Transient receptor potential ankyrin-1 has a major role in mediating visceral pain in mice

Fiore Cattaruzza,1 Ian Spreadbury,3 Marcela Miranda-Morales,3 Eileen F. Grady,1 Stephen Vanner,3 and Nigel W. Bunnett1,2

Departments of 1Surgery and 2Physiology, University of California, San Francisco, California; 3Gastrointestinal Diseases Research Unit, Division of Gastroenterology, Queen’s University, Kingston, Ontario, Canada

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Cattaruzza F. Spreadbury I, Miranda-Morales M, Grady EF, Vanner S, Bunnett NW. Transient receptor potential ankyrin-1 has a major role in mediating visceral pain in mice. Am J Physiol Gastrointest Liver Physiol 298: G81–G91, 2010. First published October 29, 2009; doi:10.1152/ajpgi.00221.2009.—The excitatory ion channel transient receptor potential ankyrin-1 (TRPA1) is prominently expressed by primary afferent neurons and is a mediator of inflammatory pain. Inflammatory agents can directly activate [e.g., hydroxyxynonalen (HNE), prostaglandin metabolites] or indirectly sensitize [e.g., agonists of protease-activated receptor (PAR2)] TRPA1 to induce somatic or visceral pain. Inflammatory agents can directly activate TRPA1, including products of oxidative stress [4-hydroxyxynonalen (HNE), 4-oxononalen (4-ONE)] and the cyclopentenone metabolites of prostaglandin (PGIE 5, 6, 41, 42). In addition, diverse inflammatory agents including those that activate GPCRs (e.g., bradykinin) and receptor tyrosine kinases (e.g., nerve growth factor) can indirectly sensitize TRPA1 by mechanisms that include activation of phospholipase C and subsequently stimulation of protein kinase C and A isoforms (26, 40), which amplifies responses to direct agonists. When TRPA1 is activated, nociceptors release SP and CGRP in the dorsal horn of the spinal cord, where peptides stimulate spinal neurons to transmit pain.

TRP channels are excitatory ion channels that are expressed by nociceptive neurons and contribute to the detection of noxious mechanical, thermal, and chemical stimuli. Members of the TRP vanilloid family, such as TRP vanilloid-1 (TRPV1) and TRPV4, are widely involved in visceral hyperalgesia (22, 38). TRP ankyrin-1 (TRPA1) is a recently identified member of the TRP family that has emerged as a major mediator of inflammatory pain. TRPA1 is selectively expressed by the peptidergic subset of sensory fibers that also express TRPV1. TRPA1 is directly activated through covalent modification of cysteine residues within its cytosolic NH2 terminus by exogenous aldehyde [e.g., mustard oil (MO), cinnamon, garlic] and environmental (e.g., acrolein, cigarette smoke) irritants. We and others recently identified endogenous agonists that similarly directly activate TRPA1, including products of oxidative stress [4-hydroxyxynonalen (HNE), 4-oxononalen (4-ONE)] and the cyclopentenone metabolites of prostaglandin (PGIE 5, 6, 41, 42). In addition, diverse inflammatory agents including those that activate GPCRs (e.g., bradykinin) and receptor tyrosine kinases (e.g., nerve growth factor) can indirectly sensitize TRPA1 by mechanisms that include activation of phospholipase C and subsequently stimulation of protein kinase C and A isoforms (26, 40), which amplifies responses to direct agonists. When TRPA1 is activated, nociceptors release SP and CGRP, which transmit pain (42). In addition, two strains of TRPA1 knockout mice display impaired responses to noxious mechanical stimulation, suggesting a role for TRPA1 in visceral pain (9, 28). Recently the role of TRPA1 in visceral mechano-sensation and its modulation by algesic stimuli have been investigated (12). However, the contribution of TRPA1 to visceral nociception during physiological and pathological conditions, such as IBD, is unknown.

Proteases are prominent mediators of inflammation and pain in many tissues, including the intestine (8, 15). Certain serine proteases can regulate cells by cleaving PARs, a family of four GPCRs. Proteolysis within the extracellular NH2 terminus exposes a tethered ligand that binds to and activates the receptor.

VISCERAL PAIN IS A COMMON and major complaint of patients with organic and functional bowel disorders, including inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Abdominal pain and discomfort in IBD are considered to be a consequence of increased visceral sensation attributable to intestinal inflammation, during which locally released inflammatory mediators sensitize primary afferent nerve fibers in the intestine (13).

A subpopulation of primary spinal afferent neurons with unmyelinated (C) or thinly myelinated (Aδ) fibers containing substance P (SP) and calcitonin gene-related peptide (CGRP) are of critical importance for pain sensation. These neurons, with cell bodies in the dorsal root ganglia (DRG), project fibers to the periphery and to the dorsal horn of the spinal cord. In peripheral tissues, such as the colon, sensory nerve endings detect diverse noxious and inflammatory stimuli that activate ligand-gated ion channels [e.g., transient receptor potential (TRP) channels], G protein-coupled receptors (GPCRs) [e.g., protease-activated receptor 2 (PAR2)], and receptor tyrosine kinases (26). Activation of these receptors stimulates the release of neuropeptides in peripheral tissues, where they cause neurogenic inflammation. Activated neurons also release SP and CGRP in the dorsal horn of the spinal cord, where peptides stimulate spinal neurons to transmit pain.
PAR2 is highly expressed in the gastrointestinal tract, including mesenteric afferent and enteric nerves, where it may be activated by proteases from mast cells (e.g., tryptase), the epithelium (e.g., trypsin IV), the lumen, and bacteria. In addition to effects on motility and ion and mucus secretion, activation of PAR2 induces visceral pain (14, 15, 18). Proteases released from colonic biopsies of patients with IBS cause hypersensitivity symptoms by activating PAR2, supporting a role for PAR2 activation peptide (PAR2-AP), and inflammatory agents (trypsin, trypsin IV, lumen, and bacteria. In addition to the mesenteric afferent and enteric nerves, where it may be activated by phospholipase C, which releases the inhibition of TRPA1 by components of the plasma membrane (19, 46).

