Effects of luminal thymol on epithelial transport in human and rat colon

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Submitted 12 November 2010; accepted in final form 1 March 2011.

Kaji I, Karaki SI, Kuwahara A. Effects of luminal thymol on epithelial transport in human and rat colon. Am J Physiol Gastrointest Liver Physiol 300: G1132–G1143, 2011. First published March 3, 2011; doi:10.1152/ajpgi.00503.2010.—Gut lumen is continually exposed to a great variety of agents, including noxious compounds. Chemical receptors that detect the luminal environment are thought to play an important role as sensors and to modulate gastrointestinal functions. Recently, it has been reported that odorant receptors (ORs) are expressed in the small intestinal mucosa and that odorants stimulate serotonin secretion. However, ion transport in the response to odorants has rarely been discussed, particularly in relation to the large intestine. In the present study, we examined the effects of the OR ligand thymol on ion transport in human and rat colonic epithelia using an Ussing chamber. In the mucosal-submucosal preparations, the mucosal addition of thymol evoked anion secretion concentration-dependently. In addition, dextran (4 kDa) permeability was enhanced by the mucosal treatment with thymol. The response to thymol was not affected by tetrodotoxin (TTX) or piroxicam treatments in human or rat colon. Thymol-evoked electrogenic anion secretion was abolished under Ca\(^{2+}\)-free conditions or mucosal treatment with transient receptor potential (TRP) A1 blocker (HC-030031). Pretreatment of thymol did not affect electrical field stimulation-evoked anion secretion but significantly attenuated short-chain fatty acid-evoked secretion in a concentration-dependent manner. OR1G1 and TRPA1 expression was investigated in isolated colonic mucosa by RT-PCR. The present results provide evidence that the OR ligand thymol modulates epithelial permeability and electrogenic anion secretion in human and rat colon. The anion secretion by luminal thymol is most likely mediated by direct activation of TRPA1 channel. We suggest that the sensing and responding to odorants in the colon also plays a role in maintaining intestinal homeostasis.

intestinal chemosensing; transepithelial transport; odorant; thymol; transient receptor potential A1

TRANSEPITHelial ION TRANSPORT in the intestine is regulated by diverse systems, including the enteric nervous system (ENS) and a variety of gut hormones and cytokines that respond to mechanical and chemical stimuli (13). Many reports have demonstrated the expression of various chemical receptors in the intestinal epithelium, such as G protein-coupled receptors (GPCRs), including free fatty acid receptors (FFAs) (10, 17, 21), taste receptors (T1Rs and T2Rs) (2, 9, 20, 41), and odorant receptors (ORs) (4). It is thought that GPCR activation by chemical stimuli from the lumen is the first important signal in fulfilling intestinal function.

Short-chain fatty acids (SCFAs), including propionate and butyrate, induce epithelial ion transport through mucosal stimulation and ENS activation (22, 43). We found that the SCFA receptors FFA2 (GPR43) and FFA3 (GPR41) are expressed in human and rat intestinal epithelia with strong density in enterodocrine cells (19, 21, 23, 38). Bacteria-derived SCFAs are continually present in the large intestine of nonruminants. We have hypothesized that further unexpected compounds, especially bacterial metabolites, may be produced in colonic lumen and therefore that various chemical receptors may be expressed by intestinal mucosa to detect luminal status.

Both gustatory and olfactory senses that detect ambient conditions depend on chemical identification by GPCRs, which comprise a large family of transmembrane receptors. Several reports have suggested that a taste signaling mechanism exists in intestinal mucosa and modulates nutrient absorption in the small intestine (31, 32). We have reported that bitter taste receptors (T2Rs) are expressed in colonic mucosa and induce anion secretion in human and rat colon (20). The bitter tastant 6-n-propyl-2-thiouracil induces anion secretion in a pathway that is prostaglandin-sensitive but tetrodotoxin (TTX)-insensitive, suggesting that chemical sensing in colonic lumen function for host defense. Other studies have detected OR expression in the small intestine of humans and rats and suggested that luminal odorants induce serotonin (5-HT) secretion in isolated duodenal EC cells and EC cell lines (4, 26). It has been reported that the thymol, a classical odorant extracted from herbs, induces luminal anion secretion in porcine small intestine (3).

Thymol is a major odor component in edible herbs and is used in oral care products. It has been reported that thymol activates certain types of the olfactory receptor OR1G1: it activates class II (terrestrial-type OR group) OR1G1 but not class I (fish-like OR group) (35). Volatile odorants, such as terpenoid derived from five carbon isoprene units, are widely produced by plants, insects, and bacteria, including gut commensal bacteria (36). Therefore, it is possible that odorants are synthesized in colonic lumen and detected by mucosal chemosensors. However, the effects of OR ligands in tissue level have rarely been discussed, particularly in relation to the large intestine. In the present study, we investigated the effects of luminal stimulation by thymol on ion transport in human and rat colon.

MATERIALS AND METHODS

Human intestinal specimens and tissue preparation. This study was approved by the Institutional Review Board of Shizuoka Saiseikai General Hospital and the University of Shizuoka. Surgical specimens of human ascending colon were obtained (following informed consent) from patients undergoing extirpation for carcinoma. The age of patients was 71.9 ± 2.1 yr (mean ± SE, n = 7, including 4 females and 3 males). A nonpathological region was cut from the specimen, and the region was then placed in ice-cold Krebs-Ringer solution (bathing solution) saturated with 95% O\(_2\)-5% CO\(_2\) and transported to the laboratory. Tissues were pinned flat on a silicone-filled petri dish with the mucosal surface down, and tissue preparations were constructed using forceps and scissors. To obtain mucosal-submucosal preparations, the
smooth muscle layer was gently removed, leaving the blood vessels in the submucosal connective tissue intact. During preparation, the tissues were bathed in ice-cold Krebs-Ringer solution (pH 7.4).

