Colitis decreases mechanosensitive $K_{2P}$ channel expression and function in mouse colon sensory neurons

Abbreviated title: Mechanosensitive $K_{2P}$ channels in colon DRG neurons

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ABSTRACT

TREK-1, TREK-2 and TRAAK are mechanosensitive two-pore domain K\(^+\) (K\(_{2p}\)) channels thought to be involved in the attenuation of mechanotransduction. Because colon inflammation is associated with colon mechano-hypersensitivity, we hypothesized that the role of these channels in colon sensory (dorsal root ganglion, DRG) neurons would be reduced by colon inflammation. Accordingly, we studied the functional expression of mechanosensitive K\(_{2p}\) channels in colon sensory neurons in both thoracolumbar (TL) and lumbosacral (LS) DRG that represent the splanchnic and pelvic nerve innervations of the colon, respectively. In colon DRG neurons identified by retrograde tracer previously injected into the colon wall, 62% of TL neurons and 83% of LS neurons expressed at least one of three K\(_{2p}\) channel mRNAs; the proportion of LS neurons expressing the TREK-1 gene was greater than in their TL counterparts. In electrophysiological studies, single channel activities of TREK-1a, TREK-1b, TREK-2 and TRAAK-like channels were detected in cultured colon DRG neuronal membranes. After trinitrobenzene sulfonic acid-induced colon inflammation, we observed significant decreases in the amount of TREK-1 mRNA, in the response of TREK-2-like channels to membrane stretch, and in the whole cell outward current during osmotic stretch in LS colon DRG neurons. These findings document that the majority of DRG neurons innervating the mouse colon express mechanosensitive K\(_{2p}\) channels and suggest that a decrease in their expression and activities contributes to the increased colon mechanosensitivity that develops in inflammatory bowel conditions.

Keywords: K\(_{2p}\) channels, TREK channels, mechanosensitive channels, dorsal root ganglia neurons, colon sensory neurons, colitis
INTRODUCTION

Two-pore domain K⁺ (K2P) channels are recently identified, structurally distinct K⁺ channels that have 4 transmembrane and 2 pore-forming domains. These channels are active across a range of physiological membrane potentials, which suggests they are background K⁺ channels playing an important role in regulating cellular excitability by setting and shaping the resting membrane potential and action potential. Six subfamilies of K₂P channels have been described to date (27). TREK-1 (K₂P2.1), TREK-2 (K₂P10.1) and TRAAK (K₂P4.1) are TREK subfamily members activated by a variety of physical and chemical stimuli such as membrane stretch, heat, intracellular acidosis (TREK-1 and -2) or alkalosis (TRAAK) and lipids (5, 24, 30-33), implicating these channels in mechano/thermo/chemosensation.

All three K₂P channels are found in sensory ganglia neurons. In human and rat dorsal root ganglia (DRG), gene expression of TREK-1 and -2 was reported to be moderate and restricted whereas TRAAK expression was strong and abundant by whole ganglia RT-PCR and in situ hybridization, respectively (35, 48). Functionally, TREK-2-like channel activity was more frequently observed in single channel recordings from cultured neonatal rat DRG neurons than the other two channels (26). In mouse DRG, in contrast, 60% of neurons were found to express TREK-1 mRNA (2) and its immunoreactivity was mostly observed in small and medium sized neurons (31). All three TREK subfamily channels were also immunohistologically detected in small sized neurons in rat trigeminal ganglia where they often colocalized with thermosensitive TRPV1 channels (53). TREK-1 and TRAAK gene transcripts (TREK-2 was not examined) are also present in nodose ganglia neurons innervating the stomach and
proximal duodenum with TRAAK mRNA preferentially detected in TRPV1-negative neurons (54). The inhibitory role of these K<sub>2p</sub> channels in mechano/thermosensation is supported by studies in TREK-1 and/or TRAAK knockout mice in which sensitivity is increased to von Frey probing of the hindpaw and to tail immersion temperatures between 46 and 50°C (2, 39).

Although the presence and role of mechano/thermosensitive K<sub>2p</sub> channels in sensory neurons has been documented, their expression profile in DRG neurons related to an innervated target has not been widely investigated. Moreover, little information is available regarding changes in their expression and properties in conditions where alterations in mechano/thermosensation occur. Accordingly, we studied the expression of TREK-1, TREK-2 and TRAAK in DRG neurons innervating the mouse colon, an organ physiologically subjected to stretch during normal function. Because the colon is innervated by two nerves with overlapping but also physiologically distinct functions (6, 8, 52)), DRG neurons at thoracolumbar (lumbar splanchnic nerve) and lumbosacral (pelvic nerve) levels were studied separately. Secondly, to test the hypothesis of the channels’ inhibitory role in mechanosensation, we examined the expression and properties of K<sub>2p</sub> channels in colon DRG neurons after colon inflammation that produces colon mechano-hypersensitivity. Portions of these data have been presented in abstract form (La and Gebhart, 2009).

**MATERIALS AND METHODS**
Adult male C57BL/6 (Taconic, Germantown, NY) mice (25–30 g) were used in this study. Mice were housed under 12/12-hr light/dark cycle. Water and food were provided *ad libitum*. All procedures were approved by and in accordance with the guidelines of the Institutional Animal Care and Use Committee, University of Pittsburgh.

