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ORIGINAL RESEARCH

Effects of ε-viniferin, a dehydrodimer of resveratrol, on transepithelial active ion transport and ion permeability in the rat small and large intestinal mucosa

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Abstract

ε-Viniferin is a dehydrodimer of resveratrol, a polyphenol synthesized in many plants, including grapevine. The present study investigated the effects of ε-viniferin and resveratrol on epithelial secretory and barrier functions in isolated rat small and large intestinal mucosa. Mucosa–submucosa tissue preparations of various segments of the rat large and small intestines were mounted on Ussing chambers, and short-circuit current (I_{sc}) and tissue conductance (G_t) were continuously measured. The mucosal addition of ε-viniferin (>10^{-5} mol/L) and resveratrol (>10^{-4} mol/L) to the cecal mucosa, which was the most sensitive region, induced an increase in I_{sc} and a rapid phase decrease (P-1) followed by rapid (P-2) and broad (P-3) peak increases in G_t in concentration-dependent manners. Mucosal ε-viniferin (10^{-4} mol/L), but not resveratrol (10^{-4} mol/L), increased the permeability of FITC-conjugated dextran (4 kDa). The mucosal ε-viniferin–evoked changes in I_{sc} (Cl^- secretion), but not in G_t, were attenuated by a selective cyclooxygenase (COX)-1 inhibitor and a selective EP_4 prostaglandin receptor. The mucosal ε-viniferin–evoked increase in I_{sc} was partially attenuated, and P-2, but not P-1 or P-3, change in G_t was abolished by a transient receptor potential cation channel, subfamily A, member 1 (TRPA1) inhibitor. Moreover, the mucosal ε-viniferin concentration-dependently attenuated the mucosal propionate (1 mmol/L)-evoked increases in I_{sc} and G_t. Immunohistochemical studies revealed COX-1–immunoreactive epithelial cells in the cecal crypt. The present study showed that mucosal ε-viniferin modulated transepithelial ion transport and permeability, possibly by activating sensory epithelial cells expressing COX-1 and TRPA1. Moreover, mucosal ε-viniferin decreased mucosal sensitivity to other luminal molecules such as short-chain fatty acids. In conclusion, these results suggest that ε-viniferin modifies intestinal mucosal transport and barrier functions.

Introduction

ε-Viniferin is a dehydrodimer of resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a polyphenol expressed in many plants, including grapevine. Resveratrol has a variety of beneficial effects, including antioxidant (Farghali et al. 2013), anti-inflammatory (Csiszar 2011), and anticancer effects (Jang et al. 1997; Aggarwal et al. 2004). Additionally, it has been reported that resveratrol oligomers (oligostilbenoids), like ε-viniferin, have a variety of beneficial effects (Xue et al. 2014). However, it is thought that resveratrol oligomers are poorly absorbed in the intestine, unlike resveratrol (Willenberg et al. 2015).

The intestinal epithelia are sensory tissues for luminal molecules (Kaji et al. 2011b; Breer et al. 2012; Furness et al. 2013). Luminal nutrient sensing in the gastrointestinal (GI) tract induces a variety of physiological responses mediated by diverse gut hormones. For example,

nutrients stimulate gastrin-containing enteroendocrine G cells to release gastrin and induce gastric acid secretion. In the large intestine, the most predominant (~100 mmol/L) anions are short-chain fatty acids (SCFAs), which are enterobacterial fermented products, that induce secretory (Yajima 1988; Karaki and Kuwahara 2011) and contractile responses (Yajima 1985; Mitsui et al. 2005) by activating the luminal side, but not the serosal side. Moreover, we previously reported that a bitter taste (Kaji et al. 2009) and an olfactory compound (Kaji et al. 2011a) evoke secretory responses by activating the luminal side of the GI tract. Therefore, it is possible that some polyphenols, including resveratrol, ε-viniferin, and other oligomers activate the luminal chemosensory system in the GI tract.

It was reported that apical administration of resveratrol stimulates cAMP-dependent chloride secretion (Cl^-) in a colonic epithelium-like monolayer-cultured cell line, T84 cells, and in the mouse jejunum (Blumenstein et al. 2005). Conversely, it was reported that resveratrol oligomers, including ε-viniferin, inhibited cAMP-dependent (forskolin-activated) Cl^- secretion (Zhang et al. 2014). Unfortunately, these earlier reports did not investigate the effects of resveratrol or its oligomers on epithelial barrier functions.

Therefore, the aims of the present study were to investigate the mucosal effects of resveratrol and ε-viniferin on epithelial secretory functions and ion permeability in isolated rat small and large intestines.

Materials and Methods

Animals and tissue preparation

Male Sprague Dawley (SD) rats (mean ± standard error of the mean [SEM], 335.2 ± 10.3 g, $n = 62$) were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and were allowed food and water ad libitum before all experiments. The animals were handled and euthanized in accordance with the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka, and the study was approved by the University of Shizuoka Animal Usage Ethics Committee. SD rats were anesthetized with isoflurane and decapitated with a guillotine. The terminal ileum, cecum, proximal colon, middle colon, distal colon, and rectum were removed and placed in ice-cold Krebs–Ringer solution saturated with O_2 and CO_2 (see Solutions). Tissues were cut along the mesenteric border, and pinned flat to silicon rubber-lined Petri dishes with the Krebs–Ringer solution. The smooth muscle layer of the tissue was removed using microscissors under a stereomicroscope, and the mucosa–submucosa preparations were mounted between the halves of Ussing flux chambers (CHM2; World Precision Instruments, Inc., Sarasota, FL) to measure transepithelial ion transport and permeability.

Ussing chamber experiments

The cross-sectional area of the mucosa–submucosa preparations in the Ussing flux chamber was 0.64 cm^2, and both sides were bathed with 10 mL of Krebs–Ringer solution, which was recirculated from a glass circulation reservoir (#5210; World Precision Instruments, Inc.) maintained at 37°C and gassed with 95% O_2/5% CO_2. The transepithelial potential difference (*PD*; the serosal electrode served as the reference) was measured using paired Ag–AgCl electrodes (EKV; World Precision Instruments, Inc.) through Krebs–agar bridges, and was clamped at 0 mV by applying a short-circuit current (I_{sc}) with another pair of Ag–AgCl electrodes (EKC; World Precision Instruments, Inc.) connected to a voltage-clamp apparatus (CEZ-9100; Nihon-Koden, Tokyo, Japan). The lumen negative electrogenic current represents a positive I_{sc} per unit area of tissue ($\mu A/cm^2$). To record tissue conductance (G_t [mS/cm^2]), voltage command pulses (10 mV, 3 sec duration) were applied at 1 min intervals, and G_t was calculated using Ohm's law as the current needed to alter the clamped voltage. The current output was continuously recorded on a data acquisition and analog-to-digital conversion system (PowerLab 4/26; ADInstruments, Cattle Hill, Australia). Before the experiments, the tissues were stabilized for 1 h and tissue viability was checked by electrical field stimulation (EFS; 25 V, 5 Hz, 0.5 msec duration for 2 min) using a pair of aluminum foil ribbon electrodes.

FD4 permeability

The permeability of fluorescein isothiocyanate (FITC)-conjugated dextran (average molecular weight 4000; FD4) in the mucosa–submucosal tissue preparation was measured under short-circuit conditions in the Ussing chamber. In this experiment, FD4 (10^{-4} mol/L) was added to the mucosal bathing solution 30 min before the addition of EtOH (10 μL; vehicle control), ε-viniferin (10^{-4} mol/L), and resveratrol (10^{-4} mol/L). Samples (100 μL) were taken from the serosal bathing solution, and fresh Krebs–Ringer (100 μL) was added at −15, 0 15, 30, 45, and 60 min (6 samples). The concentrations of FD4 were determined by measuring the fluorescence intensity of samples and standard solutions using a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Inc., Yokohama, Japan) at emission and excitation wavelengths of

520 nm and 490 nm, respectively. Based on a volume of the serosal bathing solution (V_m) of 10 mL and sample volume (V_s) of 100 μL, the total FD4 mass that crossed the tissue at each time (M_n) was:

$$M_n = C_n \times V_m + \left(\sum_{n=1}^{6} C_{n-1}\right) \times V_s$$
$$= C_n \times 10\ \text{mL} + \left(\sum_{n=1}^{6} C_{n-1}\right) \times 100\ \mu\text{L}$$

where C_1, C_2, … C_n are the sample concentrations at sampling times −15 … 60 min. The FD4 flux ($J^{m\to s}$) per unit area (0.64 cm^2) was calculated as:

$$J_n^{m\to s} = \frac{M_{n+1} - M_n}{15\ \text{min}} \times \frac{1}{0.64\ \text{cm}^2}$$

where $J_1{}^{m\to s}$, $J_2{}^{m\to s}$, … $J_{n\text{-}1}{}^{m\to s}$ are the FD4 fluxes in each period (−15–0 min, 0–15 min, … 45–60 min). Because the decrease in the mucosal concentration of FD4 during experiments is considered to be negligible, the permeability ($P_n{}^{m\to s}$) of FD4 in each period was calculated by Fick's law as follows:

$$P_n^{m\to s} = \frac{J_n^{m\to s}}{C_m - C_s} \cong \frac{J_n^{m\to s}}{C_m} = \frac{J_n^{m\to s}}{10^{-4}\ \text{mol/L}}$$

where C_m and C_s ($C_m \gg C_s$) are the concentrations of FD4 in the mucosal and serosal bathing solutions, respectively. This method was built in reference to a chapter in the textbook, Molecular Biopharmaceutics (Brodin et al. 2009).

Solutions

The Krebs–Ringer solution contained (in mmol/L) 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, and 2.5 $CaCl_2$, and was saturated with 95% O_2/5% CO_2. The Cl^--free modified Krebs–Ringer solution was prepared by replacing Cl^- with an equal concentration of $gluconate^-$, and the concentration of Ca-$gluconate_2$ was increased to 8 mmol/L to compensate for the Ca^{2+}-buffering effects of gluconate. The mucosal bathing solutions were supplemented with 5 mmol/L glucose.

