Bacterial cell products signal to mouse colonic nociceptive dorsal root ganglia neurons

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Submitted 8 December 2009; accepted in final form 17 June 2010

Ochoa-Cortes F, Ramos-Lomas T, Miranda-Morales M, Spreadbury I, Ibeakanna C, Barajas-Lopez C, Vanner S. Bacterial cell products signal to mouse colonic nociceptive dorsal root ganglia neurons. Am J Physiol Gastrointest Liver Physiol 299: G723–G732, 2010. First published June 24, 2010; doi:10.1152/ajpgi.00494.2009.—This study examined whether bacterial cell products that might gain access to the intestinal interstitium could activate mouse colonic nociceptive dorsal root ganglion (DRG) neurons using molecular and electrophysiological recording techniques. Colonic projecting neurons were identified by using the retrograde tracer fast blue and Toll-like receptor (TLR) 1, 2, 3, 4, 5, 6, 9, adapter proteins Md-1 and Md-2, and MYD88 mRNA expression was observed in laser-captured fast blue-labeled neurons. Ultrapure LPS 1 μg/ml phosphorylated p65 NF-κB subunits increased transcript for TNF-α and IL-1β and stimulated secretion of TNF-α from acutely dissociated DRG neurons. In current-clamp recordings from colonic DRG neurons, chronic incubation (24 h) of ultrapure LPS significantly increased neuronal excitability. In acute studies, 3-min superfusion of standard-grade LPS (3–30 μg/ml) reduced the rheobase by up to 40% and doubled action potential discharge rate. The LPS effects were not significantly different in TLR4 knockout mice compared with wild-type mice. In contrast to standard-grade LPS, acute application of ultrapure LPS did not increase neuronal excitability in whole cell recordings or afferent nerve recordings from colonic mesenteric nerves. However, acute application of bacterial lysate (Escherichia coli NL28) increased action potential discharge over 60% compared with control medium. Moreover, lysate also activated afferent discharge from colonic mesenteric nerves, and this was significantly increased in chronic dextran sulfate sodium salt mice. These data demonstrate that bacterial cell products can directly activate colonic DRG neurons leading to production of inflammatory cytokines by neurons and increased excitability. Standard-grade LPS may also have actions independent of TLR signaling. LPS; NF-κB; sensory neuronal excitability

THE INTESTINAL MICROBIOTA are increasingly recognized as important modulators of gastrointestinal (GI) function and may play a pivotal role in a number of GI disorders (9–11). Commensal bacterial signal to the innate immune system (2) and thereby alter the balance of pro- and anti-inflammatory cytokines. These actions may be particularly important in intestinal disorders such as inflammatory bowel disease and possibly irritable bowel syndrome (9, 22), in which increased epithelial permeability may allow bacteria access to the interstitial compartment of the intestine, thereby enhancing this signaling. Bacteria can also reach this interstitial compartment through the bloodstream, during generalized sepsis. Bacterial cell products have been shown in animal models to induce hyperalgesia (6, 25, 31) and hence could contribute to sensory disturbances in these common human conditions. Understanding the pathways that underlie the ability of intestinal bacteria to alter sensory signaling in the intestine could potentially result in a new treatment paradigm for visceral pain.

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, has been shown to alter sensory pathways in the GI tract. In animal models, LPS has been shown to produce visceral hyperalgesia through an unknown mechanism. These studies demonstrate that LPS can stimulate discharge of mesenteric afferent nerves (26, 27, 33); however, it also resulted in local cytokine release following sustained luminal stimulation with LPS (19). Thus it is unclear whether LPS directly activates nociceptive neurons to induce altered sensory signaling or whether this occurs solely as a result of secondary effects of cytokine release.

Toll-like receptors (TLRs) play a key role in the innate recognition of microbial cell products such as LPS (20, 23, 24). These are type I transmembrane protein receptors that contain a leucine-rich repeat in the extracellular domain and a Toll/IL-1 repeat homology domain in the cytoplasmic region. At present, 10 TLRs have been identified in humans and 13 in mice (20). Genetic studies have shown these TLRs respond to a host of microbial products including LPS, nucleic acids, flagellin, peptidoglycan, and lipoproteins. All TLR signaling pathways lead to activation of the transcription factor NF-κB (19), which in turn controls the activation of inflammatory cytokine genes such as TNF-α, IL-1β, and IL-6. TLRs have been identified on multiple cell types in the GI tract (9, 20, 23), including intestinal epithelia cells and macrophages. Recent studies suggest TLR receptors may be found on neurons (29), but little is known about their expression on colonic nociceptive dorsal root ganglia (DRG) neurons.

