Transient Receptor Potential Vanilloid-4 Has a Major Role in Visceral Hypersensitivity Symptoms

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Background & Aims: **The transient receptor potential vanilloid-4 (TRPV4) is an osmosensitive channel that responds to mechanical stimulation. We hypothesized that TRPV4 could be important in visceral nociception and in the development of hypersensitivity.** ***Methods:*** **TRPV4 expression was investigated by immunohistochemistry and reverse transcription–polymerase chain reaction. Calcium signaling and patch-clamp studies were performed in dorsal root ganglia (DRG) neurons validating the use of 4αPDD as a selective TRPV4 agonist. The effects of TRPV4 activation on visceral nociception were evaluated in mice that received intracolonically TRPV4 agonist (4 α-phorbol 12,13-didecanoate [4αPDD]) and in TRPV4-deficient mice in which abdominal muscle contractions in response to colorectal distention (CRD) were recorded. Intervertebral injections of TRPV4 or mismatch small interfering RNA (siRNA) were used to specifically down-regulate TRPV4 expression in sensory neurons and to investigate the role of TRPV4 in basal visceral nociception or in protease-activated receptor 2 (PAR_2) activation-induced visceral hypersensitivity.** ***Results:*** **TRPV4 agonist 4αPDD specifically activated a cationic current and calcium influx in colonic projections of DRG neurons and caused dose-dependent visceral hypersensitivity. TRPV4-targeted but not mismatched siRNA intervertebral treatments were effective at reducing basal visceral nociception, as well as 4αPDD or PAR_2 agonist-induced hypersensitivity. Effects of the TRPV4 ligand were lost in TRPV4-deficient mice.** ***Conclusions:*** **4αPDD selectively activates TRPV4 in sensory neurons projecting from the colon, and TRPV4 activation causes visceral hypersensitivity. TRPV4 activation is implicated in the nociceptive response to CRD in basal conditions and in PAR_2 agonist-induced hypersensitivity. These results suggest a pivotal role for TRPV4 in visceral nociception and hypersensitivity.**

Transient receptor potential vanilloid 4 (TRPV4) is a widely expressed cation channel of the TRP superfamily. TRP channels are intrinsic membrane receptor channels with 6 transmembrane spans and a cation permeable pore region.[1] The channel can be activated by physical stimuli, as well as by the synthetic phorbol ester 4 α-phorbol 12,13-didecanoate (4α-PDD),[2] by the bisandrographolide A,[3] and by 5′,6′-epoxyeicosatrienoic acid.[4] Its distribution in cochlear hair cells, vibrissal Merkel cells, and sensory ganglia, as well as in free nerve endings and cutaneous A- and C-fiber terminals, suggested a role in mechanotransduction beyond osmosensation.[5] TRPV4-deficient mice show an increased somatic mechanical nociceptive threshold, but a normal response to noxious heat and low-threshold mechanical stimuli.[6] TRPV4 agonists promote the release of the neuropeptides substance P and calcitonin gene-related peptide from the central projections of primary afferents in the spinal cord, suggesting a role for TRPV4 in nociception.[7] The implication of TRPV4 in a somatic chronic pain model was investigated by using intervertebral injections of antisense RNA, showing that TRPV4 activation plays a role in Taxol-induced mechanical hyperalgesia and hypotonicity-induced nociception.[8] However, no study has yet investigated the role of TRPV4 in visceral nociception.

We have recently shown the major implication of proteases and protease-activated receptor 2 (PAR_2) activation in the generation of pain symptoms by mediators released from colonic biopsies of patients with irritable bowel syndrome (IBS).[9] However, the mechanism by which PAR_2 activation and proteases are leading to visceral hypersensitivity symptoms is still unclear. Another recent study has shown that PAR_2 activation was able to potentiate the TRPV4 response to agonists in cultured dorsal root ganglia (DRG) neurons.[7] On the basis of the link that emerged between TRPV4 and PAR_2 and on the role of TRPV4 in somatic mechanical pain, we hypothesized that TRPV4 may play a major role in the regulation of visceral sensitivity. In the present study, we report the functionality of the TRPV4

Abbreviations used in this paper: 4α-PDD, 4 α-phorbol 12,13-didecanoate; 6-FAM, 6-carboxyfluorescein; CRD, colorectal distension; DRG, dorsal root ganglia; IBS, irritable bowel syndrome; mRNA, messenger RNA; PAR_2, protease-activated receptor 2; siRNA, small interfering RNA; TRP, transient receptor potential; TRPV4, transient receptor potential vanilloid 4; VMR, visceral motor response.

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channel on sensory neurons projecting to the colon and of the potent proalgesic effects of TRPV4 activation in the colon. Finally, we showed a pivotal role for TRPV4 activation in visceral sensitivity mechanisms and in PAR_2-induced visceral hypersensitivity.

