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ORIGINAL PAPER

Volume-sensitive outwardly rectifying Cl^- channels contribute to butyrate-triggered apoptosis of murine colonic epithelial MCE301 cells

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Abstract Butyrate is a fatty acid with an important role in the maintenance of colonic homeostasis. It induces apoptosis in colonic epithelial cells and contributes to the pathogenesis of ulcerative colitis. However, just how how butyrate triggers apoptosis is poorly understood. In our model system of mouse colonic epithelial MCE301 cells, whole-cell patch-clamp recordings revealed the presence of swelling-activated outwardly rectifying chloride ion (Cl^-) currents. These currents exhibited time-dependent inactivation upon strong depolarization, a low field strength anion selectivity ($I^- > Br^- > Cl^- > F^-$), and a sensitivity to 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid (DCPIB), a specific blocker of volume-sensitive outwardly rectifying (VSOR) Cl^- channels. The results of flow cytometric analysis and caspase 3/7 assays demonstrated that exposure of MCE301 cells to sodium butyrate for 16 h triggered apoptotic cell shrinkage, phosphatidylserine exposure, and caspase 3/7 activation. Importantly, the VSOR Cl^- channel blocker was able to inhibit sodium butyrate-induced apoptotic processes. These results suggest that activation of the VSOR Cl^- channel is essential for sodium butyrate-triggered apoptosis in MCE301 cells.

Keywords Cl^- channel · Apoptosis · Butyrate · Cell shrinkage · Colon

Introduction

Ulcerative colitis is an inflammatory bowel disease of unknown etiology. The butyrate-producing bacterium *Fusobacterium varium* may play a role in triggering inflammation in ulcerative colitis [1–5]. Butyrate is an abundant short-chain fatty acid produced during bacterial carbohydrate fermentation in the colon [6, 7]. It plays pivotal roles in maintaining homeostasis in the colon by regulating cell proliferation, differentiation, the cell cycle, and apoptosis [8–11]. A previous study demonstrated that mice treated with butyrate enema had a relatively higher number of apoptotic bodies in the colorectal mucosa and ulcerative colitis-like lesions in the colorectum [4]. However, the mechanism of butyrate-induced apoptosis in colonic epithelial cells is poorly known.

Cell volume remains relatively constant in ulcerative colitis, even though cells are exposed to hypotonic shock. A variety of ion transporters are essential to maintain this cell volume homeostasis [12], but in the process of apoptotic cell death, continuous cell shrinkage, called apoptotic volume decrease, is induced due to disordered ion channel activities [13, 14]. The main component of the induction of this apoptotic volume decrease has been shown to be the volume-sensitive outwardly rectifying (VSOR) chloride ion (Cl^-) channel, which contributes to cell volume regulation [13–15]. The apoptotic volume decrease triggered by activation of the VSOR Cl^- channel has also been shown to be an early prerequisite for apoptosis. The aim of the study reported here, therefore, was to investigate

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whether the VSOR Cl^- channel contributes to butyrate-induced apoptosis in colonic epithelial cells.

Materials and methods

Cell culture

Murine normal colonic epithelial MCE301 cells [16] were grown in Dulbecco's modified Eagle's medium/nutrient F-12 Ham (DMEM/F-12; Sigma, St. Louis, MO) supplemented with 5 % fetal bovine serum (Nichirei Biosciences, Tokyo, Japan), 100 U/ml penicillin (Wako, Osaka, Japan), 100 μg/ml streptomycin (Wako), and ITES (10 μg/ml insulin, 5.5 μg/ml transferrin, 2 μg/ml ethanolamine, 5 μg/ml sodium selenite), at 37 °C in a humidity-controlled incubator with 5 % CO_2. For the patch-clamp experiments to measure VSOR Cl^- currents, the cells were cultured in suspension with agitation and plated on the chamber immediately prior to the experiments.

