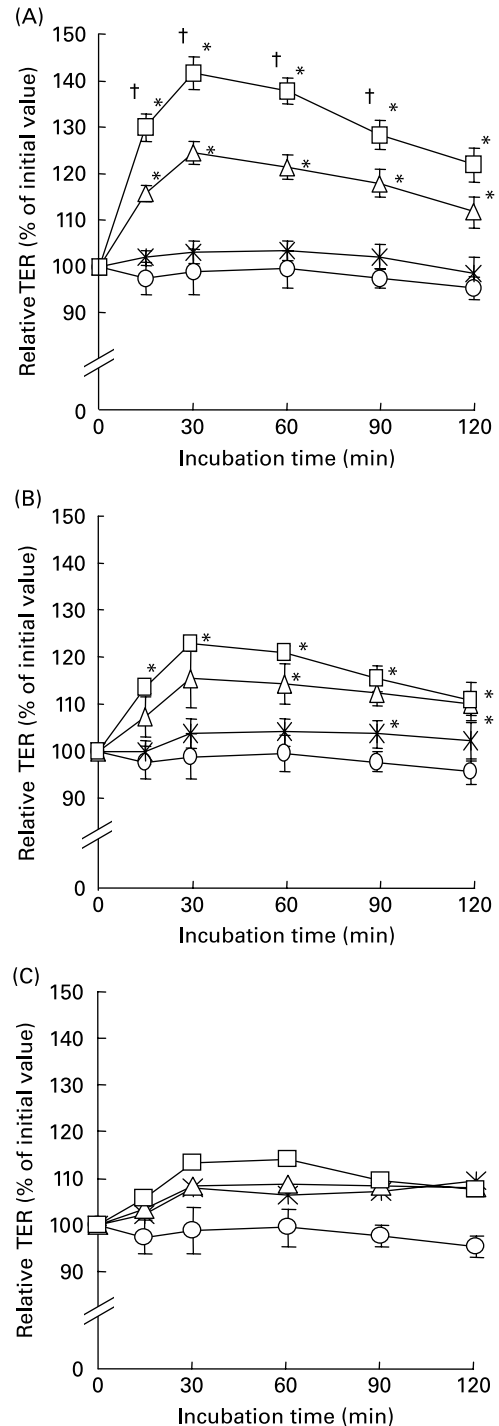

Fig. 2. Transepithelial electrical resistance (TER) in T84 cell monolayers incubated in the absence or presence of acetate (○, 0 mmol/l; ×, 8 mmol/l; △, 40 mmol/l; □, 80 mmol/l) (A), propionate (○, 0 mmol/l; ×, 4 mmol/l; △, 20 mmol/l; □, 40 mmol/l) (B) and butyrate (○, 0 mmol/l; ×, 2 mmol/l; △, 10 mmol/l; □, 20 mmol/l) (C) in the apical chambers. Values are means with their standard errors for five monolayers. *$P < 0.05$ *v.* 0 mmol/l at each time point. †$P < 0.05$ *v.* 40 mmol/l acetate (A) at each time point. For details of animals and procedures, see Materials and methods.

Table 5. Transepithelial electrical resistance (% of initial values) at 30 min in Caco-2 cell monolayers incubated in the absence or presence of acetate, propionate and butyrate†

(Mean values with their standard errors for five rats)

Treatment	Mean	SEM
Acetate		
0 mmol/l	100	1
8 mmol/l	112	1
40 mmol/l	119*	2
80 mmol/l	134*	2
Propionate		
0 mmol/l	100	1
4 mmol/l	111	1
20 mmol/l	117*	1
40 mmol/l	123*	1
Butyrate		
0 mmol/l	108	1
2 mmol/l	113	1
10 mmol/l	114	1
20 mmol/l	115	1

* $P < 0.05$ v. 0 mmol/l of each set, Duncan's multiple range test. One-way ANOVA was performed for each set (acetate, propionate and butyrate).
† For details of animals and procedures, see Materials and methods.

paracellular permeability is unclear, although the latter is consistent with the results of previous work[34].

