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Butyrate-induced colonic hypersensitivity is mediated by mitogen-activated protein kinase activation in rat dorsal root ganglia

Dabo Xu, Xiaoyin Wu, Gintautas Grabauskas, and Chung Owyang

Gastroenterology Research Unit, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA

Abstract

Objective—Increased faecal butyrate levels have been reported in irritable bowel syndrome. Rectal instillation of sodium butyrate (NaB) increases visceral sensitivity in rats by an unknown mechanism. We seek to examine the signal transduction pathways responsible for the enhanced neuronal excitability in the dorsal root ganglion (DRG) following NaB enemas and demonstrate that this is responsible for the colonic hypersensitivity reported in this animal model.

Design—Colorectal distention (CRD) studies were performed in rats treated with NaB rectal instillation with/without intrathecal or intravenous administration of mitogen-activated protein (MAP) kinase kinase inhibitor U0126. Western blot analysis and immunocytochemistry studies elucidated intracellular signalling pathways that modulate I_A . Patch-clamp recordings were performed on isolated DRG neurons treated with NaB, with/without U0126.

Results—Visceromotor responses (VMR) were markedly enhanced in NaB-treated rats. Western blot analysis of DRG neurons from NaB-treated rats showed a 2.2-fold increase in phosphorylated ERK1/2 (pERK1/2) and 1.9-fold increase in phosphorylated voltage-gated potassium channel subunit 4.2 (pKv4.2). Intrathecal or intravenous administration of U0126 reduced VMR to CRD in NaB-treated rats and prevented increases in pERK1/2 and pKv4.2. Patch-clamp recordings of isolated DRG neurons showed that NaB caused a reduction in I_A to $48.9\pm1.4\%$ of control and an increase in neuronal excitability, accompanied by a twofold increase in pERK1/2 and pKv4.2. Concurrent U0126 administration prevented these changes.

Conclusions—Visceral hypersensitivity induced by colonic NaB treatment is mediated by activation of the MAP kinase–ERK1/2 pathway, which phosphorylates Kv4.2. This results in a reduction in I_A and an enhancement of DRG neuronal excitability.

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Correspondence to: Dr Chung Owyang, Department of Internal Medicine, University of Michigan, 3912 Taubman Center, SPC 5362, Ann Arbor, MI 48109, USA; cowyang@med.umich.edu.

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INTRODUCTION

Butyrate is a short-chain fatty acid produced by bacterial fermentation of undigested dietary fibre in the colon.¹ Increased faecal butyrate levels have been reported in patients with diarrhoea-predominant irritable bowel syndrome (IBS).² This may explain why the consumption of wheat bran, an insoluble dietary fibre, aggravates IBS symptoms.³⁴ Visceral hypersensitivity is common in IBS.⁵⁶ Animal studies have shown that rectal instillation of sodium butyrate (NaB) increases visceral hypersensitivity to colonic distention and induces referred mechanical hyperalgesia.⁷ The mechanism responsible for butyrate-induced visceral hypersensitivity is unknown.

The sensitisation of primary sensory neurons in the dorsal root ganglion (DRG) may contribute to visceral hypersensitivity.⁸ The extracellular signal-regulated kinase (ERK)1/2, a member of the mitogen-activated protein (MAP) kinase family that transduces extracellular stimuli into intracellular post-translational and transcriptional responses, plays an important role in mediating neuronal excitability.⁹ Research has shown that peripheral noxious stimuli activate ERK1/2 in primary afferent DRG neurons. ERK1/2 activation, in turn, may lead to peripheral sensitisation and development of visceral hypersensitivity.¹⁰¹¹ However, the downstream mechanism responsible for peripheral sensitisation induced by ERK1/2 activation is not clear. A-type potassium channels play a pivotal role in modulating neuron excitability; in fact, their downregulation may increase pain sensation.¹² The A-type potassium channel Kv4.2 has been detected in DRG neurons and appears to be responsible for the transient A-type potassium current (I_A) recorded in small DRG neurons.¹³ Kv4.2 is a known substrate for ERK1/2.¹⁴ The phosphorylation of Kv4.2 by ERK1/2 in dorsal horn neurons underlies the induction of central sensitisation and is critical for pain modulation.¹⁵

We examined the hypothesis that rectal instillation of butyrate leads to activation of the MAP kinase (MAPK)–ERK1/2 pathway in rat DRG neurons. We aimed to show that ERK1/2 activation regulates the transient I_A and that phosphorylation of Kv4.2 results in decreased opening of the potassium channel and depolarisation of DRG neurons. The enhanced excitability of DRG neurons may be responsible for the colonic hypersensitivity reported in this animal model.