On the basis of PAR2-induced sensitization of TRPA1 and of the emerging role of TRPA1 in somatic mechanical hyperalgesia, we hypothesized that TRPA1 mediates inflammatory hyperalgesia of the colon. To test this hypothesis we 1) localized TRPA1 immunoreactivity and mRNA in DRG neurons innervating the colon, 2) examined effects of TRPA1 agonists on membrane currents in these neurons, and 3) determined whether intracolonic administration of exogenous (MO) and endogenous (HNE) TRPA1 agonists, PAR2 agonist [PAR2 activating peptide (PAR2-AP)], and inflammatory agents (trinitrobenzene sulfonic acid, TNBS) cause visceral pain, by measuring abdominal visceromotor responses (VMR) to colorectal distention (CRD) and by assessing c-fos expression in spinal nociceptive neurons. To evaluate the role of TRPA1 in colonic pain, we compared these responses in *trpa1*+/− and *trpa1*−/− mice. Our results show that TRPA1 is present in nociceptive neurons innervating the colon, where activation causes hypersensitivity to CRD. TRPA1 mediates PAR2- and TNBS-induced visceral hypersensitivity.

**MATERIALS AND METHODS**

**Animals.** Mice deficient in TRPA1 were generated as described (9). Heterozygotes were mated to produce paired, wild-type (trpa1+/+) and knockout (trpa1−/−) littermates in a C57/B6 background. Male mice (25–30 g) had free access to food and water, under a 12-h:12-h light/dark cycle. The Institutional Animal Care and Use Committees of the University of California, San Francisco approved the procedures.

In a separate series of experiments, the presence of TRPA1 currents was verified electrophysiologically on DRG neurons isolated from male 25–30-g CD1 mice, and colonic inflammation was assessed in C57/B6 male mice, in procedures approved by the Queen’s University Animal Care Committee. Mice were humanely killed using an overdose of pentobarbital sodium (200 mg/kg ip) or isoflurane, followed by cervical dislocation.

**Antibodies, agonists.** The sources of the antibodies were as follows: rabbit anti-TRPA1 (no. 110–40763; Novus Biologicals, Littleton, CO), guinea pig anti-SP (Chemicon, Temecula, CA), guinea pig anti-CGRP (Research Diagnostic, Flanders, NJ), rabbit anti c-fos (Calbiochem, San Diego, CA), goat anti-rabbit and goat anti-guinea pig IgG coupled to AlexaFluor 488 and 647 (Invitrogen, Carlsbad, CA). The TRPA1 agonist MO was from Sigma (St. Louis, MO), and HNE was from Axxora (San Diego, CA). PAR2-AP was from CPC Scientific (San Jose, CA).

**Retrograde labeling of colonic neurons.** To identify DRG neurons projecting into the colon, a retrograde tracer was injected into the colonic wall. Mice were anesthetized with ketamine and xylazine (87.5 mg/kg and 12.5 mg/kg ip, respectively). The abdominal cavity was opened by midline laparotomy, and the descending colon was exposed and placed on polyethylene film to prevent spread of tracer. The overlying viscera were kept moist using saline-soaked gauze. Under a dissecting microscope, the dicarboxylic anhydride, l,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine methanesulfonate (DiI, 50 mg/ml in methanol; Invitrogen) or Fast Blue (17 mg/ml in 0.9% saline; Cedarlane Laboratories, Homby, ON, Canada) was injected into the wall of the colon (6–8 sites, 2 μl per site) using a Hamilton syringe and a 32-gauge needle. To prevent leakage to other organs, the needle was held in place for 10 s after the injection and any excess dye carefully removed. The abdominal wall and skin were closed, and mice were allowed to recover. After 10–15 days, mice were killed, and DRG (L1-S1) were removed and fixed for immunohistochemistry or laser capture or processed for electrophysiology.

**Immunohistochemical localization of TRPA1.** Mouse DRG were immersion fixed in 4% paraformaldehyde in 100 mM PBS, pH 7.4, for 2 h. Tissues were washed, incubated in 30% sucrose in PBS overnight at 4°C, and embedded in optimal cutting temperature compound. Sections of DRG (10 μm) were cut and mounted on poly-L-lysine-coated slides. Sections were washed and incubated in 100 mM PBS, pH 7.4, containing 10% normal goat serum and 0.1% Triton X-100. Tissues were incubated with the following primary antibodies: rabbit anti-TRPA1 (1:200) and guinea pig anti-SP (1:500) or anti-CGRP (1:500) (all overnight, 4°C). We have previously established the specificity of the SP and CGRP antibodies (4). To confirm specificity of the TRPA1 antibody, diluted antibody was incubated with the peptide used for the immunization (100 μM, overnight, 4°C). Tissues were washed and incubated with goat anti-rabbit IgG coupled to Alexa 488 and with goat anti-guinea pig IgG coupled to Alexa 647 (1:1,000; 2 h, room temperature). Specimens were mounted with ProLong (Invitrogen).

