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**The G-protein on cholesterol-rich membrane microdomains mediates mucosal  
sensing of short chain fatty acid and secretory response in rat colon**

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Running title: G protein in secretory response to SCFA

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## Abstract

**Aim:** Short-chain fatty acids (SCFA) stimulate colonic contraction and secretion, which are mediated by an enteric reflex via a mucosal sensing and cholinergic mechanisms. The involvement of G-protein signal transduction was examined in the secretory response to luminal propionate sensing in rat distal colon.

**Methods:** Mucosa-submucosa and mucosa preparations were used to measure short-circuit current ( $I_{sc}$ ) and acetylcholine (ACh) release, respectively. Cholesterol-rich membrane microdomains, lipid rafts/caveolae, were fractionated using a sucrose-gradient ultra-centrifugation after detergent-free extraction of the isolated colonic crypt.

**Results:** Luminal addition of methyl- $\beta$ -cyclodextrin (10 mM) and mastoparan (30  $\mu$ M), lipid rafts/caveolae disruptors, significantly inhibited luminal propionate-induced (0.5 mM) increases in  $I_{sc}$ , but did not affect increases in  $I_{sc}$  induced by serosal ACh (0.05 mM) or electrical field stimulation (EFS). Luminal addition of YM-254890 (10  $\mu$ M), a  $G\alpha_{q/11}$ -selective inhibitor, markedly inhibited propionate-induced increase in  $I_{sc}$ , but did not affect  $I_{sc}$  responses to ACh and EFS. Both methyl- $\beta$ -cyclodextrin and YM-254890 significantly inhibited luminal propionate-induced non-neuronal release of ACh from colonocytes. Real-time PCR demonstrated that in mRNA expression of SCFA receptors, GPR 43 was far higher than that of GPR41 in the colon. Western blotting analysis revealed that the cholesterol-rich membrane microdomains that fractionated from colonic crypt cells were associated with caveolin-1, flotillin-1 and  $G\alpha_{q/11}$ , but not GPR43. Uncoupling of  $G\alpha_{q/11}$  from flotillin-1 in lipid rafts occurred under desensitization of the  $I_{sc}$  response to propionate.

**Conclusions:** These data demonstrate that the secretory response to luminal propionate

in rat colon is mediated by G-protein on cholesterol-rich membrane microdomains, provably via  $\text{G}\alpha_{q/11}$ .

## **Keywords**

$\text{G}\alpha_{q/11}$ , GPR 43, lipid lafts, mucosa-submucosa preparation, propionate, short circuit current

## **Introduction**

Chemo-sensing is ubiquitous throughout the alimentary canal, and functions to control food intake, digestion, and absorption. Food components and microbial products stimulate chemo-receptors on the epithelial surface of intestines (Furness *et al.*, 2004). G protein-coupled receptors (GPCRs) and trimeric G protein-mediated signal transduction play key roles in receptor-mediated intestinal nutrient sensing (Covington *et al.*, 2006).

Short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate, major microbial products in the large intestine, contribute not only as an energy source (Livesey and Elia, 1995), but also as chemical stimuli that affect epithelial proliferation (Sakata, 1995), mesenteric blood flow (Knock *et al.*, 2002), colonic motility (Mitsui *et al.*, 2005, Yajima, 1985) and secretion (Karaki and Kuwahara, 2011, Yajima, 1988).

Luminal addition of propionate and other SCFAs is known to result in transient chloride and bicarbonate secretions, and increased short-circuit current ( $I_{sc}$ ) and conductance in distal colon of rat and guinea pig *in vitro* (Karaki and Kuwahara, 2011, Yajima, 1988).

$I_{sc}$  responses to propionate were remarkably inhibited by luminal procaine, serosal atropine and tetrodotoxin (TTX), indicating that an enteric reflex involves both mucosal

sensory mechanisms and cholinergic systems. In combination with the contractile response, the secretory response to luminal SCFAs seems to physiologically function as a lubricant for the movement of luminal content in the colon.

Sensing of SCFAs in the gut may be mediated by GPCRs, GPR41 and GPR43, which have been identified as SCFA receptors by the agonist's selectivity in the gene-transfected mammalian cells (Brown et al., 2003, Le Poul et al., 2003). Recently, the localization of GPR43 on L-type enteroendocrine cells in rat colon have been demonstrated (Karaki *et al.*, 2006). However, whether colonic contractile and secretory responses to luminal SCFAs are mediated by the GPCRs remains to be elucidated.

Considerable evidence demonstrates that GPCRs located in cholesterol-rich membrane microdomains, known as lipid rafts and caveolae, are pivotal loci of signal transduction in cell membranes (Chini and Parenti, 2004). Disrupting lipid rafts/caveolae by extracting cholesterol from cell membranes with methyl- $\beta$ -cyclodextrin (M $\beta$ -CD) treatment is known to inhibit signal transductions by GPCRs (Smart and Anderson, 2002).

The present study aimed to examine the involvement of GPCRs and G-protein signal transduction via cholesterol-rich microdomains, lipid rafts/caveolae, in the  $I_{sc}$  response to luminal propionate in rat colon *in vitro*.

## **Materials and Methods**

### *Animals*

Male Sprague-Dawley rats (250–300 g) were used. The rats were fed a pellet diet (Type MF, Oriental Yeast Co., Tokyo, Japan) *ad libitum* with free access to water, but were starved overnight before experimentation. This study was approved by the Hokkaido

University Animal Committee, and all animals were maintained in accordance with the Hokkaido University guidelines for care and use of laboratory animals.