*Cell labeling*

The colon was exposed surgically through laparotomy (2% isoflurane anesthesia; Hospira Inc., Lake Forest, IL) and a 2% solution of retrograde tracer, 1,1’- dioctadecyl-3,3,3’,3-tetramethylindocarbocyanine perchlorate (DiI; Invitrogen, Carlsbad, CA) in absolute dimethyl sulfoxide was injected into the organ wall using a 30-gauge needle. Three to five sites were injected into the distal colon wall, each in a volume of ~5 μL. Mice were used for experiments 14–21 days after DiI injection.

*Colon Inflammation*

TNBS (2,4,6-trinitrobenzene sulfonic acid, 1%; Sigma-Aldrich, St. Louis, MO) in 50% ethanol was instilled intracolonically (200 μL) under 2% isoflurane anesthesia using a 22-gauge, 24-mm-long stainless-steel feeding needle. Mice were then kept vertical for 3 min and subsequently returned to their home cages. Nutrients and water were supplied in a gel form (DietGel®, ClearH2O, Portland, ME) and their nest area was covered with a hut to keep them warm until sacrifice at day 2 after TNBS instillation.

*DRG neuron culture*
Mice were sacrificed with overdose of pentobarbital sodium (Nembutal®, Ovation Pharmaceutical, Deerfield, IL) followed by decapitation. Thoracolumbar (T10–L1) or lumbosacral (L6–S1) DRG were dissected out and enzymatically digested with collagenase IV (200 U/ml, Worthington Biochemical, Lakewood, NJ) and dispase (7.5 U/ml, Worthington) in serum-free, advanced Dulbecco’s modified eagle medium (DMEM)/F12 containing 1% penicillin/streptomycin (Invitrogen) at 37°C in 5% CO2 for 40 min. After trituration with a fire-polished, large-bore glass Pasteur pipette, the cell suspension (~ 2ml) was transferred to a conical tube containing 8 ml of advanced DMEM/F12 with 10% fetal bovine serum (Sigma) to stop the digestion. After centrifugation at 280 g for 10 min, the pellet was dissociated in fresh medium, cells were plated on poly-D-lysine-coated coverslips (Becton Dickinson Labware, Bedford, MA) and incubated overnight at 37°C in 5% CO2. Only DiI-labeled neurons were used for subsequent experiments within 30 hr after plating.

**Single cell RT-PCR**

DiI-labeled neurons in culture were collected individually with glass pipettes, expelled into microcentrifuge tubes containing reverse transcriptase mix (15) and their first-strand cDNAs were synthesized using Oligo (dT)12-18 primer (Invitrogen) through a series of incubations: 65°C for 1.5 min, room temperature for 2 min, 37°C for 20 min after administrating 20 U SuperScript II (Invitrogen) and 65°C for 10 min. Successfully processed cells were screened by examining a transcript of a housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Reverse transcription-negative controls (cells processed without SuperScript II or a cell-free bath aspirate) were included in every screening. The first round multiplex PCR was performed using two-fifths of the
original first-strand cDNA sample as template in a 25 μL solution containing 1x GoTaq reaction buffer (Promega, Madison, WI), 0.4 μM external primers mix, 0.2 mM dNTPs, and 0.2 μL GoTaq DNA polymerase (Promega); primer sequences are listed in Table 1. Reactions consisted of initialization at 95°C for 10 min, 35 cycles at 94°C/30 s, 52°C/30 s and 72°C/30 s before a final extension step at 72°C for 10 min. Each first round PCR product served as template in the second round PCR using a channel specific internal primer pair. The second round PCR product was electrophoresed on 2% agarose gel, stained with ethidium bromide and photographed.

**Quantitative real time PCR**

The quantity of K$_{2p}$ channel transcripts in Dil-labeled colon neurons was measured adopting a single cell real time RT-PCR protocol (1, 42). Specifically, the first-strand cDNA sample of an individual neuron was preamplified by 26 cycles, which was chosen based on the linear amplification range of the reference standard GAPDH in single cells, under the multiplex PCR condition described above. After identification of the channel transcripts expressed in each neuron, the 26 cycle preamplification product from neurons expressing the channel transcript(s) was diluted six times and pooled according to the channel transcript and DRG level (TL vs. LS) of each animal. The pooled product served as template in quantitative PCR separately for a specific channel transcript and the reference standard on the ABI PRISM® 7000 system (Applied Biosystems, Foster City, CA) using ABsolute™ QPCR CYBR® Green ROX Mix (ABgene, Rochester, NY). Threshold cycle (C$_T$) and PCR efficiency was determined within the linear amplification range. The quantity of the channel transcript was
expressed relative to that of the reference standard by raising the averaged PCR efficiency to the power of the $C_T$ difference ($\Delta C_T$) between the reference standard and the channel transcript. Preamplification uniformity of the target transcripts was also assessed by calculating the difference of $\Delta C_T$ ($\Delta\Delta C_T$) in the same samples preamplified by two different cycle numbers (20 vs. 26 cycles). The $\Delta\Delta C_T$ of all channel transcripts were within $\pm 0.51$.