**Confocal microscopy.** Specimens were observed by using a Zeiss LSM510 Meta confocal microscope equipped with a Zeiss Fluar Plan Apo 40× (NA 0.8) objective. Images were acquired with an iris of <2.5 and zoom of 1, and typically 3–6 optical sections were taken at intervals of <1 μm. Acquisition parameters were determined at a submaximal level for DiI and positive controls for TRPA1. Identical parameters were used to acquire images of TRPA1-stained and control slides. Zeiss LSM510 software (version 4.2) was used for analysis of acquired images to determine percentages and size of DiI-TRPA1-positive DRG neurons. Again, parameters were optimized on the positive images and then used for analysis of control images to verify specificity of our TRPA1 antibody and also to enable discrimination of TRPA1-negative neurons. In the sections stained for TRPA1, the pixel intensity of images was quantified (0–255 pixels) and the minimum threshold set on the average background signal of each individual section to isolate discrete TRPA1 expressing neurons. We considered a neuron TRPA1 positive if it had an average pixel intensity greater than the background signal. We counted a total of 454 DRG neurons from three mice and found similar results in the sections obtained from these different mice.

**Laser-capture microdissection.** Frozen sections (8 μm) of DRG were mounted on glass slides and stored at −80°C. Retrogradely labeled neurons (100–400 per mouse) were microdissected using the PixCell II Laser-Capture Microdissection (LCM) System, and total RNA was isolated with the PicoPure RNA Isolation Kit following the manufacturer’s protocols (Arcturus, Mountain View, CA). RNA quality and integrity was assessed with the Agilent 2100 Bioanalyzer using the RNA-6000-Pico-LabChip (Agilent Technologies, Palo Alto, CA).

**RT-PCR.** RNA was reverse transcribed with oligo-dT primer using a Superscript III Reverse Transcriptase Kit (Invitrogen), cDNA (2 μl) was amplified by PCR using Taq DNA Polymerase (Invitrogen). Intron spanning primers were designed with Primer3 (http://frodo.wi.mit.edu/primer3/) on the basis of published CDS gene sequences in Genbank (accession no. for TRPA1: NM-177781). The specificity of the primers (mouse TRPA1: forward 5′-CATGATGTACCCCTTTCA-
CATAG-3; reverse 5'-ACAGGTTAGCTCCACCTTA-3') was verified using NCBI nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) online service (National Library of Medicine, http://www.ncbi.nlm.nih.gov/BLAST). PCR conditions were denaturation of 3 min at 94°C, 40 cycles at 94°C for 30 s; 55°C for 1 min, 72°C for 1 min, and elongation at 72°C for 10 min. Products were separated on a 1.5% agarose gel, stained with ethidium bromide, and identified by sequencing.

Electrophysiological recordings. Patch-clamp recordings were made from dissociated Fast Blue-labeled nociceptor neurons (<40 pF) at room temperature as described (27). Currents were amplified using an Axopatch 200B amplifier and digitized with a Digidata 1322A A/D converter (Axon Instruments, San Jose, CA). Signals were low-pass filtered at 1 kHz, acquired at 10 kHz, and stored and analyzed using pClamp 9.0 (Axon Instruments). Solutions were as follows (in mM): extracellular: 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 D-glucose, pH 7.4; pipette: 110 K-Gluconate, 30 KCl, 10 HEPES, 1 MgCl2, 2 CaCl2, pH 7.25. The liquid junction potential was taken to be 12 mV. The holding potential was −80 mV while 100 μM MO was applied for 5-s durations via a fast-flow multibarelled perfusion system.

CRD and electromyography. The placement of electrodes for electromyography (EMG) recording and the construction of balloons for CRD have been described (17). For electrode placement, mice were anesthetized using isoflurane and received buprenorphine (0.1 mg/kg sc for 2 days postsurgery) for analgesia. Electrodes were made by stripping 3 mm of insulation from the ends of Teflon-coated stainless steel wires (Cooner Wire, Chatsworth, CA) and then sutured to the abdominal musculature just superior to the inguinal ligament. Electrodes were tunneled subcutaneously to the dorsum of the neck, externalized, and secured in place. The skin was closed, and mice were allowed to recover for 7 days. During the recovery period, the mice were acclimatized to the recording chamber. On the day of testing, mice were briefly anesthetized with isoflurane, and distending balloons were placed in the rectum, 0.5 cm proximal to the anus, and secured by tape to the tail. Mice were placed in clear polycarbonate tube restrainers and connected to the CRD apparatus and amplifier. Graded distensions were generated using a compressed helium source with an ultralow flow regulator and digital pressure gauge. Mice were challenged at 15, 30, 45, and 60 mmHg distention pressures, with three 10-s trials at each pressure and a 3-min recovery period between each distention. To determine the visceromotor response to CRD, the EMG activity of the external oblique muscles was amplified and recorded via a pressure amplifier (NL 108, Digitimer, Welwyn Garden City, UK) connected in parallel with the input port. The preparation was stabilized for 30 min and was distended to an intraluminal pressure of 60 mmHg by closing the outlet port. This procedure was repeated 5 times at intervals of 10 min to test the viability of the preparation and the reproducibility to ramp distension. We use a Micro 1401 MKII interface and a PC running Spike 2 software (Cambridge Electronic Design) to obtain the relationship between volume and pressure of the segments.

