Butyrate sensitizes the release of substance P and calcitonin gene-related peptide evoked by capsaicin from primary cultured rat dorsal root ganglion neurons

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Submitted: November 21, 2006 *Accepted: November 24, 2006*

Key words: **butyrate; substance P; calcitonin gene-related peptide; vanilloid receptor 1; capsaicin; dorsal root ganglion**

Neuroendocrinol Lett 2006; **27**(6):695–701 **PMID:** 17187013 NEL270606A11

Abstract

OBJECTIVES: To investigate whether butyrate increases substance P (SP) and calcitonin gene-related peptide (CGRP) release evoked by capsaicin from primary cultured dorsal root ganglion (DRG) neurons.

METHODS: DRG was dissected out from embryonic 15-day-old Wistar rat and cultured as dissociate cells for 24 h then exposed to butyrate (0.01 mmol/L, 0.1 mmol/L, 1 mmol/L, 10 mmol/L, respectively) for another 48 h. The neurons cultured continuously in media served as normal control. All above cultured samples were processed for detecting expression of mRNA for SP, CGRP and vanilloid receptor 1 (VR1) of DRG neurons by RT-PCR, and VR1 protein expression by Western blot. SP and CGRP basal release levels were measured by radioimmunoassay (RIA). After that, the DRG cells for RIA were stimulated by capsaicin (300 nmol/L) for 5 min and the culture media were harvested for detecting SP and CGRP release levels by RIA. The neurons exposed to vehicle solution served as vehicle controls.

RESULTS: Exposure of butyrate on DRG neurons at higher concentrations (1 mmol/L, 10 mmol/L) for 48 h increased expression mRNA for SP and CGRP than that at lower concentrations (0.01 mmol/L, 0.1 mmol/L) and normal control ($P<0.001$). VR1 mRNA and VR1 protein expression increased in a dose-dependent manner after exposure of different concentrations of butyrate. Butyrate did not alter the basal release, significantly enhanced neuropeptide release evoked by capsaicin. SP and CGRP release levels in the culture media exposed by butyrate at higher concentrations (1 mmol/L, 10 mmol/L) for 48 h and then stimulated by capsaicin were higher than that at lower concentrations (0.01 mmol/L, 0.1 mmol/L) ($P<0.001$). The exposure of vehicle solution did not produce any increase of SP and CGRP release from primary cultured DRG neurons.

DISCUSSION: Butyrate may promote the expression of mRNA for SP, CGRP and increase sensitivity of capsaicin on SP and CGRP release from primary cultured rat dorsal root ganglion neurons. The promotion of VR1 mRNA and VR1 protein expression by butyrate implicated that VR1 may be involved in the mechanisms of sensory neuropeptide release evoked by capsaicin.

ORIGINAL ARTICLE

Introduction

Despite significant advances in the recognition of aetiological factors and pathological mechanisms, the pathophysiology of irritable bowel syndrome (IBS) is still not fully understood [9]. Modulation of the excitability of sensory neurones may be particularly relevant in diseases such as IBS. The involvement of substance P (SP) in visceral hyperalgesia related to intestinal inflammation has been demonstrated by the positive correlation between colonic inflammation with abdominal pain and increased concentrations of SP in chemically induced colitis [6]. And calcitonin gene-related peptide (CGRP) exerts mucosal protection during chronic experimental colitis or gastric injury [23,5]. A novel model of colonic hypersensitivity possessing several of the characteristics encountered in patients with IBS was developed by receiving enemas of a butyrate solution in rats [3]. Butyrate-induced hyperalgesia was reversed by pretreatment with capsaicin suggests the involvement of C-fibers in the butyrate-induced hypersensitivity. Butyrate is known to function as an inhibitor of histone deacetylase and affects the transcription of some genes [31]. It has been proved that butyrate can induce a hyperpolarization of the membrane as well as an increase in the intracellular Ca^{2+} concentration of rat myenteric neurons in culture [12]. Interestingly, baseline fecal butyrate concentration increased in patients with IBS [30]. But the mechanism of the butyrate-induced hypersensitivity remains unknown. The aim of the present study was to explore the relationship between butyrate and release of SP and CGRP in DRG neurons and its related mechanism involved in vanilloid receptor 1 (VR1). This study provides the first observation whether butyrate has directly or indirectly effect on neuropeptide release from primary cultured DRG neurons of the rat.