Materials and Methods

Animals

C57Bl6 male mice (6–8 weeks) from Charles River Laboratories (Montreal, Quebec, Canada), TRPV4-deficient mice ($TRPV4^{-/-}$), and wild-type littermates ($TRPV4^{+/+}$) obtained from Dr Nigel Bunnett (University of San Francisco), originally raised by Dr. Liedtke were used. All procedures were approved by the institutional Animal Care Committee (University of Calgary).

Messenger RNA Expression

Total RNA was extracted from mouse DRG neurons (T12–L6), colon, and spinal cord (T12–S1) using TRIzol (Invitrogen, Cergy Pontoise, France). RNA was reverse-transcribed with random hexamers and SuperScript III (Invitrogen, Cergy Pontoise, France). The ratio of TRPV4 to glyceraldehyde-3-phosphate dehydrogenase messenger (mRNA) was calculated for each sample after amplification with the use of primers specific to mouse TRPV4 (forward, 5′-ATCAACTCGCCCTTCAGAGA-3′; reverse, 5′-CCCAAACTTACGCCACTTGT-3′) and to GAPDH (forward, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′; reverse, 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′). After 30 cycles, products were separated by electrophoresis (2% agarose gel), detected using ethidium bromide.

Immunohistochemistry

After anesthesia, mice were transcardially perfused with 10% paraformaldehyde. DRG neurons (T10–L6), colon, and spinal cord (lumbar to sacral region) were fixed overnight at 4°C, placed in 25% sucrose for 24 hours at 4°C, embedded in OCT compound (Sakura Finetek, Torrance, CA), and sectioned at 25 μm. Sections were washed in phosphate-buffered saline containing 5% goat serum (Zymed Laboratories, San Francisco, CA) and 0.5% Triton X-100 and incubated with primary TRPV4 antibody (rabbit, 1:750; Sigma) or c-fos (1:200; Santa Cruz Biotechnology) overnight at 4°C. Sections were washed and incubated with a secondary antibody, Alexa Fluor 555 or 480 (1:1500; Molecular Probes, Eugene, OR) at room temperature for 2 hours. Washed sections were mounted in Prolong (Molecular Probes). Confocal images were acquired with a Zeiss LSM-510 META confocal inverted microscope (Carl Zeiss, Jena, Germany), ×20 objective.

TRPV4 Small Interfering RNA Treatments

Anesthetized mice (Halothane 5%) received 3 intervertebral injections (10 μL over 36 hours, every 12 hours) by subcutaneous intervertebral injection between L5 and L6 of TRPV4 small interfering RNA (siRNA): 6-FAM (6-carboxyfluorescein) 5′-UCUACCAGUACUAUGGCUUd(TT)-3′, 3′-d(TT)AGAUGGUCAUGAUACCGAA-5′, or a mismatched siRNA designed with the same percentage of GC and AT but with no corresponding sequence: 6-FAM 5′-CAUGCUAGGUUAGUACUUGd(TT)-3′; 3′-d(TT)GUACGAUCCAAUCAUGAAC-5′.[10] To localize siRNA in tissues, we followed the same protocol as for immunostaining.

Fast Blue Injections

Under anesthesia small volumes (1–2 μL) of the retrograde tracer FB (1 mg in 60 μL of sterile saline solution; Cedarlane) were injected into the wall of the exteriorized colon. Multiple injections were made with a 30-gauge needle fitted to a Hamilton syringe (total volume 15 μL). The exterior of the colon was swabbed after each injection to remove residual tracer.

Electrophysiology

Cells were held at 0 mV, and a 150-millisecond linear ramp protocol was applied (−100 mV to 100 mV every 15 seconds). Extracellular solution contained 120 mmol NaCl/L; 5 mmol KCl/L; 5 mmol $CaCl_2$/L; 2 mmol $MgCl_2$/L; 10 mmol glucose /L; 10 mmol HEPES/L, pH 7.5; 0.1 NaOH; 310 mOsm. Borosilicate glass pipettes (2–4 MΩ) were filled with internal solution that contained 110 mmol CsCl/L; 3 mmol $MgCl_2$/L; 10 mmol EGTA/L; 10 mmol HEPES/L; 3 mmol Mg-adenosine triphosphate/L; 0.6 mmol guanosine triphosphate/L; pH adjusted to 7.2 using CsOH, 315 mOsm. Recordings were performed using an Axopatch 200B amplifier (Molecular Devices). Current amplitude at −80 and 80 mV was normalized to cell capacitance to obtain current densities. Sampling frequency for acquisition was 10 kHz, and data were filtered at 2 kHz. Data were analyzed using pClamp9 (Molecular Devices).[10]