**Animals and tissue preparation.** The handling and killing of animals were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka, and this study was approved by the University of Shizuoka Animal Usage Ethics Committee. Adult male Wistar rats (SLC, Hamamatsu, Japan), weighing between 220 and 320 g, were used. Animals were fed pellet diets (type MF) with water ad libitum. They were anesthetized with ether and decapitated by guillotine. Tissue preparation was as follows, according to a previous study (43). Segments of rat distal colon (3–6 cm proximal to anus) were cut along the mesenteric border, and luminal contents were gently removed. Mucosal-submucosal preparations were constructed under a stereomicroscope using two pairs of fine forceps. During preparation, the tissues were bathed in ice-cold Krebs-Ringer solution (pH 7.4).

**Short-circuit current measurements.** The mucosal-submucosal preparations of human and rat colon with a cross-sectional area of 0.64 cm² were mounted between the halves of an Ussing flux chamber. The mucosal and serosal surfaces of the tissue were bathed with 10 ml of Krebs-Ringer solution by circulation from a reservoir maintaining at 37°C during the course of the experiments. Tissues were left to equilibrate [as judged by a stable basal short-circuit current (Isc)] and tissue conductance (GΩ) in the chambers for 1–1.5 h before conducting the experiment. Transmephalal potential difference (PD) was measured using paired Ag-AgCl electrodes via Krebs-agar bridges and clamped at 0 mV by applying a Isc with another pair of Ag-AgCl electrodes connecting a voltage-clamp apparatus (SS-1335; Nihon-Kod sen, Tokyo, Japan). To calculate GΩ, for each 1-min cycle, the clamped voltage was maintained at 0 mV for 57 s and then changed automatically to 10 mV for the remaining 3 s. Under voltage-clamp conditions, PD was calculated from Isc and GΩ by Ohm’s law. The Isc responses were continuously recorded on a chart recorder (Recti-Horitz-8K, Nihon-Denki San-ei, Tokyo Japan) and a PowerLab system (ADInstruments, Cattle Hill, Australia).

To check tissue availability, tissues were electrically stimulated by passing a current parallel to the plane of the tissue via a pair of Ag-AgCl electrodes connecting a voltage-clamp apparatus (SS-1335; Nihon-Kodsen, Tokyo, Japan). To calculate GΩ, for each 1-min cycle, the clamped voltage was maintained at 0 mV for 57 s and then changed automatically to 10 mV for the remaining 3 s. Under voltage-clamp conditions, PD was calculated from Isc and GΩ by Ohm’s law. The Isc responses were continuously recorded on a chart recorder (Recti-Horitz-8K, Nihon-Denki San-ei, Tokyo Japan) and a PowerLab system (ADInstruments, Cattle Hill, Australia).

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Fluorescein isothiocyanate (FITC)-conjugated dextran (mol wt 4000; FD-4) was used to measure paracellular permeability. After stabilization of basal Isc and GΩ in the voltage-clamp mode, 1 mM of FD-4 was added to the mucosal bathing solution. Aliquots of the samples were obtained from the serosal bathing solution at 0, 15, 30, 45, 60, and 90 min after the FD-4 application in the voltage-clamp mode. The fluorescence intensity of each 100-μl sample was analyzed at 520 nm with excitation at 490 nm using a Varioskan Flash (Thermo Fisher Scientific, Yokohama, Japan). The volume of the serosal bathing solution was maintained by adding fresh Krebs-Ringer solution.

Krebs-Ringer solution contained (in mM) 117 NaCl, 4.7 KCl, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, and 11 glucose. The solution was bubbled with a gas mixture of 95% O₂ and 5% CO₂ and buffered at pH 7.4. Cl⁻-free Krebs-Ringer solution included gluconate in place of Cl⁻. To prepare Cl⁻/HCO₃⁻-free solution, NaHCO₃ was further replaced by sodium gluconate from Cl⁻-free solution, and HEPES was added. The pH of Cl⁻/HCO₃⁻-free solution was titrated to 7.2–7.4 by 1 N NaOH. The solution was bubbled with 100% O₂, and acetazolamide (5×10⁻⁴ M) was added to inhibit endogenous HCO₃⁻ synthesis. Ca²⁺-free Krebs-Ringer solution was prepared by removal of CaCl₂ from Krebs-Ringer solution. To prepare low-Na⁺ Krebs-Ringer solution, NaCl (117 mM) was replaced by choline chloride.