Chemicals

ε-Viniferin, resveratrol, and sodium propionate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Carbachol (CCh), FD4, bumetanide, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), atropine, hexamethonium, SC-560, and NS-398 were purchased from Sigma (St. Louis, MO). TTX and HC030031 were purchased from Tocris (Ellisville, MO). Piroxicam was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was purchased from ANA SPEC (Fremont, CA). AH-6809 was purchased from Cayman Chemical (Ann Arbor, MI). ONO-8713, ONO-AE3-208, and ONO-AE3-240 were kind gifts from ONO Pharmaceutical Co., Ltd. (Osaka, Japan).

ε-Viniferin and resveratrol were dissolved in ethanol (EtOH) just before use. TTX was dissolved in citrate buffer (pH 4.8) and stored at −20°C until use. CCh, FD4, propionate, atropine, and HEX were dissolved in distilled water, and stored at 4°C until use. All of the other chemicals were dissolved in DMSO and stored at −20°C until use.

Immunohistochemistry

The isolated cecal tissues were immediately frozen with Tissue-Tek optimal cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) in a −75°C acetone bath (Histo-Tek Pino; Sakura Finetek Japan Co., Ltd.). The tissues were cut into 4-μm-thick sections on a cryostat (CM1100; Leica Microsystems, Weltzlar, Germany). The sections were placed on glass slides, fixed in 100% methanol at −20°C for 10 min, and air-dried under a cold blower for 30 min. The sections were incubated with 10% normal donkey serum and 1% Triton X-100 in phosphate-buffered saline (PBS) at room temperature for 1 h to block nonspecific antibody binding. The sections were incubated with goat anticyclooxygenase (COX)-1 antibody (×500 dilution; sc-1754; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS containing 0.3% Triton X-100 at 4°C overnight. After washing in PBS (3 × 10 min), the sections were incubated with donkey anti-goat IgG conjugated with Alexa594 (×500 dilution; A11058; Life Technologies, Carlsbad, CA) at room temperature for 1 h. The sections were washed in PBS (3 × 10 min), and coverslips were mounted on the glass slides with mounting medium (DakoCytomation, Glostrup, Denmark). Immunoreactivity for COX-1 was visualized using a fluorescence microscope (Axio Observer Z1; Carl Zeiss, Oberkochen, Germany), and the images were captured using a cooled charge-coupled device digital camera and digital imaging software (AxioCam and AxioVision; Carl Zeiss).

Data analysis and statistics

All physiologic data in the present study are expressed as the mean ± SEM. Multiple comparisons were made using Dunnett's test and Tukey's test for each group relative to

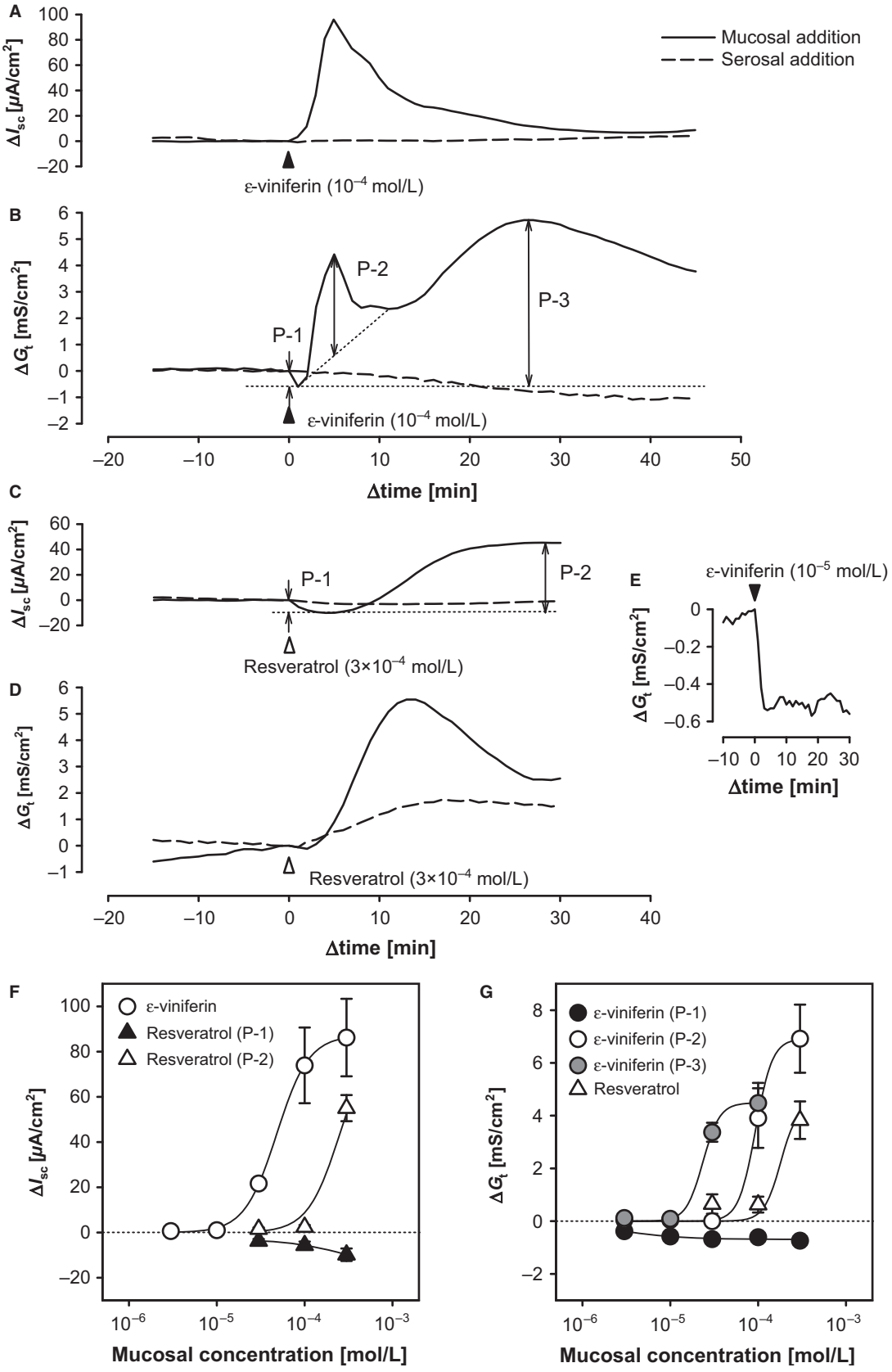

Figure 1. Concentration-dependent effects of mucosal and serosal ε-viniferin, and mucosal resveratrol on I_{sc} and G_t in the rat cecum. Mucosa–submucosal tissue preparations of rat cecum were mounted on Ussing chambers, and I_{sc} and G_t were continuously recorded. Various concentrations of ε-viniferin (3×10^{-6} to 3×10^{-4} mol/L) and resveratrol (3×10^{-5} to 3×10^{-4} mol/L) were added to the mucosal bath at 0 min (Δ). ε-Viniferin (10^{-4} mol/L) was also added to the serosal bath at 0 min. Representative traces from −15 min to 45 min showing the effects of 10^{-4} mol/L mucosal and serosal ε-viniferin (A and B) and 3×10^{-4} mol/L mucosal resveratrol (C and D) on ΔI_{sc} (A and C) and ΔG_t (B and D) relative to the values at 0 min. A representative trace showing the effects of a lower concentration (10^{-5} mol/L) of mucosal ε-viniferin on ΔG_t is shown in (E). The mean peak changes in I_{sc} (F) and G_t (G) were plotted on semi-log graphs. Data are expressed as the mean ± SEM (n = 4–6).

the control group and among all combinations of groups, respectively. Paired t tests were used to determine the statistical significance between two groups because some tissue preparations from the same animals were used in both experimental groups. In all analyses, Ps < 0.05 were considered statistically significant.

Results

Effects of mucosal and serosal ε-viniferin and resveratrol on I_{sc} and G_t in the mucosa–submucosa preparations of rat cecum

In the rat cecal mucosa–submucosal preparations, mucosal, but not serosal, addition of ε-viniferin (bath concentration, 10^{-4} mol/L) evoked a monophasic increase in I_{sc} (Fig. 1A; ΔI_{sc}: 73.93 ± 16.75 μA/cm^2, Δtime: 5.68 ± 0.52 min) and triphasic changes in G_t (Fig. 1B; P-1: ΔG_t −0.61 ± 10.07 mS/cm^2, Δtime 1.8 ± 0.2 min; P-2: ΔG_t 3.91 ± 1.13 mS/cm^2, Δtime: 5.6 ± 0.2 min; P-3: ΔG_t 4.48 ± 0.76 mS/cm^2, Δtime 20.6 ± 2.3 min; n = 5). In contrast, mucosal addition of 10^{-4} mol/L resveratrol evoked very small changes in I_{sc} and G_t (Fig. 1E and C), while mucosal, but not serosal, addition of 3×10^{-4} mol/L resveratrol evoked biphasic changes in I_{sc} (Fig. 1C; P-1: ΔI_{sc} −9.84 ± 2.76 μA/cm^2, Δtime 3.55 ± 0.42 min; P-2: ΔI_{sc} 54.98 ± 5.76 μA/cm^2, Δtime 24.62 ± 4.77 min; n = 4) and G_t, likely via ε-viniferin–evoked P-1 (ΔG_t −0.25 ± 0.09 mS/cm^2, Δtime 2.3 ± 0.5 min) and P-3 (ΔG_t 3.83 ± 0.71 mS/cm^2, Δtime 16.8 ± 1.7 min) (n = 4; Fig. 1D). At a lower concentration (10^{-5} mol/L), ε-viniferin evoked a small, but long-lasting decrease in G_t, as shown in Figure 1E. Mucosal or serosal addition of the vehicle, EtOH (10 μL), did not affect I_{sc} or G_t.