**Electrophysiological recordings**

Single channel and whole cell recordings were performed with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA). Cells on coverslips were continuously superfused with normal bath solution containing (in mM): NaCl 140, KCl 5, MgCl$_2$ 1, CaCl$_2$ 2, HEPES 10, and glucose 10 (pH 7.4, 310 mOsm) in a recording chamber. For single channel recording, borosilicate glass micropipettes (Sutter instrument, Novato, CA) with tip resistance greater than 5 MΩ were fabricated and filled with pipette solution containing (in mM) 140 KCl, 1 MgCl$_2$, 5 EGTA, 10 HEPES, 10 tetraethylammonium chloride (TEA-Cl), 1 4-aminopyridine (4-AP) and 0.01 glibenclamide (pH 7.3). The tip was coated with silicon film using Sigmacote® (Sigma) to improve signal/noise ratio. Single channel activities were recorded in an inside-out mode under symmetrical K$^+$ conditions by superfusing the pipette solution without TEA-Cl, 4-AP and glibenclamide to the cytosolic side of the membrane patch. The recorded signal was low-pass filtered at 2 kHz (80 dB/decade) at a sampling rate of 10 kHz and digitized (Digidata 1320A, Axon Instruments). For single-channel analysis, any events shorter than the filter rise time ($T_r=0.17$ ms) were ignored and those shorter than $2.5T_r$
(0.42 ms) were excluded from the current amplitude histogram. $N_{Po}$ ($N$ is the number of channels in the patch, and $Po$ is the channel open probability) was analyzed as a measure of channel activity and its changes during any stimuli were expressed relative to the activity before the stimulus application. The membrane patch was stretched by applying negative pressure inside the patch pipette through a water-filled U-shape manometer, and drugs were applied using a fast-step SF-77B superfusion system (Warner Instruments, Hamden, CT) with a three-barrel pipette placed in close proximity (100 μm) to the cell. Openings of multiple ion channels were frequently recorded in one membrane patch, hampering clear identification of single channel activity. Therefore, for channel identification experiments, we only used patches that contained no more than three types of channels distinct in their amplitude and kinetics. Changes in channel activities upon stimulation were quantified only when the patches contained one type of TREK subfamily channel.

For whole cell current recording, glass micropipettes with tip resistance 2~3 MΩ were filled with pipette solution containing (in mM) 120 K gluconate, 20 KCl, 0.2 CaCl$_2$, 2 MgATP, 10 EGTA and 10 HEPES (pH 7.2, 302 mOsm). After establishing a stable whole cell configuration, the normal bath solution was exchanged with an isotonic recording solution containing (in mM) 85 choline chloride, 5 KCl, 3 MgCl$_2$, 10 glucose, 10 HEPES, 10 TEA-Cl, 1 4-AP and 90 D-mannitol (pH 7.4, 310 mOsm). We omitted Na$^+$ and Ca$^{2+}$ from the recording solution to avoid the contamination of K$^+$ outward current by inward flux of these two ions through stretch-sensitive cation channels. Neurons were held at -60 mV and subjected to slow ramp depolarization from -110 mV to 20 mV (20 mV/s). Series resistance was compensated by >80% and leak currents were not subtracted.
Membrane stretch was done by swelling the cell with hypotonic recording solution (220 mOsm) containing a volume-activated chloride channel blocker 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, 100 μM) and measured by increase in neuronal size (μm²). Neurons under electrodes were photographed using a microscope-mounted digital camera (DFC340FX, Leica Microsystems, Bannockburn, IL). Data were sampled at 10 kHz and low-pass filtered at 5 kHz. All membrane potentials noted in this paper are liquid junction potential-corrected.

Data analysis

Data are presented as mean ± SEM with n, the number of samples and N, the number of mice. The software packages pClamp 10.0 (Axon Instruments), Origin 8 (Originlab Corporation, Northampton, MA) and SigmaPlot 9.0 (Systat Software, Inc., San Jose, CA) were used for data analyses. Chi-square tests were used for analyses of contingency tables with more than 3 categories. A set of independent contrasts was specified a priori for comparing the proportion of cells expressing each channel between two groups (e.g., TL vs. LS) and Fisher’s exact test was used for analysis of the 2X2 contingency table. For analysis of differences between means, the Mann Whitney U test or Wilcoxon signed rank test was used for comparing two groups and two-way ANOVA with the Holm-Sidak post-hoc multiple comparisons test was used for groups with two independent variables (e.g., TL vs. LS in naïve vs. TNBS-treated mice). Results were considered statistically significant when \( P < 0.05 \).
RESULTS

Gene expression of TREK subfamily channels in colon DRG neurons

In 77 TL colon DRG neurons (N=6), 62% expressed at least one of the TREK subfamily channel gene transcripts. The TREK-1 gene transcript was detected in 42%, TREK-2 in 36%, and TRAAK in 27% of TL cells; these proportions of K\textsubscript{2p} channel gene expression did not differ within the sample of TL neurons (P>0.17, $\chi^2$ test). Among all TL neurons, 12% were found to express all three channel mRNAs (Fig. 1). Cells with two TREK channel gene transcripts were also frequently encountered; 9% of colon TL DRG neurons had both TREK-1 and TREK-2, 6% had TREK-1 and TRAAK, and 4% had TREK-2 and TRAAK.