Materials and methods

DRG cell cultures

Dorsal root ganglia from embryonic 15-day-old Wistar rats obtained from the Experimental Animal Center of Shandong University of China were dissected out as described previously [32,10]. Dorsal root ganglia prior to establishment in culture were digested with 0.25% trypsin (Sigma) in D-Hanks solution at 37 °C for 10 min, centrifuged, and triturated in growth media supplemented with 2.5% fetal bovine serum (Gibco). Dissociated DRG cells were then cultured in 24-well clusters (Costar, Corning, NY, USA) for monitoring SP and CGRP levels using radioimmunoassay (RIA) or flasks (Costar, Corning, NY, USA) for detecting SP, CGRP and VR1 mRNA by RT-PCR and VR1 protein by Western blot. The clusters and flasks were precoated with poly-L-lysine prior to plating DRG cells. DRG cells were plated at 100, 000 cells/well in clusters and at a density of 5×10^5 cells/ml in flasks. All cultures were maintained in D-MEM/F-12 supplemented with 5% fetal bovine serum, 2% B-27 supplement (Gibco), insulin (0.25 μg/mL, Sigma), L-glutamine (0.1 mg/mL, Sigma), penicillin (100 U/mL), and streptomycin (100 μg/mL). All cultures were grown at 37 °C in a humidified 5% CO_2-air atmosphere.

Exposure of butyrate on DRG neurons

At 24 h of culture age, all DRG neurons both in clusters and flasks were exposed to butyrate (0.01 mmol/L, 0.1 mmol/L, 1 mmol/L, 10 mmol/L, respectively) for another 48 h. DRG cells were continuously exposed to growth media alone as normal control.

RNA extraction and RT–PCR

The mRNA levels of SP, CGRP and VR1 were analyzed by RT-PCR. The expression of β-actin was also determined as an internal control.

Total DRG cell RNA of each flask was isolated by TRIzol (Gibco) according to methods described in the previous and the mRNA level was analyzed by RT-PCR, which was performed as described previously [16]. The gene-specific primers were synthesized by use of the published cDNA sequences for SP, CGRP, VR1 and β-actin. The synthetic oligonucleotide primer sequences for SP, CGRP, VR1 and β-actin were as follows:

SP 5'-GCC CTT TGA GCA TCT TCT TC-3' (coding sense) and 5'-GTC TGA GGA GGT CAC CAC AT-3' (coding antisense).

CGRP 5'-AGG TCG GGA GGT GTG GTG AA-3' (coding sense) and 5'-ATC CGC TTG AGG TTT AGC AGA G-3' (coding antisense).

VR1 5'-CTG ACG GCA AGG ATG ACT-3' (coding sense) and 5'-CCT AAG CAG ACC ACC CAA-3' (coding antisense).

β-actin 5'-ATC ATG TTT GAG ACC TTC AAC-3' (coding sense) and 5'-CAT CTC TTG CTC GAA GTC CA-3' (coding antisense).

The predicted size of the amplified SP, CGRP, VR1 and β-actin DNA products were 450 bp, 338 bp, 372 bp and 317 bp, respectively.

PCR was carried out using a PCR (BIOMETRA T-personal, German). The cycle profile included denaturation for 45s at 94 °C, annealing for 45s at 58 °C, and extension for 60s at 72 °C. PCR was performed within the range that demonstrates a linear correlation between the amount of cDNA and the yield of PCR products.

The amplified products on agarose gels were stained with ethidium bromide, visualized by a UV transilluminator and photographed. The photographs were scanned (Pharmacia Biotech, German), and quantification was done with a Totallab.