Various concentrations of ε-viniferin (3×10^{-6} to 3×10^{-4} mol/L) and resveratrol (10^{-5} to 3×10^{-4} mol/L) were added to the mucosal bathing solution, and the mean changes in I_{sc} (Fig. 1E) and G_t (Fig. 1F) were plotted and fitted to the Hill equation. The half maximal

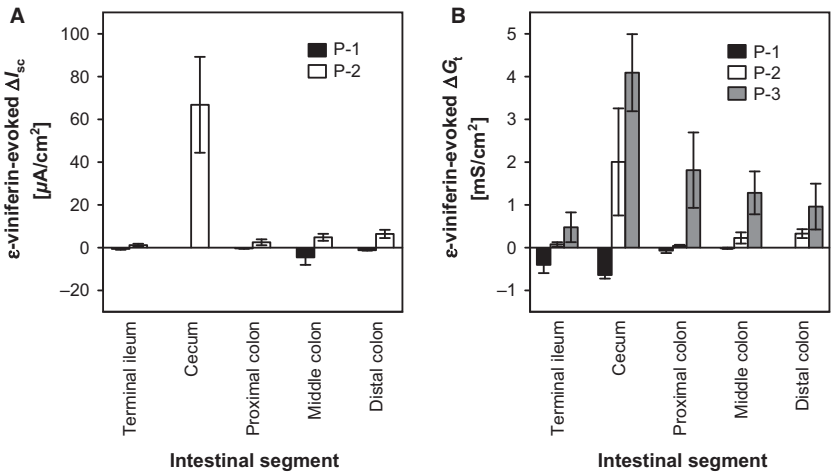

Figure 2. Segmental differences in the ε-viniferin–evoked changes in I_{sc} and G_t. Mucosa–submucosal tissue preparations of the rat terminal ileum, cecum, proximal colon, middle colon, and distal colon were mounted on Ussing chambers. ε-Viniferin (10^{-4} mol/L) was added to the mucosal bathing solution, and the peak changes in I_{sc} (A) and G_t (B) were measured. (A) ε-Viniferin evoked a decrease in the first phase (P-1) and then an increase in the second phase (P-2) in I_{sc}. (B) ε-Viniferin evoked a decrease (P-1), rapid increase (P-2), and a slow increase (P-3) in I_{sc}. Data are expressed as the mean ± SEM (n = 4).

(50%) effective concentration (EC_{50}) of the mucosal ε-viniferin–evoked ΔI_{sc} was 4.81×10^{-5} mol/L, the maximum effect (E_{max}) was 87.19 μA/cm^2, and the Hill coefficient value (n_H) was 2.37 (coefficient of determination [R^2] = 1.000). At 10^{-5} mol/L or lower concentrations, mucosal ε-viniferin only evoked a decrease in G_t, which was long-lasting. The EC_{50} of the mucosal ε-viniferin–evoked P-1 ΔG_t was 2.53×10^{-6} mol/L, E_{max} was −0.69 mS/cm^2, and n_H was 1.22 (R^2 = 0.887). Although it is unclear whether the P-2 ΔG_t evoked by 3×10^{-4} mol/L ε-viniferin was at the maximum effective concentration, the EC_{50} of ΔG_t was calculated to be 9.44×10^{-5} mol/L, when we assumed that the E_{max} was the mean value for ΔG_t at 3×10^{-4} mol/L ε-viniferin (6.92 mS/cm^2) and that the n_H was same as the that for P-3 described below (R^2 = 1.000). Therefore, the EC_{50} for mucosal ε-viniferin–evoked P-3 ΔG_t was calculated to be 2.35×10^{-5} mol/L, the E_{max} of mucosal ε-viniferin–evoked P-3 ΔG_t was 4.49 mS/cm^2, and nH was 4.53 (R^2 = 0.999).

The resveratrol-evoked P-1 and P-2 ΔI_{sc} did not seem to reach the maximum concentrations, but the EC_{50} of P-2 ΔI_{sc} was 2.52×10^{-4} mol/L (R^2 = 0.975) when we assumed that E_{max} and n_H were the same as those for ε-viniferin–evoked ΔI_{sc}. Likewise, the resveratrol-evoked increase in G_t did seem to reach the maximum concentration, but the EC_{50} was 1.82×10^{-4} mol/L (R^2 = 0.912) when we assumed the E_{max} and n_H were the same as those for ε-viniferin–evoked P-3 ΔG_t.

Although ε-viniferin may evoke more potent responses at concentrations of $>3 \times 10^{-4}$ mol/L, ε-viniferin at these concentrations evoked unlimited continuous increases in G_t. This indicates that ε-viniferin at concentrations $>3 \times 10^{-4}$ mol/L may have cytotoxic effects because the epithelial barrier function was thought to have failed. Thus, ε-viniferin was used at 10^{-4} mol/L in the following experiments.

Segmental differences in the effects of mucosal ε-viniferin in the rat small and large intestines

The effects of mucosal ε-viniferin (10^{-4} mol/L) on I_{sc} and G_t were measured and compared among the rat terminal ileum, cecum, proximal colon, middle colon, and distal colon. Mucosal addition of ε-viniferin (10^{-4} mol/L) had weak effects on I_{sc} in the intestinal tissues, except for the cecum (Fig. 2A). Regarding G_t, the ε-viniferin (10^{-4} mol/L)–evoked increase was most potent in the cecum and the magnitude of the increase gradually decreased from the proximal to distal segments of the large intestine (Fig. 2B). Thus, the other experiments in the present study were performed using the rat cecum.

Effects of mucosal ε-viniferin and resveratrol on FD4 permeability in the rat cecum

To investigate the effects of mucosal ε-viniferin and resveratrol on the barrier function against a nonionic macromolecule, the effects of mucosal ε-viniferin and resveratrol on the transepithelial permeability of FD4 from the mucosal to serosal side (FD4 $P^{m\rightarrow s}$) were measured in terms of G_t. Basal I_{sc} was 18.07 ± 1.62 μA/cm^2, basal G_t was 11.15 ± 0.41 mS/cm^2, and basal FD4 $P^{m\rightarrow s}$ was $1.26 \pm 0.20 \times 10^{-7}$ cm/s (n = 20).

Mucosal addition of ε-viniferin evoked an increase in I_{sc}, and the mean I_{sc} reached its maximum at 7 min (ΔI_{sc}: 83.56 ± 17.12 μA/cm^2, n = 8). The G_t first decreased at 3 min (P-1 ΔG_t: −0.45 ± 0.05 mS/cm^2, n = 8), the P-2 peak occurred at 8 min (P-2 ΔG_t: 4.00 ± 0.82 mS/cm^2, n = 8), and the P-3 peak occurred at 26 min (P-3 ΔG_t: 3.70 ± 0.84 mS/cm^2, n = 8). FD4 $P^{m\rightarrow s}$ increased, and reached a maximum ($3.98 \pm 0.95 \times 10^{-7}$ cm/s, n = 8) at 30–45 min after mucosal addition of ε-viniferin. This value was significantly greater ($P < 0.05$ by Dunnett's test) than the FD4 $P^{m\rightarrow s}$ after the addition of the vehicle control (EtOH; $0.138 \pm 0.025 \times 10^{-6}$ cm/s, n = 7) at 30–45 min.

After the mucosal addition of resveratrol (10^{-4} mol/L), I_{sc} decreased initially (P-1 ΔI_{sc}: −4.24 ± 2.06 μA/cm^2, n = 6, at 5 min), and then slowly increased (P-2 ΔI_{sc}: 7.40 ± 4.31 μA/cm^2, n = 6, at 37 min). G_t slowly increased (ΔG_t: 1.06 ± 0.22 mS/cm^2, n = 6, at 17 min). However, FD4 permeability did not significantly change.

Table 1. Electrophysiological parameters of the rat cecal mucosa–submucosa preparations mounted on Ussing chambers, bathed with normal Krebs–Ringer solution with Cl^--free solutions on the serosal, mucosal, and both sides of the chamber.

Krebs-Ringer solution	Basal I_{sc}	Basal G_t	n (tissue)
Normal (all tissues)	15.15 ± 0.53	12.66 ± 0.16	239
Normal (sham)	11.56 ± 1.02[a]	9.96 ± 0.74	4
Mucosal Cl^- free	−39.82 ± 4.90[b]	15.50 ± 3.56	4
Serosal Cl^- free	79.72 ± 6.85[c]	12.26 ± 1.73	4
Both sides Cl^- free	20.44 ± 0.68[a]	17.56 ± 2.99	4

Basal I_{sc} and G_t values.
Sham indicates that normal solutions of both sides of the Ussing chambers were replaced with fresh normal solution. Different superscript letters indicate statistically significant differences ($P < 0.001$, Tukey's test) among the normal solution and Cl^--free solutions on the serosal, mucosal, and both sides.

Identification of ion species involved in the ε-viniferin–evoked increase in I_{sc} and G_t in the rat cecum

To identify the ion species involved in the ε-viniferin–induced increase in I_{sc}, the bathing solutions in the serosal, mucosal, and both chambers were replaced with Cl^--free/gluconate solution. Before mounting the tissues, the fluid resistances of the normal solution and Cl^--free solution in each chamber were measured (normal: 69.5 ± 1.0 Ω; Cl^--free: 114.7 ± 0.9 Ω; n = 3). All preparations were bathed with normal solution at first. After the bathing solutions of the serosal, mucosal, or both sides were replaced with Cl^--free solution, and the compensations of fluid resistance were reset after changing the solutions. Basal I_{sc} significantly increased in the serosal Cl^--free solution, but decreased in the mucosal Cl^--free solution (Table 1). In contrast, the basal G_ts were not significantly different between the Cl^--free and sham control tests for each side (Table 1). The negative basal I_{sc} in the mucosal Cl^--free solution and the positive basal I_{sc} in the serosal Cl^--free solution are considered to be due to the liquid junction potentials between agar bridges and bathing solution.