We demonstrated that non-metabolizable acetate and propionate derivatives also induced the enhancement of barrier function in the caecum in a manner very similar to that of acetate and propionate themselves. This result indicates that metabolism of SCFA is not necessary to the induction of their promotive effects on TER. In our examination of the specificity of organic acids produced by caecal fermentation using T84 monolayers, three major fermentation products, acetate, propionate and butyrate, produced comparable effects on barrier function at the same concentration, which indicates that chain length of the fatty acids is not important in inducing TER enhancement. However, lactate and formate had less or no effect on TER enhancement. These results suggest that the hydrophobicity of side-chains in organic acids is involved in their effect on TER. Succinate markedly reduced the TER of the T84 monolayers. The mechanism underlying this effect is not clear, but it is possible that the chelating action

Table 6. Transepithelial electrical resistance (% of initial values) at 30 min in T84 cell monolayers incubated in the absence or presence of acetate, propionate, butyrate, formate, lactate and succinate in the apical chambers*

(Mean values with their standard errors for four monolayers)

Treatment	Mean	SEM
Control	111[d]	3
Acetate	135[b]	2
Propionate	144[a]	1
Butyrate	136[b]	5
Formate	115[d]	1
Lactate	127[c]	1
Succinate	42[e]	2

[a,b,c,d,e] Mean values without a common superscript letter among treatments were significantly different ($P < 0.05$, Duncan's multiple range test).
* For details of animals and procedures, see Materials and methods.

of the dicarboxylic acid is responsible for the significant reduction of TER.

The function of the tight junction is regulated by many intracellular signallings[13]. Inhibitors of phosphatidyl-inositol-3 kinase and Gq protein partially attenuated the acetate-mediated promotive effect on barrier function. Recently, cell surface G-protein coupled receptors for SCFA, GPR41 and GPR43, have been defined[35]. GPR43 has been reported to express in human and rat colon enterocytes[36,37] and couple with Gq and Gi/o proteins[35]. Activation of phosphatidylinositol-3 kinase is reportedly induced via a Gq protein-dependent pathway in airway smooth muscle cells[38]. Moreover, we found that depletion of membrane cholesterol by MCD and digestion of sphingomyelin by SMase abolished the acetate-mediated effect. It is known that cholesterol is a
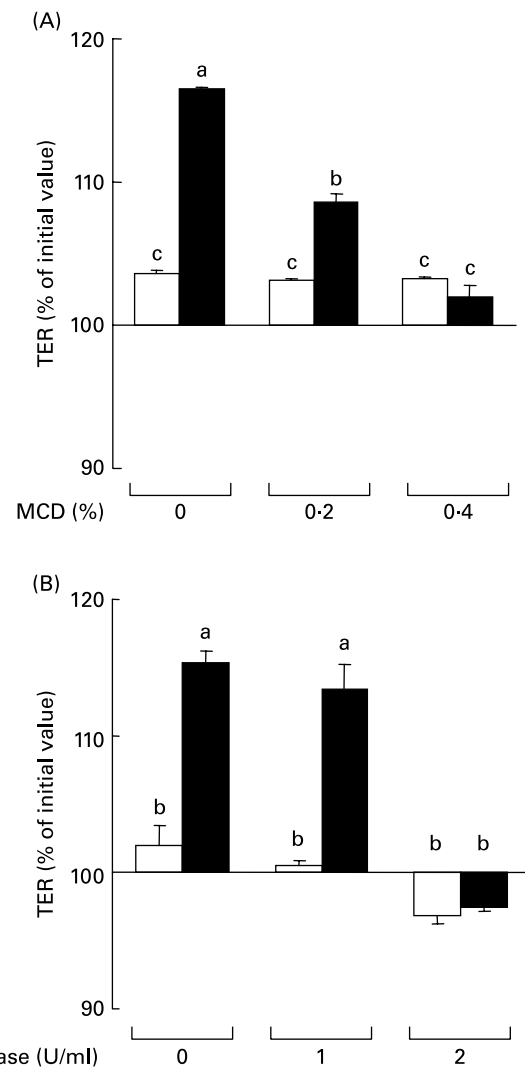