MATERIALS AND METHODS

Animal model

Experiments were performed on adult male Sprague Dawley rats (200–225 g). The animals were housed in plastic cages, three per cage, and maintained on a 12 h light:12 h dark cycle. For surgical preparations, rats were anaesthetised with a xylazine/ketamine mixture. Rats were instilled with enemas, NaB solution (1 M, pH 6.9), mannitol solution (2M, as a control for hyperosmolarity observed in NaB solution, pH was adjusted to 6.9 using sodium hydroxide) or normal saline, once daily, for 3 days. The enemas were perfused into the colon through a P100 polyethylene catheter positioned 7 cm from the anus. Each day the animals received 2 ml NaB solution, mannitol solution or normal saline. No significant inflammatory changes were observed in the colon after the NaB, mannitol or saline instillations. The visceromotor response (VMR) study (colorectal distention; CRD) was conducted on day 3, 6 h after the enema instillation. In separate studies, lidocaine jelly (20 mg of 2% lidocaine jelly in 1.0 ml, Astra USA, Inc., Westborough, Massachusetts, USA) or saline jelly was administered through a 24 gauge catheter positioned 4 cm from the anus 30 min before the VMR studies.⁶ These studies were performed to ascertain pain sensation evoked by colorectal distension is mediated by primary sensory pathways innervating the mucosa.

U0126 treatment

Intrathecal delivery of MAPK kinase MEK1/2 inhibitor U0126 (10 µg; dissolved in 10 µl 10% dimethyl sulphoxide; Calbiochem, San Diego, California, USA) was performed as previously described.¹⁶ Briefly, rats were lightly anaesthetised with 2% isoflurane and held by the pelvic girdle. A 25 gauge needle attached to a 25 µl Hamilton syringe was inserted into the tissue between the dorsal aspects of the L6-S1 spinal cord segments. In separate studies, U0126 (100 µg/kg; dissolved in 100 µl 0.4% dimethyl sulphoxide) was injected through the femoral vein. U0126 was given intrathecally or intravenously daily, for 3 days, 30 min before the enema instillation. On day 3, one additional dose was administered 30 min before the VMR study. Intrathecal or intravenous delivery of U0124 (Calbiochem), the inactive analogue of U0126, was performed as described for U0126.

VMR to CRD

The protocol for measuring VMR to CRD has been previously described.¹⁷ Briefly, a 32 gauge stainless steel wire was implanted in the external oblique pelvic muscle 4–6 days before beginning the experimental procedure. Graded CRD (20, 40, and 60 mm Hg) was produced by rapidly injecting saline into a colonic balloon over 1 s and maintaining the distention for 20 s. The raw electromyography was rectified and quantified by calculating the area under the curve (µV/s), which is the sum of all recorded data points multiplied by the sample interval (in seconds) after baseline subtraction.

Retrograde labelling of DRG neurons

Retrograde tracing of DRG neurons was performed to identify DRG neurons innervating the distal colon or stomach for immunofluorescence staining and electrophysiological recording. Rats were deeply anaesthetised with an intramuscular injection of ketamine (60 mg/ml) and xylazine (5 mg/ml). After laparotomy, crystals of the retrograde tracer 1,1'-diiodo-3,3',3'-tetramethylindocarbocyanine (Dil) (50 mg/ml in DMSO; Molecular Probes/Invitrogen, Carlsbad, California, USA) were applied to the distal colon or to the ventral and dorsal stomach walls, as previously described.¹⁸ The wounds were closed and the animals were allowed to recover for 10–15 days prior to harvesting DRG neurons.

Isolation and culture of DRG neurons

Rats were killed by CO₂ asphyxiation and L6-S1 and T9–10 DRG were dissected and placed in a 35 mm culture dish containing ice-cold Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution with penicillin and streptomycin. Desheathed ganglia were sliced into small fragments and placed in a 1.5 ml centrifuge tube containing digestion buffer (dispase II and collagenase I_A, 1 mg/ml). After a 60 min incubation at 37°C, cells were dispersed by gentle trituration through Pasteur pipettes and washed in Dulbecco's modified Eagle's medium (DMEM). The cells were resuspended in DMEM:nutrient mixture F-12 (Gibco/Invitrogen) containing 10% heat-inactivated fetal bovine serum supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and 2 mM L-glutamine at 37°C. For electrophysiological recording, neurons were plated on poly-L-lysine-coated (100 µg/ml) coverslips for 30 min and cultured for 24–48 h in DMEM with 10% fetal calf serum at 37°C.