The ε-viniferin (10^{-4} mol/L)–evoked increases in I_{sc} were almost completely abolished for Cl^--free solution on the serosal and both sides, but not the mucosal side ($P < 0.01$ by Dunnett's test, n = 4; Fig. 4A). These results indicate that the mucosal ε-viniferin–evoked increase in I_{sc} is due to electrogenic Cl^- secretion. Moreover, with serosal Cl^--free solution, mucosal ε-viniferin evoked a decrease in I_{sc} (−5.55 ± 1.66 μA/cm^2; Fig. 4A). Serosal and both side Cl^--free solutions led to unlimited increases in G_t, which made it difficult or impossible to measure the mucosal ε-viniferin–evoked changes in G_t.

To confirm that mucosal ε-viniferin evoked Cl^- secretion, a Na^+-K^+-$2Cl^-$ (NKCC) transporter inhibitor, bumetanide (10^{-4} mol/L), was added to the serosal bathing solution. Alternatively, a Ca^{2+}-dependent channel blocker, DIDS (10^{-4} mol/L), or a cAMP-dependent Cl^- channel (especially cystic fibrosis transmembrane regulator [CFTR]) blocker, NPPB (10^{-5} to 10^{-4} mol/L), were added to the mucosal bathing solution before the mucosal addition of ε-viniferin. Bumetanide (10^{-4} mol/L) and NPPB (≥5 × 10^{-5} mol/L) significantly attenuated the ε-viniferin–evoked increase in I_{sc}, but not DIDS (Fig. 5A). Although bumetanide attenuated the ε-viniferin–evoked increase in I_{sc}, it did not attenuate the G_t responses (Fig. 5B). In contrast, NPPB (5 × 10^{-5} mol/L) enhanced the ε-viniferin–evoked P-1 G_t reduction and nearly abolished the P-3 G_t change (Fig. 5B). The ε-viniferin–evoked changes in G_t in the presence of 10^{-4} mol/L NPPB were not measured because G_t gradually increased after the addition of 10^{-4} mol/L NPPB.

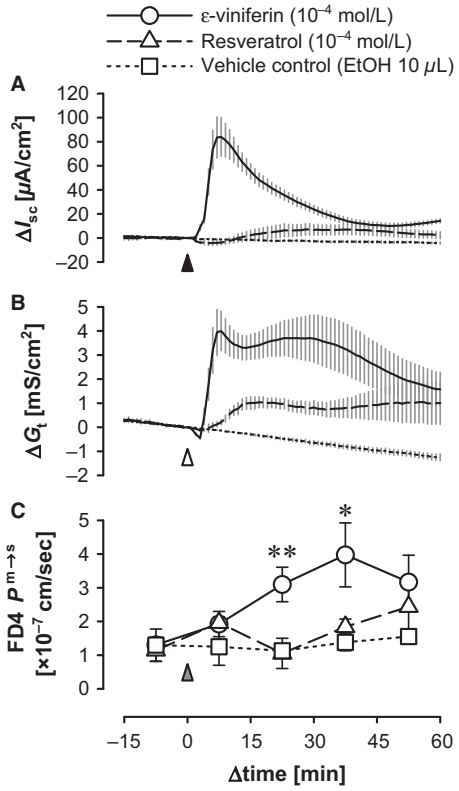

Figure 3. Time courses of mucosal ε-viniferin– and resveratrol-evoked changes in I_{sc}, G_t, and FD4 $P^{m\to s}$ in the rat cecum. Mucosa–submucosal tissue preparations of the rat cecum were mounted on Ussing chambers, and I_{sc} (A) and G_t (B) were continuously recorded. FITC-dextran (FD4; 3 kDa; 10^{-4} mol/L) was added to the mucosal bathing solution 30 min before the addition of ε-viniferin (10^{-4} mol/L; *solid traces*), resveratrol (10^{-4} mol/L; *dashed traces*), and EtOH (10 μL; as vehicle control: *dotted traces*). Samples (100 μL) were taken from the serosal bathing solution and replaced with fresh Krebs–Ringer (100 μL) every 15 min. The concentrations of FD4 in the serosal bathing solution were measured, and FD4 $P^{m\to s}$ was calculated for every 15-min time period in preparations incubated with ε-viniferin (○, *solid line*), resveratrol (Δ, *dashed line*), or the vehicle control (□, *dotted line*) (C). The time courses of I_{sc} (A) and G_t (B) every 1 min, and FD4 $P^{m\to s}$ (C) in each 15-min period are expressed as the mean ± SEM (n = 5–8). *Arrow heads* indicate mucosal addition of the stimulants. *$P < 0.05$ and **$P < 0.001$ versus the vehicle control (Dunnett's test).

Effects of mucosal resveratrol on I_{sc}, G_t, and ε-viniferin–evoked responses in the rat cecum

Although mucosal addition of 10^{-4} mol/L resveratrol evoked only a weak decrease in P-1 ΔI_{sc} (−5.66 ± 1.61 μA/cm^2) and an increase in ΔG_t

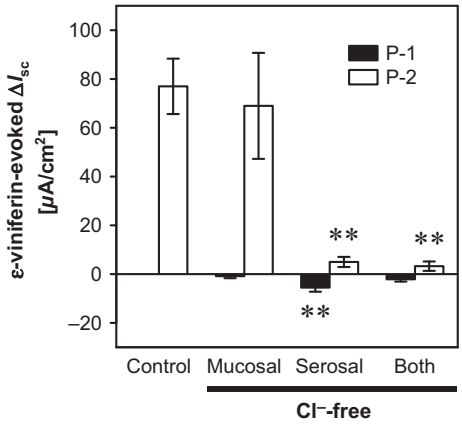

Figure 4. Effects of Cl^--free solution on the serosal, mucosal, and both sides of the chamber on ε-viniferin–evoked changes in I_{sc} in the rat cecum. The bathing solutions on the serosal, mucosal, and both sides were changed to Cl^--free solution 30 min before the mucosal addition of ε-viniferin, and the ε-viniferin (10^{-4} mol/L)–evoked changes in I_{sc} and G_t were measured. Data are expressed as the mean ± SEM (n = 4). $^{**}P < 0.01$ versus the control group (Dunnett's test).

(0.63 ± 0.30 mS/cm^2) (Fig. 6; $n = 4$), resveratrol at 3×10^{-4} mol/L elicited I_{sc} and G_t responses, which were similar to those induced by 10^{-4} mol/L ε-viniferin (Fig. 1E and F). We hypothesized that the lower concentration of resveratrol might have an antagonistic function to ε-viniferin. To investigate this hypothesis, ε-viniferin (10^{-4} mol/L) was added to the mucosal bathing solution 30 min after mucosal addition of resveratrol (at 0 to 3×10^{-4} mol/L), and the changes in I_{sc} and G_t were measured. In this experiment, resveratrol significantly attenuated the ε-viniferin–evoked changes in I_{sc} and G_t in concentration-dependent manners (Fig. 6). Mucosal resveratrol at 10^{-4} mol/L significantly attenuated the mucosal ε-viniferin (10^{-4} mol/L)–evoked increase in I_{sc} to $26.07 \pm 4.12\%$ of the control value ($P < 0.05$, $n = 4$) and P-3 ΔG_t to $29.31 \pm 8.20\%$ of the control value ($P < 0.01$, $n = 5$). When the mucosal bathing solution contained 3×10^{-4} mol/L resveratrol, the administration of ε-viniferin (10^{-4} mol/L) decreased I_{sc} (P-1 ΔI_{sc}: -8.60 ± 0.71 μA/cm^2, $P < 0.001$, $n = 4$) and abolished the P-2 response. However, there were no statistically significant differences because the P-2 response in the control conditions was weak and varied considerably between tests. Moreover, in the presence of 3×10^{-4} mol/L resveratrol, ε-viniferin (10^{-4} mol/L) caused a continuous increase in G_t, as shown in Figure 6C, so P-3 ΔG_t could not be determined.

Effects of a transient receptor potential cation channel, subfamily A, member 1 (TRPA1) inhibitor on mucosal ε-viniferin–evoked responses in the rat cecum

In our previous study, we reported that mucosal administration of a pungent principle, allyl isothiocyanate (AITC),

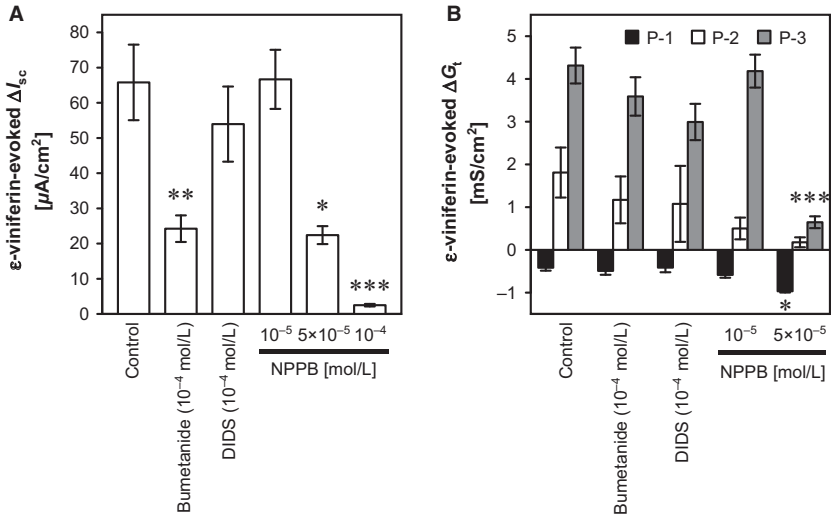

Figure 5. Effects of bumetanide, DIDS, and NPPB on the ε-viniferin–evoked changes in I_{sc} and G_t in the rat cecum. ε-Viniferin (10^{-4} mol/L) was added to the mucosal bathing solution in the Ussing chambers 30 min after the addition of serosal bumetanide (10^{-4} mol/L), mucosal DIDS (10^{-4} mol/L), or mucosal NPPB (10^{-5} to 10^{-4} mol/L), and the ε-viniferin–induced peak changes in I_{sc} (A) and G_t (B) were measured. ΔG_t was not determined in the presence of 10^{-4} mol/L of NPPB, because G_t continuously increased in these conditions. Data are expressed as the mean ± SEM (n = 3–7). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the control group (Dunnett's test).