Fig. 3. Transepithelial electrical resistance (TER) at 30 min in Caco-2 cell monolayers incubated with or without acetate (control, ☐; 80 mmol/l acetate, ■). Cells were pre-treated with or without methyl-β-cyclodectrin (MCD; (A)) or sphingomyelinase (SMase; (B)) for 1 h prior to incubation. Values are means with their standard errors for five monolayers. [a,b,c] Mean values without a common superscript letter among treatments are significantly different ($P < 0.05$). For details of animals and procedures, see Materials and methods.
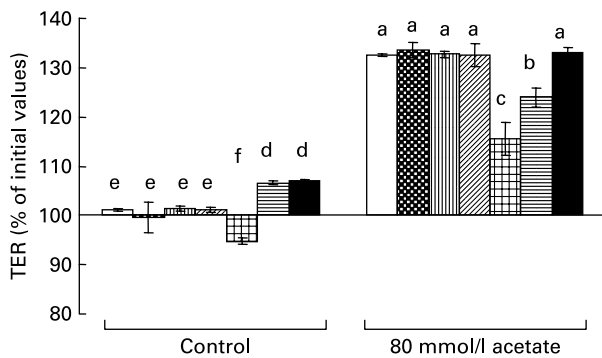
Fig. 4. Transepithelial electrical resistance (TER) at 30 min in Caco-2 cell monolayers incubated with or without acetate in the presence or absence of various signalling inhibitors (□, control; ▨, Y27632; ▥, genistein; ▧, Calphostin C; ▦, LY294002; ▤, YM-254690; ■, ML-7). Values are means with their standard errors for five monolayers. [a,b,c,d,e,f] Mean values without a common superscript letter among treatments were significantly different ($P < 0.05$). For details of animals and procedures, see Materials and methods.

key component of lipid microdomain[39] and sphingomyelin forms tight hydrophobic interactions with cholesterol and has an important role in the formation of lipid microdomains[40]. Cholesterol-rich microdomains, such as raft or caveolae, are known to play an important role in intracellular signal transduction[41]. It is possible that these SCFA receptors are involved in the mechanisms for the promotion of barrier function through the lipid microdomain-dependent signal transduction. However, we have to consider also the other alternative mechanism underlying the SCFA effect, because the inhibitory effect of these two signalling blockers is partial. Nusrut *et al.* have reported that the hyperphosphorylated forms of the tight-junction proteins occludin and ZO-1 are associated with lipid rafts isolated from T84 cells[42]. This has led to the suggestion that lipid microdomains directly play a role in promoting tight junction assembly and integrity. Protonated SCFA, which can easily penetrate plasma membrane, may directly interact with lipid raft and promote the function of tight junctions. This may be supported by the result that lower pH of media enhances the acetate-mediated promotive effect.

In conclusion, physiological concentrations of acetate and propionate enhance the barrier function in the rat caecum, T84 and Caco-2 cell monolayers and the pH-dependent

Table 7. Concentration of [³H] mannitol (pmol/l) in rat serum collected from the caecal vein 30 min after the injection of solutions with or without mixed SCFA and acetate†

(Mean values with their standard errors for six rats)

Treatment	Mean	SEM
Mixed SCFA		
0 mmol/l	30·4	7·5
70 mmol/l	22·3	3·6
140 mmol/l	15·0*	2·1
Acetate		
0 mmol/l	30·4	7·5
40 mmol/l	25·6	7·0
80 mmol/l	20·2	3·0

*$P < 0.05$ *v.* 0 mmol/l of each set, Duncan's multiple range test. One-way ANOVA was performed for each set (mixed SCFA and acetate).
† For details of animals and procedures, see Materials and methods.

penetration of SCFA into epithelial cells is involved in the enhancing effect via cholesterol-rich microdomain in the plasma membrane.

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British Journal of Nutrition

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