**RT-PCR analysis.** Scraped mucosa from human and rat colonic segments were immediately immersed in RNA stabilization regent (RNA later; Qiagen, Tokyo, Japan). The fixed tissues (~20 mg) were then transferred to new 2.0-ml tubes and homogenized using a bead homogenizer Bead Beater Mini (Wakenyaku, Kyoto, Japan). Total RNAs were isolated using the RNeasy Mini Kit (Qiagen) and were reacted with RQ1 RNase-free DNase (Promega, Madison, WI) to remove contaminative genome DNA. cDNA was synthesized by the RNA samples using a First Strand cDNA Synthesis Kit for RT-PCR [AMV] (Roche Applied Science, Mannheim, Germany). Synthesized cDNA and primers were stored at −20°C until use. The primer pair for human (h) OR1G1 was according to a previous study (4). Primer pairs for hTRPA1, rat (r) OR1G1, and rTRPA1 were designed based on the mRNA and genome sequences [NCBI; hTRPA1, NM_007332; rOR1G1 (Olr430), gene ID 296691; rTRPA1, NM_207608]. To confirm the absence of genome DNA contamination, the primer pairs for TRPA1 and β-actin were designed to span the intron region, and negative control PCR was performed with RNA as template. All primer sequences used in the present study are shown in Table 1. The sequence specificities of designed primers were tested by NCBI BLAST. PCR was performed using a Premix Taq (Takara Ex Taq Version; Takara Bio, Shiga, Japan) and a Takara PCR Thermal cycler MP (TP3000; Takara Bio). The reaction mixture was 50 μl including 20–50 ng of cDNA and 0.4 μM of forward and reverse primers. Before PCR, reaction mixtures were incubated at 94°C for 5 min to completely denature the template. PCR cycles consisted of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1min (35 cycles), followed by final extension at 72°C for 4 min; amplification products were stored at 4°C. PCR products and DNA ladder marker (100-bp DNA ladder; Bio Medical Science, Tokyo, Japan) were separated by electrophoresis on 2% agarose (Agarose 21;
Nippon Gene, Tokyo, Japan) gel and stained by ethidium bromide. Gel images were viewed by the Printgraph AE6932GX gel documentation system (ATTO, Tokyo, Japan).

**Chemicals.** Thymol, dimethyl sulfoxide (DMSO), bumetanide, FD-4, 3-tropanyl-3,5-dichlorobenzoate, and SB-204070 were from Sigma (St. Louis, MO); allyl isothiocyanate (AITC) and cinnamaldehyde (CA) were from Kanto Chemical (Tokyo, Japan); and TTX, 6-iodonordihydrocapsaicin (6-I-CAP), and HC-030031 were from Tocris (Ellisville, MO). FD-4 was dissolved in distilled water, and TTX was dissolved in citrate buffer (pH 4.8) while other drugs were dissolved in DMSO and stored at −20°C. The volumes of dissolved drugs that were added to the bathing solutions did not exceed 100 μl (1% of bathing solution). It was confirmed that 1% DMSO had no effect on ion transport in human and rat distal colon.

Data analysis and statistics. All data are expressed as means ± SE. The n values represent the numbers of human patients or animals. Student’s t-test or multiple comparisons following ANOVA were used to examine the effects of inhibitors. P ≤ 0.05 was considered statistically significant.

**RESULTS**

Basal electrical parameters were measured when basal $I_{sc}$ and $G_t$ stabilized. In human ascending colon, the average PD, $I_{sc}$, and $G_t$ were $−7.7 ± 1.4$ mV, $48.5 ± 8.4$ μA/cm², and $7.0 ± 0.7$ mS/cm², respectively ($n = 7$). The increase in $I_{sc}$ induced by EFS was $52.5 ± 5.1$ μA/cm² ($ΔI_{sc} n = 7$). These results were consistent with that in the previous study (20) indicating that tissue conditions were available to investigate physiological responses. In rat distal colon, the average PD, $I_{sc}$, and $G_t$ were $−2.7 ± 0.1$ mV, $20.5 ± 0.8$ μA/cm², and $8.2 ± 0.2$ mS/cm², respectively. The increase in $I_{sc}$ induced by EFS was $106.7 ± 2.8$ μA/cm² ($ΔI_{sc} n = 103$).

**Effect of thymol on electrical activity in human ascending colon and rat distal colon.** The addition of thymol ($10^{-3}$ M) to the mucosal bathing solution evoked transient increases in $I_{sc}$ and $G_t$ in both human and rat colon (Fig. 1A). In human ascending colon, the single application of $10^{-3}$ M thymol to the mucosal bathing solution gradually increased $I_{sc}$ to a peak value at $7.5 ± 1.9$ min ($20.3 ± 3.3$ μA/cm², $ΔI_{sc}$), whereas a $G_t$ peak was observed at $17.6 ± 1.5$ min ($9.0 ± 3.7$ mS/cm², $ΔG_t$; $n = 5$) (Fig. 1A, human). The $I_{sc}$ returned to the basal level 15–18 min after the addition while $G_t$ was sustained for >1 h. In rat distal colon, after the addition of $10^{-3}$ M thymol to the mucosal bath, $I_{sc}$ increased to a peak at $3.2 ± 0.4$ min ($28.6 ± 4.0$ μA/cm², $ΔI_{sc}$) and returned the basal level 20–25 min after the addition. The $G_t$ also increased gradually for $6.7 ± 1.1$ min ($11.7 ± 2.6$ mS/cm², $ΔG_t$; $n = 10$) and did not return to the basal level, even in >30 min (Fig. 1A, rat).

Concentration-dependent effects of thymol on basal $I_{sc}$ and $G_t$ in human colon were investigated by cumulative application (Fig. 1B, human). The levels of the response to thymol ($10^{-3}$ M) were similar to that of the single application. In rat colon, $10^{-6}$ to $10^{-3}$ M of thymol was individually added (Fig. 1B, rat). The concentration-response curves of the response to thymol in the human and rat colon showed a similar pattern (Fig. 1B).

**Effect of TTX and piroxicam on thymol-evoked ion transport in human and rat colon.** This experiment was designed to investigate the involvement of the ENS or prostaglandin synthesis in the response to mucosal application of thymol. The...
neutral Na⁺ channel blocker TTX or cyclooxygenase inhibitor piroxicam was used. TTX (10⁻⁶ M) was added to the serosal bathing solution, whereas piroxicam (10⁻³ M) was added to both mucosal and serosal bathing solutions. When basal isc stabilized, thymol (10⁻³ M) was added to the mucosal bathing solution.