Patch-clamp experiments

Whole-cell recordings were performed using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA) at room temperature. The Clampex 10 Data Acquisition Module (Molecular Devices) was used for the control and recording of pulses and data acquisition. Clampfit 10 and WinASCD sofware (kindly provided by Dr. G. Droogmans, KU Leuven, Belgium) were utilized for data analysis. Currents were filtered at 1 kHz in an Axopatch 200B amplifier and digitalized at 5 kHz. Patch electrodes had a resistance of 2–4 MΩ when filled with a pipette solution. The access resistance (<10 MΩ) was electrically compensated by 70 % to minimize voltage errors. The time course of current activation and recovery was monitored by repetitively applying alternating pulses from a holding potential of 0 to ±40 mV every 15 s, respectively. To observe the voltage dependence of current profiles, we applied step pulses ranging from −100 to +100 mV in 20-mV increments with a pre-potential of −100 mV and a post-potential of −60 mV. To measure reversal potentials of the current, we used ramp pulses ranging from −100 to +100 mV.

The isotonic bathing solution contained (in mM) CsCl (110), $MgSO_4$ (5), HEPES (12), Tris (7), and D(−)-mannitol (110; pH 7.4, 340 mOsmol/kg H_2O). For the hypotonic bathing solution, we reduced the concentration of D(−)-mannitol to 40 mM (pH 7.4, 270 mOsmol/kg-H_2O). The standard pipette (intracellular) solution contained (in mM) CsCl (110), $MgSO_4$ (2), Na_2-ATP (1), Na-HEPES (15), HEPES (10), ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA; 1), and D(−)-mannitol (50; pH 7.3, 300 mOsmol/kg-H_2O). The osmolality of solutions was measured using a freezing-point depression osmometer (model 3320; Advanced Instruments, Norwood, MA). To investigate anion selectivity, we substituted NaCl for CsCl in the bathing solutions. NaCl in the hypotonic bathing solution was similarly substituted with NaF, NaBr, NaI, and Na-gluconate.

Apoptosis assays

Apoptosis was assessed with fluorescence-activated cell sorter (FACS) analysis using the TACS® Annexin V-FITC kit (Trevigen, Gaithersburg, MD). MCE301 cells plated in 6-well plates were incubated in the absence and presence of the test compounds for 16 or 48 h. For the staining, harvested cells were suspended in the binding buffer containing fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI). The samples were analyzed on a BD FACSCant™ II or a BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA). At least, 10,000 cells were counted for the analysis.

Caspase assays

MCE301 cells cultured in 6-well plates were incubated in the absence and presence of the test compounds for 16 h. Caspase activities were measured using the Caspase-Glo 3/7 Assay kit (Promega, Madison, WI). The luminescence of each sample was measured in a multifunction microplate reader (GENios; Tecan, Männedorf, Switzerland).

Statistics

Data are presented as mean ± standard error of the mean (SEM) of *n* observations. Statistical differences in the data were evaluated by Student's *t* test or one-way analysis of variance. The *P* values were considered to be significant when $P < 0.05$.

Results

VSOR Cl^- channels in MCE301 cells

Our first step was to examine whether VSOR Cl^- channels are functionally expressed in MCE301 cells. As shown in Fig. 1a, cell swelling after the application of the hypotonic solution increased whole-cell currents, which were decreased by reperfusion with isotonic solution. The swelling-activated currents exhibited a mild outward rectification and a time-dependent inactivation at increased depolarized potentials (Fig. 1b, c).