Agonist administration. MO, HNE, PAR2-AP, TNBS, or vehicle (control) were administered to mice immediately after VMR baseline was measured. MO (2.5%) was dissolved in olive oil; HNE (10−3 M) was dissolved in 1% DMSO in saline; PAR2-AP (1 mg/ml) was dissolved in saline; TNBS (2 mg) was dissolved in 50% ethanol in saline. Under brief isoflurane anesthesia, agonists or vehicles (50–100 μl) were injected by enema into the lumen of the colon 2.5 cm proximal to the rectum using a PTFE 24 catheter attached to a 1-ml syringe. While anesthetized, mice were held inverted for 5 min to prevent leakage. After treatment (2 h for MO, 3 h for HNE, 6 h for PAR2-AP, and 3 days for TNBS) VMR to CRD or compliance were assessed.

Activation of spinal nociceptive neurons. To assess activation of nociceptive neurons in the spinal cord, c-fos was localized using immunohistochemistry. Lumbar spinal cord was subdivided in three segments (L1-2; L3-4; L5-S1), placed in 4% paraformaldehyde for 2 h at room temperature, and cryoprotected by incubation overnight in 30% sucrose in PBS at 4°C. Sections (40 μm) were cut in the transverse plane using a sliding microtome and placed in 100 mM PBS, pH 7.4. Samples were incubated in 3% normal goat serum in PBS, 1 h, room temperature, and then with c-fos antibody (1:20,000; 16 h, room temperature). Sections were washed and incubated with biotinylated goat anti-rabbit antibody (1:2,000; 1 h, room temperature), followed by an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Sections were treated with 1% hydrogen peroxide as the chromogen. Images of the spinal cord were captured using a 10× objective. c-fos-Stained nuclei in laminae I and II of dorsal horns were counted in four sections per segment per animal with a computer-assisted image analysis system (NIH Image software; National Institutes of Health, Bethesda, MD), and mean data were determined for each mouse.

TNBS-induced macroscopic damage score. Immediately after death, the colon of mice treated with TNBS (2 mg dissolved in 50% ethanol in saline; 3 days) or its vehicle was removed and rinsed with saline. Macroscopic colonic damage was evaluated by an investigator (E. Grady) unaware of the experimental group, scoring (0–4) the following features: 1) hemorrhage, 2) edema, 3) structure, 4) erythema, 5) mucous, and 6) diarrhea. The scores were tabulated and the mean value defined as the macroscopic damage score. The colon was then fixed in 10% formalin overnight. Paraffin-embedded sections (4–6 μm) were stained with hematoxylin and eosin. Sections were observed, and representative images were acquired using a Zeiss Axiolab microscope equipped with a 20× NeoFluor objective and a SPOT camera. Images were assembled and labeled in Photoshop, but the raw images were unmodified.

Statistical analysis. Data are presented as means ± SE. Differences among treatments in CRD, spinal nociceptive activation, and macroscopic damage score were examined using one-way ANOVA followed by Dunnett’s post hoc test while compliance data were analyzed using two-way ANOVA and c-fos data in mice treated with HNE or vehicle using Student’s two-tailed t-test. All data were analyzed using GraphPad Prism 5 software package (GraphPad, San Diego, CA). P < 0.05 was considered statistically significant.

RESULTS

TRPA1 is coexpressed with SP and CGRP by a subset of DRG neurons innervating the colon. TRPA1 is coexpressed with TRPV1 by a subpopulation of primary afferent neurons containing SP and CGRP that mediates pain and neurogenic inflammation (19, 39). However, it is not known whether TRPA1 is expressed by murine DRG neurons innervating the colon and
whether these neurons also coexpress SP and CGRP. To identify neurons innervating the colon, we injected the retrograde tracer DiI into the wall of the descending colon of mice. We detected DiI in 38 ± 11% of neurons in DRG from the lumbosacral regions (n = 3 mice). TRPA1-like immunoreactivity (LI) was detected in 33 ± 4% (of 454 neurons) of all DRG neurons (Fig. 1A). TRPA1 was detected in some, but not all, DiI-positive neurons. Among the DiI-positive DRG neurons innervating the colon, 52 ± 5% also expressed TRPA1. Most of the measured DiI-TRPA1-positive neurons were either small or medium sized (diameter between 10–40 μm, Fig. 1E), thus meeting the criteria for pain-sensing neurons. Moreover, most DiI-positive neurons that expressed TRPA1 also expressed CGRP-LI and SP-LI (Fig. 1, C and D). Preadsorption of the TRPA1 antibody with the peptide used for immunization (Fig. 1B) or omission of the primary antibody (not shown) abolished staining. Thus TRPA1 is present in a large subset of neurons innervating the colon, some of which are peptidergic, nociceptive neurons.