Patch-clamp electrophysiology

Prior to electrophysiological recording, retrogradely labelled colonic DRG neurons were identified by their red-orange colour under Hoffman Contrast Optics (400×) in fluorescent light with a rhodamine filter (excitation wavelength, 546 nm; barrier filter, 580 nm). Only Dil-labelled neurons were studied. All measurements were made in a physiological saline solution, as previously described.¹⁸ Whole-cell patch-clamp recordings were performed using borosilicate glass electrodes (3–6 MΩ, A-M Systems, Carlsborg, Washington, USA).

Current and voltage recordings of isolated DRG neurons were obtained using the Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, California, USA) and filtered at 2 kHz using a four-pole low-pass Bessel filter. For data analysis, signals were digitised using the analogue-to-digital converter Digidata 1322B (Molecular Devices) and stored and analysed on a personal computer running pCLAMP 9 (Molecular Devices), as previously described.¹⁸

Western blot analysis

The L6-S1 dorsal root ganglia were obtained and pooled for western blot analyses.¹⁸ The neurons were homogenised in ice-cold lysis buffer, as previously described.¹⁸ The homogenate was centrifuged at 14 000 g for 10 min. Protein samples were run on Ready Gel 10% Tris-HCl (Bio-Rad, Hercules, California, USA) for 1.5 h at 80 V. Proteins were transferred to polyvinylidene difluoride membranes for 1 h at 80 V. The membranes were blocked with StartBlock buffer T20 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 1 h at room temperature, probed with primary antibodies against phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 (Cell Signaling Technology, Danvers, Massachusetts, USA) at 1:3000 dilution or with antibodies against phosphorylated Kv4.2 (pKv4.2) and total Kv4.2 (Santa Cruz Biotechnology, Santa Cruz, California, USA) at 1:1000 dilution at 4°C overnight, and then washed in Tris-buffered saline for 1 h. The membranes were probed with corresponding horseradish peroxidase-conjugated secondary antibodies at 1:2500 dilution for 1 h at room temperature, and the bands were visualised by electrochemiluminescence (PerkinElmer, Waltham, Massachusetts, USA). Signals were quantified using ImageJ (National Institutes of Health, Bethesda, Maryland, USA) and normalised to controls.

Immunohistochemistry

For immunohistochemical staining, coronal sections of T9–10 and L6-S1 DRG were cut with a cryostat (10 µm; Leica CM1800, Leica Microsystems, Wetzlar, Germany) and permeabilised in phosphate-buffered saline with 0.3% Triton X-100 for 10 min. After blocking with 10% goat serum (Vector Laboratories, Burlingame, California, USA) in phosphate-buffered saline with 0.3% Triton X-100, DRG sections were incubated with the following antibodies: mouse monoclonal anti-Kv4.2 K57/1 (1:500; NeuroMab, Davis, California, USA), rabbit polyclonal antiphospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (1:1000, Cell Signaling Technology) and chicken polyclonal antimicrotubule-associated protein 2 (MAP2) (1:500; Millipore, Billerica, Massachusetts, USA). MAP2 was used as a neuronal marker. Alexa Fluor 488-conjugated donkey antimouse IgG (1:250; Molecular Probes) was used to detect anti-Kv4.2; Cy3-conjugated donkey antirabbit antibody (1:500; Jackson ImmunoResearch, West Grove, Pennsylvania, USA) and Alexa Fluor 488-conjugated donkey antirabbit IgG (1:250; Molecular Probes) were used as secondary antibodies to detect phospho-p44/42 MAPK (ERK1/2) antibody; and AMCA-conjugated donkey antichickn antibody (1:100; Jackson ImmunoResearch) was used to detect anti-MAP2.

Statistical analyses

Differences of quantified western blot and immunoreactive signals between groups were compared using one-way ANOVA followed by a post hoc Dunnett test or a Student t test if only two groups were applied. VMR was compared using two-way repeated-measures ANOVA, followed by multiple comparisons adjusted by the Bonferroni test using baseline values as a covariate and two main factors (ie, distention level as the repeated factor and group as the independent factor). Single comparisons were performed using Student t tests.

Results are expressed as means $\pm$ SEM. p Value <0.05 was considered statistically significant.

RESULTS

Butyrate enemas increase VMR to CRD

Both control and NaB-treated rats showed pressure-dependent increases in VMR to CRD (figure 1A). These responses were enhanced significantly in NaB-treated rats. Graded CRD (20, 40 and 60 mm Hg) caused an increase in abdominal muscle electromyogram mean amplitude in NaB-treated rats compared with control rats, which was statistically significant at pressure levels of 40 ($p<0.05$) and 60 mm Hg ($p<0.01$). Since 1 M of NaB is hyperosmolar, to control for the hyperosmolarity we showed there was no difference in VMR to CRD when the rats were treated with intracolonic infusion of mannitol (2 M). This suggests that NaB treatment induced visceral hypersensitivity to colorectal distension, which is independent from the osmolarity of the butyrate enemas.