In the human colon, TTX or piroxicam itself gradually decreased basal isc (−12.9 ± 4.2 and −20.5 ± 4.1 μA/cm², Δisc, respectively) without the changes of basal Gi. Thymol-evoked increases in isc and Gi in TTX (n = 6) or piroxicam (n = 6)-pretreated tissues did not significantly differ from nontreated tissues.

In the rat colon, two preparations of distal colon from one rat were used for the control and experimental groups. TTX was added to the serosal bathing solution 15 min before the application of thymol. TTX did not affect basal isc and Gi or the response to thymol (n = 5). Pretreatment of the tissue with piroxicam decreased basal isc (−11.0 ± 1.7 μA/cm², Δisc; n = 3). However, thymol-evoked increases in isc and Gi were not reduced by piroxicam.

Because the property of the response to thymol in rat distal colon was similar to that in human colon, further study was performed in rat distal colon.

Participation of Cl⁻ and HCO₃⁻ on thymol-evoked ion transport in rat distal colon. This experiment was designed to investigate the ionic basis for the increases in isc and Gi evoked by thymol. The response to the mucosal application of thymol (10⁻³ M) was measured under the Cl⁻-free or Cl⁻/HCO₃⁻-free conditions. Both mucosal and serosal bathing solutions were replaced by these modified Krebs-Ringer solutions, and EFS (25 V, 5 Hz, 0.5-ms duration, 2 min) was performed to confirm the absence of the anion(s). The removal of Cl⁻ decreased basal Gi, but not isc, from 7.7 ± 1.3 to 4.8 ± 0.4 mS/cm² (P < 0.05, n = 5). Under the Cl⁻/HCO₃⁻-free conditions, both isc and Gi were significantly decreased from 27.3 ± 3.3 to 11.7 ± 2.6 μA/cm² and 9.4 ± 0.8 to 5.8 ± 0.6 mS/cm², respectively (P < 0.05, n = 4). The EFS-evoked isc increase was 66% reduced by the absence of Cl⁻ (20.7 ± 4.5 μA/cm², Δisc, P < 0.01 vs. control in the Tukey-Kramer test) and 96% reduced by the absence of Cl⁻/HCO₃⁻ (2.7 ± 0.7 μA/cm², Δisc, n = 4) compared with control tissue (60.7 ± 8.7 μA/cm², Δisc, n = 6). Thymol was added when basal isc and Gi stabilized. The thymol-evoked increase in isc was reduced by 60% in the absence of Cl⁻ (9.5 ± 0.9 μA/cm², Δisc, P = 0.15 vs. control in Tukey-Kramer test) and completely abolished in the absence of Cl⁻/HCO₃⁻ (−0.33 ± 1.0 μA/cm², Δisc, P < 0.05 vs. control) (Fig. 2, A and B). The Gi increases were almost completely abolished by the absence of Cl⁻ (1.1 ± 0.3 mS/cm², ΔGi, P < 0.01 vs. control) and the absence of Cl⁻/HCO₃⁻ (2.6 ± 1.4 mS/cm², ΔGi, P < 0.05 vs. control) (Fig. 2, A and C).

Effect of bumetanide on thymol-evoked ion transport in rat distal colon. To investigate whether Na⁺-K⁺-2Cl⁻ cotransporter (NKCC) contributed in the anion secretion induced by thymol, the NKCC1 inhibitor bumetanide was used. Bumetanide (10⁻⁴ M) was added to the serosal bathing solution 15 min before the application of thymol (10⁻³ M). In the presence of bumetanide, the thymol-evoked increase in isc was significantly reduced from 32.2 ± 7.2 to 11.2 ± 4.6 μA/cm² (Δisc; P < 0.05, n = 5; Fig. 3A), but the thymol-evoked increase in Gi was not affected (Fig. 3B).

Effect of Na⁺ replacement by choline on thymol-evoked ion transport in rat distal colon. The increases in isc and Gi evoked by thymol were investigated under low-Na⁺ conditions. After replacement of Krebs-Ringer solution on both the mucosal and the serosal side by low-Na⁺ Krebs-Ringer solution, basal isc,
but not \( G_i \), significantly increased from 19.5 ± 3.3 to 37.4 ± 5.5 \( \mu A/cm^2 \) (\( P < 0.01, n = 4 \)). The mucosal addition of thymol (10\(^{-3}\) M) increased \( I_{sc} \) to the same level in control tissues (Fig. 4, A and B). Thymol-evoked \( G_i \) increases were significantly reduced by the \( Na^+ \) replacement by choline (1.3 ± 0.4 mS/cm\(^2\), \( \Delta G_i, P < 0.05, n = 4 \)) compared with control (16.5 ± 3.0 mS/cm\(^2\)) (Fig. 4, A and C). When basal \( I_{sc} \) and \( G_i \) had stabilized, EFS (25 V, 5 Hz, 0.5-ms duration, 2 min) was performed to confirm the removal of \( Na^+ \). The EFS-evoked \( I_{sc} \) increase under the low-\( Na^+ \) condition (21.3 ± 6.6 \( \mu A/cm^2 \), \( \Delta I_{sc}, P < 0.01, n = 4 \)) was reduced by 77% of that in control (60.7 ± 8.7 \( \mu A/cm^2 \), \( \Delta I_{sc} \)).