#### *Tissue preparation*

Rats were asphyxiated with CO<sub>2</sub> gas and exsanguinated. The colonic segments were removed and opened along the mesenteric border, and the luminal contents were washed out with warm Krebs bicarbonate saline solution. For mucosa-submucosa preparation, which histologically consist of mucosa and most of the submucosa (data not shown), tunica muscularis was removed using fine forceps under a stereomicroscope (Andres et al., 1985, Yajima, 1988).

Epithelial sheets were made using the mucosa-submucosa preparations. Two sheets were prepared using the distal colon for measuring electrical activity and were mounted in an Ussing chamber with a window size of 0.5 cm<sup>2</sup>. For acetylcholine (ACh) release experiments, two sheets of the mucosa preparation (Yajima, 1988) were prepared from the distal colon, and mounted in an Ussing chamber with a window size of 0.98 cm<sup>2</sup> and a volume of 5 ml. Of these two sheets, one was treated with inhibitors of the propionate response. The other was treated only with the solvent for the drug and served as control.

#### *Short-circuit current measurement*

In the Ussing chambers, each side of the mucosa was bathed in 5 ml of bathing solution containing the following: 119 mM NaCl; 1.25 mM CaCl<sub>2</sub>; 1 mM MgCl<sub>2</sub>; 2.2 mM K<sub>2</sub>HPO<sub>4</sub>; 0.2 mM KH<sub>2</sub>PO<sub>4</sub>; 21 mM NaHCO<sub>3</sub> and 10 mM Glucose. The solution was bubbled with a gaseous mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4, 37 °C). The tissues were short-circuited using a voltage clamp (Nihon Koden, Tokyo, Japan) at zero

potential automatically with compensation for the solution resistance.  $I_{sc}$  was continuously recorded, and tissue conductance ( $G_t$ ) was measured every min. A positive  $I_{sc}$  was defined as current necessary to compensate a transepithelial potential difference with the serosal side positively charged. The current was recorded using the Power Lab system (ADInstruments, Bella Vista, Australia).

#### *Propionate, acetylcholine and electrical field stimulations*

Tissues were left for about 40 min to allow  $I_{sc}$  to stabilize before the effects of propionate and other drugs were studied. 25  $\mu$ l of sodium propionate (100 mM or 1.0 M) and acetylcholine (ACh) chloride (10 mM) was added to the mucosal and serosal sides of the Ussing chambers, respectively. Appropriate concentrations of other drugs in volumes of 10-30  $\mu$ l were added to the mucosal or serosal sides 10-60 min prior to adding propionate or ACh. Electrical field stimulation (EFS) was performed as previously described (Yajima, 1988). A pair of aluminum-foil electrodes was attached to the output of an electrical stimulator with a stimulus-isolator unit (Model SEM, Nihon Koden, Tokyo, Japan). Single polar rectangular pulses with duration 1 ms, amplitude 5 mA, and frequency 10 Hz were applied for 90 s.

#### *ACh release and measurement*

The ACh release experiments were performed as previously described (Yajima et al., 2011). A volume of 500  $\mu$ l from serosal fluid was taken at 5 min before and after propionate stimulation to measure ACh concentration. After sampling, 500  $\mu$ l of bathing solution was added to the serosal fluid. The samples were stored at -80 °C until measurement of ACh. The concentration of ACh in the samples were measured as

previously described (Yajima et al., 2011).

#### *RNA extraction and reverse transcription*

Pieces of tissue were sampled from the cecum, proximal colon, middle colon and distal colon, according to the previous procedures (Yajima, 1985). The tissues were weighed and stored at -80 °C until real-time PCR (RT-PCR). Total RNA was extracted from these samples using the QuickGene RNA tissue kit SII (FUJIFILM, Tokyo, Japan), an RNA extraction kit for use with a semi-automated nuclear acid extraction machine (QuickGene-810; FUJIFILM).

Concentration of total RNA extracted was measured using a Nano-Drop ND1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA) and 150 ng of the total RNA was used for reverse transcription using a PrimeScript® RT reagent kit (Perfect Real Time) (TAKARA, Osaka, Japan). All procedures were performed according to the manufacturer's instructions.

#### *Real-time quantitative PCR*

Real-time PCR was performed using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Tokyo, Japan). The primers and Taqman probes used in this study are listed in Table 1.

Amplification was performed in a 10 µl reaction volume containing 5 µl *Premix Ex Taq™* (Perfect Real Time) (TAKARA), 0.4 µl cDNA, 0.9 µM of each primer and 0.25 µM Taqman probe. The thermal cycling profile was 10 s at 95 °C followed by 40 cycles of 5 s at 95 °C and 34 s at 60 °C. The gene for GAPDH was amplified for use as a housekeeping gene in the same manner as with the GPR41 and GPR43, but except that

SYBR *Premix Ex Taq*<sup>TM</sup> (Perfect Real Time) (TAKARA) was used instead of *Premix Ex Taq*<sup>TM</sup>.

To prepare a standard curve, plasmids containing the PCR product of each gene were generated in pGEM-T easy vectors (Promega, Tokyo, Japan) and included in every run. The transcript copy number of each sample was calculated from the standard curve and normalized to the copy number of GAPDH.

#### *Crypt isolation*

The distal colon was removed according to previously described anatomical divisions (Yajima, 1985), opened longitudinally, and rinsed with warm PBS. Each colon was incubated in 10 ml Hank's balanced solution (Sigma Chemical Co, St. Louis, MO, USA) containing 5 mM dithiothreitol, 0.1% BSA, 1 mM glutamine, and 30 mM EDTA (pH 7.4) and shaken for 15 min at 37 °C. After incubation, the colons were gently scraped with a rubber policeman to isolate crypts, which were then centrifuged at 100 g for 3 min at 4 °C. The crypt pellets were washed twice with cold PBS, weighed and stored at –80 °C until fractionation of the cholesterol-rich membrane microdomains.