To investigate whether the primary nociceptive afferent neurons in the mucosal layer are involved in the mediation of visceral hypersensitivity, 2% lidocaine jelly was used as a topical anaesthetic. This method has previously been shown to reverse the visceral hypersensitivity induced by intracolonic trinitrobenzene sulphonic acid treatment in rats.¹⁹ As shown in figure 1, intracolonic administration of 2% lidocaine blocked the increase in VMR to CRD at pressures of 40 and 60 mm Hg in NaB-treated rats ($p<0.05$) (figure 1B). Our observations suggest that primary nociceptive afferent neurons in the mucosal layer mediate visceral hypersensitivity evoked by NaB. However, it does not completely rule out the involvement of mechano-sensitive afferents in the muscular layer, since lidocaine jelly may remain in contact with the colonic mucosa for a longer duration and reach deeper layers in small animals such as rats.

Enhanced expression of pERK1/2 and pKv4.2 in DRG of NaB-treated rats

Compared with control rats, ERK1/2 phosphorylation in L6-S1 DRG increased significantly in rats after 3 days of butyrate enemas. Figure 2 shows an example of a western immunoblot using polyclonal antibodies against pERK1/2 and total ERK1/2. Although total ERK1/2 did not differ between the two groups, densitometric quantification of the signals showed that pERK1/2 increased significantly to $220\pm33\%$ of control after 3 days of butyrate enemas ($p<0.01$). Similarly, pKv4.2 increased $190\pm15\%$ after 3 days of butyrate enemas ($p<0.05$).

Retrograde tracing of colonic and gastric afferent DRG neurons with Dil was performed for immunofluorescence double staining with pERK1/2 to determine if increased ERK1/2 phosphorylation was specific to colonic afferents (figure 3A,B). In all, $15\pm5\%$ of T9–10 DRG neurons were labelled with Dil applied to the stomach and $12\pm4\%$ of L6-S1 DRG neurons were labelled with Dil applied to the distal colon. After butyrate treatment, pERK1/2 immunoreactivity was significantly increased in $54\pm8\%$ of Dil-labelled L6-S1 DRG neurons (figure 3; $p<0.05$) but was present in only $6\pm3\%$ of the gastric DRG neurons (figure 3), which was similar to the saline-treated rats, indicating specific increase in ERK1/2 phosphorylation in distal colonic afferents after butyrate treatment.

Immunohistochemistry was performed to verify that the increase of ERK1/2 phosphorylation after NaB treatment occurred in a subpopulation of L6-S1 DRG neurons expressing Kv4.2. Using anti-MAP2 as a marker to assess the total number of DRG neurons, we showed that Kv4.2 immunoreactivity was present in $33\pm8\%$ and $30\pm10\%$ of L6-S1 DRG neurons from control and NaB-treated rats. Figure 3C shows immunostained DRG neurons from a control and a NaB-treated rat. Most pERK1/2 immunoreactivity colocalised with Kv4.2 in NaB-treated rats. In control rats, pERK1/2 immunoreactivity was present in

3.1%±0.5% of Kv4.2-expressing DRG neurons. Butyrate enema instillation markedly increased ERK1/2 activation, with 21.5%±2.5% of Kv4.2-immunoreactive DRG neurons labelled for pERK1/2, representing a 6.9-fold increase ($p<0.05$). Taken together, these observations indicate that NaB-induced visceral hypersensitivity is accompanied by ERK1/2 activation in Kv4.2-expressing DRG neurons that innervate the distal colon.

Intrathecal and intravenous administrations of MEK inhibitor reduce colonic hypersensitivity induced by butyrate enemas

To determine if increased ERK1/2 activation is responsible for the development of visceral hypersensitivity, we examined the effects of intrathecal and intravenous administration of MEK inhibitor U0126 on the VMR in controls and the enhanced VMR induced by butyrate enemas. U0126 given intravenously does not cross the blood–brain barrier, but can access DRG neuron somas and afferent fibres.²⁰ On the other hand, when U0126 is delivered intrathecally, it affects DRG neurons as well as dorsal horn neurons.²⁰ As shown in figure 4, intrathecal U0126 administration significantly abolished the increase in VMR to CRD at pressures of 40 and 60 mm Hg in NaB-treated rats (figure 4A). Intrathecal U0126 administration also reduced VMR to noxious CRD at 60 mm Hg in control rats (figure 4B). In separate studies, we showed that intravenous U0126 administration, which normalised VMR to 40 and 60 mm Hg CRD in butyrate-treated rats, did not significantly inhibit VMR in controls (figure 4C,D). Intrathecal or intravenous injection of U0124 (inert control for U0126) did not significantly affect VMR. These observations suggest that ERK1/2 activation in L6-S1 DRG neurons is associated with the development of visceral hypersensitivity that occurs after the instillation of butyrate enemas in rats. On the other hand, stimulation of ERK1/2 in the dorsal horn neurons may play a role in regulating colorectal sensitivity under normal physiological conditions. We observed no adverse effects on consciousness, motor function, gait and behaviour such as grooming or eating following intrathecal administration of U0126.