**Effect of thymol on paracellular permeability in rat distal colon.** This experiment was designed to investigate whether the increase in nonelectrolyte paracellular permeability was increased by thymol similar to \( G_i \). FD-4 (1 mM) was added to the mucosal bathing solution 30 min before the application of thymol (10\(^{-3}\) M), and concentrations of transferred FD-4 in the serosal bathing solution were measured. In the control tissues (absence of thymol), FD-4 gradually moved to the serosal side, and 313.8 ± 37.0 nM was detected at 90 min after the addition of FD-4. Thymol significantly enhanced the FD-4 transfer through mucosal epithelia (\( P < 0.01 \) by 2-way ANOVA from time 0 to 60 min, \( n = 6 \)). The same experiment was performed using \( Cl^- \) free Krebs-Ringer solution. Under the \( Cl^- \) free condition, FD-4 permeability was enhanced significantly by thymol application (682.7 ± 187.4 nM at 90 min after the addition of FD-4) compared with that in the absence of thymol (302.3 ± 29.9 nM, \( P < 0.05 \) by 2-way ANOVA from time 0 to 60 min, \( n = 6 \)).

**Effect of 5-HT receptor antagonists on the response to thymol in rat distal colon.** To investigate whether 5-HT was involved in the responses to thymol, 5-HT receptor antagonists were used, 3-tropanyl-3,5-dichlorobenzoate for 5-HT3 receptor and SB-204070 for 5-HT4 receptor. Pretreatment of serosal bathing solution with each antagonist (10\(^{-5}\) M) individually did not affect the response to thymol. Even with the coapplication of antagonists, thymol-evoked \( I_{sc} \) and \( G_i \) increases (28.9 ± 29.9 \( \mu A/cm^2 \), \( \Delta I_{sc} \); 21.9 ± 10.7 mS/cm\(^2\), \( \Delta G_i \)) did not differ from the control (34.3 ± 8.1 \( \mu A/cm^2 \), \( \Delta I_{sc} \); 14.3 ± 0.8 mS/cm\(^2\), \( \Delta G_i \); \( n = 3 \)).
Effect of TRP channel blockers on the response to thymol.

Several transient receptor potential (TRP) channels are considered to be activated by thymol (29, 42). To investigate their involvement in the increases in $I_{sc}$ and $G_t$ by thymol, a potent competitive TRPV3 antagonist (6-I-CAP) and a TRPA1 channel blocker (HC-030031) were used. The mucosal addition of 6-I-CAP ($10^{-4}$ M) increased basal $I_{sc}$ to a peak at 9.5 ± 1.8 min (21.5 ± 7.5 μA/cm², $\Delta I_{sc}$; n = 4). When $I_{sc}$ stabilized, thymol ($10^{-3}$ M) was added to the mucosal bathing solution. The response to thymol in the presence of 6-I-CAP did not differ from that in the control.

The addition of HC-030031 ($10^{-4}$ M) to the serosal or mucosal bathing solutions had no effect on basal $I_{sc}$ and $G_t$ themselves (Fig. 6A, traces on middle and bottom). Pretreatment with mucosal HC-030031 significantly decreased ($P < 0.01$ vs. control in Tukey-Kramer’s test, n = 5–10) the thymol-evoked $I_{sc}$ increases (Fig. 6B). The $G_t$ increases were enhanced by the serosal and mucosal pretreatment with HC-030031 (Fig. 6C). There was no significant difference between the effects of serosal and mucosal applications of HC-030031.

Effect of Ca$^{2+}$ removal and U-73122 on the response to thymol in rat distal colon. To investigate whether the response to thymol depends on extracellular Ca$^{2+}$ and phospholipase C (PLC) activity, Ca$^{2+}$-free Krebs-Ringer solution and PLC inhibitor U-73122 were used. Both mucosal and serosal bathing solutions were replaced by Ca$^{2+}$-free solution, and increases in $I_{sc}$ and $G_t$ by the addition of thymol ($10^{-3}$ M) were measured. The removal of Ca$^{2+}$ did not affect the basal $I_{sc}$ and $G_t$. The thymol-evoked increase in $I_{sc}$ was reduced by 77% under Ca$^{2+}$-free conditions (n = 3, P < 0.01; Fig. 7, A and B) while the thymol-evoked $G_t$ increase did not differ from control (n = 3; Fig. 7, A and C).

The serosal addition of U-73122 ($10^{-5}$ M) itself gradually increased basal $I_{sc}$ to a peak at 16.7 ± 4.2 min (26.3 ± 10.7 μA/cm², $\Delta I_{sc}$; n = 3). When $I_{sc}$ stabilized, thymol ($10^{-3}$ M) was added to the mucosal bathing solution. The response to thymol in the presence of U-73122 did not differ from that in the control.

Effect of thymol on EFS- and SCFA-evoked increases in $I_{sc}$ in rat distal colon. EFS induces anion secretion, represented by an increase in $I_{sc}$, via ENS. To examine whether thymol affects ENS activity, EFS-evoked $I_{sc}$ responses were measured in the presence or absence (control) of thymol ($10^{-3}$ M). In the presence of thymol, EFS-evoked increase in $I_{sc}$ (82.7 ± 12.2 μA/cm², $\Delta I_{sc}$; n = 4) did not differ from the response in the control (104.9 ± 6.3 μA/cm², $\Delta I_{sc}$; n = 4).

SCFAs are the main anion components in colonic lumen. Luminal applications of butyrate (C-4) or propionate (C-3), but not acetate (C-2), are known to induce chloride secretion in rat distal colon (43). To examine whether thymol affects chloride secretion evoked by SCFAs, the propionate-evoked $I_{sc}$ response was measured in the presence or absence of thymol ($10^{-3}$ M). In the absence of thymol, $5 \times 10^{-3}$ M propionate induced a biphasic $I_{sc}$ response similar to the previous study (22, 43).