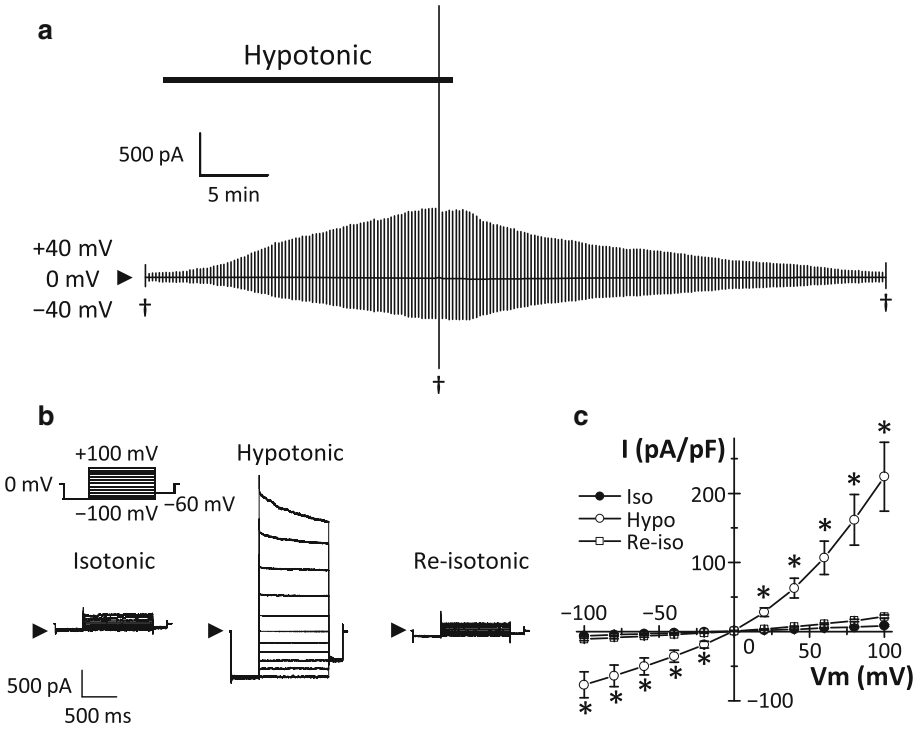

Fig. 1 Swelling-activated whole-cell currents in mouse colonic epithelial MCE301 cells. **a** Representative whole-cell currents induced by hypotonic stimulation. Alternating pulses of ±40 mV and step pulses from −100 to +100 mV in 20-mV increments (with a pre-pulse to −100 mV and a post-pulse to −60 mV) were applied from a holding potential of 0 mV. *Daggers* Time points at which step pulses were applied, *arrowhead* the zero current level. **b** Expanded traces of current responses to step pulses (*inset*) applied in **a** under isotonic (*left*), hypotonic (*middle*), and re-isotonic (*right*) conditions. **c** Instantaneous current–voltage (I–V) relationships of swelling-activated currents under isotonic, hypotonic, and re-isotonic conditions. *I (pA/pF)*: current density, *Vm*: membrane potential. Each data point represents the mean ± standard error of the mean (SEM; *vertical bar*) of five experiments. $*P < 0.05$ compared to the currents under isotonic conditions

When extracellular Cl^- was equally replaced with gluconate$^-$ after the stable activation of swelling-activated channels, the outward current at +40 mV was decreased (Fig. 2a) and the reversal potential of the current was rightward shifted (Fig. 2b), indicating that the current is Cl^--selective. Moreover, the Cl^- channel had the anion selective sequence of iodine (I^-) > bromine (Br^-) > chlorine (Cl^-) > fluorine (F^-) ≫ gluconate (gluconate$^-$) when calculated by the reversal potential shift (Fig. 2b). The data are summarized in Table 1.

We next investigated the effects of Cl^- channel blockers on swelling-activated Cl^- currents, such as 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid (DCPIB) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB). DCPIB (2.5 μM) rapidly suppressed the Cl^- currents in a voltage-independent manner (Fig. 3a–c). NPPB (10 μM) similarly inhibited the Cl^- currents (Fig. 3d).

As shown in Electronic Supplementary Material (ESM) Fig. 1, sodium butyrate (8 mM) had no significant effects on swelling-activated Cl^- currents—at least not in the 5-min perfusion.

VSOR Cl^- channels contribute to sodium butyrate-induced apoptosis in MCE301 cells

To investigate whether sodium butyrate triggers apoptotic cell death in MCE301 cells, we performed FACS analysis by using annexin V and PI. Annexin V binds to phosphatidylserine on the outer leaflet of the plasma membrane, whereas PI is membrane-impermeable but binds to DNA. It is considered that those cells which only stain with annexin V are in the early apoptotic stage, whereas those which stain with both annexin V and PI are in the late apoptotic stage. Figure 4a shows dot-plots of FITC-conjugated annexin V- versus PI-stained cells. The application of sodium butyrate (8 mM) for 2 days increased the percentage of cells stained with both annexin V and PI, but the sodium butyrate-induced late apoptosis was inhibited by DCPIB (2.5 μM; Fig. 4a, b) and NPPB (10 μM; Fig. 4c).