#### *Detergent-free fractionation of cholesterol-rich membrane microdomains*

Cholesterol-rich membrane microdomains were fractionated using sucrose-gradient ultracentrifugation after performing a detergent-free extraction of crypts isolated from the distal colon at below 4 °C according to the previously described methods (Song *et al.*, 1996). Pellets of isolated crypts were suspended into 2 ml 500 mM sodium carbonate (pH 11.0), and homogenized using an Ultra-Turrax tissue grinder (three, 10 s bursts separated by 1 min cooling periods; IKA-T25, IKA Works Co., Germany), and

sequentially sonicated (three, 20 s bursts separated by 1 min cooling periods; Astrason 3000, Misonix Inc., New York, USA). The homogenate was then adjusted to 45% sucrose by adding 2 ml 90% sucrose prepared in MES buffer solution (25 mM MES, pH 6.5, 0.15 M NaCl) and placed at the bottom of an ultracentrifugation tube (12.5 ml). A discontinuous sucrose gradient was made by placing 4 ml 35% sucrose and then 4 ml 5% sucrose in MES buffer solution containing 250 mM sodium carbonate, and centrifuged at 200,000 g for 18 h in a SW41Ti rotor (Beckman Instruments, CA. USA). Twelve fractions of 1.0 ml were collected from the top of the gradients.

#### *Immunoblotting analysis*

Samples containing 2 µg protein from each fraction were denatured and lysed by adding NuPAGE 4×LDS sample buffer and 10×sample reducing agent (Invitrogen, Tokyo, Japan). The lysates were then loaded on a 12% NuPAGE Bis-Tris polyacrylamide gel (Invitrogen) in NuPAGE MOPS SDS Running Buffer (Invitrogen) under reducing conditions. After electroblotting the lysates onto nitrocellulose membranes and blocking them with a PBS solution containing 5% skim milk, the membranes were probed with one of the following primary antibodies: rabbit anti-GPR43 (1:10000) (Karaki *et al.*, 2006), mouse anti-caveolin-1, mouse anti-flotillin-1 (BD Biosciences, Tokyo, Japan; 1:1000), or rabbit anti-G $\alpha_{q/11}$  (Upstate, NY, USA; 1:2000). The membranes were washed three times with PBS and incubated with either anti-rabbit IgG or anti-mouse IgG as the secondary antibody (Dako; 1:2000). After three rinses PBS, immunoreactive bands were detected using the ECL Western blot detection kit (GE Healthcare Bioscience, Tokyo, Japan). Analysis of the band intensities were performed using MULTIGAUGE software (FUJIFILM).

### *Drugs*

Sodium propionate was obtained from Kanto Kagaku Co. Ltd (Tokyo, Japan). Acetylcholine chloride, dithiothreitol, EDTA, eserine hemi-sulphate, glutamine, mastoparan methyl- $\beta$ -cyclodextrin and tetrodotoxin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). YM-254890 was a gift from Astellas Pharma Inc. (Tokyo, Japan). YM-254890 was dissolved in DMSO at a concentration of 10 mM.

### *Statistics*

Values are presented as mean  $\pm$  S.E. Significance of difference between data of control and experimental groups was determined using Student's t-test. Differences were considered significant when  $P < 0.05$ .

## **Results**

### *Effects of lipid rafts/caveolae disruptors on $I_{sc}$ response to propionate*

To examine the involvement of lipid rafts/caveolae in the  $I_{sc}$  response to propionate, M $\beta$ -CD, a strong drug for extracting cholesterol from cell membranes, was added to the luminal side of the mucosa-submucosa preparations of distal colon that were mounted onto the Ussing chambers. A 1 h treatment with 10 mM M $\beta$ -CD significantly inhibited propionate-induced (0.5 mM) increases in  $I_{sc}$  following luminal additions, but did not significantly affect the  $I_{sc}$  responses to serosal addition of ACh (0.05 mM) or EFS (Fig. 1A). Base-line  $I_{sc}$  and tissue  $G_t$  slightly increased from  $17.8 \pm 0.9$  to  $21.9 \pm 2.1\mu\text{A cm}^{-2}$  and from  $7.8 \pm 0.4$  to  $8.9 \pm 0.7 \text{ mS cm}^{-2}$ , respectively, ( $n = 10$ ) after 60 min treatment with M $\beta$ -CD, but these increases were not significant, indicating that the apical

membranes of colonocytes were not damaged by the luminal addition with M $\beta$ -CD.

Mastoparan, a wasp venom peptide toxin, has been widely used as a pharmacological tool not only as a G $\alpha_1$  activator (Higashijima *et al.*, 1988) but also as a G $\alpha_s$  signaling inhibitor by altering the localization of G $\alpha_{q/11}$  and G $\alpha_s$  in lipid rafts (Sugama *et al.*, 2005, Sugama *et al.*, 2007). Luminal addition of mastoparan (30  $\mu$ M) significantly inhibited propionate-induced (0.5 mM) increase in  $I_{sc}$ , but no effects were observed on the  $I_{sc}$  responses to serosal addition of ACh (0.05 mM) or EFS (Fig. 1B). Base-line  $I_{sc}$  and tissue  $Gt$  increased from  $19.7 \pm 2.3$  to  $26.7 \pm 2.0 \text{ } \mu\text{A cm}^{-2}$  and from  $8.0 \pm 0.4$  to  $10.7 \pm 0.8 \text{ mS cm}^{-2}$ , respectively, ( $n = 6$ ,  $P < 0.05$ ) following treatment with mastoparan.