Calcium Imaging

DRG neurons were perfused with an extracellular solution of 120 mmol NaCl/L; 5 mmol KCl/L; 5 mmol $CaCl_2$/L; 2 mmol $MgCl_2$/L; 10 mmol glucose/L; 10 mmol HEPES/L, pH 7.5; 0.1 NaOH, 310 mOsm. For calcium imaging, cells were loaded with Fluo-4 AM (0.3 μmol/L for 15 minutes; Molecular Probes). After loading, neurons were perfused (2 mL/min) for 20 minutes. Band-limited excitation (420–495 nm) was provided by a mercury arc lamp and filter. Neurons were imaged using an inverted microscope (Nikon) and a 20× 0.5 NA objective. Images were acquired using a CCD camera (Zeiss) at an effective sampling rate of 1 Hz. Acquisition variables were kept constant within each experiment.

Capsaicin was used at 2 μmol/L and 4αPDD at 50 μmol/L in bath solution at 37°C. Data were analyzed as described previously.[11] Briefly, regions of interest were fitted around the perimeter of Fast Blue–labeled cells, and intensity variations for each region of interest were

corrected for background levels and expressed in relation to a baseline fluorescence level preceding the stimulus resulting in ΔF/F fluorescence intensity values.

Colorectal Distention and Electromyography Recording

Groups of electrodes were implanted in the abdominal external oblique musculature of anesthetized mice as previously described.[9] Colorectal distension (CRD) was performed 5 days after the electrode implantation[9] by insertion of a balloon (10-mm long) into the colon at 5 mm from the anus. The balloon was inflated to different pressures, and 10-second distensions were performed in triplicate at each pressure, with 5-minute intervals.

In a first set of experiments, 2 groups of mice were intervertebrally injected with TRPV4-targeted siRNA or mismatched siRNA; from day 1 to 6 after the intervertebral injection, we performed a series of CRD. In a second set of experiments, mice received intracolonic injection of 100 μL of TRPV4 agonist 4αPDD (10, 100, and 500 μmol/L). At various times after 4αPDD administration (0.5, 2, 4, and 6 hours), we performed a series of CRD to establish a kinetic and a dose-effect relation. In a third set of experiments, we used 4 groups of 8 mice, administrated with 4αPDD (100 μmol/L), capsaicin (10 μmol/L), or their vehicle (40% ethanol) and pretreated with intervertebral injection of TRPV4-targeted siRNA or mismatched siRNA; CRD was performed 1 hour after treatment. We performed a study on TRPV4-deficient mice compared with littermates, using 5 groups of 8 mice. Mice were treated with 4αPDD (100 μmol/L) or capsaicin (10 μmol/L), and CRDs were performed, one before the administration and one 1 hour after. In a fourth set of experiments, mice were treated intracolonically with 100 μL of PAR_2 agonist SLIGRL (75 μmol/L, 150 μmol/L, and 1.5 mmol/L). After SLIGRL administration (0.5, 2, 4, and 6 hours), we performed a series of CRDs to establish a kinetic and a dose-effect relation. Three groups of 8 mice were given a PAR_2 agonist (SLIGRL; 150 μmol/L) or its vehicle (40% ethanol) and were pretreated with an intervertebral injection of TRPV4-targeted siRNA or mismatched siRNA. CRD was performed 6 hours after SLIGRL administration. In a last set of experiments 4 groups of mice were administrated intracolonically with SLIGRL (75 μmol/L) or its vehicle (40% ethanol) and with 4αPDD (10 μmol/L) or its vehicle (40% ethanol). CRD was performed 6 hours after SLIGRL administration.[9] All experimental procedures are schematically described in Supplementary Figure 1 (see supplemental material online at www.gastrojournal.org).

Chemicals

The selective PAR_2-AP SLIGRL-NH_2 was dissolved in 40% ethanol and was prepared by solid-phase synthesis (Peptide Synthesis Facility, University of Calgary). Capsaicin and 4αPDD were obtained from Sigma and dissolved in 40% ethanol and 60% saline. SiRNAs were synthesized by Core DNA Services of the University of Calgary and dissolved in diethyl pyrocarbonate water.

Statistical Analysis

Data are presented as means ± standard error of the means. Analyses were done by running the GraphPad Prism 3.0 software (GraphPad, San Diego, CA). All data were normally distributed. Between-group comparisons were performed by the Student's unpaired *t* test. Multiple comparisons within groups were performed by repeated-measures one-way analysis of variance, followed by Tukey's post test. Statistical significance was accepted at $P < .05$.