Fig. 1. Localization of transient receptor potential ankyrin-1 (TRPA1) in colonic dorsal root ganglia (DRG). Colonic neurons from mice were retrogradely labeled with 1,1-dioctadecyl-3,3,3,3-tetramethlindocarbocyanine methanesulfonate (DiI), and TRPA1-like immunoreactivity (LI), calcitonin gene-related peptide (CGRP)-LI, and substance P (SP)-LI were localized. A: TRPA1-LI was expressed by DiI-labeled neurons (arrows). B: in controls, the signal was abolished by preadsorption of the primary antibody with the peptide used for immunization. C and D: TRPA1 was coexpressed with CGRP and SP by a subset of DRG neurons innervating the colon (arrows). Scale bar = 50 μm. E: DiI-TRPA1-positive DRG neurons (neurons = 88) were small (<25 μm diameter) or medium (25–40 μm diameter) in size; thus they are likely involved in sensing pain.
TRPA1 mRNA and currents are present in DRG neurons innervating the colon. To further examine the expression of TRPA1 in colonic DRG, we used LCM to collect Fast Blue-labeled neurons innervating the colon and amplified TRPA1 by RT-PCR. Analysis of the PCR product revealed a single band of expected size (216 bp), which was identified by sequencing (Fig. 2A). To confirm expression of functional TRPA1, we measured membrane currents in Fast Blue-labeled neurons in response to TRPA1 agonists. Dissociated small-diameter DRG neurons (<40 pF capacitance) were tested for the presence of TRPA1 currents using the perforated-patch configuration of the patch clamp technique under voltage-clamp mode. Applications of the TRPA1 agonist MO produced inward currents (150.78 ± 62.53 pA) in 38% of the neurons tested, including those labeled as colonic projecting with Fast Blue (Fig. 2B). Repeat applications produced lasting desensitization of the currents, consistent with the behavior reported for TRPA1 in sensory neurons (2). Thus DRG neurons innervating the colon express TRPA1 mRNA and respond to the TRPA1 agonist MO, providing functional evidence for the expression of this channel.

TRPA1 is not required for distention-induced pain in the colon. Transient distention of the colon and rectum of mice induces VMR that is indicative of pseudo-affective visceral pain (31). To investigate the role of TRPA1 in colonic mechanical sensation in mice, we compared VMR to CRD in trpa1+/+ and trpa1–/– mice at baseline. As expected, in both groups, transient and graded CRD (15, 30, 45, 60 mmHg) caused a linear trend toward increasing VMR, assessed by measuring EMG activity of the abdominal muscle. The VMR to all distending pressures was identical in trpa1+/+ and trpa1–/– mice (Fig. 3A). Thus TRPA1 is not required for distention-induced pain sensation in mice under basal conditions.

Direct activation of TRPA1 by exogenous and endogenous agonists causes hyperalgesia to colonic distention. To examine the role of TRPA1 in visceral pain, we compared VMR to CRD in trpa1+/+ and trpa1–/– mice 2 h after intracolonic administration of TRPA1 agonist MO or vehicle (control). In trpa1+/+ mice, MO significantly enhanced the VMR to the higher distending pressures (45 and 60 mmHg) (Fig. 3C), compared with baseline responses. In marked contrast, intracolonic injection of MO in trpa1–/– mice did not affect the magnitude of the VMR at any distending pressure, suggesting a specific role of TRPA1 in modulating visceral mechanical hyperalgesia. In control experiments, intracolonic administration of the vehicle did not affect the VMR to CRD in both trpa1+/+ and trpa1–/– mice (Fig. 3B). Thus intracolonic administration of MO causes a robust and sustained increase in the VMR to CRD in trpa1+/+ but not in trpa1–/– mice, indicative of visceral mechanical hyperalgesia.

Fig. 2. TRPA1 mRNA and TRPA1 currents in colonic DRG. A: amplification of TRPA1 transcript in Fast Blue-labeled colonic DRG, dissected by laser-capture microdissection and amplified by RT-PCR. B: representative trace of inward current of Fast Blue-labeled DRG neurons recorded in voltage-clamp mode. Application of mustard oil (MO) (100 μM, 5 s, produced at −80 mV) depolarized 38% of the tested neurons (n = 16).

Fig. 3. Visceromotor responses (VMR) to graded colorectal distension (CRD) in trpa1+/+ and trpa1–/– mice in response to intracolonic administration of MO. A: under baseline conditions (no agonist), CRD induced similar VMR in trpa1+/+ and trpa1–/– mice. B: in trpa1+/+ and trpa1–/– mice, intracolonic administration of vehicle (VEH, 100 μl of olive oil) did not alter VMR to CRD distention measured 2 h later, compared with baseline. C: in trpa1+/+ mice, intracolonic MO (100 μl, 2.5% MO in olive oil) robustly increased the VMR to CRD at 45 and 60 mmHg measured 2 h later, compared with baseline. Note lack of response to MO in trpa1–/– mice. n = 6–7 mice per group, *P < 0.05 and **P < 0.01 compared with baseline.
We recently demonstrated that HNE, a downstream product of arachidonic acid generated during oxidative stress, is an endogenous agonist of TRPA1 (42). To further characterize the role of TRPA1 in visceral pain, we compared the acute effects of HNE or vehicle in \textit{trpa1}\textsuperscript{−/−} mice. After 3 h, intracolonic administration of HNE, but not its vehicle, caused 1.5- and 2-fold increases in VMR to CRD at the highest distending pressures (45 and 60 mmHg, respectively) compared with baseline (Fig. 4). Given the lack of significance in these data, we did not repeat the experiments in \textit{trpa1}\textsuperscript{−/−} mice.