In LS colon DRG neurons, however, the number of cells expressing TREK-1 mRNA was greater (P<0.0001, $\chi^2$ test, standardized residual >2.5) than those expressing TREK-2 or TRAAK mRNA. In 81 samples (N=6), the TREK-1 gene transcript was found in 73% of neurons, TREK-2 in 42% and TRAAK in 43%. Seventeen percent of LS neurons were found to be devoid of any TREK channel gene transcripts, whereas 22% expressed all three channel mRNAs. Fourteen percent of LS colon DRG neurons were positive to both TREK-1 and -2, 16% to TREK-1 and TRAAK, and 1% to TREK-2 and TRAAK.

Two major differences were observed between TL and LS colon DRG neurons in the proportion of channel-expressing cells. More LS colon DRG neurons expressed TREK-1 mRNA than their TL counterparts (P<0.0001, Fisher’s exact test) and the proportion of neurons not expressing any TREK subfamily channel mRNA was significantly less in the LS population (P<0.005, Fisher’s exact test). When quantified in
neurons grouped by the channels expressed, TREK-1 mRNA was most abundant and no difference was detected between TL and LS colon DRG neurons in the content of channel mRNAs (Fig. 2).

We tested commercial antibodies to immunolocalize the three channel proteins in DRG tissue sections and culture, but none of them provided convincing results in our experiment conditions (data not shown).

*TREK subfamily-like single channel activities in colon DRG neurons*

To examine the functional expression of TREK subfamily channels in colon DRG neurons, single channel activity was recorded in inside-out patches under symmetrical K⁺ conditions (140 mM) at membrane potentials ranging from -80 mV to 80 mV. Isolation of the TREK subfamily channels was facilitated by including TEA, 4-AP and glibenclamide in the pipette solution, all of which suppress the activities of Kv channels and KCa channels without inhibition of TREK subfamily channels activities (27).

We observed four mechanosensitive K⁺ channels with distinct single channel current-voltage (I-V) relationships that allowed us to differentiate them by their chord conductances at ±60 mV (Fig 3). The first and second type showed relatively linear I-V relationships with 140 pS and 40 pS conductances, respectively, at ±60 mV. The third and fourth type showed weak inward rectification. The chord conductance of the third type was 50 pS at 60 mV and 110 pS at -60 mV and that of the fourth was 55 pS at 60 mV and 80 pS at -60 mV. At least one of the 4 channels was encountered in 39% of patches in TL and 40% in LS colon DRG neurons.
As shown in Fig 4, the activities of these 4 different channels were increased upon membrane stretch, by arachidonic acid (AA, 20 μM), and by decrease in intracellular pH for the first, second and third type or by increase in the intracellular pH for the fourth type, all of which are characteristic properties of TREK subfamily channels. The first type was similar to classical TREK-1 (TREK-1a), the third to TREK-2, and the fourth to TRAAK recorded in rat neurons and COS-7 cells transfected with each channel gene (20, 26). The second type resembled the TREK-like channel iK_{AA} originally reported in rat supraoptic nucleus magnocellular neurons (20), and TREK-1b, the splice variant of TREK-1, observed in rat cardiac ventricular muscle and TREK-1 gene-transfected HEK293 cells (51).

Channel activities of two or more of these TREK subfamily-like channels were often observed in a single patch, especially in LS colon DRG neurons. The observation frequency of each channel is summarized in Table 2. Because of the limited number of patches that contained only one channel type, necessary for an unconfounded analysis, no statistical comparison of channel activities was made between TL and LS neurons, and the responses of each channel found in both TL and LS DRG neurons were pooled for presentation.

Effect of colon inflammation

When applied into the lumen of mouse colon, TNBS dissolved in ethanol induces inflammation accompanied by mechanical hypersensitivity to colorectal distension (29, 49).
The percentage of colon DRG neurons expressing each channel gene was not significantly changed 2 days after intracolonic instillation of TNBS (Fig 1), although there was a strong tendency toward a decrease in the number of TREK-1 gene-expressing cells (P=0.0796, Fisher’s exact test; P<0.02 by χ² test) and an increase in the proportion of cells not expressing any of the three TREK channel mRNAs (P=0.0689, Fisher’s exact test; P<0.01 by χ² test) in TL neurons. The differences in the gene expression of TREK subfamily channels between TL and LS colon DRG neurons were again observed in TNBS-treated mice; the proportion of LS colon DRG neurons expressing TREK-1 or TRAAK mRNA was greater than that of TL neurons in TNBS-treated mice (P<0.0001 for TREK-1, P<0.05 for TRAAK, Fisher’s exact test) and the proportion of neurons devoid of any TREK subfamily channel mRNA was smaller in the LS population (P<0.001, Fisher’s exact test).

When measured in colon DRG neurons from mice 2 days after TNBS treatment, the quantity of TREK-1 mRNA (relative to the amount of reference standard) was decreased from 20.5±6.5% (n=48, N=5) to 11.1±3.0% in LS neurons (n=45, N=5, P<0.05 by Holm-Sidak multiple comparison test). The Cₜ value of reference standard (GAPDH mRNA) in colon DRG neurons did not differ between naïve and TNBS-treated mice (F₁,2₄=1.32, P>0.26 for TL colon DRG neurons, F₁,2₄=1.20, P>0.28 for LS colon DRG neurons).