Intrathecal administration of MEK inhibitor reduces expression of pERK1/2 and pKv4.2 induced by butyrate enemas

Western blot analysis of DRG neurons showed that intrathecal U0126 administration prevented ERK1/2 activation induced by butyrate enema instillation (figure 5A,C). Similarly, Kv4.2 phosphorylation at Thr602 was reduced to basal (figure 5B,C). As a result, the enhanced VMR to CRD in butyrate-treated rats treated was prevented.

NaB induces rapid phosphorylation of ERK1/2 and Kv4.2 in primary cultured DRG neurons

To determine if NaB can directly stimulate DRG neurons, we examined the effect of NaB on isolated L6-S1 DRG neurons in culture. Western blots were used to evaluate the effectiveness and time course of NaB on ERK1/2 phosphorylation in primary cultured DRG neurons. Western blot analysis showed rapid ERK1/2 phosphorylation in DRG neurons exposed to 1 mM NaB, peaking at 10 min, declining gradually, but remaining activated over basal at 1 h (figure 6A,B).

Densitometry, which was used to quantify signals of the immunoblot bands, showed that pERK1/2 increased significantly to 206%±23% of control in primary cultures of DRG neurons after 30 min of NaB treatment (figure 6E, $p<0.01$). Pretreatment of cultured DRG neurons with U0126 (10 μ M) completely blocked ERK activation by NaB ($p<0.01$). The 30 min 1 mM NaB exposure also caused a 194%±24% increase in Kv4.2 phosphorylation (figure 6E, $p<0.05$), which was attenuated by U0126 pretreatment to 109%±5% of basal ($p<0.05$). These results show that ERK1/2 activation is responsible for Kv4.2 phosphorylation induced by NaB.

NaB reduces I_A and enhances depolarisation in DRG neurons

To investigate the functional significance of NaB-induced ERK1/2 activation and Kv4.2 phosphorylation, we performed whole-cell patch-clamp recordings on retrogradely labelled L6-S1 DRG neurons and evaluated the effects of butyrate and U0126 on A-type current and neural firing in response to depolarising current stimulation. NaB (1 mM) superfusion enhanced the number of action potentials evoked by double rheobase depolarising current stimulation (4.3 ± 0.9 APs/500 ms) compared with the control (1.3 ± 0.4 APs/500 ms) (figure 7A,C, $p < 0.01$). NaB (1 mM) superfusion also caused a significant, time-dependent decrease in A-type current amplitude. The decrease, which began at the 5 min time point and bottomed out at the 15 min time point, was $48.9\% \pm 1.4\%$ of control in A-type current after the bath application. Pretreatment with U0126 (10 μ M) prevented the reduction in A-type current induced by NaB and caused an increase of $187.5\% \pm 17.2\%$ of control in A-type current in DRG neurons compared with neurons not treated with U0126 (figure 7B, D, $p < 0.01$). This suggests that butyrate modulates A-type current in DRG neurons by way of ERK1/2 activation.

DISCUSSION

In this study, we showed that short-term butyrate enemas induced rapid activation of MAPK–ERK1/2 in rat DRG neurons. This was accompanied by enhanced phosphorylation of the A-type potassium channel Kv4.2, which may contribute to the increased response to CRD. Inhibition of ERK1/2 activity with MEK inhibitor U0126 attenuated Kv4.2 phosphorylation and prevented the development of butyrate enema-induced colonic hypersensitivity. In vitro study showed that NaB directly induced ERK1/2 activation and increased Kv4.2 phosphorylation, resulting in I_A reduction in rat DRG neurons. These events were prevented by the concurrent application of U0126. Thus, visceral hypersensitivity induced by colonic NaB treatment appears to be mediated by activation of ERK1/2, which phosphorylates the Kv4.2 channel, resulting in a reduction in I_A and an enhancement of DRG neuronal excitability.