To determine whether bacterial cell products can signal directly to nociceptive DRG neurons, we employed the retrograde marker fast blue dye to enable colonic DRG neurons to produce visceral hyperalgesia through an unknown mechanism. We therefore employed molecular and perforated patch electrophysiological recording techniques to determine whether bacterial cell products activate these neurons.

MATERIALS AND METHODS

Male CD1 and C57BL/6 mice (25–30 g) were obtained from Charles River Laboratories (Montreal, Quebec, Canada). C3H/HeJ TLR4 knockout and C3H/HeOuJ control background mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Experiments were performed according to the guidelines of the Canadian

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http://ajpgi.physiology.org/ by 10.220.32.246 on November 4, 2016
Council of Animal Care and were approved by Queen’s University Animal Care Committee.

**Fast Blue Injection**

Mice were anesthetized with ketamine-xylazine (0.15/0.01 mg/g wt ip) and subjected to midline laparotomy (3), the colon was exposed, and the retrograde marker fast blue (1.7% wt/vol in sterile water) was injected (volume: 1–2 μl) at multiple sites along the colon. The gut was sutured after each injection to remove seepage and prevent indiscriminate labeling before being replaced in the abdomen, which was closed by suturing.

**Primary Neuronal Culture**

At least 7 days postsurgery, animals were anesthetized with ketamine-xylazine combination (0.15/0.01 mg/g wt ip) and underwent transcald perfusion of 50 ml ice-cold HBSS over 3 min, preceded by an injection of 0.1 ml heparin into the left ventricle. The spinal column was removed and DRG from thoracic vertebra T9 to T13 were isolated bilaterally and placed into ice-cold HBSS. DRGs were dissociated as described by Malin et al. (28); briefly, a sequential 10 min/37°C treatment with two enzymatic solutions (initially: papain, 69 U activated with 1 mg l-Cys and 3 μl saturated NaHCO3/1.5 ml HBSS, followed by 12 mg collagenase and 14 mg dispase/3 ml HBSS). Enzymes were removed and DRGs washed with 2 ml prewarmed F12 medium containing 10% FCS. After trituration neurons were placed onto round coverslips precoated with sterile laminin/poly-D-lysine in medium containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) and main- tained at 37°C in humidified atmosphere of 5% CO2 until they were retrieved for electrophysiological experiments.

**Electrophysiological Experiments**

Electrophysiological experiments were conducted on DRG neurons after ~24 h of culture. Nociceptive neurons were identified by their small size (~30 μm or ~40 pF) and colonic projecting neurons by the blue fluorescence emitted under short-duration exposure to ultraviolet light on an inverted microscope (Observer A1, Carl Zeiss, Toronto, Ontario, Canada). Neuronal activity was recorded by using the amphotericin-perforated patch-clamp technique, as previously described (18). Patch pipettes were made by using thin-wall glass capillaries (Warner Instruments), pulled with a Narishige PP-830 puller, and polished with a Narishige MF-830 microforge to a final resistance of 2–5 MΩ.

Membrane potential at rest and in the presence of LPS was recorded via a Multiclamp 700B amplifier and digitized by a Digidata 1440A AD converter, for storage on a PC using pClamp 10.1 software (all by Molecular Devices, Sunnyvale, CA).

The standard solutions had the following compositions (in mM), pipette solution: 110 K-glucionate, 30 KCl 10 HEPES, 1 MgCl2, and 2 CaCl2; external solution: 100 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1 MgCl2, and 2 CaCl2. The pH of solutions was adjusted to 7.25 with KOH (pipette solution) and to 7.3–7.4 with NaOH (external solution). The liquid junction potential was calculated to be 12 mV and was corrected during analysis.

The recording chamber was continuously superfused with external solution at 2 ml/min. Rapid changes in the external solution were made by using lateral movements of a system with eight parallel constantly flowing tubes producing laminar flows of differing solutions. Only cells with a resting potential more negative than ~40 mV were used.