SP and CGRP release experiments on DRG neurons

After 48 h of butyrate incubation, DRG neuron cultures were washed with DMEM/F-12 and incubated for 5 min at 37 °C in DMEM/F-12 plus 0.1% ethanol (capsaicin vehicle) to measure basal SP and CGRP release. Fresh DMEM/F-12 containing capsaicin (300 nmol/L) was added for an additional 5 min to measure capsaicin evoked SP and CGRP release. After each incubation, the

culture media were removed and measured by RIA for SP and CGRP release from DRG neurons.

The RIA technique for the measurement of SP and CGRP was similar to the technique as reported previously [19,33]. The samples were reconstituted in PBS. Standards of synthetic SP and CGRP (rat amino acid sequence) ranging from 2.5 to 1280 pg/assay tube and 4 to 240 pg/assay tube dissolved in a volume of 0.2 mL PBS, respectively. The dissolved SP and CGRP were then incubated at 4 °C with 0.1 ml of anti-SP antibody (anti-rat SP antibody) for 24 h and 0.1 ml of anti-CGRP antibody (antihuman CGRP II antibody) for 48 h, respectively. Anti-SP antibody cross-reacts 100% with rat SP and shows, <0.01% cross-reactivity with neuropeptide K, neurokinin A and 0% cross-reactivity with neurokinin B, somatostatin (data from Department of Neurobiology, Second Military Medical University, China). Anti-CGRP antibody cross-reacts 100% with rat CGRP and shows 0% cross-reactivity with endothelin, motilin, bone gla protein, C-parathyroid hormone (C-PTH), and calcitonin (data from Sun Biomedical Technology Co., Ltd., Beijing, China). The mixture was then incubated for an additional 24 h at 4 °C with 0.1 ml of ^{125}I-labeled SP (20,000 counts/min/tube) and CGRP (17,000 counts/min/tube) in PBS. Free and bound neuropeptides were separated by adding 0.5 ml PR separating agent 45min for SP and 20min for CGRP. The RIA test tubes were centrifuged (4000rpm at 4 °C, 20 min) for SP and (3500rpm at 4 °C, 25 min) for CGRP. After removal of the supernatant fraction, the RIA test tubes were counted for iodine-125 remaining in the tubes.

Western blot analysis for VR1 protein expression

Western blot was performed as described previously [34]. Fresh and cultured DRG neurons at 72 h of culture age were homogenized in 10 mM Tris homogenization buffer (pH 7.4) with protease inhibitors (1 tablet in 50 ml; Sigma). The samples were centrifuged at 12,000 rpm for 20 min and the supernatant collected for Western blot. After determining the protein concentrations of the supernatants (BCA method, standard: BSA), 50 μg protein of each sample was loaded onto the 8% SDS gel, separated by electrophoresis and transferred to nitrocellulose membrane. The membranes were incubated with goat anti-rat VR1 polyclonal IgG (1:800, Santa Cruz Biotechnology). After being washed three times for 10 min