We instilled 1 M NaB solution into the distal colon to induce visceral hypersensitivity. A previous study showed that the total short chain fatty acids (SCFA) concentration in the human colon varies from 80 to 131 mmol/kg.²¹ A study of patients with self-reported food hypersensitivity reported an SCFA concentration in stool between 120 and 133 mmol/kg.²² Bourdu and colleagues⁷ showed that NaB (8–1000 mM) induced concentration-dependent colonic hypersensitivity. Although we observed a similar dose–response relationship (data not shown), we chose the maximal dose because it produced the most suitable signals in the DRG for the MEK inhibitor study. It is difficult to determine precisely the concentrations of butyrate at the sensory nerve terminals of the bowel wall. There are many factors including epithelial barrier and metabolism, diffusion and blood flow which may significantly influence the final concentrations of butyrate in the bowel wall. Furthermore, butyrate may also increase intestinal permeability in vitro as well as ex vivo.^{23–25} For the in vitro study we used 1 mM NaB, a similar concentration to that used in other in vitro studies.^{26–28} Further justification for using this dose was the report of an SCFA in excess of 1000 μ M in the pig portal vein after the ingestion of rye bread.²⁸

We provided direct evidence that colorectal exposure to butyrate results in activation of the MAPK–ERK1/2 pathway in DRG neurons of the L6-S1 spinal cord segments, which innervate the descending colon and rectum.²⁹ Previous studies have shown that ERK1/2 activation plays an important role in the induction and maintenance of heat and mechanical hyperalgesia after tissue and nerve injury or noxious gastric or colonic distention.¹⁰¹¹³⁰³¹ MEK activation is also known to play a key role in the regulation of central sensitisation resulting in long-term pain hypersensitivity.³² We have reported that diabetic visceral

hypersensitivity is associated with the activation of mitogen-activated kinase in rat DRG.¹⁸ In the current study, we showed that colonic administration of NaB induces ERK1/2 activation in L6-S1 DRG neurons of rats. This was accompanied by induction of visceral hypersensitivity. This action was not related to mucosal inflammation, the osmolarity of enemas or local mechanical distention. There was no significant inflammatory change in the mucosa and hyperosmolar mannitol did not induce colonic hypersensitivity to colorectal distension. Furthermore, the increased phosphorylation of ERK1/2 was not observed with saline enemas. Topical application of lidocaine prevented colonic hypersensitivity induced by NaB. This indicates that local primary nociceptive afferent neurons innervating the mucosa are involved in the mediation of visceral hypersensitivity. Further, our *in vitro* studies showed that a similar increase in ERK1/2 activation was observed with direct exposure of DRG neurons to NaB. This suggests that butyrate is capable of activating MAPK in DRG neurons.

How does ERK1/2 activation affect neuronal excitability? It is well documented that activation of MAPK enhances nociceptive transmission by post-translational modification of target proteins, such as pronociceptive receptors and ion channels.⁹¹⁴¹⁵ It may also stimulate gene transcription and protein synthesis.^{33–35} The spectrum of interacting ionic currents in different types of neurons appears to be an important determinant of neuronal excitability.^{36–38} One example is the transient I_A , which participates in the transduction of graded stimulating currents into graded firing rates.³⁸ Kv4.2 channels, which are the primary molecular correlates of I_A in sensory neurons, are prime targets for modulation.¹²³⁹

The Kv4.2 channel is a substrate for MAPK–ERK¹⁴ and is required for modulating the excitability of nociceptive neurons.¹⁵⁴⁰⁴¹ In this study, we observed a 2.2-fold increase in ERK1/2 activation and a 1.9-fold increase in pKv4.2 expression in the DRG after 3 days of butyrate enema treatment. This was accompanied by a marked increase in the VMR to CRD at 40 and 60 mm Hg. This suggests the development of colorectal hypersensitivity, resulting in hyperalgesia, to mechanical stimulation after colonic exposure to butyrate.

The development of visceral hypersensitivity after NaB enema instillation is likely mediated by the activation of the MAPK pathway, resulting in phosphorylation of Kv4.2 and reduction in the amplitude of I_A in DRG neurons. This possibility is supported by our electrophysiological studies showing that butyrate significantly inhibited the I_A component of the potassium current, accompanied by an increase in DRG neuron excitability. The inhibition of I_A was prevented by the concurrent application of MEK inhibitor U0126. Most importantly, the enhanced VMR to CRD in butyrate-treated rats was prevented by intrathecal and intravenous administration of U0126. U0126 delivered intravenously does not cross the blood–brain barrier, but it can access DRG neuron somas and afferent fibres.²⁰ Thus, intravenous injection of U0126 should not affect dorsal horn neurons in the spinal cord. This supports our hypothesis that the development of visceral hypersensitivity after NaB enemas is likely to be mediated by the activation of the MAPK pathway in L6-S1 DRG neurons. It should be noted that intrathecal administration of U0126, which inhibits activation of MAPK pathways in DRG as well as dorsal horn neurons, reduced the VMR (CRD 60 mm Hg) to values below those observed in controls. This suggests that the MAPK–Kv4.2 pathway in the dorsal horn and possibly the DRG neurons may play a role in the regulation of colorectal sensitivity in rats under normal physiological conditions.