We further assessed apoptotic processes at an earlier stage by FACS analysis. The population of MCE301 cells showing a low forward scatter, reflecting cell shrinkage, and intense annexin V staining was defined as early apoptotic cells. As shown in Fig. 5a, b, application of

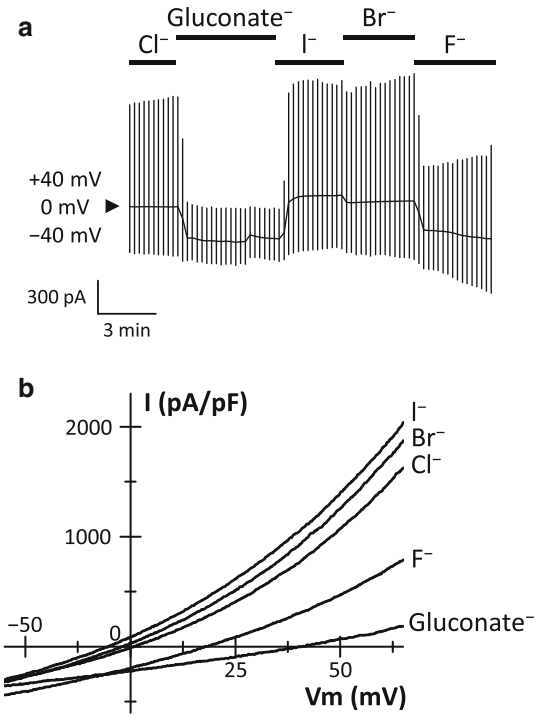

Fig. 2 Anion selectivity of swelling-activated currents in MCE301 cells. **a** Effects of extracellular chloride ion (*Cl^-*) substitution on swelling-activated currents. Extracellular Cl^- was replaced with ions of iodine (*I^-*), bromine (*Br^-*), fluorine (*F^-*), and gluconate (*gluconate$^-$*). Alternating pulses of ±40 mV were applied from a holding potential of 0 mV. *Arrowhead* Zero current level. **b** shifts of the reversal potential of swelling-activated currents under various extracellular anion conditions. Ramp pulses from −100 to +100 mV were applied