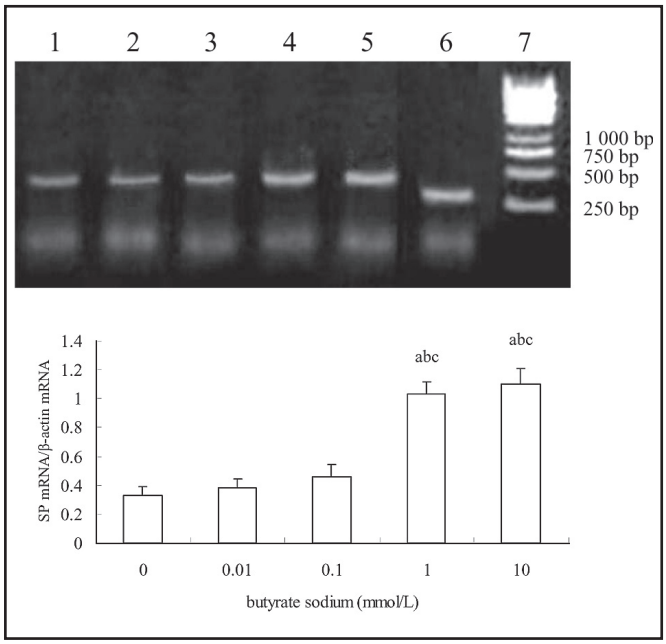

Figure 1. Effects of butyrate on SP mRNA expression in primary cultured DRG neurons. SP and β-actin mRNA were analyzed by RT-PCR. Lane 1: Normal control. Lane 2: Butyrate 0.01 mmol/L. Lane 3: Butyrate 0.1 mmol/L. Lane 4: Butyrate 1 mmol/L. Lane 5: Butyrate 10 mmol/L. Lane 6: Internal control. Lane 7: DNA marker. The quantitative analysis of the results are as follows: DRG cells were cultured continuously in growth media as normal control (SP mRNA/β-actin mRNA = 0.3304±0.0593). DRG cells were treated with 0.01 mmol/L butyrate (SP mRNA/β-actin mRNA = 0.3808±0.0659). DRG cells were treated with 0.1 mmol/L butyrate (SP mRNA/β-actin mRNA = 0.4566±0.0886). DRG cells were treated with 1 mmol/L butyrate (SP mRNA/β-actin mRNA = 1.0344±0.0835). DRG cells were treated with 10 mmol/L butyrate (SP mRNA/β-actin mRNA = 1.1030±0.1068). Bar graphs with error bars represent mean ± SD (n=5). ^{a}P<0.001 *vs* control, ^{b}P<0.001 *vs* 0.01 mmol/L, ^{c}P<0.001 *vs* 0.1 mmol/L.

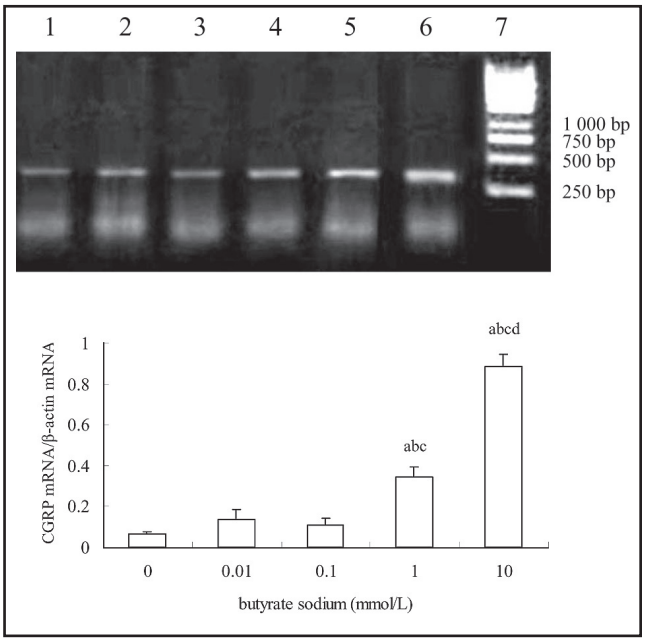

Figure 2. Effects of butyrate on CGRP mRNA expression in primary cultured DRG neurons. CGRP and β-actin mRNA were analyzed by RT-PCR. Lane 1: Normal control. Lane 2: Butyrate 0.01 mmol/L. Lane 3: Butyrate 0.1 mmol/L. Lane 4: Butyrate 1 mmol/L. Lane 5: Butyrate 10 mmol/L. Lane 6: Internal control. Lane 7: DNA marker. The quantitative analysis of the results are as follows: DRG cells were cultured continuously in growth media as normal control (CGRP mRNA/β-actin mRNA = 0.0655±0.0106). DRG cells were treated with 0.01 mmol/L butyrate (CGRP mRNA/β-actin mRNA = 0.1355±0.0479). DRG cells were treated with 0.1 mmol/L butyrate (CGRP mRNA/β-actin mRNA = 0.1091±0.0315). DRG cells were treated with 1 mmol/L butyrate (CGRP mRNA/β-actin mRNA = 0.3427±0.0482). DRG cells were treated with 10 mmol/L butyrate (CGRP mRNA/β-actin mRNA = 0.8853±0.0622). Bar graphs with error bars represent mean ± SD (n=5). ^{a}P<0.001 *vs* control, ^{b}P<0.001 *vs* 0.01 mmol/L, ^{c}P<0.001 *vs* 0.1 mmol/L, ^{d}P<0.001 *vs* 1 mmol/L.