In electrophysiological recordings of DRG neuronal membrane patches, the observation frequencies of the TREK-subfamily-like channels in colon neurons from TNBS-treated mice did not differ from their counterparts encountered in colon neurons from naïve mice except that TREK-1b-like channel activities were not observed in TL
colon neurons from TNBS-treated mice (Table 2). Interestingly, the TREK-2-like channel in colon DRG neurons from TNBS-treated mice showed a significant decrease in the response to membrane stretch (negative pressure) of -4 cmH2O (n=7 in naïve, 8 in TNBS-treated, N=4 each, P<0.03, Mann-Whitney U test), and a strong tendency toward decreased response to intracellular acidosis (n=8 in naïve, 7 in TNBS-treated, N=4 each, P=0.0541).

Because the findings described above in TNBS-treated mice pointed to an overall reduction in the expression and activity of mechanosensitive K2P channels in colon DRG neurons, we expected to find a decrease in the osmotic membrane stretch-induced whole cell K+ current in colon DRG neurons from TNBS-treated mice. Hypotonic cell swelling effectively activates TREK subfamily channels, resulting in an increased whole cell K+ current (5, 41).

Neuron size (area) was increased by exposure to hypotonic solution (220 mOsm). The sizes of TL colon DRG neurons from naïve and TNBS-treated mice were significantly increased to 121.4±1.7% (n=12, N=4, P<0.001, Wilcoxon signed rank test) and 124.6±3.8% (n=7, N=3, P<0.05) of their original size, respectively, and their LS counterparts to 124.2±2.0% (naïve, n=21, N=4, P<0.001) and 129.3±3.1% (TNBS-treated, n=16, N=3, P<0.001). The degree of swelling was not significantly different either between TL and LS colon DRG neurons (F(1,52)=1.75, two-way ANOVA; P>0.19) or between naïve and TNBS-treated mice (F(1,52)=2.14, two-way ANOVA; P>0.14).

Under recording conditions where the influx of Na+ and Ca2+ was blocked, and voltage-activated K+ channels and volume-activated Cl− channels were inhibited, the osmotic stretch-induced change in outward current density was reversible and
dichotomous (Fig. 5). When measured at 20 mV membrane potential, 50% (6/12) of colon TL neurons from naïve mice (N=4) showed an increase in current density and the other 50% showed a decrease. Comparatively, 43% (3/7) of colon TL neurons from TNBS-treated mice (N=3) showed an increase while 57% (4/7) showed a decrease.

Quinine (Qn, 0.3 mM) and L-methionine (L-Met, 1 mM), both of which are known to inhibit TREK channels (4, 27) further reduced the outward current (n=3 for Qn, n=4 for L-Met, N=2, Fig 6A & C) when the current had been decreased, or inhibited the current back to control level (n=3 for Qn, n=2 for L-Met, N=2, Fig 6B & D) when the current had been increased by osmotic stretch.

The magnitude of changes in current density upon osmotic stretch had no correlation with cell size (capacitance), degree of swelling, or current magnitude at 20 mV under isotonic recording conditions (Fig 7). Neither the proportion of cells showing an increased outward current during osmotic stretch (P=1, Fisher’s exact test) nor the magnitude of current increase (P>0.38, Mann-Whitney U test) or decrease (P>0.47) significantly differed between naïve and TNBS-treated mice.

Dichotomous responses to osmotic stretch were also observed in LS colon neurons. In LS colon neurons from naïve mice (N=4), 48% (10/21) showed an increase and the remaining 52% (11/21) showed a decrease in outward current. In contrast, the majority (75%, 12/16) of colon LS neurons from TNBS-treated mice (N=3) showed a decrease in the outward current during osmotic stretch. Although the difference in proportion of LS colon neurons showing increased outward current did not reach statistical significance (P=0.1908, Fisher’s exact test) and the magnitude of current increase did not differ between neurons from naïve and TNBS-treated mice (P>0.94,
Mann-Whitney U test), the magnitude of current decrease was greater in neurons from TNBS-treated mice (P<0.029, Mann-Whitney U test). This difference did not appear to be due to sampling bias because their electrophysiological properties examined (resting membrane potential, capacitance and whole cell current density at 20 mV under isotonic recording condition) did not significantly differ (Fig 8).

We further examined whether the whole cell K⁺ current response to AA was decreased in colon DRG neurons from TNBS-treated mice. Unexpectedly, the effect of AA (10 or 20 μM) on whole cell K⁺ current (in the isotonic recording solution) was predominantly inhibitory, suggesting complexity of its mechanisms and/or sites of action at the whole cell level. No difference was detected either between TL and LS or between naïve and TNBS-treated mice in the whole cell current response to AA (Fig 8D).

A decrease in whole cell K⁺ current by hypotonic swelling or AA was also noted in hippocampal neurons (47), oligodendrocytes (46) and myocytes (45). We did not pursue elucidating its inhibitory mechanism in colon DRG neurons because it was not the immediate focus of the present study.