Results

TRPV4 Expression

In wild-type mice, we detected TRPV4 expression on approximately 47% of the DRG neurons and brush-bordered epithelial cells but not mucus-secreting epithelial cells stained for TRPV4 and unidentified cells of submucosal and muscular layers of the colon and in the gray matter of the spinal cord (Figure 1*A*–*C*). We confirmed the specificity of the anti-TRPV4 antibody in tissues from TRPV4-deficient mice, in which no staining in DRG neurons, colon, or spinal cord was observed (Figure 1*A*). Mice that received intervertebral injections of TRPV4-targeted siRNA showed a significant reduction of TRPV4 protein and mRNA expression in DRG neurons 3 and 4 days after the first injection of siRNA (Figure 1*B* and *C*). Intervertebral injections of mismatched siRNA had no effect on TRPV4 expression (data not shown). The siRNA labeled by a 6-FAM tag, was detected in the DRG neurons, but it was absent from the colon or from the spinal cord (Figure 1*B*). Intervertebral injection of TRPV4-targeted siRNA had no effect on TRPV4 protein or TRPV4 mRNA expression in the colon and spinal cord (Figure 1*B* and *C*). These results validate the use of subcutaneous injections between L5 and L6 of TRPV4-targeted siRNA to decrease the expression of TRPV4 expression in DRG neurons.

TRPV4 Calcium Signaling in Colon-Innervating DRG Neurons

To investigate the activation of TRPV4 in DRG neurons innervating the colon, we investigated calcium mobilization in response to TRPV4 agonists in colonic DRG neurons labeled by an intracolonic injection of retrograde Fast Blue marker. Six days after in vivo Fast Blue injection, a heterogeneous population of DRG neurons was labeled with Fast Blue, including small (<20 μmol/L) and medium (>20 μmol/L, <40 μmol/L) sized DRG neurons (Figure 2*A*, *right panel*). For all the following experimentation, only Fast Blue–labeled neurons were considered. Stimulation of these neurons with the TRPV4 agonist 4αPDD induced calcium mobilization,