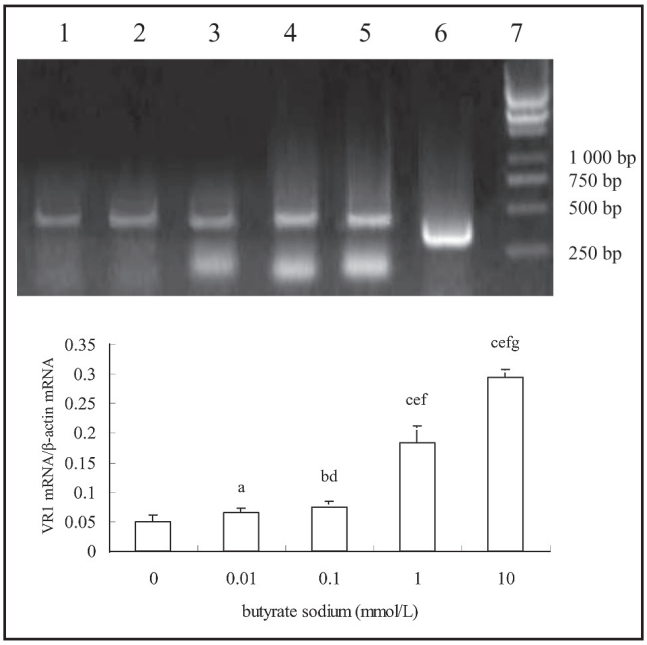

Figure 3. Effects of butyrate on VR1 mRNA expression in primary cultured DRG neurons. VR1 and β-actin mRNA were analyzed by RT-PCR. Lane 1: Normal control. Lane 2: Butyrate 0.01 mmol/L. Lane 3: Butyrate 0.1 mmol/L. Lane 4: Butyrate 1 mmol/L. Lane 5: Butyrate 10 mmol/L. Lane 6: Internal control. Lane 7: DNA marker. The quantitative analysis of the results are as follows: DRG cells were cultured continuously in growth media as normal control (VR1 mRNA/β-actin mRNA = 0.0439±0.0108). DRG cells were treated with 0.01 mmol/L butyrate (VR1 mRNA/β-actin mRNA = 0.0656±0.0078). DRG cells were treated with 0.1 mmol/L butyrate (VR1 mRNA/β-actin mRNA = 0.0879±0.0117). DRG cells were treated with 1 mmol/L butyrate (VR1 mRNA/β-actin mRNA = 0.1844±0.0284). DRG cells were treated with 10 mmol/L butyrate (VR1 mRNA/β-actin mRNA = 0.2938±0.0148). Bar graphs with error bars represent mean ± SD (n=5). $^{a}P<0.05$ *vs* control, $^{b}P<0.01$ *vs* control, $^{c}P<0.001$ *vs* control, $^{d}P<0.05$ *vs* 0.01 mmol/L, $^{e}P<0.001$ *vs* 0.01 mmol/L, $^{f}P<0.001$ *vs* 0.1 mmol/L, $^{g}P<0.001$ *vs* 1 mmol/L.