DISCUSSION

The present report documents the expression of mechanosensitive K₂p channels, both at the gene and functional single channel level, in DRG sensory neurons innervating mouse colon and, importantly, their down-regulation after colon inflammation. Specifically, we found that the majority of colon DRG neurons (62% in TL and 83% in LS) expressed at least one gene transcript of TREK subfamily channels TREK-1, TREK-
2 and TRAAK with frequent coexistence of two or more channel mRNAs in one neuron (31% in TL and 53% in LS). We observed that the proportion of TREK-1 gene expressing cells was greater in LS than in TL DRG and, further, that the proportion of neurons devoid of any TREK subfamily channel mRNA was smaller in LS than in TL. These differences between the two pathways of innervation of the same organ, in this case the colon, adds to a growing literature establishing significant functional differences between lumbar splanchnic and pelvic nerve afferent pathways innervating pelvic organs (6, 8, 11, 13, 14, 52). In addition, we confirmed electrophysiologically in colon DRG neurons activities of channels (39% of patches in TL and 40% in LS) showing characteristic biophysical and chemical properties of all TREK subfamily channels. The lower detection frequency of TREK subfamily-like channels in electrophysiological recordings than in gene transcripts assay may be accounted for by the higher detection sensitivity of the latter, the proportional difference in the quantities between mRNAs and translated channel proteins functional in the plasma membrane or native TREK subfamily channels existing with their known channel properties modified.

The presence of mechanosensitive TREK subfamily channels in colon DRG neurons has implications relative to colon physiology; the colon regularly experiences deformation of its wall during functioning as a reservoir and peristaltic passage of luminal contents. This structural deformation has been shown to be detected by sensory neurons and encoded into changes in neuronal activities. DRG neurons co-cultured with and in contact with colonic myocytes showed a rise of [Ca^{2+}]] upon light touch of the myocyte membrane (16). In addition, Raybould et al (43) reported that ~40% of colon DRG neurons were responsive to direct mechanical stimulation of their soma. Channels
involved in mechanotransduction, such as TRPV1, TRPV4, TRPA1 and ASIC3 are also found in colon DRG neurons with observations that disruption of their gene expression results in reduced mechanosensitivity of colon afferents or decreased behavioral response to colorectal distension (7, 9, 10, 12, 22, 23, 36, 40, 44). Considering the prevalent expression of these channels in colon DRG neurons (TRPV1 ~80%, TRPV4 ~52%, TRPA1 ~58% and ASIC3 ~75%), there must be some overlap in colon DRG neurons in the expression of these nonselective cation channels and mechanosensitive K<sub>2p</sub> channels, suggesting that K<sup>+</sup> outflow through TREK subfamily channels potentially counteracts the depolarizing effect of cation influx through TRP and ASIC channels when colon sensory neurons are mechanically stimulated. Therefore, one could speculate that a down-regulation of TREK subfamily channels in colon DRG neurons would cause a decrease in hyperpolarizing drive upon mechanical stimulation, which in turn increases mechanotransduction of colon wall deformation.

This idea is supported by the present findings in TNBS-treated mice. TNBS has long been used to model ulcerative colitis in rodents, which develop hypersensitivity to colorectal distension in the active inflammatory phase (17, 19, 29, 49, 50). When examined two days after intracolonic TNBS treatment in the present study, a down-regulation of TREK subfamily channels was apparent in colon DRG neurons: 1) the amount of TREK-1 gene transcripts was significantly reduced in LS neurons and the proportion of TREK-1 mRNA-expressing cells showed a tendency toward a decrease in TL neurons, indicating their gene expression is subject to plastic changes in inflammation as reported in other pathological conditions such as deafness by cochlear nerve ablation (21) and bladder hypertrophy by partial bladder outlet obstruction (3). The expression of
TREK subfamily channels monitored was decreased in cochlear nucleus and in bladder smooth muscle, respectively. 2) The response of TREK-2-like channel to membrane stretch was diminished in DRG neurons from TNBS-treated mice. The mechanosensitivity of TREK-2 is regulated by its C-terminus (28) and phosphorylation of the domain by protein kinase C decreases channel activity (25). Therefore, it seems not unlikely that in addition to decreased channel gene expression, post-translational modulation occurs after inflammation to regulate channel activity. The tendency of the TREK-2-like channel toward decreased sensitivity to intracellular acidosis after intracolonic TNBS treatment suggests an inhibitory modulation through changes in the C-terminus because this domain also confers proton-sensitivity on TREK-2 (28). 3) The inhibition of whole cell outward current by osmotic membrane stretch was greater in LS colon DRG neurons from TNBS-treated mice. Although caution is needed in interpretation of these results because of the nonspecific nature of osmotic stimulation (activating stretch-activated channels as well as suppressing stretch-inhibited channels), our findings suggest that a decrease in function/expression of TREK subfamily channels in colon DRG neurons augmented the inhibitory effect of hypotonic swelling on whole cell outward current and accounted for the tendency toward a decrease in the proportion of DRG neurons showing current increase upon osmotic stretch in TNBS-treated mice. Together, these outcomes suggest that the down-regulation of TREK subfamily channels contributes to the heightened mechanosensitivity to colorectal distension in the presence of colon inflammation. Supporting evidence for the correlation between down-regulation of these channels and visceral hypersensitivity to mechanical stimulation could be provided by experiments using a method to inhibit the channels’ activities in vivo. Such
efforts have been hindered largely by lack of pharmacological tools that selectively interfere with TREK subfamily channels. Alternative strategies would include silencing TREK subfamily channel gene expression to examine the effect of their absence/reduction on behavioral nociceptive responses to colorectal distension and/or analyzing the expression pattern of these channels in other models of visceral hypersensitivity in which inflammation is not present.