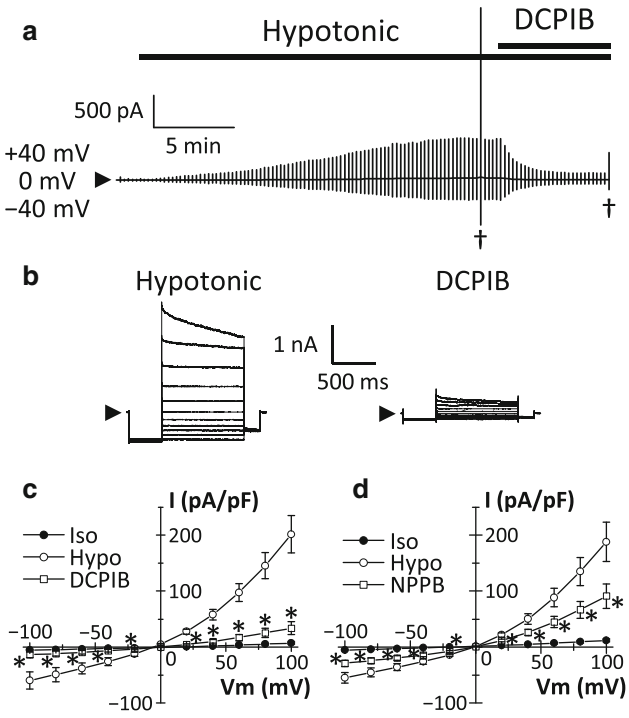

Fig. 3 Effects of Cl^- channel blockers on swelling-activated Cl^- currents in MCE301 cells. **a** Representative swelling-activated Cl^- currents before and during the application of 2.5 μM 4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid (*DCPIB*). Alternating pulses of ±40 mV and step pulses from −100 to +100 mV in 20-mV increments (with a pre-pulse to −100 mV and a post-pulse to −60 mV) were applied from a holding potential of 0 mV. *Daggers* Time points at which step pulses were applied, *arrowhead* the zero current level. **b** Expanded traces of current responses to step pulses before (*left*) and during (*right*) DCPIB application. **c** Instantaneous I–V relationships of swelling-activated Cl^- currents in the absence and presence of DCPIB. Each data point represents the mean ± SEM (*vertical bar*) of four experiments, $*P < 0.05$ compared to swelling-activated Cl^- currents in the absence of DCPIB. **d** Instantaneous I–V relationships of swelling-activated Cl^- currents in the absence and presence of 10 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid (*NPPB*). Each data point represents the mean ± SEM (*vertical bar*) of seven experiments. $*P < 0.05$ compared to swelling-activated Cl^- currents in the absence of NPPB. *Iso* Isotonic, *Hypo* hypotonic

Table 1 The anion selectivity of swelling-activated chloride ion channels

Anion[a]	Reversal potential shift (mV)	PX^-/PCl^-[b]	*n*
I^-	−4.9 ± 0.9	1.27 ± 0.04	6
Br^-	−0.3 ± 0.7	1.07 ± 0.02	10
Cl^-	0.4 ± 1.1	1	11
F^-	12.7 ± 2.1	0.66 ± 0.06	9
Gluconate$^-$	36.5 ± 3.6	0.26 ± 0.05	6

[a] Anions: I^-, Iodine; Br^-, bromine; Cl^-, chlorine; F^-, fluorine

[b] Relative permeability ratio

sodium butyrate (8 mM) for 16 h increased early apoptotic cells. The number of early apoptotic cells was significantly decreased by DCPIB (2.5 μM; Fig. 5a) and NPPB (10 μM, Fig. 5b).

We also investigated the effects of Cl^- channel blockers on caspase 3/7 activities, which are elevated during apoptosis. In MCE301 cells exposed to sodium butyrate (8 mM) for 16 h, caspase 3/7 activities were markedly increased (Fig. 5c, d). DCPIB (2.5 μM; Fig. 5c) and NPPB (10 μM; Fig. 5d) significantly suppressed the sodium butyrate-induced caspase 3/7 activation, while they had no effects on caspase 3/7 activity in the absence of sodium butyrate (Fig. 5c, d).

Discussion

The results of our study demonstrate that the VSOR Cl^- channel is functionally expressed in mouse colonic epithelial MCE301 cells and that sodium butyrate induces apoptosis in these cells. Interestingly, sodium butyrate-triggered apoptosis was inhibited by the VSOR Cl^- channel blockers. These results are the first to demonstrate that