The addition of propionate induced a decreasing (−70.2 ± 14.3 μA/cm², $\Delta I_{sc}$) and further increasing (112.0 ± 12.3 μA/cm², $\Delta I_{sc}$; n = 14) $I_{sc}$ responses in the control tissue (Fig. 8A, trace on top). Although EFS induced an increase in $I_{sc}$ in the presence of thymol, both the first and second phases of the response to propionate were almost completely abolished to $−0.19 ± 0.1$ and $1.0 ± 0.9$ μA/cm², respectively (n = 5; Fig. 8A, trace on bottom). The responses to propionate were reproduced by washing the tissues: bathing solution was replaced by fresh Krebs-Ringer solution three times within 20 min (Fig. 8B). Thymol decreased the response to propionate in a concentration-dependent manner (Fig. 8C).

Effect of TRPA1 agonists on electrical activity in rat distal colon. Experiments were designed to investigate whether well-known TRPA1 agonists have a secretory effect similar to...
addition of 10−3 M AITC or CA to the serosal bathing solution evoked increases in 
transient ScFAs in the large intestine are likely to be modulated by 
luminal odorant compounds.

Thymol-induced ion transport. We previously reported that 
luminal bitter tastant induced anion secretion by a TTX-
resistant and tissue PG-dependent mechanism (20). The pres-
ent study indicates that thymol-evoked anion secretion is 
independent of neural and PG synthesis pathways. These results 
suggest that distinct mechanisms detecting ambient chemicals 
exist in the colonic mucosa. In both human and rat colon, the 
addition of thymol to the mucosal bath induced increases in 
Isc and Gi in a concentration-dependent manner. In addition, we 
have demonstrated OR1G1 mRNA expression in the colonic 
mucosa of humans and rats (Fig. 10). It has been reported that 
10−4 to 2.5 × 10−4 M of thymol stimulates 5-HT secretion in 
duodenal EC cell and EC cell lines and that this response is 
hindered by the PLC inhibitor U-73122 (4, 26). In our present 
study, the anion secretory response to thymol at the tissue level 
was inhibited at >10−4 M (Fig. 1), and the effect of thymol was 
insensitive. These observations suggest that the sen-
sitivity of isolated EC cells might be higher than that under 
physiological conditions. Additionally, the transit time of 
chyme in the small intestine is known to be shorter than in the 
large intestine. Therefore, we speculate that odorant concentra-
tion in the large intestine is higher than that in the small 
intestine; it might be the reasons why ranges of chemical 
detection are different among intestinal regions. After transient 
increases in the Isc and Gi response to thymol, the value of Gi 
returned to the basal level slower than Isc. This indicates that 
the mechanisms of increase in Isc and Gi are different.

Pretreatment of the tissues with bumetanide or Cl−-free 
solution attenuated the thymol-evoked increase in Isc by 60− 
65% (Figs. 2B and 3A). Consistent with a previous study (20), 
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discussion

The present study has demonstrated several actions of the 
odorant thymol in colonic lumen. Thymol concentration-de-
pendently evoked anion secretion in both human and rat colon 
and greatly increased both Gi and nonelectrolyte transepithelial 
permeability in rat distal colon. This study has also shown the 
involvement of the TRPA1 channel in the mechanism of 
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gests that luminal odorants stimulate ion secretion in colonic 
epithelia and that TRPA1 functions in colonic mucosa. Fur-

Fig. 7. Effect of Ca2+-free solution on Isc and Gi responses to mucosal thymol in rat distal colon. The mucosal and serosal bathing solutions were replaced by 
Ca2+-free Krebs-Ringer solution after the check of basal electrical parameters. After stabilization of basal Isc and Gi, thymol (10−3 M) was added to the mucosal 
bathing solution. A: representative traces showing the effect of thymol in the presence or absence of Ca2+. B and C: thymol-evoked Isc (B) and Gi (C) increases. 
The increases in Isc were significantly inhibited by Ca2+ removal (*P < 0.05 by paired t-test), but Gi increases were not affected. Peak values are expressed as means ± SE, n = 3.

Fig. 8. Expression of OR1G1 and TRPA1 in RT-PCR analysis. This experiment was designed to examine the mRNA expression of OR1G1 and TRPA1, which possibly detect thymol and 
mediated the secretory response in human and rat colonic mucosa. RT-PCR analysis was performed with RNA isolated 
from the mucosal layer without submucosa and muscle. Specific amplicons of the expected sizes (Table 1) for mRNA of 
OR1G1 and TRPA1 were detected in both human and rat 
colon mucosa (Fig. 10).

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However, thymol affected luminal SCFA-induced ion secre-
tion. This suggests that the physiological effects of luminal 
SCFAs in the large intestine are likely to be modulated by 
luminal odorant compounds.