#### *Effects of G protein inhibitor on $I_{sc}$ response to propionate*

The G $\alpha_{q/11}$ -selective inhibitor YM-254890 (10  $\mu$ M) (Takasaki *et al.*, 2004), markedly inhibited propionate-induced (0.5 mM) increase in  $I_{sc}$  following luminal addition, but did not significantly affect the  $I_{sc}$  response to the serosal addition of ACh (0.05 mM) or EFS (Fig. 1C). On the other hand, the serosal addition of YM-254890 (10  $\mu$ M) completely inhibited  $I_{sc}$  responses to luminal propionate and serosal ACh ( $n=4$ ). Base-line  $I_{sc}$  and tissue  $Gt$  slightly increased from  $11.0 \pm 0.8$  to  $12.8 \pm 1.3 \text{ } \mu\text{A cm}^{-2}$  and from  $9.3 \pm 1.9$  to  $10.4 \pm 2.1 \text{ mS cm}^{-2}$ , respectively, ( $n=5$ ) following treatment with luminal YM-254890, but these increases were not significant.

#### *Effects of M $\beta$ -CD and YM-254890 on ACh release induced by propionate*

Recently, we have demonstrated that luminal propionate induces non-neuronal release of ACh which stimulates muscarinic receptors to result in the increase of  $I_{sc}$  in rat colon

(Yajima et al., 2011). So, we examined whether the luminal treatment with M $\beta$ -CD (10 mM) or YM-254890 (10  $\mu$ M) inhibit the non-neuronal release of ACh induced by propionate in the presence of TTX (1  $\mu$ M). As shown in Fig. 2, both drugs significantly inhibited ACh release following luminal propionate stimulation into the serosal side in the mucosa preparations of distal colon.

#### *mRNA expression of GPR41 and GPR43 in the large intestine*

Real-time PCR demonstrated that mRNA expression of GPR43 was far higher than that of GPR41 in the caecum and three parts of the colon (Fig. 3). The gene copy number of GPR41 was less than 800 in every parts of the large intestine.

#### *Cholesterol-rich membrane microdomain of colonic crypt cells*

Cholesterol-rich fractions (4 and 5) were obtained by sucrose gradient ultracentrifugation of the colonic crypt lysates (Fig. 4A). The concentration of cholesterol in the fractions 4 and 5 decreased from  $70.9 \pm 6.2$  to  $40.4 \pm 4.8 \mu\text{g ml}^{-1}$  ( $n = 6$ ,  $P < 0.01$ ) following the treatment with 10 mM M $\beta$ -CD for 1 h. Western blot analysis revealed that the major bands of caveolin-1, a marker protein of caveolae, and flotillin-1, a marker protein of lipid rafts, were associated with the cholesterol-enriched fractions 4 and 5 (Fig. 4B). These findings suggest that crypt cells isolated from rat distal colon contain the same structures as lipid rafts and caveolae on their membrane surfaces.

When the fractions were analyzed by probing for G $\alpha_{q/11}$  and GPR43, almost all of G $\alpha_{q/11}$  was recovered in fractions 4 and 5, on the other hand, major bands for GPR43 were detected in fractions 9–12 (Fig. 4B).

*Desensitization of the secretory response to propionate uncouples G $\alpha_{q/11}$  from flotillin-1*

The  $I_{sc}$  response to luminal addition of propionate (1 mM) was desensitized against the following stimulation (Fig. 5A). Increases in  $I_{sc}$  induced by the second propionate stimulation were significantly reduced compared to increase induced by the first propionate stimulation (Fig. 5B). Therefore, uncoupling of G-protein from flotillin-1 was analyzed in lipid rafts/caveolae using Western blotting. Ten-minute stimulation with luminal addition of propionate (5 mM) in the isolated colonic loops caused reductions in the immunoreactivities of G $\alpha_{q/11}$  in lipid rafts fractions (Fig. 5C). This significant decrease in the ratio of G $\alpha_{q/11}$  to flotillin-1 after propionate stimulation indicates that uncoupling of G $\alpha_{q/11}$  from flotillin-1 occurred in crypt cells isolated from the distal colon (Fig. 5D).

## Discussion

Cholesterol plays a pivotal role in maintaining the structure of lipid rafts and caveolae on plasma membrane of cells. M $\beta$ -CD efficiently extracts cholesterol from cell membranes, resulting in disruption of the structure of lipid rafts/caveolae (Smart and Anderson, 2002). It has been reported that the treatment with 10 mM M $\beta$ -CD induces the release of 50 - 90 % of the cholesterol contained in the membrane of mouse fibroblast after 1–2 h of incubation at 37 °C (Kilsdonk *et al.*, 1995). In the present study, treatment with 10 mM M $\beta$ -CD for 1 h on the mucosal surface of isolated colon segments caused the release of  $42.1 \pm 6.7\%$  ( $n = 6$ ) cholesterol from the cholesterol-rich membrane fractions obtained from crypt cells. Using the same M $\beta$ -CD treatment on the luminal side of the Ussing chamber, propionate-induced increase in  $I_{sc}$  were significantly inhibited in the mucosa-submucosa preparations of rat distal colon, suggesting that the structures of the

lipid rafts/caveolae are required for the propionate-induced secretory response on the luminal side. A similar disruption in the function of caveolae by treatment with M $\beta$ -CD was observed in studies on muscarinic receptor-mediated bronchoconstriction (Schlenz *et al.*, 2010).