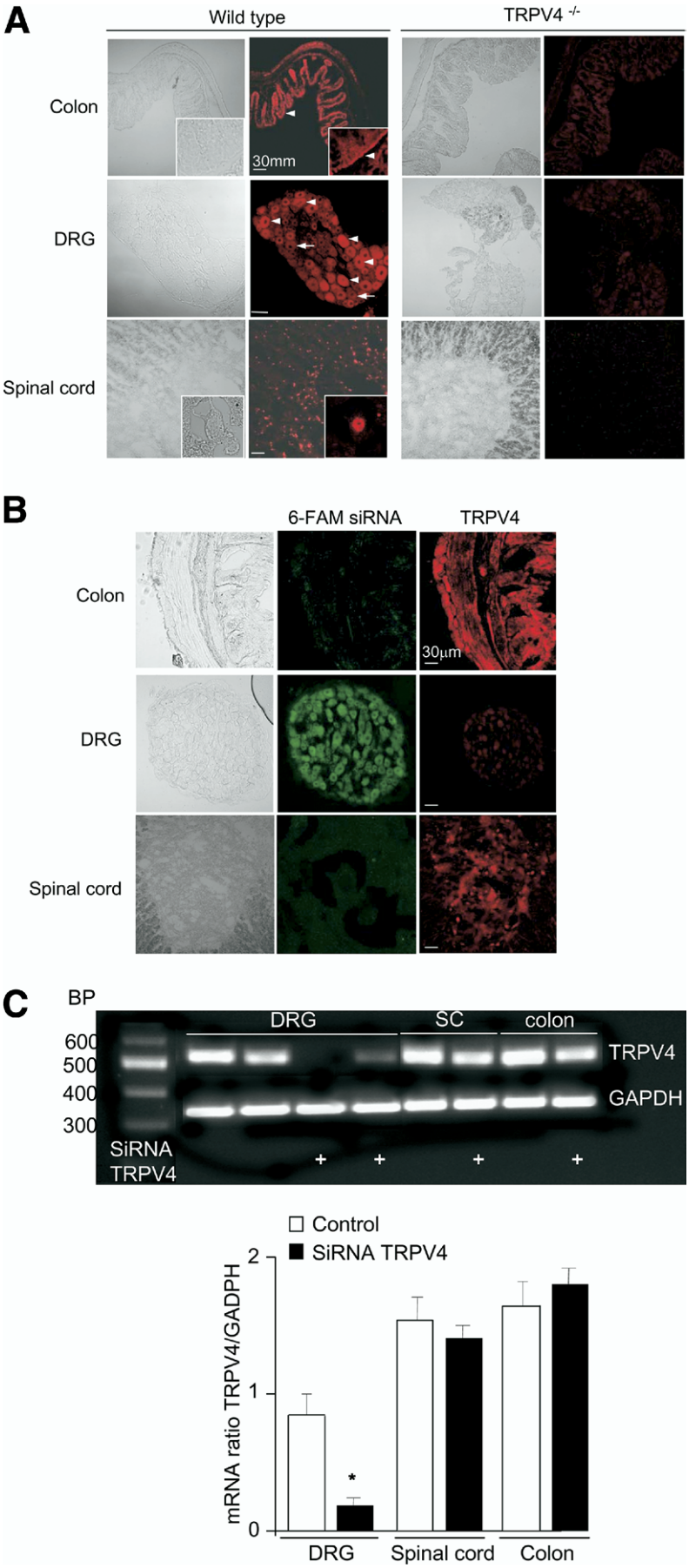

Figure 1. Immunostaining of TRPV4 in the DRG neurons, colon, and spinal cord. (*A*) TRPV4 was detected in the colon, principally on the apical side of the epithelial cells (*arrow head*), and in neurons from the DRG (*arrows heads* point to labeled neurons and *arrows* to unlabeled neurons) and spinal cord of wild-type but not TRPV4-deficient mice. (*B*) 6-FAM–labeled TRPV4 siRNA was injected intervertebrally, and the DRG neurons, colon, and spinal cord were collected after 4 days to verify uptake of fluorescent 6-FAM siRNA and to localize immunoreactive TRPV4. TRPV4 6-FAM siRNA was detected in most of the cells of the DRGs, including neurons, but not in the spinal cord and in colon tissues. Immunoreactive TRPV4 was poorly detected in DRGs, but not in colon or spinal cord after intervertebral injection of TRPV4 siRNA. (*C*) Reverse transcription–polymerase chain reaction of DRGs, colon, and spinal cord from mouse untreated or treated with TRPV4 SiRNA, showing amplification of transcripts for TRPV4 and glyceraldehyde-3-phosphate dehydrogenase (GADPH). Values are mean ± SEM, n = 6 per group, $^*P < .05$ compared to control group.

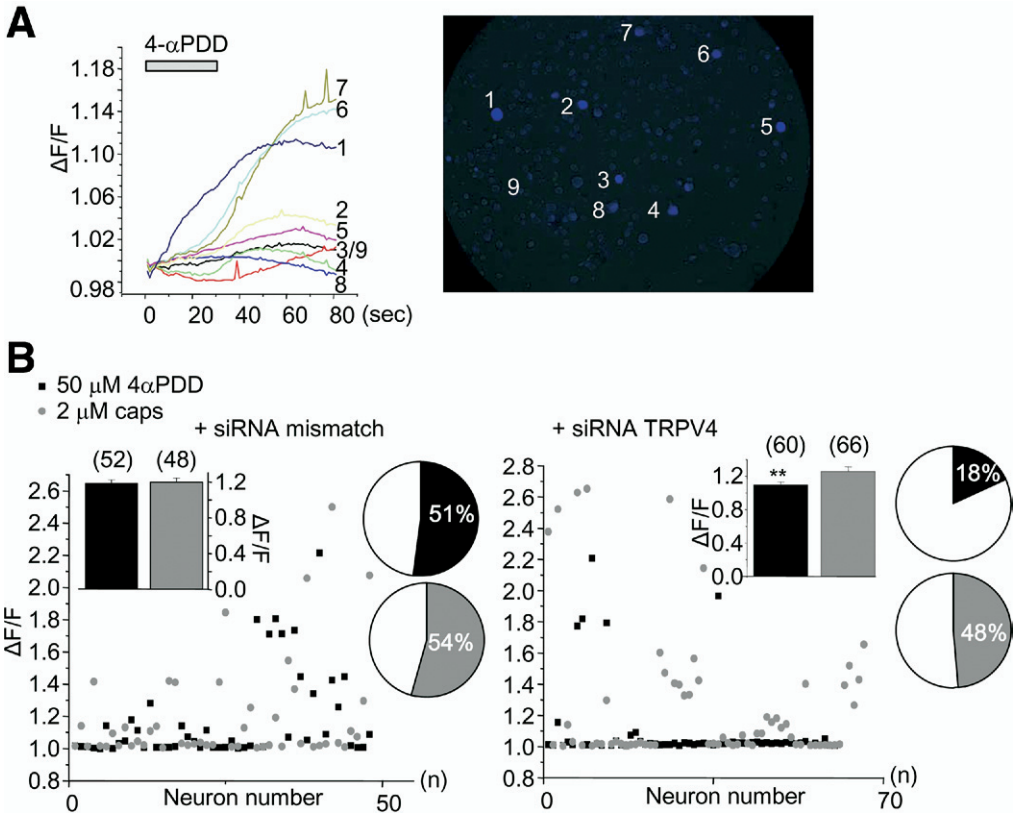

Figure 2. Calcium influx mediated by TRPV4 activation in Fast Blue–positive DRG neurons innervating the mouse colon. (*A*) Time course of relative fluorescent intensity (ΔF/F) evoked by 4αPDD (*left panel*) in the corresponding Fast Blue–labeled DRG neurons (*right panel*). (*B*) Measure of relative fluorescent intensity (normalized to baseline) in response to 4αPDD (50 μmol/L) or capsaicin (2 μmol/L) stimuli in animals treated with siRNA mismatch (*left*) or siRNA TRPV4 (*right*). Histograms represent the mean of relative fluorescent intensity associated to 4αPDD (*black*) and capsaicin (*gray*) stimuli. The pie graph represents the percentage of cells responding to 4αPDD or capsaicin; response was scored if the ratio increased a minimum of 10% from baseline (n = number of cells, $^{**}P < .01$, *t* test).