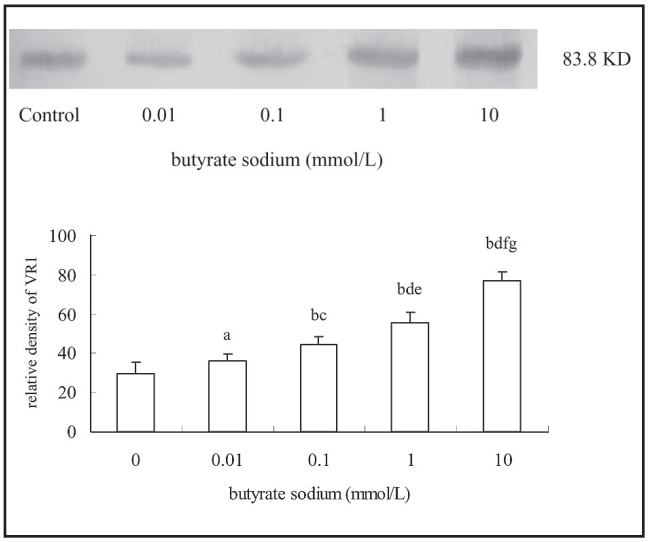

Figure 4. Effects of butyrate on VR1 protein expression in primary cultured DRG neurons. VR1 protein were analyzed by Western blot. The relative density values of VR1 protein are as follows: DRG cells were cultured continuously in growth media as normal control (29.77±5.84). DRG cells were treated with 0.01 mmol/L butyrate (36.04±3.41). DRG cells were treated with 0.1 mmol/L butyrate (44.60±3.64). DRG cells were treated with 1 mmol/L butyrate (55.58±5.44). DRG cells were treated with 10 mmol/L butyrate (77.04±4.88). Bar graphs with error bars represent mean ± SD (n=5). $^{a}P<0.05$ *vs* control, $^{b}P<0.001$ *vs* control, $^{c}P<0.01$ *vs* 0.01 mmol/L, $^{d}P<0.001$ *vs* 0.01 mmol/L, $^{e}P<0.01$ *vs* 0.1 mmol/L, $^{f}P<0.001$ *vs* 0.1 mmol/L, $^{g}P<0.001$ *vs* 1 mmol/L.

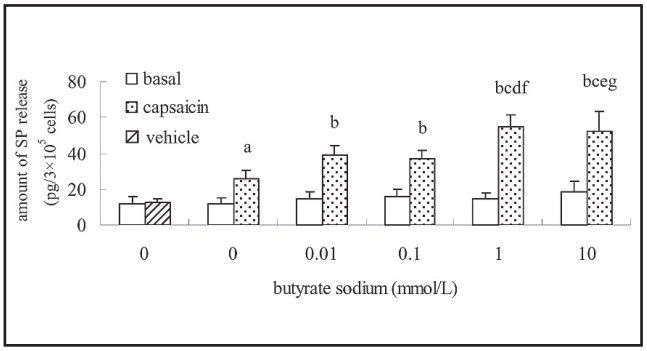

Figure 5. Effects of butyrate on SP release from DRG neurons induced by capsaicin. Butyrate at different concentrations did not alter the basal SP release ($P<0.05$). Butyrate at higher concentrations (1 mmol/L, 10 mmol/L) significantly enhanced SP release evoked by capsaicin. $^{a}P<0.01$ *vs* vehicle, $^{b}P<0.001$ *vs* vehicle, $^{c}P<0.001$ *vs* control, $^{d}P<0.01$ *vs* 0.01 mmol/L, $^{e}P<0.05$ *vs* 0.01 mmol/L, $^{f}P<0.01$ *vs* 0.1 mmol/L, $^{g}P<0.05$ *vs* 0.1 mmol/L.