In conclusion, our studies showed that increased DRG neuronal excitability is mediated by activation of MAPK–ERK1/2, which phosphorylates Kv4.2, leading to a reduction in I_A . This provides a cellular mechanism to explain the enhanced sensory neurotransmission in butyrate-induced visceral hypersensitivity and potential therapeutic targets for this subgroup of patients with IBS.

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Significance of this study

What is already known on this subject?

- Increased faecal butyrate levels have been reported in patients with diarrhoea-predominant irritable bowel syndrome (IBS).
- Rectal instillation of sodium butyrate induces visceral hypersensitivity in rats.
- Visceral hypersensitivity is common in IBS.
- Mechanism responsible for butyrate-induced visceral hypersensitivity is unknown.

What are the new findings?

- Butyrate enemas induce rapid activation of MAP kinase–extracellular signal-regulated kinase (ERK)1/2 and enhanced phosphorylation of Kv4.2 channel in rat dorsal root ganglion (DRG) neurons.
- Inhibition of ERK1/2 activity with MEK inhibitor U0126 attenuates Kv4.2 phosphorylation and prevents the development of colonic hypersensitivity.
- Visceral hypersensitivity induced by colonic butyrate treatment is mediated by activation of ERK1/2 resulting in a reduction in I_A and an enhancement of DRG neuronal excitability.

How might it impact on clinical practice in the foreseeable future?

- This study provides a cellular mechanism to explain the enhanced sensory neurotransmission in butyrate-induced visceral hypersensitivity.
- This new knowledge may have therapeutic implications as it strengthens the causal relationship between colonic butyrate levels and irritable bowel syndrome (IBS) symptoms.
- MEK inhibitors could provide novel approaches to treating a subset of painful IBS patients.

Figure 1.

Visceromotor responses (VMR) to graded colorectal distention (CRD) in rats instilled with saline, sodium butyrate (NaB) or mannitol enemas. At basal conditions (CRD, 0 mm Hg), there was no significant difference in VMR between control and NaB-treated rats. (A) Mean amplitude of abdominal muscle contractions expressed as area under the curve (AUC) after baseline subtraction in saline-, NaB- or mannitol-treated rats. (B) Electromyogram (EMG) mean amplitude in saline-treated rats with intracolonic administration of saline jelly and NaB-treated rats with intracolonic administration of 2% lidocaine or saline jelly 30 min before CRD. Values are means $\pm$ SE, n=6–8 per group. *p<0.05, compare with saline-treated rats with intracolonic administration of saline jelly; #p<0.05, compare with NaB-treated rats with intracolonic administration of 2% lidocaine jelly, two-way repeated-measures ANOVA/Bonferroni post-test.

Figure 2. Phosphorylated ERK1/2 (pERK1/2) expression in L6-S1 dorsal root ganglion (DRG) of control and sodium butyrate (NaB)-treated rats. (A) Representative immunoblots show pERK1/2, total ERK1/2 and GAPDH expression. Duplicated lanes shown for each group. Western blot shows a significant increase in pERK1/2 expression in DRG of rats treated with NaB enemas for 3 days. (B) Densitometric quantification of pERK1/2 expression in DRG of NaB-treated rats (percentage of controls). Each bar represents mean $\pm$ SEM. n=5 per group. *p<0.05, significantly different from control group.

Figure 3. Phosphorylated ERK1/2 (pERK1/2) immunoreactivity (IR) in dorsal root ganglion (DRG) neurons. (A) Representative images of double labelling for pERK1/2 (green) and retrograde tracer Dil (red) in L6-S1 DRG neurons innervating the distal colon (Aa-c) and T9-10 DRG neurons innervating the stomach (Ad-f) of sodium butyrate (NaB)-treated rats. Enhanced pERK1/2 expression (arrow) was predominant in Dil-labelled (arrow) L6-S1 DRG neurons but not T9-10 DRG neurons. (B) Quantification of pERK1/2-labelled DRG neurons innervating the distal colon and stomach in NaB-treated rats. Each bar represents mean $\pm$ SEM, n=5 rats per group, *p<0.05, significantly different from control group, scale bar =100 μ M. (C) Representative images of Kv4.2-labelled L6-S1 DRG neurons and pERK1/2-

stained neurons in control (*Ca-c*) and NaB-treated rats (*Cd-f*). pERK1/2 expression was increased after NaB treatment. Merged images show that most pERK1/2-IR was localised in Kv4.2-labelled DRG neurons (arrows). pERK1/2-IR increased significantly in Kv4.2-IR positive neurons of NaB-treated rats. (D) Quantification of the percentage of pERK1/2-labelled neurons in Kv4.2-IR positive and negative DRG neurons (L6-S1) from control and NaB-treated rats. Each bar represents mean $\pm$ SEM, n=5 rats per group. *p<0.05, significantly different from control group. Scale bar =100 μ m.