ACh is a potent secretagogue of Cl<sup>-</sup> by the stimulation of muscarinic receptors on the basolateral membrane of the enteric epithelium (Hirota and McKay, 2006). In rat colonic epithelial cells, cholinergic activation of Cl<sup>-</sup> secretion is mediated by muscarinic M1 and M3 receptors on the basolateral membranes (Haberberger *et al.*, 2006, O'Malley *et al.*, 1995). The  $I_{sc}$  response to the serosal addition of ACh as well as EFS was not significantly affected by luminal treatment with M $\beta$ -CD (Fig. 1A), indicating that the actions of M $\beta$ -CD were restricted only by the apical membrane of the mucosa in this study. Because the secretory response to propionate in rat colon is mainly mediated by the release of ACh from cholinergic systems (Hubel and Russ, 1993, Yajima, 1988, Yajima *et al.*, 2011), these results suggest that the structures of the lipid rafts/caveolae in the apical membrane of colonic mucosa are required for luminal propionate-induced secretory response. This hypothesis has been confirmed by the evidences that the propionate induced-ACh releases from colonic epithelial cells were significantly inhibited by the luminal treatments with not only M $\beta$ -CD also YM-254890.

GPR41 and GPR43 are known to be activated by SCFAs (Brown *et al.*, 2003), and are expressed in L-type enteroendocrine and mucosal mast cells in rat and human colons (Karaki *et al.*, 2006, Tazoe *et al.*, 2008). This study confirmed remarkable expression of GPR43 compared to GPR41 in rat colon using RT-PCR. To explore the involvement of GPCRs in the secretory responses to propionate, a pharmacological approach using mastoparan (Fig. 1B), a versatile drug that affects G protein functions by changing the

cellular localization of  $G\alpha_q$  and  $G\alpha_s$  in lipid rafts/caveolae (Sugama et al., 2005, Sugama et al., 2007), revealed that propionate-induced  $I_{sc}$  increases are mediated by  $G\alpha$  proteins. In addition to the inhibitory effects of M $\beta$ -CD on the  $I_{sc}$  response to propionate, the inhibitory effects of mastoparan on the secretory responses to propionate supported the notion that the structure of the lipid rafts/caveolae on the luminal surface of colonic mucosa should be involved in the secretory response to propionate. Significant increase in tissue  $Gt$  following mastoparan treatment seems to be due to the cytotoxic activities of this drug, which results in the loss of cellular components like lactate dehydrogenase and eventually cell death (Sugama et al., 2005). No significant changes in the  $I_{sc}$  responses to ACh or EFS following mastoparan treatment were noted in this study, indicating that the secretory functions of colonic epithelial cells were not damaged following mastoparan treatment.

Furthermore, the marked inhibition by the  $G\alpha_{q/11}$ -selective inhibitor, YM-254890, supports the involvement of G protein in the secretory response to propionate. In this study, 10  $\mu$ M YM-254890 was added to the luminal side of the colonic mucosa and treated for 30 min. This treatment resulted in slight increase in tissue  $Gt$ , but the  $I_{sc}$  response to ACh was not significantly reduced, suggesting that the inhibitory actions of YM-254890 are restricted to the apical membrane of colonocytes as  $Cl^-$  secretion by the M1 and M3 receptors on the basolateral membrane of colonocytes is linked to  $G\alpha_{q/11}$  (Lanzafame et al., 2003). This speculation was supported by the evidences that serosal addition of 10  $\mu$ M YM-254890 abolished the  $I_{sc}$  response to both luminal propionate and serosal ACh.

The structure and organization of lipid rafts and/or caveolae regulate functions of the apical membrane of epithelial cells in the small intestine, including digestion and

absorption of nutrients, and its function as a portal for pathogens (Danielsen and Hansen, 2006). Furthermore, caveolae have been fractionated from the isolated crypts of mouse and rat colons, using a detergent-free procedure, in which caveolin-1 was enriched (Ma *et al.*, 2004). Western blots of detergent-free, sucrose-gradient fractionations of cholesterol-rich membrane microdomains from the isolated crypts showed the major bands of caveoline-1 and flotillin-1 are associated with the cholesterol-rich fractions, indicating the presence of caveolae and lipid rafts structures in colonocytes (Fig. 4B). The same placement of  $G\alpha_{q/11}$  with caveolin-1 and flotillin-1 in the cholesterol-rich fractions suggests that the cholesterol-rich membrane microdomains, such as lipid rafts and caveolae, on the colonocytes are the primary site of G protein signal transduction.

Uncoupling of  $G\alpha_{q/11}$  from flotillin-1 in crypt cells isolated from the distal colon (Fig. 5D) during the desensitization of the secretory response evoked by luminal propionate stimulation strongly supports the involvement of  $G\alpha_{q/11}$  in the secretory response to luminal propionate. If the secretory response to luminal propionate is possibly mediated by GPCRs, GPR43 is a plausible candidate because mRNA expression of GPR43 is far higher than that of GPR41 in rat colon. The evidence that GPR43 is coupled with both  $G\alpha_{i/o}$  and  $G\alpha_q$  protein families may support the involvement of GPR43 in the secretory response to propionate (Le Poul *et al.*, 2003). In the present study, however, Western blot of GPR43 did not indicate the association of GPR43 with  $G\alpha_{q/11}$  in cholesterol-rich membrane microdomains (Fig. 4B).