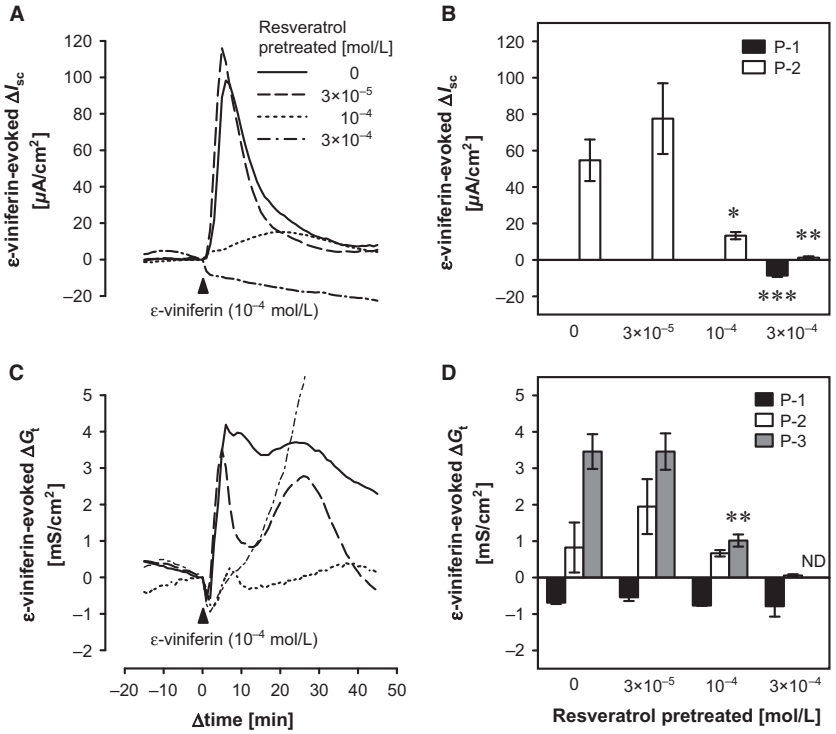

Figure 6. Effects of mucosal resveratrol on the mucosal ε-viniferin–evoked changes in I_{sc} and G_t in the rat cecum. Representative traces of the mucosal ε-viniferin (10^{-4} mol/L)–evoked changes in I_{sc} and G_t in the presence of mucosal resveratrol (0 mol/L and 3×10^{-5} to 3×10^{-4} mol/L) are shown in (A) and (C), respectively, and the mean ± SEM (n = 4–5) values were shown in (B) and (D), respectively. In the presence of 3×10^{-4} mol/L resveratrol, the ε-viniferin–evoked ΔG_t was not determined (ND), because the G_t continuously increased in these conditions. *$P < 0.05$ and **$P < 0.001$ versus the control group (Dunnett's test).

evoked anion secretion by activating TRPA1 channels (Kaji et al. 2012). Therefore, we investigated whether the ε-viniferin–evoked changes in I_{sc} and G_t were mediated via TRPA1 channels, like those evoked by AITC. In this setting, a TRPA1 inhibitor, HC030031 (10^{-4} mol/L), significantly reduced the ε-viniferin–evoked increase in I_{sc} from 60.95 ± 10.70 μA/cm^2 to 38.04 ± 8.02 μA/cm^2 ($P < 0.05$, paired t test, n = 4; Fig. 7B), and the peak time after the addition of ε-viniferin was significantly delayed from 6.71 ± 0.93 min to 14.68 ± 2.05 min ($P < 0.05$, paired t test, n = 4; Fig. 7A). For G_t, the ε-viniferin–evoked P-1 and P-3 responses were not affected, but the P-2 response was almost completely abolished by HC030031, as shown in Figure 7A and B ($P < 0.01$, n = 4).

Lack of effects of neural blockade and cholinergic antagonists, and the involvement of COX activity in the mucosal ε-viniferin–evoked I_{sc} and G_t responses in the rat cecum

The neural reflex pathways in the submucosal plexus in the gut wall play important roles in luminal stimuli-evoked secretory responses in the intestinal mucosa (Cooke 1998; Karaki and Kuwahara 2004). Thus, we investigated whether neural and cholinergic pathways are involved in the ε-viniferin–induced I_{sc} and G_t responses by adding TTX (a neural blocker; 10^{-6} mol/L), HEX (a nicotinic ACh receptor antagonist; 10^{-4} mol/L), or atropine (a muscarinic ACh receptor antagonist) to the serosal bathing solution 30 min before the addition of ε-viniferin (10^{-4} mol/L). However, TTX, HEX, and atropine did not affect the ε-viniferin–evoked changes in I_{sc} and G_t (n = 4, Fig. 8A, B).

In our previous studies, some mucosal stimulants including a bitter tastant (6-*n*-propyl-2-thiouracil) (Kaji et al. 2009), an odorant (thymol) (Kaji et al. 2011a), and a pungent principle (AITC) (Kaji et al. 2012) increased I_{sc}, and these effects were mediated by COX because piroxicam attenuated these effects. Therefore, we hypothesized that the ε-viniferin–evoked I_{sc} and G_t responses were dependent on COX activity. To test this hypothesis, a nonselective COX inhibitor (piroxicam; 10^{-5} mol/L), a selective COX-1 inhibitor (SC-560; 10^{-5} mol/L), a selective COX-2 inhibitor (NS-398; 10^{-5} mol/L), or a combination of SC-560 and NS-398 were added to the serosal

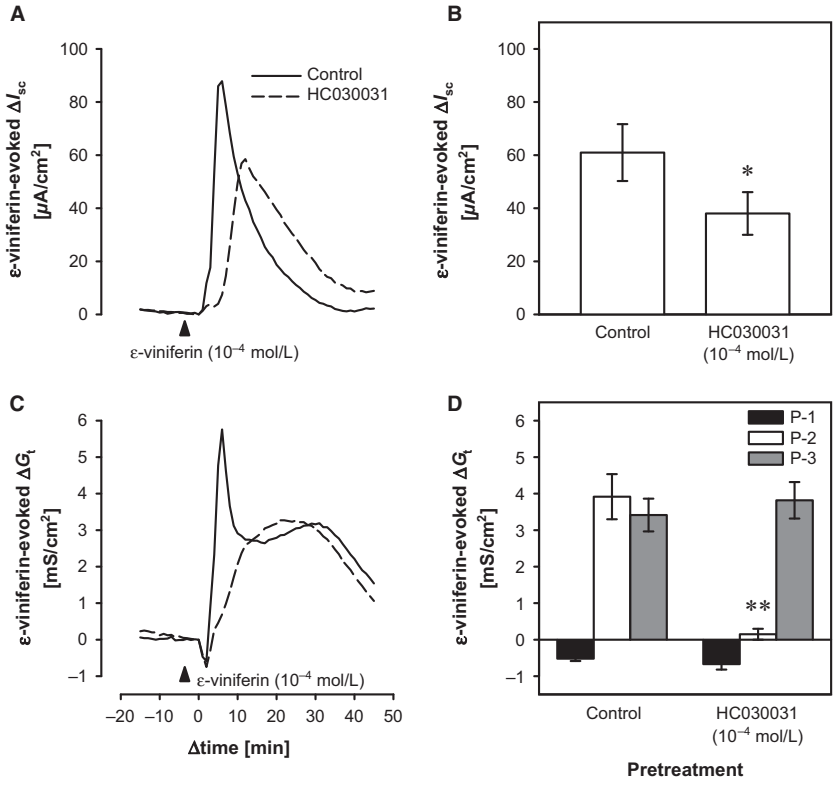

Figure 7. Effects of a TRPA1 inhibitor, HC030031, on the ε-viniferin–evoked changes in I_{sc} and G_t in the rat cecum. Representative traces of the mucosal ε-viniferin (10^{-4} mol/L)-evoked changes in I_{sc} and G_t in the presence and absence of mucosal HC030031 (10^{-4} mol/L) are shown in (A) and (C), respectively, and the mean ± SEM (n = 4) values are shown in (B) and (D), respectively. *P < 0.05 and **P < 0.001 versus the control group (paired t test).

bathing solution 30 min before the mucosal addition of ε-viniferin (10^{-4} mol/L). Piroxicam, SC-560, and the combination of SC-560 and NS-398, but not NS-398 alone, significantly attenuated the ε-viniferin–evoked increase in I_{sc} from the control value of 59.40 ± 10.53 μA/cm^2 (n = 6) to 13.33 ± 2.79 μA/cm^2 with piroxicam (n = 4; P < 0.01, Dunnett's test), to 23.10 ± 6.36 μA/cm^2 with SC-560 (n = 6; P < 005, Dunnett's test), and 22.92 ± 7.70 μA/cm^2 with the combination of SC-560 and NS-398 (Fig. 8B). These results indicate that the ε-viniferin–evoked increase in I_{sc} is mediated by COX-1 production of prostaglandin (P)G. In contrast, piroxicam, SC-560, and NS-398 did not significantly affect the ε-viniferin–evoked changes in G_t (Fig. 8B).