TRPA1 agonists MO and HNE activate spinal nociceptive neurons. \textit{c-fos}, an inducible transcription factor, is a quantifiable marker for identifying neuronal populations activated by noxious stimuli in the spinal cord (23). To investigate whether intracolonic administration of TRPA1 agonists activates spinal nociceptors, we compared \textit{c-fos} expression in superficial laminae (I and II) of the dorsal horn gray matter of mice intracolonically treated with MO and HNE or their vehicles. After 3 h from its administration, MO caused a robust 2.5-fold increase in \textit{c-fos}-LI of L3-4 and L5-S1 levels in \textit{trpa1}\textsuperscript{+/+} but not in \textit{trpa1}\textsuperscript{−/−} mice (Fig. 5A). As expected, control mice from both genotypes showed no important change in \textit{c-fos} expression at any lumbosacral levels. Similarly, intracolonic administration of HNE significantly increased \textit{c-fos} expression, as indicated by the corresponding increased \textit{c-fos}-LI in L1-2 and L5-S1 levels, when compared with control mice injected with vehicle (Fig. 5, B and C). Thus intracolonic administration of TRPA1 agonists activates spinal nociceptors.

TRPA1 agonist MO does not affect colonic compliance. Altered colonic tone and accommodation to distension may contribute to the altered colonic sensitivity observed in functional gastrointestinal disorders (20). To investigate whether the increased visceral hypersensitivity we observed in mice treated with TRPA1 agonist was attributable to alterations in colonic compliance, we used an in vitro model to compare the pressure-volume relationship of colonic segments of mice pretreated with intracolonic instillation of MO or vehicle. The pressure-volume relationship was unaffected by MO, and

![Fig. 4. VMR to graded CRD in \textit{trpa1}\textsuperscript{+/+} mice in response to intracolonic administration of hydroxynonenal (HNE). At 45 and 60 mmHg, HNE (100 µl, 10\textsuperscript{−3} M in 1% DMSO in saline) causes a 1.5- and 2-fold increase in VMR to CRD measured 3 h later, compared with baseline; \textit{n} = 7–9 mice per group.](http://ajpgi.physiology.org/)

![Fig. 5. Activation of spinal nociceptive neurons after intracolonic administration of TRPA1 agonists. \textit{c-fos} expression in the nucleus of spinal neurons in laminae I-II of the dorsal horn of L1-2, L3-4, and L5-S1 2 h after intracolonic administration of MO (100 µl, 2.5% MO in olive oil) (A) and 3 h after intracolonic administration of HNE (100 µl, 10\textsuperscript{−3} M in 1% DMSO in saline) (B and C). MO administration caused a robust increase in \textit{c-fos}-LI of L3-4 and L5-S1 levels in \textit{trpa1}\textsuperscript{+/+} but not in \textit{trpa1}\textsuperscript{−/−} mice. Control mice received vehicle and did not show any change in \textit{c-fos}-LI. HNE significantly increased \textit{c-fos}-LI in L1-2 and L5-S1 levels compared with controls in \textit{trpa1}\textsuperscript{+/+}; \textit{n} = 5–8 mice per group, *\textit{P} < 0.05 and **\textit{P} < 0.01 compared with control \textit{trpa1}\textsuperscript{+/+} mice.](http://ajpgi.physiology.org/)
TRPA1 mediates PAR2-induced colonic mechanical hyperalgesia. Intracolonic administration of PAR2 agonists causes hypersensitivity to colonic distention (18). In addition, in mice, TRPV4 mediates PAR2-induced colonic hypersensitivity (14, 38). PAR2 agonists can also sensitize TRPV1 and TRPA1 (3, 19). To investigate the role of TRPA1 in PAR2-induced visceral hyperalgesia, we compared the effects of intracolonic administration of PAR2-AP in *trpa1*+/+ and *trpa1*−/− mice. When PAR2-AP was administered to the colon of *trpa1*+/+ mice, VMR measured 6 h later to all distention pressures were increased compared with baseline, the effect becoming significant at 30 and 60 mmHg (Fig. 7). In marked contrast, PAR2-AP did not affect the magnitude of VMR to CRD in *trpa1*−/− mice at any distending pressure (Fig. 7). Thus TRPA1 mediates PAR2-induced hyperalgesia to distention of the colon.

Deletion of TRPA1 ameliorates visceral hyperalgesia and spinal neuron activation during TNBS-induced colitis. TNBS-induced colitis is associated with oxidative stress (37), production of prostaglandins (1, 47), and increased visceral hypersensitivity produced during inflammation (19, 32, 40). Thus TNBS-induced colitis is associated with oxidative stress (37), production of prostaglandins (1, 47), and increased visceral hypersensitivity (30). TRPA1 can be directly activated by several products of oxidative stress (5) and indirectly sensitized by inflammatory mediators produced during inflammation (19, 32, 40). Thus TNBS-induced colitis is a suitable model to determine the role of TRPA1 in inflammatory hyperalgesia of the colon. IBD is also associated with high production of inflammatory mediators and reactive oxygen species (36), but the role of TRPA1 activation in visceral nociceptive modulation in an experimental model of IBD is unknown. We compared the VMR to CRD in *trpa1*+/+ and *trpa1*−/− mice after induction of colitis with TNBS. First, we confirmed the presence of colonic inflammation by evaluating the macroscopic damage score. TNBS, but not its vehicle (50% ethanol), significantly increased macroscopic damage in both *trpa1*+/+ and *trpa1*−/− mice to a similar extent (Fig. 8A). Histological assessment revealed an intact mucosa and external muscularis with myenteric neurons in the proximal colon of *trpa1*+/+ mice treated with vehicle (Fig. 8B, top, left). Similarly, normal histology was apparent in *trpa1*−/− mice treated with vehicle (Fig. 8B, bottom, left). Thus, in our C57/B6 mice, deletion of TRPA1 does not alter baseline appearance of the large intestine. Treatment with TNBS caused a marked inflammation in *trpa1*+/+ mice (Fig. 8B, top, right). In the representative section shown, an intact mucosa is apparent; however, in other animals, ulcers were seen with the typical skip pattern expected with TNBS treatment. There was a striking infiltration of neutrophils (black arrow) and monocytes (black arrowhead) in the submucosa, which had marked edema. A dramatic influx of neutrophils and monocytes was seen in the lamina propria (white arrows). The ongoing nature of this inflammation was evident as additional nucleated cells were being recruited from the bloodstream (white arrowhead). A thickening of the muscularis externa occurred. Similar results were observed in *trpa1*−/− mice (Fig. 8B, bottom, right). Thus both macroscopic and microscopic analyses indicate that TNBS induced colonic inflammation that was unaffected by deletion of TRPA1.