In conclusion, we have demonstrated in the present study that the secretory response to luminal propionate sensing in rat colon is mediated by the G-protein in cholesterol-rich membrane domains, provably via  $G\alpha_{q/11}$  in membrane lipid rafts. The far higher mRNA expression of GPR43 than GPR41 in the colon suggests that GPR43

is a potential receptor for the secretory response to SCFAs, but further study is needed to elucidate a clear and functional relationship between SCFA receptor stimulation and secretory response.

### **Conflicts of interest**

There are no potential conflicts of interest to report.

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## Figures legends

Fig. 1. Inhibitory effects of M $\beta$ -CD, mastoparan and YM-254890 on the  $I_{sc}$  response to

propionate in mucosa–submucosa preparations of rat distal colon.

M $\beta$ -CD (10 mM), mastoparan (30  $\mu$ M) and YM–254890 (10  $\mu$ M) were added to the luminal fluid of the Ussing chamber after the base-line  $I_{sc}$  stabilized. (A) 10 mM M $\beta$ -CD treatment for 60 min (n = 10). (B) 30  $\mu$ M mastoparan treatment for 10min (n = 6). (C) 10  $\mu$ M YM–254890 treatment for 30 min (n=5). After the treatments with drugs, maximal increases in the  $I_{sc}$  responses to luminal addition of propionate (0.5 mM), serosal addition of ACh (0.05 mM) and EFS (10 Hz, 5 mA, 90 s) were calculated. Values are mean  $\pm$  S.E. \*P<0.05 compared between control and drug treatment by Student's *t* test.

Fig. 2. Effects of M $\beta$ -CD and YM-254890 on non-neuronal ACh release induced by luminal propionate in mucosa preparations of rat distal colon.

M $\beta$ -CD (10 mM) and YM–254890 (10  $\mu$ M) were added to the luminal fluid of the Ussing chambers 60 min and 30 min, respectively, prior to luminal propionate (0.5 mM) stimulation. TTX (1  $\mu$ M) and eserine (0.1 mM) were added to the serosal side and both sides of the Ussing chambers, respectively. The concentration of ACh in the serosal fluid was measured at 5 min after the luminal propionate stimulation. Values are mean  $\pm$  S.E (n=5). \*P<0.05 compared between control and drug treatment by Student's *t* test.

Fig. 3. mRNA expression of GPR41 and GPR43 in the large intestine of rat.

Real-time PCR was performed on the caecum, proximal colon, middle colon, and distal colon (n = 12).

Fig. 4. Fractionation of cholesterol-rich membrane microdomains and Western blots of

caveolin-1, flotillin-1,  $G\alpha_{q/11}$ , and GPR43.

(A) Percentage total cholesterol in the 12 sucrose-density gradient fractions ( $n = 6$ ). (B) Immunoblots were performed with polyclonal antibodies to caveolin-1, flotillin-1,  $G\alpha_{q/11}$ , and GPR43.

Fig. 5. . Desensitization of the secretory response to propionate and uncoupling of  $G\alpha_{q/11}$  from flotillin-1 in cholesterol-rich membrane microdomains.

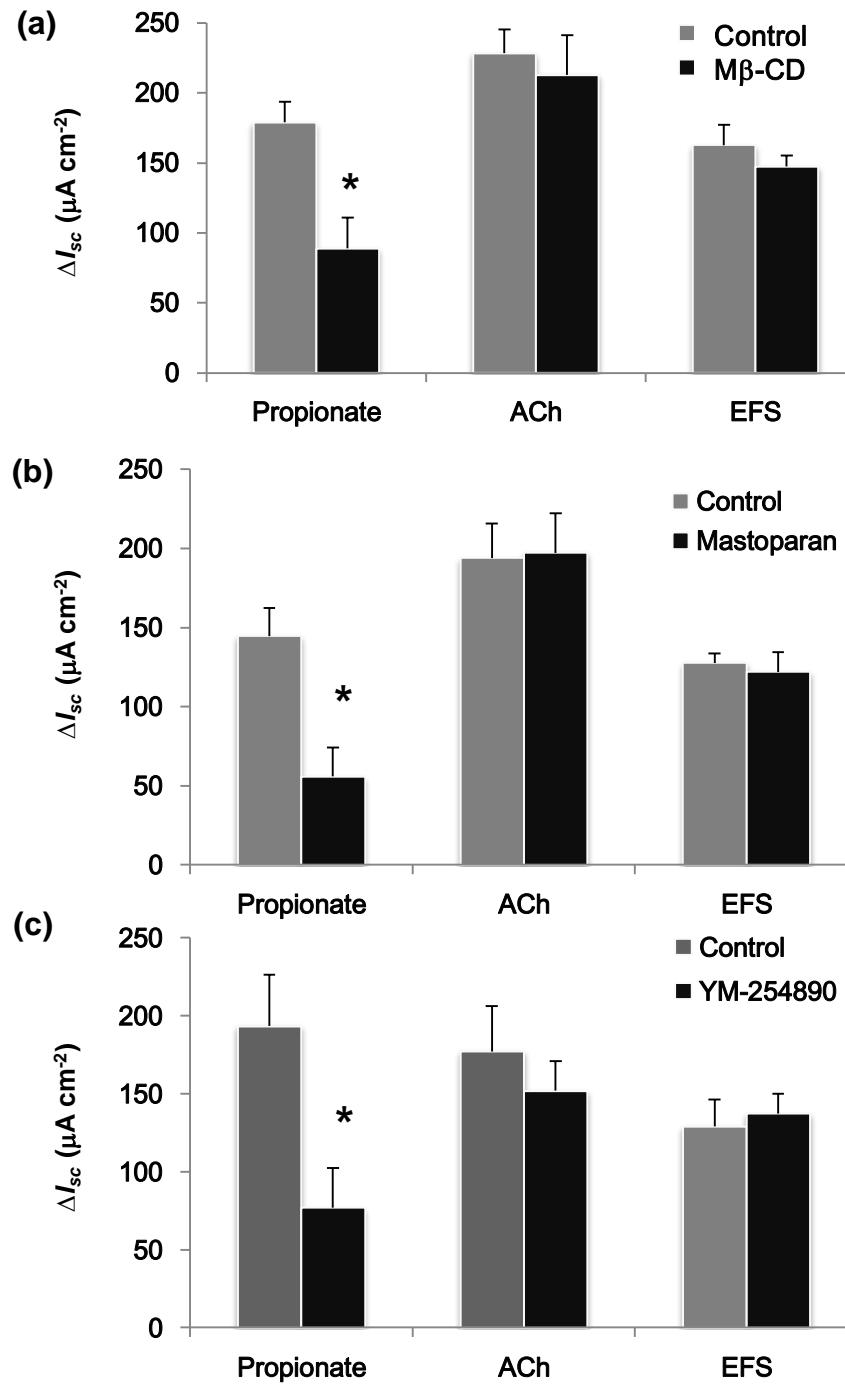
(A) A typical  $I_{sc}$  response to repeated additions of propionate (1 mM) in rat distal colon. The deflections shown at every min are the results of the measurement of tissue conductance. (B) Changes in the  $I_{sc}$  response produced by repeated addition of propionate ( $n = 6$ ). Values are mean  $\pm$  S.E. \* $P < 0.001$  compared between first propionate and second propionate stimulations by Student's *t* test. (C) Immunoblots of  $G\alpha_{q/11}$  and flotillin-1 in sucrose-density gradient fractions 4 and 5 obtained from tissues of the control and experimental groups stimulated with propionate. The isolated loops (5-7 cm) of distal colon filled with bathing solution (control groups) or 5 mM propionate-bathing solution (experimental groups) were incubated for 10 min in 50 ml of bathing solution bubbled with 5% CO<sub>2</sub> gas in O<sub>2</sub> at 37 °C. The crypts from colonic segments of both groups were isolated, and then the cholesterol-rich membrane microdomains were fractionated as described in Materials and Methods. (D) Concentration ratio of  $G\alpha_{q/11}$  to flotillin-1 determined by immunoblots of fractions of 4 and 5 obtained from tissues of the control and experimental groups stimulated with propionate ( $n = 6$ ). Values are mean  $\pm$  S.E. \* $P < 0.001$  compared between control and propionate treatment by Student's *t* test.