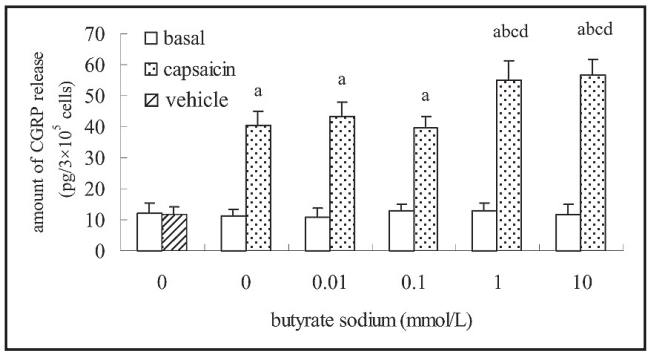

Figure 6. Effects of butyrate on CGRP release from DRG neurons induced by capsaicin. Butyrate at different concentrations did not alter the basal CGRP release ($P<0.05$). Butyrate at higher concentrations (1 mmol/L, 10 mmol/L) significantly enhanced CGRP release evoked by capsaicin. $^{a}P<0.001$ *vs* vehicle, $^{b}P<0.001$ *vs* control, $^{c}P<0.01$ *vs* 0.01 mmol/L, $^{d}P<0.001$ *vs* 0.1 mmol/L

with washing solution, the membranes were incubated with bovine anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology). The immunoreactive bands were visualized by an ECL Western blotting detection kit (Amersham Pharmacia Biotech) on light sensitive film.

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was evaluated by one-way ANOVA followed by SPSS software. Significance was accepted at $P<0.05$.

Results

Effects of butyrate on SP and CGRP mRNA expression

The effects of butyrate on SP and CGRP mRNA expression in primary cultured DRG neurons were investigated by RT-PCR. As shown in Figure 1, 2, butyrate at higher dosages promoted SP and CGRP mRNA expression in primary cultured DRG neurons.

Effects of butyrate on VR1 mRNA and VR1 protein expression

The effects of butyrate on VR1 mRNA and VR1 protein expression in primary cultured DRG neurons were investigated by RT-PCR and Western blot, respectively. As shown in Figure 3, 4, an increase expression of VR1 mRNA and VR1 protein in primary cultured DRG neurons induced by butyrate in a dose-dependent manner.

Butyrate increases sensitivity of capsaicin on SP and CGRP release

To test whether butyrate can increase sensitivity of capsaicin on the release of SP or CGRP from DRG neurons *in vitro*, DRG neurons were incubated with butyrate at different concentrations for 48 h and then stimulated by 300 nmol/L capsaicin for 5 min. Butyrate at different concentrations could not alter the basal neuropeptide release. Butyrate at higher concentrations (1 mmol/L, 10 mmol/L) significantly enhanced neuropeptide release evoked by capsaicin as compared with that at lower concentrations (0.01 mmol/L, 0.1 mmol/L) of butyrate and without butyrate. Vehicle solution could not increase neuropeptide release as compared with basal release (Figure 5, 6).

Discussion

In the present study, chronic exposure (48 h) of butyrate at higher concentrations (1mmol/L, 10mmol/L) sensitized the release of excitatory neuropeptides SP and CGRP induced by capsaicin from primary cultured DRG neurons of the rat. Butyrate at lower concentrations (0.01 mmol/L, 0.1 mmol/L) did not have this effect. The effect of butyrate on neuropeptide release induced by capsaicin from primary cultured DRG neurons was not a dose-dependent manner. And butyrate alone did not trigger neuropeptide release.