**Acute application experiments.** Experiments were performed at room temperature (~23°C). Neuronal excitability was measured before (control), during (90 s after the start of a 3-min bacterial product application), and 30 s and 5 min after washout. The following assays of neuronal excitability were recorded: changes in resting membrane potential, rheobase, and number of action potentials at two times rheobase or during a 250-pA single pulse. Only one cell per coverslip was used to avoid artifacts through desensitization.

**Chronic incubation experiments.** Equal volumes of dissociated neurons were plated at high density on coverslips and incubated for 24 h at 37°C with culture medium alone (control) or with ultrapure LPS 055:B5 10 μg/ml. Neuronal excitability was assessed by patch-clamp recordings in both groups of neurons.

Stock solution of LPS from *Escherichia coli* 055:B5 strain (“standard-grade”) (Sigma-Aldrich, Oakville, Ontario, Canada), and ultrapure LPS from *E. coli* 0111:B4 (InvivoGen via Cedarlane, Hornby, Ontario, Canada), and 055:B5 (Sigma-Aldrich) strains were dissolved into injectable water to 5 mg/ml, aliquoted and stored at ~20°C. *E. coli* NLM28 preparations were a kind donation from Dr. Nancy Martin, Queen’s University. Briefly, a single bacterial colony was inoculated in 5 ml Luria-Bertani (LB) medium and incubated overnight at 37°C. One milliliter was transferred to 50 ml LB and cultured at 37°C until the bacteria reached a concentration of ~10⁸ cells/ml. The bacterial culture was spun down at 13,000 rpm for 2 min, and the pellet washed once with LB, before resuspending and resuspending the same amount of fresh LB or deionized water. Half of the preparation was sonicated (~4 × 5 s pulses, being kept on ice for 10 s in between pulses). Cells and lysate were aliquoted and stored at ~20°C until used at a final concentration of 10⁶ cells/ml. All other bacterial products were acquired from InvivoGen. Amphotericin B (Sigma-Aldrich) was made fresh daily to 60 μg/μl in DMSO. HBSS and F12

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### Table 1. List of primers

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medium were purchased from Invitrogen (Carlsbad, CA). Poly-d-lysine was purchased from VWR (Mississauga, Ontario, Canada). Papain was purchased from Worthington Biochemical (Lakewood, NJ). Dextran sulfate sodium salt (DSS), molecular mass 36–50 kDa, was purchased from MP Biomedicals (Solon, OH) and dissolved at 2% wt/vol in tap water. All other substances were purchased from Sigma-Aldrich.

ELISA Measurement of LPS-Stimulated TNF-α in Culture Supernatants

Equal volumes of dispersed DRG neurons were seeded onto laminin/poly-d-lysine coated coverslips in a 24-well plate containing 1 ml culture media per well; 10 μg/ml ultrapure LPS (E. coli O55:B5) was added and incubated at 37°C in a humidified 5% CO₂ incubator. After overnight incubation, the culture supernatants were harvested and stored at –80°C for ELISA measurement of released TNF-α. Mouse TNF-α ELISA kits were obtained from R&D Systems, Minneapolis, MN. Assay of samples and standards were performed simultaneously according to the manufacturer’s instruction. Briefly, polyclonal anti-mouse TNF-α antibodies were used as capturing antibodies and horseradish-conjugated polyclonal anti-mouse TNF-α antibodies as the detecting antibody. Stabilized hydrogen peroxide and chromogen were added as color reagents. Optical densities of each well were determined by using a microplate reader–TiterTek Multiskan Plus photometer set at 450 nm within 30 min the color reactions were stopped. All steps were performed at room temperature and samples were assayed in duplicate.

Cell Culture for Western Blotting and PCR

DRGs were isolated and triturated as described above. Next, to remove nonneuronal cells (1), the pellet was resuspended in HBSS with 50% Percoll gradient and centrifuged at 2,500 for 7 min. The supernatant containing nonneuronal cells was discarded; the neurons were washed with HBSS and pelleted by centrifugation at 2,000 rpm for 5 min. The pellet was resuspended in F-12 medium plus FBS, penicillin-streptomycin, and 10 μM β-arabinofuranosylcytosine and plated on poly-d-lysine- and laminin-coated 17-mm coverslips.