Another potential function of TREK subfamily channels in colon DRG neurons could be inhibition of spontaneous neuronal activity in response to colon wall deformation. When colorectal distension was used as a search stimulus to identify neurons receiving mechanosensory inputs from colorectum, a group of TL and LS spinal dorsal horn neurons were found to cease their spontaneous action potential firing during colorectal distension (37, 38). Although there is yet no evidence of spontaneously active colon DRG neurons that respond in such a fashion, it would be interesting to examine the possible link between TREK subfamily channels and neural substrates involved in inhibitory reflexes triggered by visceral organ distension.

It is noteworthy that there is both qualitative and quantitative heterogeneity of mechanosensitive colon sensory fibers. In single fiber recordings using an in vitro colon preparation with lumbar splanchnic or pelvic nerves attached, five different fiber types were identified based on their responses to mechanical stimuli (mucosal stroking, blunt probing and circumferential stretch), including two different stretch-sensitive fiber groups based on their high and low response thresholds to mechanical stimulation (8, 18). Similarly, sharp electrode recordings of colon DRG neurons also revealed two populations that are distinct in their mechanical thresholds and action potential firing
frequencies upon colon distension (34). Mapping the expression pattern of the three mechanosensitive K$_{2P}$ channels in sensory neurons in conjunction with studies such as these would extend our understanding of the underlying mechanisms that define the neuronal heterogeneity in mechanosensitivity.

In summary, we found that all three mechanosensitive K$_{2P}$ channels, TREK-1, TREK-2 and TRAAK, are expressed in colon DRG neurons that represent two different visceral afferent pathways, lumbar splanchnic and pelvic nerves. Their expression (TREK-1 in LS colon neurons) and activity (TREK-2) was significantly decreased in the presence of colon inflammation. These findings suggest a potential inhibitory role for these channels in sensory coding of colon distension and in the manifestation of visceral hyper-mechanosensitivity in gut inflammation.
ACKNOWLEDGEMENTS

The work described in this manuscript was supported by National Institutes of Health (NIH) awards R01 NS 19912 and UL1 RR024153 from the National Center for Research Resources (NCRR), a component of the NIH, NIH Roadmap for Medical Research, and by the Office of the Senior Vice Chancellor for the Health Sciences, University of Pittsburgh. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH. Information on NCRR is available at http://www.ncrr.nih.gov/. Information on Re-engineering the Clinical Research Enterprise can be obtained from http://nihroadmap.nih.gov/clinicalresearch/overview-translational.asp." We thank Michael Burcham for preparation of figures.


47. **Somjen GG.** Low external NaCl concentration and low osmolarity enhance voltage-gated Ca currents but depress K currents in freshly isolated rat hippocampal neurons. *Brain Res* 851: 189-197, 1999.


FIGURE LEGENDS

Fig 1. Differential gene expression of TREK subfamily K$_{2p}$ channels between TL and LS colon DRG neurons from naïve and TNBS-treated mice. (A) Representative photographs of RT-PCR products of TREK subfamily channels from 3 colon DRG neurons (lanes 1~3, no template in lane 4). The expected amplicon sizes were 229 bp (TREK-1), 254 bp (TREK-2) and 109 bp (TRAAK). (B) In both naïve and TNBS-treated mice, the proportion of cells expressing the TREK-1 gene was significantly greater in LS than in TL colon DRG neurons whereas the percentage of neurons not expressing any of these three channels (None) was greater in TL than in LS colon neurons. Error bars (SEM) represent animal-to-animal variations in percentage. *P<0.0125 and **P<0.0025 between TL and LS colon DRG neurons by Fisher’s exact test.

Fig 2. Quantity of TREK subfamily K$_{2p}$ channel mRNAs in TL and LS colon DRG neurons from naïve and TNBS-treated mice. TREK-1 mRNA was most abundant in both TL and LS colon neurons, but the relative amount of channel mRNA was not different between TL and LS colon neurons from naïve mice. Two days after intracolonic treatment with TNBS, the quantity of TREK-1 mRNA was significantly reduced in LS colon DRG neurons. *P<0.05 between channel transcripts. *P<0.05 between naïve and TNBS-treated mice (N=5 each).

Fig 3. Single channel activities of TREK subfamily-like channels in cultured colon DRG neurons. (A) TREK-1a, (B) TREK-1b, (C) TREK-2 and (D) TRAAK-like channel activities were detected in inside-out membrane patches under symmetrical K$^+$ (140 mM)
condition. The I-V relationship of each channel did not differ between naïve and TNBS-treated mice.

**Fig 4. Single channel responses of TREK subfamily-like channels to membrane stretch, changes in intracellular pH and arachidonic acid.** (A) TREK-1a, (B) TREK-1b, (C) TREK-2 and (D) TRAAK-like channel activities were increased by membrane stretch and arachidonic acid (AA; cytosolic side superfusion) at -60 mV membrane potential. Intracellular acidosis (pH 6.3) activated TREK-1- and TREK-2-like channels whereas alkalosis (pH 8.3) stimulated TRAAK-like channels. In the examples shown in the left traces, the vertical bars indicate 5 pA and horizontal bars, 10 s. The middle and the right traces are 1-s segments of the left traces marked by arrow heads that represent the channel activities before and during stimulus application (dashed line). The responses of TREK-2-like channels to membrane stretch (-4 cmH2O negative pressure) and intracellular acidosis (pH 6.3) were decreased in TNBS-treated mice (panel C, rightmost). The difference in the responses of the other channels between naïve and TNBS-treated mice was not statistically tested because of limited sample size. *P<0.05 by Mann-Whitney U test.