Effects of selective prostaglandin E_2 receptor antagonists on mucosal ε-viniferin–evoked responses in the rat cecum

To determine which PG receptor subtype mediates the ε-viniferin–evoked increase in I_{sc}, a selective EP_1 receptor antagonist (ONO-8713; 10^{-5} mol/L), an EP_1 and EP_2 receptor antagonist (AH-6809; 10^{-5} mol/L), a selective EP_3 receptor antagonist ($EP_3 > EP_4$; ONO-AE3-240; 10^{-6} to 10^{-5} mol/L), or a selective EP_4 receptor antagonist ($EP_4 > EP_3$; ONO-AE3-208; 10^{-7} to 10^{-5} mol/L) were added to the serosal bathing solution 30 min before the mucosal addition of ε-viniferin (10^{-4} mol/L). In this experiment, neither ONO-8713 nor AH-6809 affected the ε-viniferin–evoked increase in I_{sc}, but ONO-AE3-208 at concentrations ≥10^{-6} mol/L significantly attenuated the ε-viniferin–evoked increase in I_{sc} (from 84.47 ± 18.14 μA/cm^2 to 17.81 ± 10.21 μA/cm^2 at 10^{-6} mol/L; P < 0.05, n = 4; Fig. 9A). These results indicate that the ε-viniferin–evoked increase in I_{sc} is mediated by EP_4 receptors.

Inhibitory effects of mucosal ε-viniferin on the mucosal propionate-evoked I_{sc} response

Short-chain fatty acids, including acetate (two carbons), propionate (three carbons), and butyrate (four carbons), are the predominant anions in the large intestine and exist at concentrations of ≥100 mmol/L. They are produced by bacterial fermentation of indigestible dietary

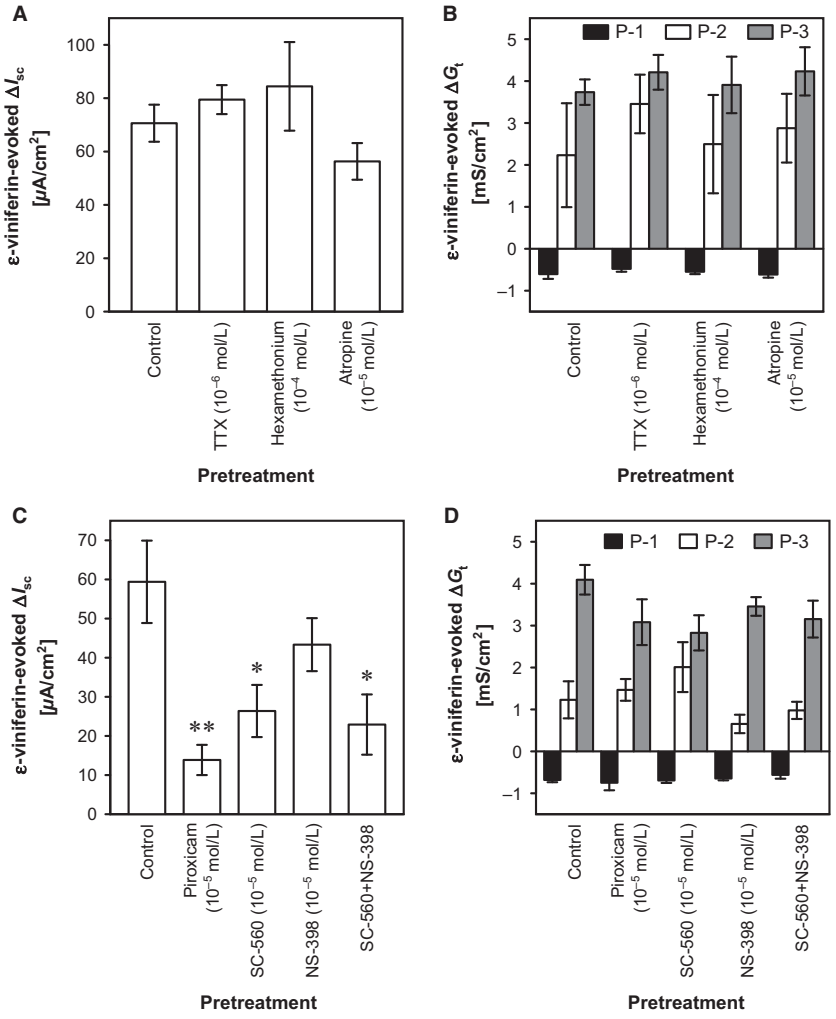

Figure 8. Effects of neural blockade, cholinergic antagonists, and COX inhibitors on the ε-viniferin–evoked changes in I_{sc} and G_t in the rat cecum. ε-Viniferin (10^{-4} mol/L) was added to the mucosal bathing solution 30 min after the serosal addition of a neural blocker (TTX; 10^{-6} mol/L), nicotinic acetylcholine receptor antagonist (hexamethonium; 10^{-4} mol/L), or muscarinic AChR antagonist (atropine; 10^{-5} mol/L), and the ε-viniferin–evoked changes in I_{sc} (A) and G_t (B) were measured. The effects of a nonselective COX inhibitor (piroxicam; 10^{-4} mol/L), a selective COX-1 inhibitor (SC-560; 10^{-5} mol/L), a selective COX-2 inhibitor (NS-398; 10^{-5} mol/L), or both SC-560 (10^{-5} mol/L) and NS-398 (10^{-5} mol/L) on the ε-viniferin–evoked changes in I_{sc} (C) and G_t (D) were also determined. Data are expressed as the mean ± SEM (n = 3–6). *P < 0.05 and **P < 0.001 versus the control group (Dunnett's test).

fibers and oligosaccharides. Mucosal SCFAs (with a potency order of propionate ≥ butyrate ≫ acetate) are known to stimulate the large intestinal mucosa to secrete transepithelial anions (Yajima 1988; Karaki and Kuwahara 2011). In our previous studies, luminal thymol and AITC attenuated propionate-evoked anion secretion in the rat colon (Kaji et al. 2011a, 2012). Therefore, we hypothesized that ε-viniferin would attenuate the mucosal propionate-evoked secretory responses. To confirm this hypothesis, mucosal propionate (10^{-3} mol/L)-evoked I_{sc} responses were measured 30 min after the addition of ε-viniferin at a variety of concentrations, as described earlier.

In the absence of ε-viniferin, propionate (10^{-3} mol/L) transiently increased I_{sc} (ΔI_{sc}: 222.33 ± 17.45 μA/cm^2; Fig. 11A) and G_t (ΔG_t: 6.39 ± 0.67 mS/cm^2; Fig. 11B) (n = 6). However, the mucosal propionate (10^{-3} mol/L)-evoked increases in I_{sc} and G_t were attenuated by mucosal ε-viniferin in a concentration-dependent manner at ε-viniferin concentrations ranging from 3×10^{-6} to 3×10^{-4} mol/L (Fig. 11). Pretreatment with 3×10^{-4} mol/L ε-viniferin nearly abolished the propionate response. The concentration–response curves of ε-viniferin for inhibiting the propionate-evoked I_{sc} and G_t responses were drawn by fitting the data to the inhibitory Hill equation, as described in Figure 11. In this analysis,

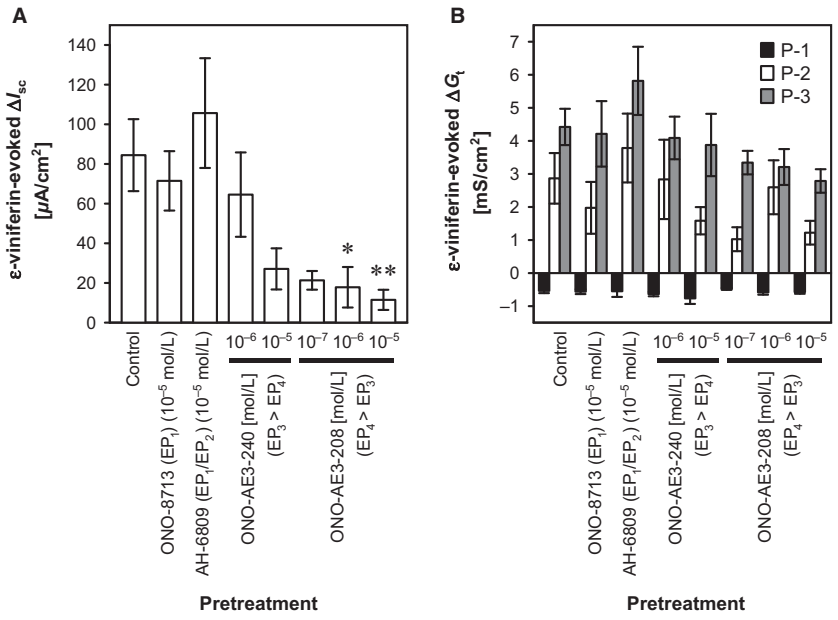

Figure 9. Effects of PGE_2 receptor antagonists on the ε-viniferin–evoked changes in I_{sc} and G_t in the rat cecum. ε-Viniferin (10^{-4} mol/L) was added to the mucosal bathing solution 30 min after the serosal addition of a selective EP_1 receptor agonist (ONO-8713; 10^{-5} mol/L), a selective EP_1/EP_2 receptor antagonist (AH-6809; 10^{-5} mol/L), an EP_3 receptor antagonist ($EP_3 > EP_4$; ONO-AE3-240; 10^{-6} and 10^{-5} mol/L), an EP_4 receptor antagonist ($EP_4 > EP_3$; ONO-AE3-208; 10^{-7} to 10^{-5} mol/L), or EtOH as a vehicle control (10 μL), and the ε-viniferin–evoked peak changes in I_{sc} (A) and G_t (B) were measured. Data are expressed as the mean ± SEM (n = 3–10). *P < 0.05 and **P < 0.01 versus the control group (Dunnett's test).

the half-maximal inhibitory concentration (IC_{50}) of ε-viniferin for ΔI_{sc} was 4.12×10^{-5} mol/L and the n_H was 2.11 (R^2 = 0.998). For G_t, the IC_{50} was 2.68×10^{-5} mol/L and n_H was 1.68 (R^2 = 0.985), where 119.4%, which was the mean value of the propionate-evoked G_t in 3×10^{-6} mol/L ε-viniferin, was used as the value for the maximal effect.