Western Blotting

Standard protocols for Western blotting were used to examine the expression of NF-κB, phospho-NF-κB, and β-actin in cultured DRG neurons of CD-1 mice. The harvested neurons (3 mice) were lysed in Trizol reagent (Invitrogen), and the final pellet was diluted in modified Laemmli buffer (21 mM Tris, 6.6% glycerol, 0.6% SDS, 1.66% β-mercaptoethanol) supplemented with Complete protease inhibitor cocktail (Roche Molecular Biochemicals, West Sussex, UK), and heated at 95°C for 5 min, and kept at –20°C. The protein was quantified by using the Micro BCA protein assays kit (Thermo Scientific). Total protein samples (40 μg) were run in each lane of SDS-PAGE gels (10% gels). Proteins were blotted onto PVDF membranes (Immobilon-P) and were detected by using the horseradish-conjugated polyclonal antibodies. Total protein samples (40 μg) were run in each lane of SDS-PAGE gels (10% gels). Proteins were blotted onto PVDF membranes (Immobilon-P) and were detected by using the horseradish-conjugated polyclonal antibodies.

Fig. 1. Toll-like receptors (TLRs) in colonic dorsal root ganglia (DRG) neurons. Laser capture-microdissected fast blue-labeled mouse DRG neurons express diverse TLRs and other receptors involved in pathogen-associated molecular pattern signaling, as detected by RT-PCR and agarose gel electrophoresis. Representative of 3 separate experiments from different animals.

Fig. 2. Ultrapure LPS activates signaling pathways in DRG neurons. Western blot, RT-PCR, and ELISA data showing LPS-induced changes in inflammatory signaling in cultured DRG neurons from thoracic vertebrae T9–T13. A: Western blot showing increased phospho Ser536 p65 after exposure to 1 μg/ml ultrapure LPS. Representative of 3 separate experiments. B: exposure to ultrapure LPS also enhanced TNF-α and IL-1β transcript. Representative of 3 separate experiments. C: overnight incubation in 10 μg/ml ultrapure LPS significantly increased the secretion of TNF-α by cultured DRG neurons (ELISA, P = 0.013, n = 3).

Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific).

Semi-quantitative RT-PCR

RT-PCR was used to determine the levels of TNF-α, IL-1β, and GAPDH mRNAs in cultured DRG neurons. Total mRNA was obtained using Trizol reagent (Invitrogen). cDNA was synthesized from 0.5 μg total RNA with oligo(dT) and SuperScript III (Invitrogen) for 50 min at 50°C, and 0.5 μl cDNA was used as a template for PCR amplification in a 25-μl reaction volume containing 2.5 μl buffer 10X, 200 nM dNTPs, 1.5 mM MgCl₂, 0.2 pm of each primer, and 0.1 μl Taq DNA polymerase (Invitrogen). The reaction conditions were as follows: TNF-α, 38 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min; IL-1β, 38 cycles, 94°C for 30 s, 61°C for 30 s, and 72°C for 1 min; and GAPDH, 35 cycles, 94°C for 30 s, 61.5°C for 30 s, and 72°C for 2 min. All had a final 8-min 72°C extension. Primers were obtained from Invitrogen. See Table 1.

Colonic Afferent Responses to LPS

Experiments were performed on control and DSS treated colons from CD1 or C57BL/6 mice, as previously described (18). Briefly, the colon was removed with attached mesentery and placed in a Sylgard-lined organ chamber continually perfused with oxygenated Krebs solution (in mM: 118.4 NaCl; 24.9 NaHCO₃; 1.9 CaCl₂; 1.2
MgSO₄·7H₂O; 1.2 KH₂PO₄, and 11.7 D-glucose) at a flow rate of 6–7 ml/min and maintained at 33–34°C. Proximal and distal ends of the bowel were securely attached to an input and outlet port. The input port was connected to a perfusion syringe pump, which allowed continuous intraluminal perfusion of Krebs solution through the segments (0.2 ml/min). The mesenteric bundle was pinned out on the base of the chamber, and a mesenteric nerve was dissected out from the bundle and drawn into a suction electrode. The electrical activity was recorded by a Neurolog headstage (NL 100, Digitimer), amplified (NL104), filtered (NL125 band pass 0.2–3 kHz), and acquired (20 kHz sampling rate) via a Micro 1401 MKII interface to a PC running Spike 2 software (Cambridge Electronic Design).