suggesting that colonic DRG neurons express functional TRPV4 channels (Figure 2*A*, *left panel*). Exposure of colonic DRG neurons to capsaicin also evoked calcium influx (Figure 2*B*). In animals treated with mismatch siRNA, we observed that the same proportion of DRG neurons responded to capsaicin and 4αPDD (54%, n = 52 for capsaicin compared with 51.9%, n = 48 for 4αPDD responders) and the mean of relative fluorescence (ΔF/F) was not different between the 2 groups (1.19 ± 0.04 for capsaicin and 1.18 ± 0.04 for 4αPDD) (Figure 2*B*, *left panel*). In contrast, injection of TRPV4-targeted siRNA drastically reduced the number of colonic-labeled DRG neurons responding to 4αPDD (from 51% to 18.3%, n = 60; Figure 2*B*, *right panel*), without affecting their response to capsaicin (48%, n = 66). Finally, the mean of relative fluorescence was reduced in response to 4αPDD, but not in response to capsaicin after TRPV4 siRNA treatment, compared with the fluorescence observed in mismatch SiRNA-treated neurons (Figure 2*C*). Altogether, our results suggest that functional TRPV4 channels are present on colon-innervating DRG neurons. Treatment with TRPV4 siRNA reduced efficiently 4αPDD-mediated but not capsaicin-mediated calcium responses in these neurons.

Electrophysiologic Evidences of the Efficiency of In Vivo TRPV4 siRNA Treatments

We validated the specificity of TRPV4 siRNA treatments on whole-cell TRPV4 agonist-induced current. We performed electrophysiologic recordings on small DRG neurons isolated from mice that had received an intervertebral injection of siRNA. In the whole-cell configuration, we recorded current on application of a voltage ramp from −100 to 100 mV. In DRG neurons from animals injected with mismatch siRNA, perfusion of 10

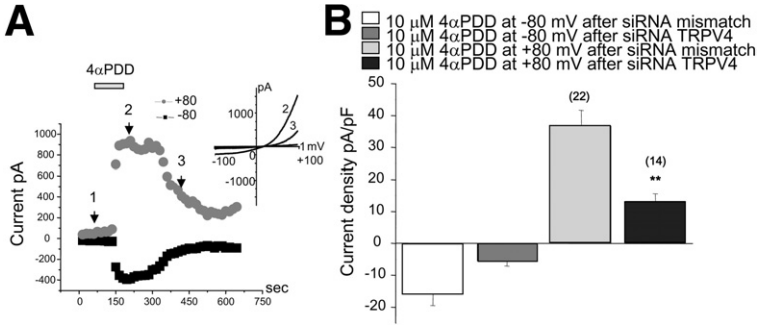

Figure 3. Silencing of TRPV4 current in DRG neurons by in vivo siRNA treatments. (*A*) Representative time course of whole-cell current obtained from voltage ramp (−100 mV to 100 mV every 15 seconds) and measured at −80 and 80 mV, from mismatched siRNA-treated DRG neurons. Cells were held at 0 mV to inactivate voltage-gated calcium and sodium channels. (*Inset*) I–V curve was obtained from linear voltage ramp measured at the time indicated on the time course (1, 2, or 3). (*B*) Whole-cell recordings of peak current densities measured at −80 mV and 80 mV of DRG neurons from mismatched siRNA– or TRPV4-targeted siRNA–treated mice and treated with 4alphaPDD (10 μmol/L) ($^{**}P < .01$; *t* test).