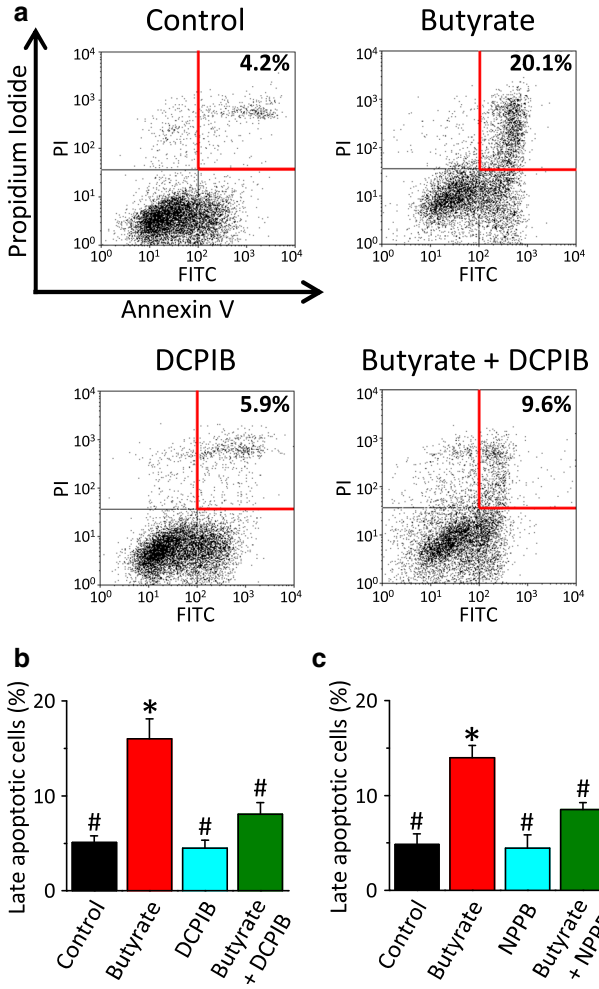

Fig. 4 Effects of Cl^- channel blockers on apoptotic processes of MCE301 cells treated with sodium butyrate for 2 days. **a** Representative dot-plots showing the distribution of cells stained with annexin V and/or propidium iodide (*PI*). Annexin V- and PI-positive cells (*upper right*) are defined as late apoptotic cells. **b**, **c** Quantification of late apoptotic cells: 2.5 μM DCPIB (**b**) and 10 μM NPPB (**c**) decreased the number of late apoptotic cells after exposure to 8 mM sodium butyrate for 2 days. Each *column* represents the mean ± SEM (*vertical bar*) of five experiments. $^*P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to butyrate. *FITC* Fluorescein isothiocyanate

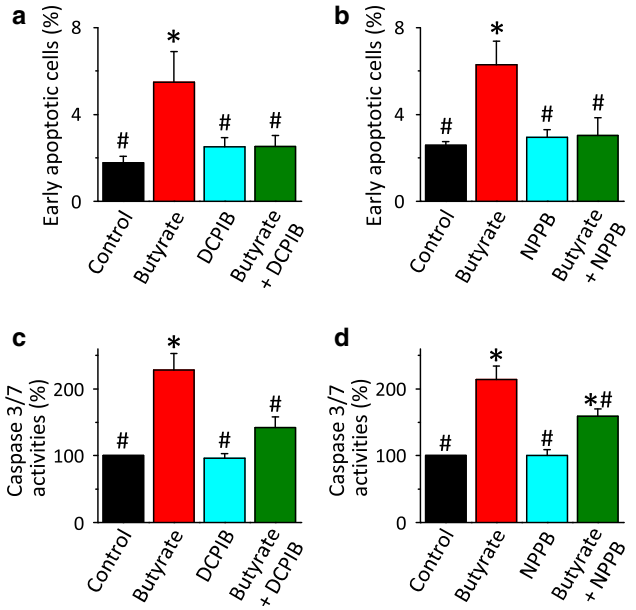

Fig. 5 Effects of Cl^- channel blockers on apoptotic processes of MCE301 cells treated with sodium butyrate for 16 h. **a**, **b** Quantification of apoptotic cells. Annexin V-positive cells with low forward scatter (*shrinkage*) are defined as early apoptotic cells: 2.5 μM DCPIB (**a**) or 10 μM NPPB (**b**) decreased the number of apoptotic cells after exposure to 8 mM sodium butyrate for 16 h. Each *column* represents the mean ± SEM (*vertical bar*) of five experiments. $^*P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to butyrate. **c**, **d** Sodium butyrate-induced caspase 3/7 activation: 2.5 μM DCPIB (**c**) or 10 μM NPPB (**d**) inhibited caspase 3/7 activation in cells exposed to 8 mM sodium butyrate for 16 h. Each *column* represents the mean ± SEM (*vertical bar*) of five (**c**) and seven (**d**) experiments. $^*P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to butyrate

activation of the VSOR Cl^- channel is a prerequisite for apoptosis in MCE301 cells treated with sodium butyrate.