**Table**

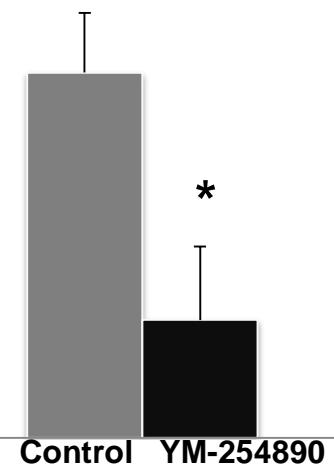
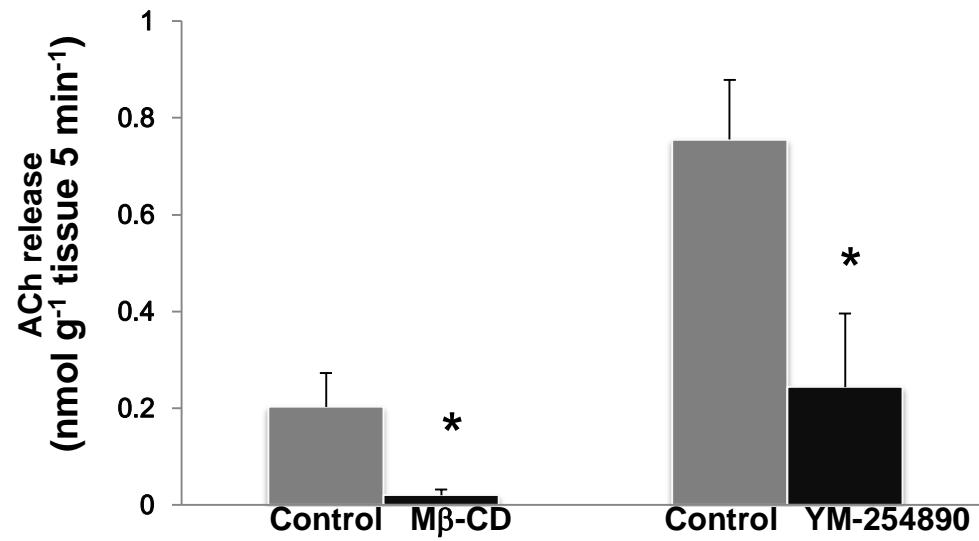
Table 1. The primers and probes used in this study

Gene Name	Genbank Accession #		Sequence 5'-3'
GAPDH	NM_017008	Forward	GTCTTCACTACCACATGGAGAAGG
		Reverse	TCATGGATGACCTTGGCCAG
GPR41	NM_001108912	Forward	TGACGGTGAGCATAGAACGTTT
		Reverse	GCCGGGTTTGACACAGT
		Probe	TGAGCGTAGCCTACC
GPR43	NM_001005877	Forward	TCGTGGAAGCTGCATCCA
		Reverse	GCGCGCACACGATCTTT
		Probe	CTTCCGCTGGTACCTAC