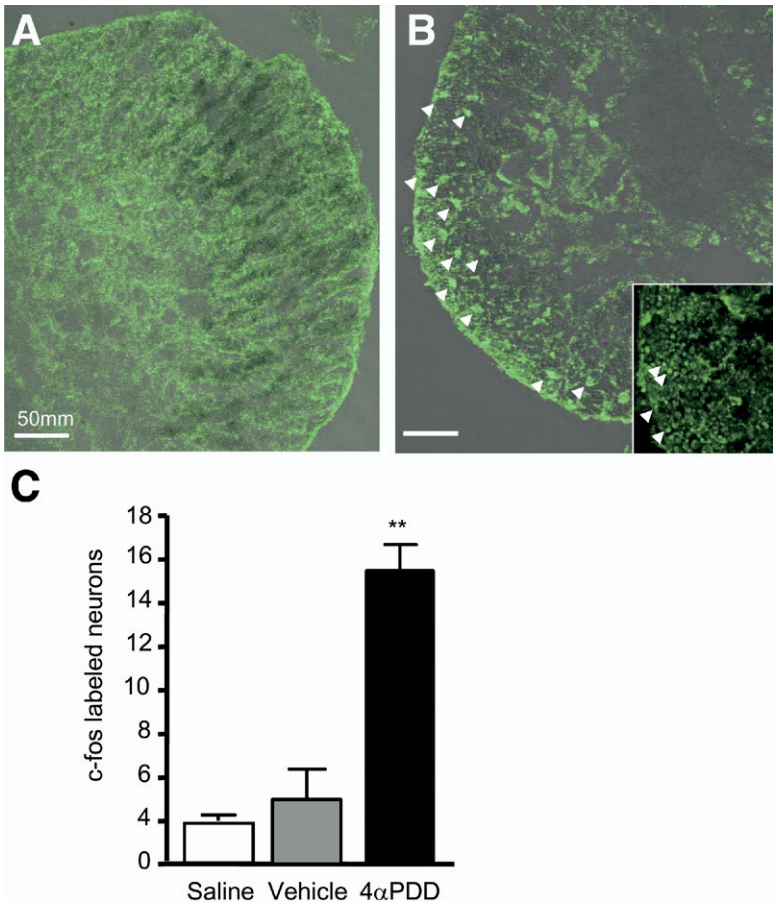

Figure 4. Intracolonic administration of 4αPDD (100 μmol/L) increased fos expression in the spinal cord. (*A*,*B*) Photomicrographs of fos immunoreactivity in spinal cord tissues 2 hours after the intracolonic administration of (*B*) 4αPDD in 40% ethanol or (*A*) 40% ethanol. Scale bar: 20 μm. Insert pictures represent higher magnification of spinal neurons labeled for Fos (*arrow head*) and unlabeled (*arrow*). (*C*) Quantitative changes in fos-like immunoreactivity in dorsal horn (L4–L6 levels) of mice, 2 hours after intracolonic injection of 4αPDD (100 μmol/L; *black bar*) or vehicle (40% ethanol; *white bar*). Values are mean + SEM, n = 5 mice per group. *$P < .05$ compared with the 40% ethanol-injected group.

μmol/L 4αPDD activates an outwardly rectifying current characteristic of TRPV4 activation (Figure 3*A*) with a reversal potential that was shifted toward positive potentials (Figure 3*A*, inset). When current densities were measured, DRG cells from animals injected with TRPV4 siRNA exhibited reduced current densities compared with DRG neurons obtained from animals injected with mismatch siRNA (−15.77 ± 3.77 pA/pF and 36.67 ± 4.99 for mismatch, n = 22 compared with −5.46 ± 1.57 pA/pF and 13.18 ± 2.41 pA/pF for TRPV4 siRNA, n = 14 at −80 and 80 mV, respectively; Figure 3*B*). Although there was a clear diminution in current densities at −80 mV, the decrease of the inward current was not significant. This could be explained by a smaller contribution of the inward current during TRPV4 activation, because it has been reported previously.[12] In contrast, the large outward current was significantly reduced after TRPV4 siRNA treatment. These results validate the efficiency of siRNA on TRPV4 current in DRG sensory neurons and provide us with a specific tool to explore the role of this channel in the visceral pain model of colonic distension.

Effects of Colonic Administration of 4αPDD on Spinal Neuron Activation

The number of Fos-like immunoreactive nuclei per section of dorsal horn gray matter at the L5–S1 level of the spinal cord was significantly increased after intracolonic administration of 4αPDD (100 μmol/L), compared with mice injected with vehicle (40% ethanol) (Figure 4). Fos expression was detected principally in superficial laminae (I and II) of the dorsal horn. These results show that TRPV4 agonist injected into the colon activates neurons at the spinal level.