Immunohistochemistry for COX-1 in the rat cecum

It was hypothesized that luminal ε-viniferin might stimulate some epithelial sensory cells expressing COX-1, because luminal ε-viniferin evoked I_{sc} and G_t responses, but serosal ε-viniferin did not. An earlier study revealed that COX-1–expressing epithelial cells are scattered throughout the crypts in the rat large intestine (Shao et al. 1999). However, there are no previous reports describing COX-1 expression in the rat cecum. Thus, we performed immunohistochemical analysis of COX-1 expression in the rat cecum. Figure 10 shows the images of COX-1 immunoreactivity in the rat cecal crypt. COX-1 immunoreactivity was detected on scattered cells in the crypt epithelial cells (Fig. 10).

Discussion

The results of the present study show that ε-viniferin, a dehydrodimer of resveratrol, stimulated the rat intestinal mucosa, especially the cecal mucosa, from its luminal side, whereas a 10 times higher concentration of resveratrol was needed to evoke the same effect. Mucosal ε-viniferin had three major effects: (1) induce transepithelial electrogenic Cl^- secretion by activating EP_4 receptors via PGs produced by COX-1; (2) elicit a rapid decrease and a sustained increase in transepithelial ion permeability; and (3) increase transepithelial permeability of a nonionic macromolecule, FD4. In addition, mucosal ε-viniferin inhibited mucosal propionate-evoked Cl^- secretion. These results indicate that ε-viniferin stimulates the intestinal epithelium from the luminal side and effects mucosal barrier functions.

Mucosal ε-viniferin– and resveratrol-evoked changes in I_{sc} and G_t

Mucosal ε-viniferin at concentrations $\geq 10^{-5}$ mol/L elicited a concentration-dependent monophasic positive change in I_{sc} and triphasic changes in G_t, which comprised an abrupt decrease (P-1) followed by fast (P-2)

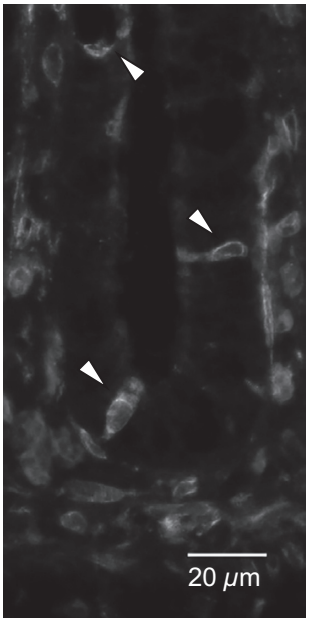

Figure 10. COX-1 immunohistochemistry in the rat cecum. Four-μm thick cryostat sections of fresh cecal tissues were fixed with cold methanol, and immunostained with a goat anti-COX-1 primary antibody and a donkey anti-goat IgG antibody conjugated to Alexa594. COX-1 immunoreactive crypt cells in the rat cecum are indicated by arrowheads.

and sustained (P-3) increases in the rat cecal mucosa, whereas serosal administration of 10^{-4} mol/L ε-viniferin did not (Fig. 1A and B). On the other hand, 10^{-4} mol/L resveratrol evoked little, but 3×10^{-4} mol/L resveratrol evoked biphasic changes in I_{sc}, which included a weak negative ΔI_{sc} (P-1) and a sustained positive ΔI_{sc} (P-2), and a single sustained increase in G_t. In contrast, serosal administration of 3×10^{-4} mol/L resveratrol did not affect I_{sc} and a small decrease (P-1) and increase (P-2) in G_t (Fig. 1C and D) were observed. These results suggest that ε-viniferin stimulates apical targets on the epithelium with a potency 10 times greater than that of resveratrol. Moreover, the first negative ΔI_{sc} evoked by ε-viniferin was apparently masked by the positive phase ΔI_{sc}. In fact, the ε-viniferin–evoked negative ΔI_{sc} was observed when the positive ΔI_{sc} was inhibited, as in the serosal Cl^--free condition described below.

At lower concentrations ($\leq 10^{-5}$ mol/L), ε-viniferin evoked the long-lasting decreases in P-1 G_t, as shown in Figure 1E. This suggested that, at higher concentration ($>3 \times 10^{-5}$ mol/L), the ε-viniferin–evoked changes in G_t consisted of an abrupt and long-lasting decrease in ion permeability and subsequent increases in ion permeability. At lower concentration, ε-viniferin may enhance epithelial barrier function, and at higher concentrations ε-viniferin may further enhance ionic and nonionic permeability (as mentioned below) to induce secretory and inflammatory functions as part of a host defense mechanism.

Segmental differences in the effects of mucosal ε-viniferin

The most potent effects of ε-viniferin in terms of the I_{sc} and G_t responses occurred in the cecal mucosa (Fig. 2). The physiologic meaning of these findings might be related to the primary function of the cecum as a fermentation tank. The gut microbiota in adult humans, especially in the colon, consists of more than 100 trillion microbes of at least 400 species (Bourlioux et al. 2003). The cecum in rodents, including rats, has not atrophied like that in humans, and is the major reservoir of microbiota in the gut. Thus, a variety of compounds produced by fermentation, including organic acids, alcohols, aldehydes, and phenols (Garner et al. 2007), are thought to accumulate in the cecal lumen owing to the activity of cecal microbiota. We hypothesize that the cecal epithelial membrane monitors the luminal fermentation conditions by sensing the luminal content. This is because maintaining adequate luminal conditions for microbiota within the major fermentation site is likely to be extremely important for gut homeostasis. Accordingly, the cecal mucosa might be the most sensitive region for sensing luminal chemicals, including ε-viniferin.

Mucosal ε-viniferin–evoked increase in FD4 $P^{m\rightarrow s}$

Mucosal ε-viniferin (10^{-4} mol/L), but not resveratrol (10^{-4} mol/L), elicited a transient (15–45 min after addition) increase in FD4 $P^{m\rightarrow s}$ (Fig. 3). In our previous study, mucosal addition of thymol also increased I_{sc}, G_t, and FD4 $P^{m\rightarrow s}$ in the rat colon (Kaji et al. 2011a). In the present study, ε-viniferin (10^{-4} mol/L) also increased FD4 $P^{m\rightarrow s}$ in the rat cecum. These findings suggest that luminal ε-viniferin directly and/or indirectly affects epithelial tight junctions, and increases paracellular permeability of nonionic macromolecules. However, the mechanism underlying the ε-viniferin–evoked increase in FD4 $P^{m\rightarrow s}$ needs to be examined in future studies.

Identification of ion species involved in the mucosal ε-viniferin–induced changes in I_{sc} and G_t

The ε-viniferin–induced increase in I_{sc} was due to transepithelial Cl^- secretion, because these responses were attenuated by Cl^--free solution on the serosal and both sides, but not on the mucosal side (Fig. 4A and B). It has

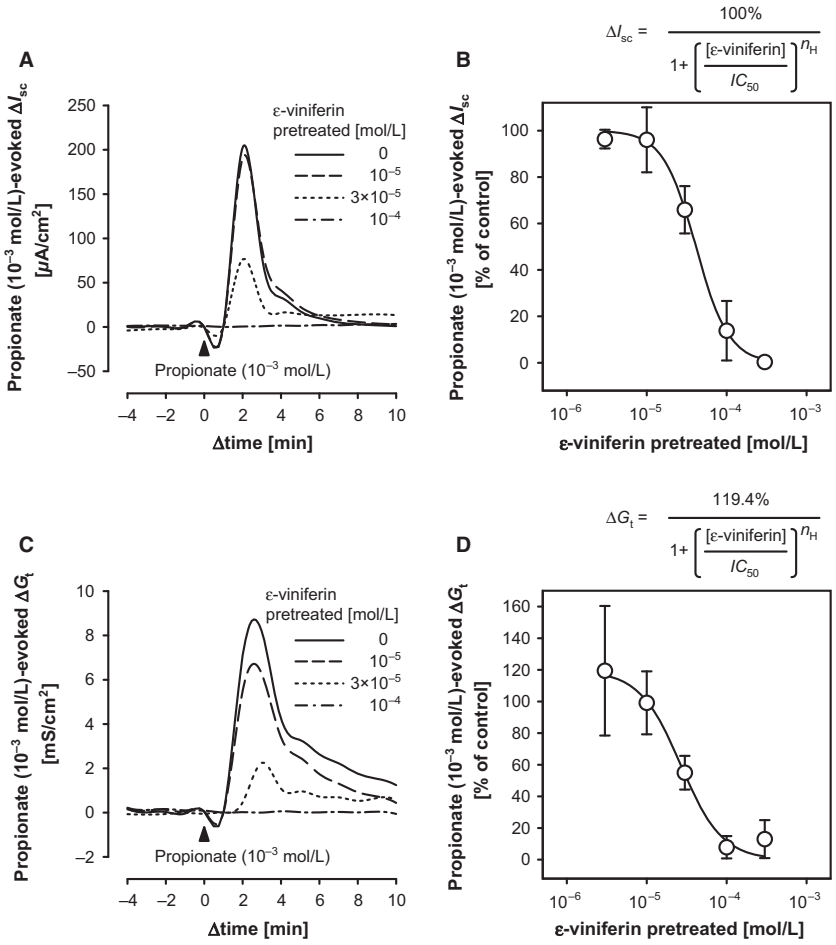

Figure 11. Inhibitory effects of ε-viniferin on the mucosal propionate-evoked changes in I_{sc} and G_t in the rat cecum. Sodium propionate (10^{-3} mol/L) was added to the mucosal bathing solution (▲) 1 h after the mucosal addition of ε-viniferin (3×10^{-6} to 3×10^{-4} mol/L) or the vehicle control (10 μL of EtOH; Δ). Representative traces of the propionate-evoked increase in I_{sc} and G_t in the presence (10^{-5} to 10^{-4} mol/L) of ε-viniferin or the vehicle control are shown in (A) and (B), respectively. The percent changes relative to the control group were plotted and fitted using the nonlinear square procedure to the inhibitory Hill equation. Data are expressed as the mean ± SEM (n = 3–6).