Using the TNBS model of colitis, we examined the role of TRPA1 in inflammatory visceral pain. TNBS increased VMR to CRD at all distending pressures in *trpa1*+/+ mice, compared with baseline responses (Fig. 9A). In striking contrast, TNBS-induced colitis did not affect VMR to CRD in *trpa1*−/− mice, suggesting a role of TRPA1 in mediating visceral hyperalgesia in pathological conditions. Moreover, TNBS-induced colitis increased the number of neurons expressing c-fos-LI in lamina I/II of the spinal cord in *trpa1*+/+ mice but not in *trpa1*−/− or in control mice (Fig. 9, B and C). Thus TRPA1 deletion ameliorates pain in this IBD model, suggesting that TRPA1 plays a major role in inflammatory visceral hyperalgesia.

**DISCUSSION**

Recently, it has been shown that TRPA1 is preferentially expressed by nociceptive sensory neurons where, upon activation, it enhances release of nociceptive peptides (SP and CGRP), thereby inducing neurogenic inflammation and somatic pain (19, 42). However, the same role for TRPA1 may not be applicable to visceral nociception given that mechanisms of visceral pain differ from those involved in somatic pain (16). We report the novel findings that TRPA1 is expressed by DRG neurons that innervate the colon and that direct activation or indirect sensitization of TRPA1 activates
spinal nociceptive neurons and enhances VMR to CRD, indicative of mechanical hyperalgesia, without affecting colonic compliance. We also report that TRPA1 mediates inflammatory hyperalgesia of the colon.

We found that TRPA1-LI is expressed by ~52% of small- to medium-sized colonic DRG neurons and that TRPA1 colocalizes with SP and CGRP in the majority of these neurons, which are thus nociceptive neurons that mediate neurogenic inflammation and transmit pain. These findings agree with other reports of TRPA1 expression in peptidergic DRG neurons and in retrogradely labeled gastrointestinal neurons (12, 19). We confirmed expression of TRPA1 in these neurons by amplifying TRPA1 mRNA from microdissected neurons innervating the colon. Patch-clamp recordings from the same neurons revealed inward currents in response to the TRPA1 agonist MO, which confirms expression of functional channels. Given that functional TRPA1 is expressed by small- to medium-sized colonic DRG neurons, which are polymodal nociceptors, TRPA1 is appropriately localized to mediate visceral pain.

TRPA1 activation induces colonic mechanical hyperalgesia and spinal nociceptive activation. We observed that intracolonic administration of MO, but not its vehicle, caused a robust hyperalgesia to CRD. This response was not observed in trpa1−/− mice and is thus completely dependent on TRPA1. These findings agree with other reports that intracolonic MO increases visceral hypersensitivity and referred hyperalgesia (29, 35). We also observed that intracolonic administration of MO induced c-fos expression in spinal nociceptive neurons of the lumbosacral levels of the spinal cord in trpa1+/+ mice but not in trpa1−/− mice, which corroborates the effects of MO on pain-related behavior. The same results, but with less dramatic effects on both visceral hypersensitivity and spinal nociceptive activation, were mimicked by intracolonic administration of the endogenous agonist HNE in trpa1+/+ mice. Intracolonic instillation of HNE caused a significant increase in c-fos expression in superficial laminae of the spinal cord. Although HNE increased visceral hypersensitivity to CRD, this effect was not statistically significant. The diminished effect of HNE compared with MO on pain-related behavior and activation of nociceptive neurons may be explained by the selected dose and the rapid metabolism of HNE. HNE reportedly accumulates at the plasma membrane during oxidative stress at concentrations that range between 10 μM and 5 mM (43), whereas we used 1 mM. The half life of free HNE in tissues is only ~4 s (11), which might be too short a period for HNE to penetrate the mucosa and activate nociceptors. A recent report suggests that 4-ONE, another electrophilic downstream product of arachidonic and linoleic acid that shares the same immediate precursor of HNE (4-hydroperoxy-2-nonalen) (10), is a better candidate for TRPA1 activation of C fibers. In that study, 100 μM HNE could not evoke action potential discharge or tachykinin release from bronchopulmonary C-fiber terminals (41). In contrast, 4-ONE, at 10-fold lower concentration (10 μM), caused substantial action potential discharge and tachykinin release from the same terminals. This dramatic increase in the electrophilic reactivity of 4-ONE, attributable to a ketone group in position C4, also increases its potential to form Michael adducts and makes 4-ONE a more potent endogenously produced agonist of TRPA1. The study concluded that, at equivalent concentrations, 4-ONE is more likely than HNE to be involved in the activation of afferent C fibers at sites of oxidative stress. Further studies are required to determine whether 4-ONE or other endogenous agonists of TRPA1, such as cyclopentenone PGs, are endogenous agonists of TRPA1 in the inflamed colon.