The preparation was stabilized for 60 min and we performed experiments in the presence of bacterial lysate, standard-grade LPS, or ultrapure LPS 30 μg/ml applying the drug into the bath for 5 min and washing it out for 30 min.

**Chronic DSS Inflammatory Model**

C57BL/6 mice underwent three cycles of alternating 5-day periods receiving 2% DSS in drinking water and 5-day periods of normal drinking water. Mice had access to food and fluids ad libitum. Subcutaneous injections of 0.5 ml lactated Ringer’s solution were administered daily to DSS-treated mice to avoid dehydration, and weight loss was closely monitored, with 15% loss as the threshold for euthanasia to avoid unnecessary suffering. Following the final water cycle, mice were euthanized and their colons removed, as described above, to assess colonic afferent responses to bacterial lysate.

**Antibodies**

The polyclonal anti-rabbit anti-NF-κB p65 (no. 3034), monoclonal anti-rabbit anti-phospho-NF-κB p65 (no. 3033), and anti-rabbit secondary antibody (no. 7074) were from Cell Signaling Technology (Danvers, MA). The rabbit anti-β-actin antibody (no. A5316) was from Sigma-Aldrich.

**Statistical Analysis**

Data were analyzed with Prism 5 by GraphPad (La Jolla, CA). Results are expressed as means ± SE. Student’s t-test and one- or two-way ANOVA with Dunnett’s or Bonferroni’s posttests were used.
where appropriate. P values of 0.05 or less were considered statistically significant and are represented at plots as asterisks (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001).

RESULTS

Colonic Projecting DRG Neurons Express PRRs

Colonic projecting neurons labeled with fast blue were isolated by laser capture microdissection (see Refs. 18, 21) and expression of pattern recognition receptors (PRRs) determined by RT-PCR. Transcript for TLRs 1, 2, 3, 4, 5, 6, 9, nucleotide-binding oligomerization domain containing receptors (Nod) 1–2, and the adapter proteins MyD88, Md1, and Md2 were detected (Fig. 1; representative of 3 separate experiments).

LPS Activates NF-κB and Stimulates Cytokine Release

Incubation of acutely dissociated colonic projecting DRG neurons from spinal segments T9-T13 with 1 μg/ml LPS (E. coli 055:B5) increased expression of phospho Ser536 p65 (NF-κB) in a time-dependent manner (Fig. 2A; representative Western blot from 3 separate experiments). No differences were observed between ultrapure and standard-grade LPS. To test whether activation of NF-κB was associated with increased cytokine expression in DRG neurons, changes in TNF-α and IL-1β mRNA levels were examined following acute application of 1 μg/ml LPS (E. coli 055:B5; ultrapure). LPS caused a time-dependent increase in transcript (Fig. 2B; representative of 4 different experiments). Acutely dissociated neurons were

Fig. 4. Acutely applied standard-grade LPS increases DRG neuronal excitability. Current-clamp recordings of fast blue-labeled colonic DRG neurons and neuronal excitability in the presence of 30 μg/ml standard-grade LPS. A: example traces from a typical DRG neuron to show the effects of standard-grade LPS on the rheobase and the number of action potentials fired with a 250-pA current injection before, during 30 μg/ml standard-grade LPS, and after washing. B: mean number of action potentials was increased by standard-grade LPS (P < 0.001 and < 0.0001 for “during” and “30-s washout,” respectively, n = 21). C: standard-grade LPS decreased mean rheobase (P < 0.0001, n = 21). D: the mean membrane potential hyperpolarized during 30 μg/ml standard-grade LPS (P < 0.0001, n = 21). E: the mean input resistance was reduced by 30 μg/ml standard-grade LPS (P < 0.01, n = 21). **P ≤ 0.01; ***P ≤ 0.001.
also incubated in LPS and levels of TNF-α in the medium measured by ELISA; 1 μg/ml LPS induced a ~40% increase in TNF-α (n = 3; P = 0.013).

**Bacterial Cell Products Increase Colonic DRG Neuronal Excitability**

*Chronic incubation in ultrapure LPS.* Neuronal excitability was assessed in a series of experiments in which high densities of plated DRG neurons were incubated for 24 h with 10 μg/ml ultrapure LPS 055:B5.