Figure 4.

Effects of intrathecal and intravenous injection of MEK inhibitor U0126 on visceromotor responses induced by graded colorectal distention (CRD). (A) Electromyogram (EMG) mean amplitude in sodium butyrate (NaB)-treated rats after intrathecal treatment with U0126 or U0124, an inert control for U0126. (B) EMG mean amplitude in saline-treated rats after intrathecal treatment with U0126 or U0124. (C) EMG mean amplitude in NaB-treated rats after intravenous injection with U0126 or U0124. (D) EMG mean amplitude in saline-treated rats after intravenous injection with U0126 or U0124. Values are means $\pm$ SEM, n=6 per group. *p<0.05, compared with controls treated with U0124, two-way repeated-measures ANOVA/Bonferroni post-test. AUC, area under the curve.

Figure 5. Effects of MEK inhibitor U0126 on phosphorylated ERK1/2 (pERK1/2) and pKv4.2 expression in L6-S1 dorsal root ganglion (DRG) neurons of sodium butyrate (NaB)-treated rats. Representative immunoblots show expression of (A) pERK1/2, total ERK1/2 and GAPDH and (B) pKv4.2 and GAPDH. One control lane from rats treated with saline enemas is shown along with a lane from rats treated with NaB enemas and intrathecal saline injection and a lane from rats treated with NaB enemas and intrathecal U0126 injection. (C) Densitometric quantification of pERK1/2 and pKv4.2 expression in the DRG of rats treated with saline or NaB enemas and intrathecal injection of saline, U0126 or U0124 (percentage of controls). Each bar represents mean $\pm$ SEM. n=5 rats in each group. *p<0.05, significantly different from control group.

Figure 6.

The effect of sodium butyrate (NaB) on the induction of ERK phosphorylation. (A) Representative immunoblots of primary cultured L6-S1 dorsal root ganglion (DRG) neurons at various time points after exposure to 1 mM NaB. (B) Histogram summarises results from three experiments. * $p < 0.05$ compared with unstimulated state. (C–E) Effects of pretreatment with intrathecal administration of U0126 on phosphorylated ERK1/2 (pERK1/2) and pKv4.2 expression in primary cultured DRG neurons treated with 1 mM NaB. (C, D) Representative immunoblots show expression of (C) pERK1/2 and GAPDH and (D) pKv4.2 and GAPDH. (E) Densitometric quantification of pERK1/2 and pKv4.2 expression in cultured DRG neurons exposed to 1 mM NaB with or without U0126 pretreatment (percentage of controls). Each bar represents mean $\pm$ SEM. $n = 5$ rats in each group. * $p < 0.05$, significantly different from control group.

Figure 7.

Sodium butyrate (NaB) stimulates neural firing and inhibits I_A in L6-S1 dorsal root ganglion (DRG) neurons. (A) Representative current-clamp traces of action potentials in response to depolarising current pulse (double rheobase) in a retrogradely labelled neuron before (left) and 15 min after 1 mM NaB superfusion (right). NaB superfusion caused a marked increase in the number of spikes evoked by depolarising current stimulation. (B) Representative voltage-clamp recordings of I_A in a retrogradely labelled L6-S1 DRG neuron elicited by voltage depolarising test pulses before and after 1 mM NaB application (top row). NaB significantly inhibited the I_A component of the K^+ current. The lower row shows the effect of U0126 on I_A amplitude before and after NaB superfusion. Pretreatment with U0126 produced a significant increase in A-type current and prevented I_A inhibition evoked by NaB superfusion. (C) Number of spikes elicited by depolarising current stimulation (double rheobase, 500 ms). The number of spikes from retrogradely labelled DRG neurons treated with 1 mM NaB increased significantly, $p < 0.05$. (D) Effect of NaB and U0126 on I_A amplitudes. NaB superfusion inhibited I_A in retrogradely labelled DRG neurons. U0126 administration increased I_A and prevented I_A inhibition evoked by NaB superfusion. $n = 5$ per group. * $p < 0.05$, compared with control.