Thymol-induced ion transport. We previously reported that 
luminal bitter tastant induced anion secretion by a TTX-
resistant and tissue PG-dependent mechanism (20). The pres-
ent study indicates that thymol-evoked anion secretion is 
independent of neural and PG synthesis pathways. These results 
suggest that distinct mechanisms detecting ambient chemicals 
exist in the colonic mucosa. In both human and rat colon, the 
addition of thymol to the mucosal bath induced increases in 
Isc and Gi in a concentration-dependent manner. In addition, we 
have demonstrated OR1G1 mRNA expression in the colonic 
mucosa of humans and rats (Fig. 10). It has been reported that 
10−4 to 2.5 × 10−4 M of thymol stimulates 5-HT secretion in 
duodenal EC cell and EC cell lines and that this response is 
hindered by the PLC inhibitor U-73122 (4, 26). In our present 
study, the anion secretory response to thymol at the tissue level 
was inhibited at >10−4 M (Fig. 1), and the effect of thymol was 
U-73122-insensitive. These observations suggest that the sen-
sitivity of isolated EC cells might be higher than that under 
physiological conditions. Additionally, the transit time of 
chyme in the small intestine is known to be shorter than in the 
large intestine. Therefore, we speculate that odorant concentra-
tion in the large intestine is higher than that in the small 
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Pretreatment of the tissues with bumetanide or Cl−-free 
solution attenuated the thymol-evoked increase in Isc by 60− 
65% (Figs. 2B and 3A). Consistent with a previous study (20), 
the absence of HCO3− and Cl− completely abolished the Isc
response to thymol and EFS. On the other hand, the thymol-evoked increase in $G_t$ was abolished under the Cl$^-$-free or low-Na$^+$ conditions (Figs. 2C and 4C) but not by treatment with bumetanide (Fig. 3B). These results suggest that the thymol-induced increase in $I_{sc}$ consists of electrogenic anion secretion that is dependent on NKCC1, whereas the increase in $G_t$ is not related to NKCC1. Because bumetanide decreased basal $I_{sc}$ under the low-Na$^+$ conditions (unpublished observations), the replacement of Na$^+$ by choline was unlikely to inhibit NKCC1 activity. This might be the reason why the low-Na$^+$ condition did not alter the thymol-evoked increase in $I_{sc}$. It has been reported that transepithelial permeability of particular ions is regulated by the expression of distinct Claudins, members of a tight junction component protein family,
independently of nonelectrolyte permeability (1, 14, 39). If thymol modulated the tight junction structure, an increase in $G_t$ should be composed of a summation of distinct ion permeability. However, removal of Cl$^-$/HCO$_3^-$ or Na$^+$/HCO$_3^-$ almost completely abolished the thymol-evoked increase in $G_t$; therefore, the $G_t$ increase is more likely the result of increases in transcellular Cl$^-$/HCO$_3^-$ and Na$^+$/HCO$_3^-$ permeability, not paracellular permeability. Our dextran flux experiments further showed that the mucosal application of thymol enhanced permeability of nonionic macromolecules (Fig. 5). Although the $G_t$ increase induced by thymol was transient (Fig. 5B), dextran continually flowed at a higher level in the thymol-treated tissue than in the control tissue. Even under the Cl$^-$/HCO$_3^-$-free conditions, dextran permeability was significantly enhanced by thymol, indicating that thymol induces an increase in paracellular nonelectrolyte permeability by a different mechanism from ion transport.

It has been reported that thymol-induced electrogenic anion secretion is mediated by the cholinergic neural pathway in porcine small intestine (3). Although the present study showed similar results in human and rat colon, the mechanisms of this thymol-induced secretion are likely different in the small and large intestine for the following reasons. In the present study, TTX did not affect thymol-evoked anion transport in human or rat colon. The previous study suggested the involvement of ORs in 5-HT secretion from duodenal EC cells and cell lines (4, 26). Because it has been reported that 5-HT$_3$ and 5-HT$_4$ receptors contribute to colonic Cl$^-$/HCO$_3^-$ secretion via neural and nonneural pathways in rat distal colon (6), we tested the effect of 5-HT receptor antagonists. However, tissue treatment with these antagonists had no effect on the thymol-evoked responses. In addition, thymol did not attenuate the EFS-evoked response (Fig. 8). These observations suggest that luminal

**Fig. 9.** Effect of allyl isothiocyanate (AITC) and cinnamaldehyde (CA) on ion transport in rat distal colon. A and B: representative traces showing the effects of mucosal (A) and serosal (B) applications of AITC ($10^{-4}$ M) and CA ($3 \times 10^{-3}$ M). C and D: AITC- and CA-induced increases in $I_{sc}$ (C) and $G_t$ (D). The responses to the mucosal addition were significantly greater (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by unpaired t-test) than that to the serosal addition. Peak values are expressed as means ± SE, $n = 5–7$.

**Fig. 10.** Expression of OR1G1 and TRPA1 in the human and rat colonic mucosa. RT-PCR was performed using the specific primers listed in Table 1 to detect the expression of OR1G1 and TRPA1 in the mucosa of human and rat colon. RT-PCR for $\beta$-actin was performed as a positive control; RT$^-$, PCR performed with RNA as a negative control to verify the absence of genome contaminations.
thymol evokes anion secretion mediated via nonneural and nonserotonic pathways in human and rat colon. It has been reported that odorant stimulation leads to an increase in intracellular Ca2+ concentration ([Ca2+]i) in olfactory neurons and in other OR-expressing cells depending on extracellular Ca2+ (4, 11, 12). The present study showed that thymol-evoked Isc increases were significantly inhibited under the Ca2+-free conditions (Fig. 7). We previously reported that substance P induced increases in [Ca2+]i in colonic crypts and that its secretory effect depended on extracellular Ca2+ stimulated by tachykinin receptors coupled to Gq/11 (18). From these observations, we speculate that extracellular Ca2+ is required to increase the intracellular Ca2+ response to thymol in colonic epithelial cells in rat distal colon.

Possible role of TRPA1 for thymol-induced anion secretion. Several odor molecules, especially those in spices, are known to be ligands not only of GPCRs but also of TRP channels. It has been reported that thymol activates TRPV3 and TRPA1 in cell expression systems (29, 40, 42). TRP channels are often functionally associated with GPCR and linked with [Ca2+]i signaling. For example, TRPM5 has been well studied in gustatory and olfactory sensing (30, 44) and in intestinal luminal chemosensing (2, 25). In the gastrointestinal tract, it has been reported that TRPA1 activity is involved in the motility of the small intestine (33, 34). However, the physiological function of TRPA1 in epithelial transport in relation to the large intestine has rarely been studied. In the present study, the mucosal thymol-evoked Isc response was decreased by 88% due to the mucosal application of the TRPA1 selective blocker HC-030031 (Fig. 6) and by 80% due to Ca2+ removal from the bathing solution (Fig. 7). In addition, we showed that other well-known TRPA1 agonists, AITC and CA, increased Isc when they were added to the mucosal side (Fig. 9). Furthermore, the mRNA expression of TRPA1 was detected in the isolated mucosa of humans and rats (Fig. 10) similar to that of a previous study (37). Taken together, these results suggest that thymol-evoked electrogenic Cl-/HCO3- secretion is mediated via the TRPA1 channel.