Mechanisms other than activation of TRPA1 on sensory nerves may contribute to TRPA1-induced visceral pain. Altered colonic accommodation can contribute to colonic hypersensitivity in patients with functional gastrointestinal disorders (20). However, we observed the TRPA1 agonist MO did not affect colonic compliance because the pressure-volume relationship of the colon was unaffected by administration of MO. Activation of serotonergic pathways may also contribute to
TRPA1-mediated visceral mechanical hyperalgesia. TRPA1 is expressed by enterochromaffin cells throughout the small and large intestine and regulates gastrointestinal motility through release of serotonin upon luminal stimulation, including distension (33). Serotonin activates different receptor subtypes, in particular 5HT3 receptors that activate primary afferent fibers and cause visceral mechanical hypersensitivity. The contribution of this mechanism to inflammatory pain remains to be determined.

TRPA1 mediates PAR2-induced colonic mechanical hyperalgesia. Proteases released from colonic tissues of patients with IBS can activate nociceptive visceral sensory nerves (8) and produce hypersensitivity symptoms in mice (15). Thus proteases and PAR2 may represent major mediators of hypersensitivity associated with IBS. However, the mechanisms by which proteases and PAR2 activation generate visceral hypersensitivity are not fully understood. Mechanisms may include PAR2-induced sensitization of TRPV1 and TRPV4, resulting in enhanced sensitivity of nociceptors to direct activation (14, 22, 38). Our results show that visceral mechanical hypersensitivity induced by PAR2 is also mediated by TRPA1 activation. Indeed, intracolonial administration of PAR2-AP caused a delayed visceral mechanical hypersensitivity to colonic distention in trpa1+/+ mice that was completely absent in trpa1−/− mice. These findings agree with other reports that activation of PAR2 sensitizes TRPA1 in DRG neurons and sensory afferents (19, 46). In contrast, a recent study did not find evidence to support an interaction of TRPA1 and PAR2 in splanchnic colonic afferents (12). This difference may be related to differences in the source or TRPA1-deficient mice or differences in the experimental systems. Thus, whereas electrophysiological recordings from splanchnic afferents did not reveal that PAR2 sensitizes TRPA1 (12), behavioral studies of awake animals did reveal sensitization. However, this was the only contradicting evidence, as our findings agree and support their conclusions in considering TRPA1 critical in visceral mechanosensation and in underlying alterations of mechanical responsiveness of visceral afferents by inflammatory and algic stimuli.

Given the role of TRPA1 as mechanoreceptor, it is noteworthy that mice lacking functional TRPA1 responded similarly to all graded distention pressures as trpa1+/+ mice at the basal state. This suggests that other molecular transducers, such as purinergic P2X receptors (44), acid-sensing ion channels (24, 34), and TRPV4 (14), may play primary roles in sensing mechanical stimuli under basal conditions. Our observation that TRPA1 is required for PAR2-induced visceral hyperalgesia suggests that TRPA1 may be important in high-threshold or silent afferents that are activated during inflammation.

We observed that deletion of TRPA1 completely prevented PAR2-induced hyperalgesia to colonic distention. Similarly, deletion of TRPV4 also prevents PAR2-induced colonic hyperalgesia (38). These results suggest that TRP channels may functionally interact. It is likely that they are coexpressed by the same nociceptors because PAR2 has been reported to be coexpressed in peptidergic DRG neurons with TRPV4 (38) and with TRPA1 (19). Thus they may interact in sensitizing the same nociceptors and thereby amplifying visceral hypersensitivity. Another speculative mechanism for explaining their similar effects in mediating PAR2-induced visceral pain could be redundancy, given that these (and other ion channels such as acid-sensing ion channel) overlap in their contribution in colonic afferent. Further investigations are required to resolve these issues.

TRPA1 mediates visceral hyperalgesia and activation of spinal nociceptive neurons during TNBS-induced colitis. We observed that TNBS-induced colitis dramatically increased mechanical hypersensitivity of the colon and spinal nociceptive activation in trpa1+/+ mice. In striking contrast, both responses were completely absent in trpa1−/− mice. Our findings
in trpa1−/− mice agree with those from other studies showing that TNBS-induced colitis produces visceral hyperalgesia, as documented by the increase of VMR to CRD (12, 30). Together, these findings suggest that TRPA1 plays a major role in hyperalgesia of TNBS-induced colitis in mice and confirm results shown in other studies (12, 45). The agents that activate or sensitize TRPA1 in TNBS-induced colitis remain to be identified. Possibilities include products of oxidative stress such as HNE and ONE, metabolites of PGs, or proteases that activate PAR2 and other GPCRs.

In summary, our results reveal a major role for TRPA1 in mechanical hyperalgesia of the colon. TRPA1 is expressed by nociceptive neurons innervating the colon, and activation and sensitization of TRPA1 induces visceral hypersensitivity to colorectal distention. These findings, together with the knowledge that visceral hypersensitivity of nociceptive pathways contributes to painful symptoms in IBS and IBD, suggest that TRPA1 is a new potential therapeutic target for abdominal pain.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