been proposed that intestinal fluid secretion is induced by transepithelial electrogenic anion, especially Cl^-, secretion elicited by the collaborative functions of basolateral and apical transporters and channels (Karaki and Kuwahara 2004). In particular, the basolateral NKCC1 cotransporter promotes Cl^- uptake from the basolateral membrane, whereas anion channels, especially CFTR channels in the apical membrane, release anions, especially Cl^-, into the luminal side (Karaki and Kuwahara 2004). The present results indicate that the mucosal ε-viniferin–evoked positive ΔI_{sc} was attenuated by serosal bumetanide, a NKCC inhibitor, and by mucosal NPPB, a cAMP-dependent Cl^- channel (including CFTR) blocker (Fig. 5). Accordingly, the ε-viniferin–evoked positive ΔI_{sc} may be driven by Cl^- secretion. In the serosal Cl^--free condition, mucosal ε-viniferin evoked a negative ΔI_{sc}, as did resveratrol (Fig. 5). This suggests that the initial ε-viniferin–evoked negative ΔI_{sc} was masked in normal conditions. When both sides contained Cl^--free solutions, ε-viniferin did not evoke significant negative ΔI_{sc} (Fig. 5). This suggests that the initial ε-viniferin–evoked negative ΔI_{sc}, which was masked in normal conditions, is due to the presence of mucosal Cl^- and might be based on electrogenic Cl^- absorption. However, little is known about electrogenic Cl^- absorption in the mammalian intestine. Therefore, further studies are necessary to clarify the mechanism involved in the initial ε-viniferin– or resveratrol-evoked negative ΔI_{sc}.

Although the ε-viniferin–evoked P-1, P-2, and P-3 ΔG_t were not significantly affected by serosal bumetanide (10^{-4} mol/L) and apical DIDS (10^{-4} mol/L), the apical administration of NPPB at concentrations ≥5×10^{-5} mol/

L significantly enhanced P-1 and significantly attenuated P-3 ΔG_t (Fig. 5B). The enhancement of the ε-viniferin–evoked P-1 ΔG_t by NPPB might be shown by the NPPB-mediated abolishment of the ε-viniferin–evoked increase in G_t. The ε-viniferin–evoked change in G_t was likely due to the change in the condition of tight junctions. However, the mechanism by which mucosal NPPB inhibited the ε-viniferin–evoked P-2 and P-3 ΔG_t was unclear, so further studies are need to clarify the mechanism underlying the ε-viniferin–evoked changes in G_t.

Antagonistic effects of resveratrol on ε-viniferin–evoked changes in I_{sc} and G_t

Although mucosal resveratrol at 10^{-4} mol/L hardly affected I_{sc} or G_t, it inhibited the mucosal ε-viniferin–evoked increases in I_{sc} and the increase in G_t (Fig. 6). This suggests that 10^{-4} mol/L resveratrol has antagonistic effects against the receptors for ε-viniferin on the apical membrane of the epithelium without causing a tissue response. However, mucosal resveratrol did not inhibit the initial ε-viniferin–evoked decrease in G_t (Fig. 6C and D). These results suggest that the ε-viniferin–evoked increase and decrease in G_t are mediated via different mechanisms.

Moreover, we hypothesize that the apical membrane of cecal epithelial cells express some receptors, on which ε-viniferin acts as an agonist, and resveratrol acts as an antagonist at $\leq 10^{-4}$ mol/L or as an agonist at $>10^{-4}$ mol/L. However, the identity of these receptors and the molecular mechanisms involved in the binding of ε-viniferin and resveratrol to these receptors are still unknown. Future studies should address these questions.

The mucosal ε-viniferin–evoked increase in I_{sc} and P-2 increase in G_t was mediated via TRPA1

We previously reported that a luminal stimulant, AITC, a TRPA1 agonist, induced anion secretion via EP_4 receptors (Kaji et al. 2012). Therefore, the ε-viniferin–evoked Cl^- secretion following the release of PGs may occur via the same signaling pathway to the AITC-evoked response. The peak ΔI_{sc} of the ε-viniferin–evoked increase in I_{sc} was significantly, but not completely, inhibited by a TRPA1 blocker, HC030031, and the peak time after the addition of ε-viniferin was significantly delayed in the presence of HC030031 (Fig. 7A and B). Moreover, the ε-viniferin–evoked P-2 ΔG_t was completely abolished by HC030031 (Fig. 7C and D). Therefore, the ε-viniferin–evoked increase in I_{sc} was partially due to TRPA1 and the increase in P-2 ΔG_t was completely due to TRPA1. There are currently no reports showing that ε-viniferin activates TRPA1, but mucosal ε-viniferin may activate apical TRPA1 expressed on epithelial cells (Kaji et al. 2012).

Mechanisms involved in mucosal ε-viniferin–evoked Cl^- secretion and changes in ion permeability

The present results suggest that ε-viniferin–evoked Cl^- secretion is due to the production of PG by COX-1, and that the G_t response is independent of PG (Fig. 8C). ONO-AE3-208, a selective EP_4 ($EP_4 > EP_3$) antagonist, significantly attenuated ε-viniferin–evoked Cl^- secretion (Fig. 9A). Therefore, it appears that luminal ε-viniferin stimulates COX-1 activity and PG production in sensory epithelial cells in the cecum. An earlier study revealed that COX-1–expressing epithelial cells are scattered throughout the crypts in the rat colon (Shao et al. 1999), and we confirmed the existence of these cells in the rat cecal epithelium by immunohistochemistry (Fig. 10). Accordingly, it appears that PGs activate EP_4 receptors on the secretory epithelial cells, ultimately inducing Cl^- secretion. It has been reported that EP_2 and EP_4 receptors, but not EP_1 and EP_3 receptors, mediate PGE_2-evoked Cl^- secretion via the cAMP pathway (Mosa et al. 2008).

In addition, the ε-viniferin–evoked changes in ion permeability did not involve neural pathways or PG-related pathways. Therefore, it seems likely that the ε-viniferin–evoked changes in ion permeability occurred through direct effects of ε-viniferin on the epithelial cells.

Inhibitory effects of mucosal ε-viniferin on mucosal propionate-evoked Cl^- secretion

Bacterial fermentation in the lumen of the large intestine produces numerous metabolites. The predominant molecules are SCFAs, particularly acetate, propionate, and butyrate. Propionate and butyrate, but not acetate, were reported to induce anion secretion in the rat (Yajima 1988) and guinea pig (Karaki and Kuwahara 2011) colon. Therefore, some molecules, which modulate the effects of SCFAs in the colon, critically affect the physiologic and/or pathophysiologic conditions of the large intestine. The present study showed that mucosal ε-viniferin attenuated the luminal propionate (1 mmol/L)-evoked increase in I_{sc} and G_t in a concentration-dependent manner (Fig. 11). This indicates that ε-viniferin does not inhibit the secretory functions of epithelial cells, but instead suggests that ε-viniferin might affect the mechanism for sensing propionate. Although the mechanism by which ε-viniferin may inhibit the propionate-evoked I_{sc} response is unclear, the data suggest that the inhibitory effects of ε-viniferin on the propionate-evoked responses are mediated by positive

cooperative binding because n_H was >1. The propionate-evoked response is thought to be mediated by its receptors, namely free fatty acid receptor 2 (FFA2 or GPR43) and/or FFA3 (GPR41) (Karaki et al. 2006, 2008; Tazoe et al. 2009; Karaki and Kuwahara 2011). Thus, ε-viniferin may allosterically bind to these receptors, with a possible stoichiometry of 2:1 because the n_H was nearly 2. Nevertheless, further studies are necessary to confirm this hypothesis. Moreover, at the lower concentration of 3×10^{-6} mol/L, ε-viniferin very weakly enhanced the propionate-evoked increase in G_t (Fig. 11C). Therefore, the effects of lower concentrations of ε-viniferin on transepithelial ion permeability need to be investigated in future studies.

Physiologic relevance of sensing and responding to mucosal ε-viniferin in the intestinal mucosa

In the gastrointestinal lumen, especially in the large intestine, the luminal microbiota can synthesize a variety of compounds and some of these compounds may have cytotoxic effects. Thus, the intestinal mucosa has protective roles, including enhancing the integrity of the epithelial barrier and fluid secretion when the mucosa senses potentially cytotoxic chemicals. We hypothesized that the tissue concentrations of PGs may constitute an alarm-response system, in which an increase in the PG concentration might enhance the protective functions of the mucosa in host defense (Karaki and Kuwahara 2004). Therefore, ε-viniferin may activate COX-expressing cecal crypt cells, increase the tissue PG level as part of a tissue alarm-response system, and enhance the tissue's host defense functions.

Conclusion

The present study shows that administration of ε-viniferin to the mucosal side of the rat intestine modulates transepithelial ion transport, ion permeability, and the permeability of nonionic macromolecule as well as the effects of the other luminal molecules, such as SCFAs. These results also imply that ε-viniferin has beneficial effects on intestinal functions by enhancing the mucosal host defense mechanism.

Conflict of Interest

A. Kuwahara have received research fund from FANCL Corporation. I. Ishikawa is an employee of FANCL Corporation.

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