In contrast, the thymol-evoked increase in Gt was enhanced by the TRPA1 blocker HC-030031 (Fig. 6C). Additionally, tissue treatment with bumetanide or with Ca2+-free buffer did not affect the thymol-evoked increase in Gt (Figs. 3B and 7C), although the increase in Isc was inhibited. Furthermore, AITC and CA did not increase Gt as much as thymol, although the increase in Isc was to a similar degree to thymol (Fig. 9). From the above-mentioned results, we suggest that the ion transport systems that are modulated by thymol cannot be explained solely by the TRPA1-mediated pathway. The reason why HC-030031 enhanced the thymol-induced increase in Gt is unclear, but increases in Isc and Gt responses to thymol are possibly regulated by distinct mechanisms.

The colonic epithelium consists of many different kinds of cells, such as absorptive, goblet, enteroendocrine, and caveolated (brush) cells, etc.; therefore, future studies should be done to identify sensor cells expressing ORs and TRPA1. It has also been reported that afferent nerves expressing TRPA1 are involved in colorectal mechanosensation in mice (5). However, the addition of AITC or CA to the serosal bathing solution had significantly small effects compared with the mucosal addition (Fig. 9), indicating that TRPA1 is involved in local secretory function in the apical membrane in colonic epithelium. It is still unclear whether OR1G1 is directly involved in thymol-evoked anion secretion and whether ORs link with TRPA1. Although the direct mediators of the anion secretory systems remain unclear, the present data show the novel possibility that nonneural TRPA1 functions in colonic epithelia and is involved in electrogenic Cl− and/or HCO3− secretion induced by the odorant thymol.

Physiological role of odorant sensing in large intestine. Because previous reports have suggested that bacteria are able to synthesize isoprene units (28), and even that Escherichia coli possess terpenoid biosynthesis enzymes (16), it is possible that active odor molecules that are similar to thymol may be produced in mammalian colon. Indeed, a great variety of volatile compounds (including acids, alcohols, aldehydes, terpenoid, etc.) were detected in human feces (15). It was reported that the concentration range of fecal indole was 0.5–1 mM in healthy men (24, 45). Taken together, these reports suggest that colonic mucosa is exposed to a high concentration of various volatile odorants. Although identification of sensory cells detecting odor molecules would be important, the present study provides considerable evidence that the putative receptors of thymol are expressed in human and rat colonic mucosa. The present study has also shown the inhibitory effect of luminal thymol on SCFA-evoked secretion, albeit with a low anion secretion response to thymol itself (Fig. 8B). The inhibitory effect of thymol was a concentration-dependent and reversible pattern; the propionate-evoked response was restored by washing thymol out of the luminal bathing solution (Fig. 8B). These response properties suggest that thymol may bind to the receptors located in the apical membrane. Although it has been reported that both propionate- and EFS-induced anion secretion processes are dependent mainly on the cholinergic pathway (8, 22, 27, 43), in the present study, the EFS-evoked response was not attenuated by thymol (Fig. 8A). Therefore, the present results suggest that thymol inhibits the detection process of SCFAs but does not inhibit secretagogue transduction or epithelial secretory mechanisms. We previously reported that SCFA receptors FFA2 and FFA3 are expressed in the colonic mucosa of some species (21–23, 38) and that propionate-induced biphasic ion secretion consisted of K+, Cl−, and HCO3− in guinea pig colon (22). The time course of response to propionate in the present study (Fig. 8) was similar to that in guinea pig colon, indicating that propionate possibly induces rapid K+ secretion, followed by anion secretion in rat distal colon. Thymol concentration-dependently inhibited both phases, suggesting that it may function as a competitive antagonist for SCFA receptors FFA2 and/or FFA3. Under physiological conditions, commensal bacteria-produced SCFAs exist as luminal anions and have multiple functions (7). The present results suggest that luminal odorants, which may be produced by microflora, can modulate SCFA function in colonic lumen and that bacterial metabolites may be detected by colonic mucosa and involved in colonic physiological function. We previously reported that luminal bitter tastants induced anion secretion independent of neural reflexes in human and rat colon. Because bitter taste and irritant odor are considered danger signals for animals, these chemical receptors and distinct mechanisms also play an important role in the luminal surface of the colon in the host defense.

In conclusion, the present study suggests that the OR ligand thymol modulates epithelial ion and nonelectrolyte permeabil-
ity and electronic anion transport in human and rat colon. Although OR1G1 expression is detectable in colonic mucosa, the anion secretion by luminal thymol is most likely mediated by direct activation of the TRPA1 channel.

ACKNOWLEDGMENTS

We thank Philip Hawke (University of Shizuoka Global Center of Excellence Program) for checking the English.

GRANTS

This study was supported by funding from a Grant-in-Aid for the Japan Society for the Promotion of Science (JSPS) Fellows to I. Kaji (no.22-5372) and by grants from the JSPS and by the Yamazaki Spice Promotion Foundation (JSPS) for checking the English.

DISCLOSURES

No conflicts of interest are declared by the authors.

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