VSOR Cl^- channels are reported to be functionally expressed in a variety of cells, contributing to important physiological processes such as cell volume regulation, differentiation, migration, and death [12, 17, 18]. Electrophysiological properties of the VSOR Cl^- channel have been well characterized by mild outward rectification, time-dependent inactivation at large positive potentials, and a low field strength anion selectivity ($I^- > Br^- > Cl^- > F^-$) [12, 18, 19]. In our study, we observed swelling-activated Cl^- currents exhibiting similar biophysical fingerprints in MCE301 cells (Figs. 1, 2) that were subject to inhibition by DCPIB (Fig. 3), a specific VSOR Cl^- channel blocker [20]. These results demonstrate that the VSOR Cl^- channel is endogenously expressed in MCE301 cells.

Sodium butyrate is reported to induce apoptotic cell death in a variety of cells, including colonic epithelial cells [21, 22]. One of the hallmarks of early apoptosis is cell shrinkage, referred to as apoptotic volume decrease. Activation of the VSOR Cl^- channel has been shown to be essential for the induction of apoptotic volume decrease and subsequent apoptotic processes [13–15]. In MCE301 cells treated with sodium butyrate for 16 h, we observed apoptotic volume decrease, phosphatidylserine exposure to the outer leaflet of the plasma membrane, and caspase 3/7 activation (Fig. 5)—all of which are major characteristics of apoptosis. These results suggest that sodium butyrate induces apoptosis in normal colonic epithelial MCE301 cells. Importantly, apoptotic processes observed 16 h after exposure of the cells to sodium butyrate were inhibited by the potent VSOR Cl^- channel blockers DCPIB and NPPB

(Fig. 5), suggesting that activation of the VSOR Cl^- channel is required for the induction of apoptotic processes following exposure to sodium butyrate. In addition, FACS analysis demonstrated that 2 days after exposure to sodium butyrate the cells were stained by both annexin V and PI (Fig. 4), suggesting that they were in the late apoptotic stage. The VSOR Cl^- channel blockers also inhibited this late apoptosis.

Sodium butyrate is known to act as an inhibitor of histone deacetylases (HDAC) [23], and it has been suggested that gene modification by HDAC inhibition blocks the transition from the G1 phase of the cell cycle to the S phase, triggering apoptosis. Interestingly, regulation of the VSOR Cl^- channel by HDAC inhibitors such as trichostatin A (TSA) and apicidin has previously been reported [24, 25]. In these studies, the authors examined cisplatin-resistant KCP-4 cells derived from human epidermoid cancer KB cells which lacked functional VSOR Cl^- channel activities and cisplatin-sensitive KB cells which exhibited typical VSOR Cl^- currents. Surprisingly, VSOR Cl^- currents were partially restored in the cisplatin-resistant KCP-4 cells treated with TSA or apicidin for more than 1 day; TSA-treated KCP-4 cells were also sensitive to cisplatin. These results suggest that increased VSOR Cl^- channel activities by HDAC inhibitors are able to overcome acquired cisplatin resistance. In our study, we observed that sodium butyrate-induced early apoptosis was inhibited by VSOR Cl^- channel blockers (Fig. 5). Therefore, it is likely that sodium butyrate affects VSOR Cl^- channel activities, but not HDAC activities, to trigger early apoptotic processes, sodium butyrate did not directly act on the VSOR Cl^- channel (ESM Fig. 1). Further studies are needed to clarify the apoptotic signaling pathway induced by sodium butyrate in MCE301 cells.

Butyrate is produced by the bacterium *F. varium* in colonic mucosa. Since it has been demonstrated that an antibiotic combination therapy targeting the bacterium achieves a significant remission of active ulcerative colitis in humans [1–3] and that luminal administration of butyrate into the rectum triggers ulcerative colitis-like lesions in mice [4], it would appear that butyrate produced by this bacterium is (at least) partly associated with the ulcerative colitis condition. If so, selective VSOR Cl^- channel blockers might be candidates for the therapy of ulcerative colitis. However, further studies are needed to clarify the pathophysiological function of butyrate in the development of ulcerative colitis.

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Conflicts of interest The authors declare that they have no conflict of interest.

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