In 12 cells studied for each of the control and LPS incubated groups, neither rheobase (Fig. 3, A, left and B) (control, 80.83 ± 11.25 pA; LPS, 70.83 ± 11.58 pA), membrane potential (control, -60.18 ± 1.18 mV; LPS, -60.23 ± 1.71 mV), nor input resistance (control, 1,085 ± 235.2 MΩ; LPS, 931.9 ± 133.3) was altered. However, the action potential number from a 500-ms current injection of 250 pA was significantly increased in the ultrapure LPS-incubated group of neurons, (2.1 ± 1.0 action potential vs. 6.4 ± 1.8 action potential, control and LPS-incubated, respectively, P = 0.04, Fig. 3, A, right, and C).

**Acute LPS effect.** The possibility that bacterial cell products may alter the excitability of colonic DRG neurons was also examined by studying the effects of acute application of standard-grade LPS. A 3-min application of LPS 30 μg/ml (*E. coli* 055:B5) hyperpolarized the membrane potential (mean control = -64.4 ± 0.9 mV vs. mean LPS effect = -71.2 ± 0.9 mV, n = 21; Fig. 4D) with an associated decrease in input resistance (mean control = 1.2 ± 0.1 GΩ vs. mean LPS = 1.0 ± 0.1 GΩ, P < 0.0001; Fig. 4E). To measure neuronal excitability, changes in rheobase and action potential discharge were examined at the membrane potential evoked by the LPS application. LPS reduced the rheobase up to 40% (Fig. 4, P < 0.0001; n = 21) and increased the action potential discharge at 10% (Fig. 4, P < 0.0001; n = 21) during (at 1.5 min) and at 30 s following the 3-min application of LPS. LPS (0.3–30 μg/ml) effects on rheobase, action potential discharge, and membrane hyperpolarization were concentration dependent (Fig. 5).

**TLR4 knockout mice.** To examine the pathways mediating the effects of LPS, the effects of LPS 30 μg/ml (*E. coli* 055:B5) on fast blue-labeled DRG neurons were studied in TLR4 knockout mice (C3H/HeJ) and compared with wild-type animals. LPS induced hyperpolarization of the membrane potential during a 3-min application (mean LPS-mediated change in membrane potential in TLR4 knockout animals = -63.7 ± 0.98 to -70.28 ± 1.0 mV, n = 17 and mean LPS-mediated change in membrane potential in wild-type animals = -62.25 ± 1.2 to -67.28 ± 1.5 mV; n = 13) and LPS-mediated decrease in input resistance (1.51 ± 0.2 to 1.16 ± 0.19 GΩ in knockout, 1.25 ± 0.19 to 0.98 ± 0.11 in wild-type) were similar in the two groups of animals. LPS significantly decreased the rheobase in the wild-type animals (Fig. 6A; n = 13) but not the knockout animals, although there was a trend toward a decrease (Fig. 6A, n = 17). There was no significant change in the action potential discharge in either the wild-type or the knockout mice with the application of LPS (Fig. 6A).

**Ultrapure LPS.** A 3-min application of ultrapure LPS 30 μg/ml (*E. coli* 055:B5) on fast blue-labeled DRG neurons from CD1 mice had no effect on rheobase or action potential discharge rate (Fig. 6B, n = 8). Similarly, ultrapure LPS 30 μg/ml (*E. coli* 0111:B4) had no effect (data not shown). In multimini afferent recordings from the mouse colon, standard-grade LPS 30 μg/ml (*E. coli* 055:B5) increased afferent nerve discharge (Fig. 6C; n = 3) whereas ultrapure LPS (*E. coli* 055:B5) had no effect (n = 3).

**Bacterial cell lysate.** To test the possibility that bacterial cell products may signal in concert to colonic DRG neurons, the effect of a 3-min application of bacterial cell lysate from *E. coli* NLM28 was tested and compared with control LB medium. Acute application (at 1.5 min) caused a mean increase of action potential discharge over 60% compared with baseline (Fig. 7A, P < 0.01; n = 10). No change was observed in the rheobase. The vehicle (LB medium) alone had no effect on the rheobase or action potential frequency (Fig. 7B). However, acute application of vehicle alone depolarized the membrane potential whereas the bacterial cell lysate (in medium) had no effect on membrane potential (mean depolarization = 2.92 ± 0.92 mV vs. 0.1 ± 0.64 mV, respectively, P = 0.021; data not shown), suggesting that the action of the lysate countered the depolarization evoked by the medium alone. In a different set of experiments, we investigated the effects of bacterial cell lysate on colonic afferent nerve activity from control and chronic DSS mice. There was a small increase in afferent firing rate during a 5-min application of bacterial lysate to control tissues (0.624 ± 0.42 imp/s, n = 8); however, this response was significantly increased for tissue from chronic DSS mice (4.143 ± 1.74 imp/s, n = 5, P = 0.033) (Fig. 7C).