And also, chronic exposure (48 h) of butyrate at higher concentrations (1 mmol/L, 10 mmol/L) promoted the expression of mRNA for SP and CGRP in primary cultured DRG neurons. SP is a member of the tachykinin family of peptide neurotransmitters that are derived from preprotachykinin gene by alternative splicing [7]. SP is found in sensory nerves innervating peripheral tissues [24] including the gastrointestinal tract [8,14]. SP, apart from its role in nociception, has been found also to participate in inflammatory actions like vasodilation, plasma extravasation [25], release of cytokines and recruitment and proliferation of inflammatory cells [18]. SP may be involved in nociceptive and/or inflammatory actions in this experimental model. The mechanisms of these actions still await clarification. CGRP is a 37-amino acid-long peptide widely distributed in the central and peripheral nervous system, including the primary sensory neurons and its peripheral and central branches [15]. CGRP is released upon appropriate stimulation and plays an important role in pain signal transmission by potentiating nociceptive signaling and pain-related behavior mediated by SP [2]. CGRP is also known to have proinflammatory effects through vasodilation and promotion of protein extravasation [4,11]. The role of SP and CGRP has been demonstrated in several inflammatory or noninflammatory bowel models [3,21,29]. Whether expression or release of sensory neuropeptides is a cause or consequence of IBS remains unkmown. In this study, chronic exposure (48 h) of butyrate at higher concentrations (1mmol/L, 10mmol/L) sensitized the release of SP and CGRP induced by capsaicin from primary cultured DRG neurons may be part of the SP and CGRP mRNA expression evoked by butyrate. Because butyrate at lower concentrations (0.01 mmol/L, 0.1 mmol/L) did not sensitize the release of SP and CGRP induced by capsaicin in coincidence with expression of mRNA for SP and CGRP by butyrate at the same concentrations. These results demonstrated that the effects of butyrate on the sensitivity of capsaicin to the release of SP or CGRP and the promotion of SP and CGRP mRNA expression need a higher dosage but are not a dose-dependent manner.

Interestingly, butyrate also enhanced the expression of VR1 mRNA and VR1 protein. VR1 is a molecular sensor for detecting adverse stimuli and a key element for inflammatory nociception [17]. VR1 can be activated by capsaicin, the main ingredient in hot chili peppers, which elicits a sensation of burning pain by selectively activating sensory neurons that convey information about noxious stimuli to the central nervous system [28]. DRG neurones in culture can share characteristics with nociceptors in vivo and are frequently used to investigate the ion channels that underlie the transduction of noxious stimuli into electrical activity during sensory processing [22]. A variety of chemical and environmental stimuli which might cause pain *in vivo* also cause stimulation on cultured DRG neurons [26]. In the present study, primary cultured DRG neurons were used as an experimental model of sensory neurons. Chronic exposure (48 h) of butyrate at a variety of concentrations and acute exposure (5 min) of capsaicin were as the stimuli in the

present experiment. The results showed that they had some interactions on cultured DRG neurons.

Recently, it has been demonstrated that several intracellular effectors (potassium and bradykinin) were involved in the regulation of SP release evoked by capsaicin [27]. And also, a new teleocidin analog [14-O-(N-acetylglucosaminyl) teleocidin A (GlcNAc-TA)] sensitizes the release of SP induced by capsaicin from primary cultured DRG neurons of the rat and GlcNAc-TA alone also triggers SP release in a dose-dependent manner [20]. A potent prostanoid, prostacyclin-mediated actions may involve activation or sensitization of sensory neurons to enhance release of neuroactive peptides evoked by capsaicin [13]. An immunosuppressant drug FK506 can activite DRG neurons directly and increase the firing rate of heat-sensitive and bradykinin-sensitive C-fibers and may also have indirect stimulation of nerve fibers [26]. Inflammatory proteases cleave protease-activated receptor 2 (PAR2) sensitizes VR1 through protein kinase C (PKC) and PAR2 activation also potentiated capsaicin sensitivity [1]. Chronic exposure of butyrate may be as a stimulating effector on primary cultured DRG neurons to promote VR1 expression and then elevate sensitivity of capsaicin on SP and CGRP release from DRG neurons. The mechanisms by which butyrate elevates the expression of SP, CGRP and VR1 mRNA and VR1 protein are not known at the moment. More detailed studies are required to delineate the mechanisms involved in butyrate on SP, CGRP or VR1 expression and sensitivity of capsaicin on DRG neurons.

Disclosure statement

This Project is supported by the Foundation for Excellent Young Scientists in Shandong Province of China (No.02BS091) and Foundation for Natural Sciences in Shandong Province of China (No. Z2006C06, Z2006D05).

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