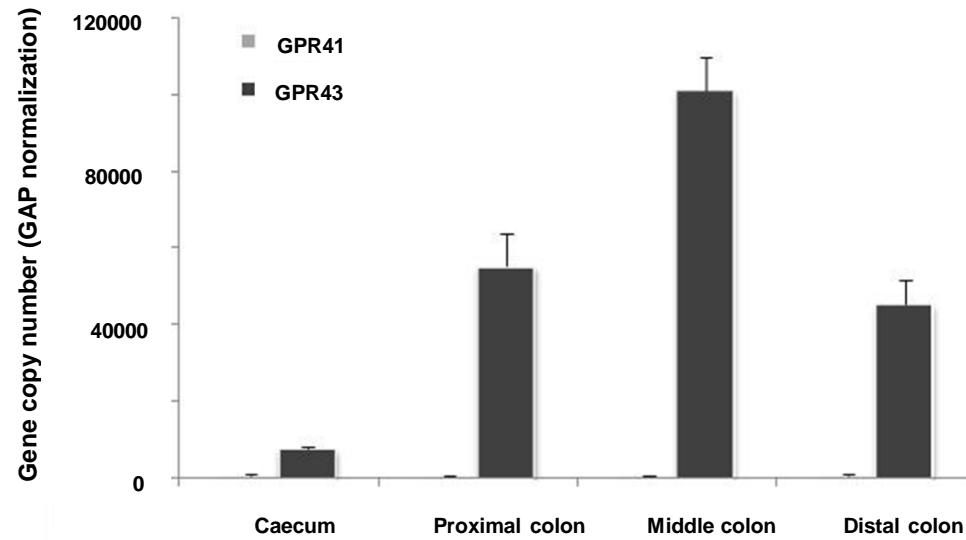
**Figure 1**



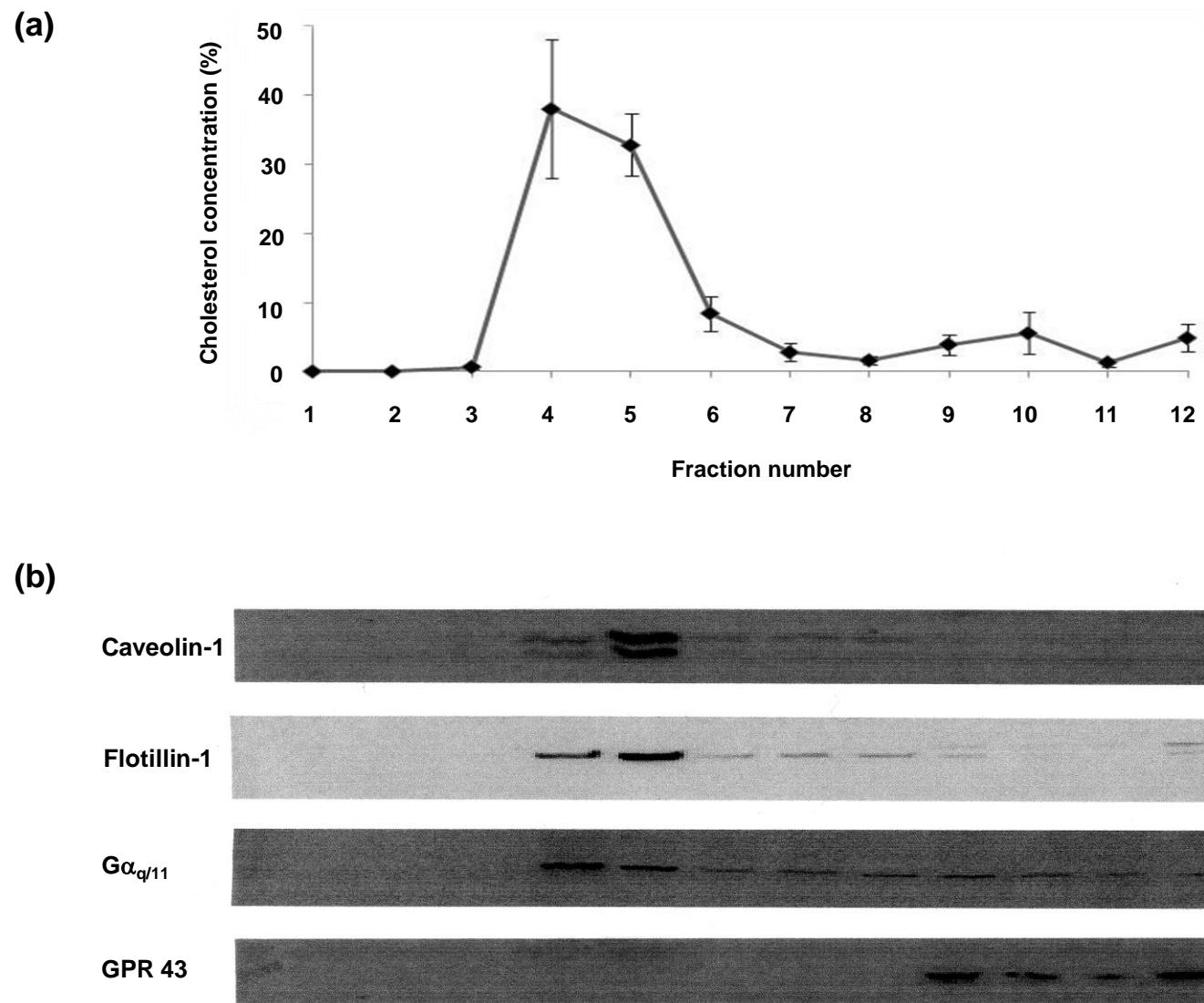
**Figure 2**



**Figure 3**



**Figure 4**



# Figure 5