**Fig 5. Whole cell current response of colon DRG neurons to osmotic stretch by hypotonic cell swelling.** The whole cell current by a slow ramp depolarization was reversibly increased (A: left trace) or decreased (right) in response to osmotic stretch. The magnitude of decrease was significantly greater in LS colon DRG neurons from TNBS-treated mice than that from naïve mice. *P<0.05, Mann-Whitney U test.
Fig 6. Effect of TREK subfamily channel inhibitors on the outward current during osmotic membrane stretch. Quinine (Qn, 0.3 mM in A and B) and L-methionine (L-Met, 1 mM in C and D), nonselective but effective blockers of TREK subfamily channels, decreased the magnitude of outward current elicited under the hypotonic solution-induced membrane stretch.

Fig 7. Correlation of neuronal properties with the magnitude of changes in outward current density upon osmotic membrane stretch. No statistically significant correlation was detected between the current response to osmotic stretch and (A) cell size, (B) degree of swelling or (C) current density at 20 mV under isotonic recording condition.

Fig 8. Electrophysiological properties of colon DRG neurons used for whole cell current measurement. No differences were detected in (A) resting membrane potentials, (B) cell capacitance and (C) outward current density at 20 mV between samples. (D) The effect of arachidonic acid (AA) on whole cell K⁺ current was predominantly inhibitory.
**Figure 1**

**Panel A**: Gel electrophoresis showing bands for TREK-1, TREK-2, and TRAAK. Bands are labeled with numbers 1 to 4.

**Panel B**: Bar graphs comparing mRNA expression of TREK-1, TREK-2, TRAAK, and all three combined under Naïve and TNBS-treated conditions. Significance levels are indicated with * for p=0.08 and ** for p=0.07.

**Legend**

- TL: Total Length
- LS: Labeled Neurons
- Naïve
- TNBS-treated

**Graphs**

- TREK-1: Naïve (p=0.08)
- TREK-2: TNBS-treated
- TRAAK: Naïve
- All 3: TNBS-treated
- None: Naïve (p=0.07)
mRNA amount (% of Reference Standard)

TL

TREK-1

TREK-2

TRAACK

LS

Naive

TNBS-treated

P=0.27

*
A TREK-1a-like  B TREK-1b-like  C TREK-2-like  D TRAAK-like

(mV) 60 0 -60

(pA) 14 7 -7 -14

Naïve  TNBS-treated

5 mV  100 ms
A  TREK-1a-like
-4cmH\textsubscript{2}O  
\(\text{pH}_i\) 6.3  
AA 20 \(\mu\text{M}\)

B  TREK-1b-like
-4cmH\textsubscript{2}O  
\(\text{pH}_i\) 6.3  
AA 20 \(\mu\text{M}\)

C  TREK-2-like
-4cmH\textsubscript{2}O  
\(\text{pH}_i\) 6.3  
AA 20 \(\mu\text{M}\)

D  TRAAK-like
-4cmH\textsubscript{2}O  
\(\text{pH}_i\) 8.3  
AA 20 \(\mu\text{M}\)
Figure A: Current (nA) response to changes in membrane potential (mV) under isotonic and hypotonic conditions. The hypotonic wash out is indicated.

Figure B: Current density during osmotic stretch (% of control) for Naïve and TNBS-treated groups in TL and LS. The Naïve group is shown on the left, and the TNBS-treated group on the right. The asterisk indicates a significant difference (*) between the groups.
Current Density at 20 mV

Capacitance (pF)

Degree or Swelling (% or Initial Size)

(% or control) during Osmotic Stretch

Naïve TL

TNBS TL

Naïve LS

TNBS LS
Table 1. Primer Pairs used for PCR (5’→3’)

<table>
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<th>Gene</th>
<th>External Primers</th>
<th>Internal Primers</th>
<th>Accession No.</th>
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<tr>
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<td>TREK-1</td>
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### Table 2. Observation frequencies (% of patched neurons) of TREK subfamily-like channels in colon DRG neurons

<table>
<thead>
<tr>
<th></th>
<th>TREK-1a-like</th>
<th>TREK-1b-like</th>
<th>TREK-2-like</th>
<th>TRAAK-like</th>
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<td>TL</td>
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<tr>
<td>Naïve (26)</td>
<td>8</td>
<td>15</td>
<td>19</td>
<td>0</td>
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<tr>
<td>TNBS-treated (38)</td>
<td>13</td>
<td>0*</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>LS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naïve (35)</td>
<td>9</td>
<td>14</td>
<td>23</td>
<td>6</td>
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<tr>
<td>TNBS-treated (36)</td>
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<td>17</td>
<td>39</td>
<td>11</td>
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</table>

N=6 for naïve and 8 for TNBS-treated mice. Numbers in brackets indicate the number of total cells analyzed in corresponding group. *P<0.05 vs. naïve mice by Fisher’s exact test.