**Bacterial cell products.** We tested selected bacterial products reported to act at TLR 1 (15, 16, 25), 2 (7), 3, and 7 (5,
Normalized rheobase and action potential number at twice rheobase (Fig. 7D) are shown in response to a 1.5-min application of synthetic tripalmitoylated lipopeptide bearing a Cys-SerLys 4 (Pam3SCK4, 1 μg/ml, n = 4), peptidoglycan from E. coli 0111:B4 strain (PGNEB; 1 μg/ml, n = 10), E. coli K12 RNA complexed with LyoVec (ecRNA; 1 μg/ml, n = 7), and ultrapure lipopolysaccharide from Porphyromonas gingivalis (PGLPS; 1 μg/ml, n = 6). None of these agents given alone had any effect on these parameters of neuronal excitability.

DISCUSSION

This study examined whether bacterial cell products can directly signal to colonic DRG neurons. Retrograde fast blue labeling was employed to identify colonic DRG neurons, and small cells were examined (<40 pF) in electrophysiological studies because these cells exhibit properties of nociceptors (4, 18). We found that bacterial products can activate the NF-κB pathway and also alter the intrinsic excitability of these neurons by signaling through other pathways. The implication of this action is that acute exposure to bacterial cell products, which could occur when intestinal permeability is increased, could magnify the responses of nociceptive DRG neurons in the colon when simultaneously activated by other stimuli.

The apical membrane of the colonic epithelium is lined by up to 10^14 bacteria/g tissue (9, 18). This microbiome exerts a tremendous influence on intestinal responses by activating TLRs, including the expression of pain (2). To date, 13 TLRs have been identified in mice and 10 in humans (20, 24). Their respective ligands have also been identified, based on genetic studies (20). For example, LPS activates TLR4, lipopeptides TLR 1, 4, 6, ds RNA TLR3, flagellae TLR5, and bacterial and viral CpG DNA motifs that recognize TLR9 (9). There is also evidence that molecules such as heat shock proteins released from cells undergoing oxidative stress or degradation during inflammation may also act as TLR ligands (8). We employed laser capture microdissection to isolate fast blue-labeled DRG neurons (i.e., colonic neurons) and examine TLR mRNA expression. We identified transcript for TLR 1, 2, 3, 4, 5, 6, 9,
and the Md and MyD88 adapter molecules. This finding suggests that these sensory neurons have the capacity to respond to a wide array of molecular epitopes or pathogen-associated molecular patterns (PAMPs).

TLRs activate a common signaling pathway culminating in the induction of the nuclear transcription factor NF-κB (19), as well as other pathways. Activation of NF-κB directs the synthesis of inflammatory cytokines such as TNF-α, IL-1β, and IL-6. We tested this possibility in acute dissociated DRG neurons, removing nonneuronal cells including microglia by isolating neurons via centrifugation and treating the cultures with β-ARAC (see MATERIALS AND METHODS). We found evidence that the TLR4 ligand LPS activates p65 heterodimeric NF-κB and increased transcript of TNF-α and IL-1β. Using an ELISA we also provided direct evidence for secretion of inflammatory cytokines (TNF-α) from DRG neurons. These results suggest a potential new paradigm in which DRG neurons participate directly in the inflammatory response mediated by PAMPs. This release of inflammatory cytokines could have a paracrine and/or autocrine effect, leading to enhanced sensitization of axon terminals of DRG neurons in the intestine. Further studies should also explore the possibility that release
of inflammatory mediators from the soma within the ganglia themselves could also enhance signaling between adjacent neurons. This could include direct neuronal signaling as well as the activation of the microglia, which in turn signal to adjacent neurons in the ganglia (29). We have found that chronic incubation of ultrapure LPS with acutely dissociated DRG neurons for 24 h markedly increased the excitability of these neurons, consistent with the activation of one or more of these TLR-mediated pathways.

Previous studies have also suggested that acute application (i.e., minutes) of bacterial cell products can directly increase the excitability of dorsal root ganglia neurons (17, 33), although changes in passive and active membrane properties of identified neurons by patch-clamp electrophysiological techniques had not been completed. Using this latter approach we have provided direct evidence that bacterial cell products can increase the excitability of the colonic DRG neurons through direct signaling to the neurons. These actions, however, appear to involve a number of ionic mechanisms, some of which are not TLR4 mediated and would have competing effects on neuronal excitability. We found that LPS (standard reagent grade) caused a hyperpolarization of DRG neurons with an associated decrease in input resistance, implying the associated opening of “leak” currents. This action would serve to decrease the excitability of the neurons. Simultaneously, LPS induced a decrease in rheobase and increase action potential discharge (i.e., increase in excitability), implying the modulation of voltage-gated Na⁺ and/or K⁺ currents (4, 32). The mechanism(s) underlying these actions is not clear, but the hyperpolarizations do not appear to involve a TLR4-mediated pathway given they were still evident in the TLR4 knockout mice. The role of TLR4 in the LPS-mediated effects on the rheobase and action potential discharge were less clear given that the LPS effect on action potential discharge was not observed in the wild-type animals whereas the effect on rheobase appeared to be less in the knockout animals (see Fig. 5A). We therefore examined the actions of the acute application of ultrapure LPS and found that, in contrast to standard-grade LPS, ultrapure LPS had no effect on the membrane properties of DRG neurons in patch-clamp recordings and similarly failed to excite multiunit colonic afferent nerves. The explanation for these differences is not entirely clear but could be due to the actions of other bacterial cell products in the standard-grade LPS and/or possibly impurities in the standard-grade LPS (13, 16), such as adenosine or glutamate.

In an effort to exclude the possibility that impurities contributed to the electrophysiological actions of standard-grade LPS, we examined the effect of the acute application of a bacterial lysate prepared from a nonpathogenic strain of E. coli (NLM28). We found that lysate increased action potential discharge and that this could not be accounted for actions of the culture media alone (see Fig. 6). Interestingly, we also found that this lysate acutely applied activated significantly more colonic afferents from inflamed colons following chronic DSS-treated mice than controls. Whether this difference is the result of inflammation-induced increase in expression of TLRs (14, 30), altered tissue access of the lysate, or another mechanism such as altered intrinsic firing threshold of the axons is unclear. However, these studies do argue against a sole role for impurities but the mechanism underlying the actions of the cell products involved remains to be determined. We examined the individual actions of membrane lipoproteins and RNA that have been reported to signal through TLRs but did not observe a change in neuronal excitability. Thus it appears that either other products are involved and/or multiple products act in concert to mediate the observed actions of the bacterial cell lysate on neuronal excitability.

In summary, we have found that bacterial cell products have the potential to directly increase colonic nociceptive signaling through several pathways, in addition to their well-recognized effects on pronociceptive cytokine release from immune cells. We found that they activate NF-κB pathways in colonic DRG neurons and stimulate the production and secretion of inflammatory cytokines from these cells. This could amplify the levels of pronociceptive cytokines, which in turn sensitize and activate axon terminals of DRG neurons in the colon. We also found that bacterial cell products alter the intrinsic excitability of nociceptive DRG neurons and thereby have the potential to acutely modulate pain signaling. Further studies are needed to address the multiple factors which could influence the magnitude of this effect, including the ability of bacteria to directly access the DRG axons in the interstitium in conditions such as inflammatory bowel disease, the expression of TLRs, which may be markedly increased during inflammation (9, 10), and the nature of the bacterial cell products that translocate into the tissues (12, 21, 27). These studies must also exclude possible contributions of impurities in commercially available bacterial cell products, including LPS.

GRANTS
This study was supported by an operating grant from the Crohn’s and Colitis Foundation of Canada (CCFC). S. Vanner is supported by a CCFC scientist award. F. Ochoa-Cortes, M. Miranda-Morales, and T. Ramos-Lomas were supported by Consejo Nacional de Ciencia y Tecnologia.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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