Effects of Colonic Administration of 4αPDD on Visceral Nociception

Visceral motor responses (VMRs) to CRD of mice that received intracolonic saline or vehicle (40% ethanol) were similar (not shown). The intracolonic administration of 4αPDD (100 μmol/L), but not its vehicle, provoked an increase in the VMR to CRD. 4αPDD provoked allodynia, characterized by an increased VMR to 15 mm Hg, a normally innocuous pressure of distension (Figure 5*A*, *top left panel*). Hyperalgesia was also observed after intracolonic administration of 4αPDD, and it was characterized by an increased VMR to noxious pressures (30, 45, and 60 mm Hg) of distension (Figure 5*A*). The maximal effects of 4αPDD treatment on allodynia were observed at 2 hours after the TRPV4 agonist administration, whereas maximal hyperalgesia was observed from 30 minutes to 4 hours after the intracolonic administration of 4αPDD (100 μmol/L) (Figure 5*A*). Both allodynia

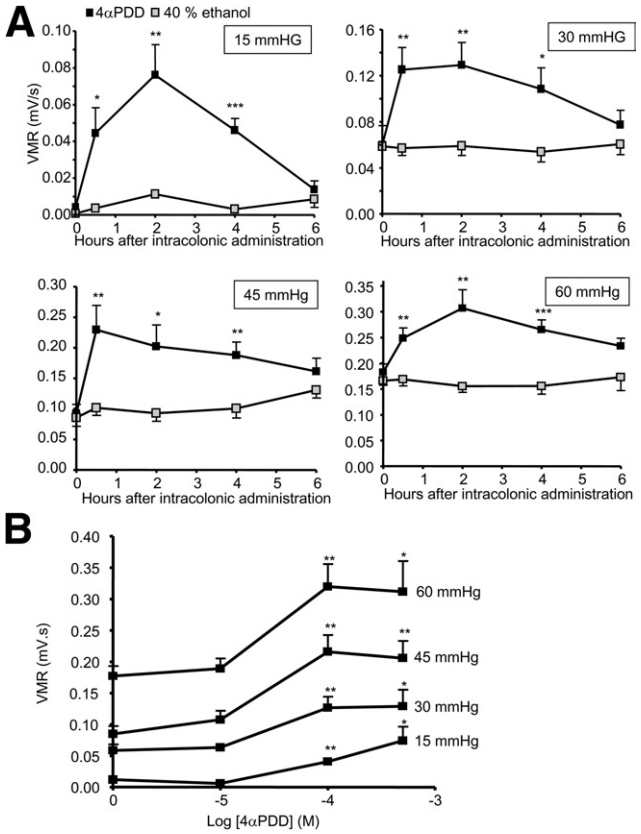

Figure 5. Intracolonic administration of 4αPDD (100 μmol/L) induced visceral hypersensitivity in response to CRD. (*A*) Mice received an intracolonic administration of 4αPDD in 40% ethanol (100 μmol/L; *black square*) or 40% ethanol (*gray square*) and were submitted to CRD 30 minutes and 2, 4, and 6 hours after the intracolonic injection. VMR to CRD was recorded in response to pressure of distension or 15, 30, 45, and 60 mm Hg. Time 0 corresponds to VMR recorded in response to CRD, before 4αPDD administration. Values are mean + SEM, n = 5 mice per group. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ compared with the 40% ethanol-injected group. (*B*) Dose-response curves of VMR induced by the intracolonic administration of 4αPDD (10, 100, or 500 μmol/L) in response to CRD (15, 30, 45, and 60 mm Hg).

(response to 15 mm Hg) and hyperalgesia (response to 30, 45, and 60 mm Hg) provoked by 4αPDD were dose dependent when measured 1 hour after intracolonic administration (Figure 5*B*).

Effects of TRPV4 Blockade on 4αPDD-Induced Allodynia and Hyperalgesia

We have previously performed a kinetic analysis of TRPV4 expression and 6-FAM labeling in DRG neurons after intervertebral injection of TRPV4-targeted siRNA, and we had determined that 3 days after intervertebral injection of TRPV4-targeted siRNA, the inhibition of TRPV4 expression and the expression of 6-FAM–labeled siRNA were maximal (data not shown). Therefore, we used the time point of 3 days after intervertebral injection of TRPV4 or mismatched siRNA for all of the following functional studies. The allodynia and hyperalgesia observed 1 hour after intracolonic injection of 4αPDD (100 μmol/L) was prevented by an intervertebral injection of TRPV4-directed siRNA 3 days before the experiment (Figure 6*A*). In contrast, intervertebral injection of the mismatched siRNA 3 days before the experiment had no effect on 4αPDD-induced hyperalgesia and allodynia (Figure 6*A*). In TRPV4-deficient mice, 4αPDD treatment had no effect on the VMR to CRD (Figure 6*B*). Capsaicin was used as a positive control in TRPV4 siRNA-treated and TRPV4-deficient mice. Intracolonic administration of capsaicin (100 μmol/L) provoked allodynia and hyperalgesia in response to CRD (increased VMR to CRD at 15 mm Hg and 30, 45, 60 mm Hg, respectively). Deficiency of TRPV4 expression in TRPV4$^{-/-}$ mice (Figure 6*B*) or TRPV4 siRNA-treated mice (Figure 6*A*) had no effect on the visceral sensitivity increase induced by capsaicin. Taken together, these results further validate the use of 4αPDD as a selective and specific TRPV4 agonist.

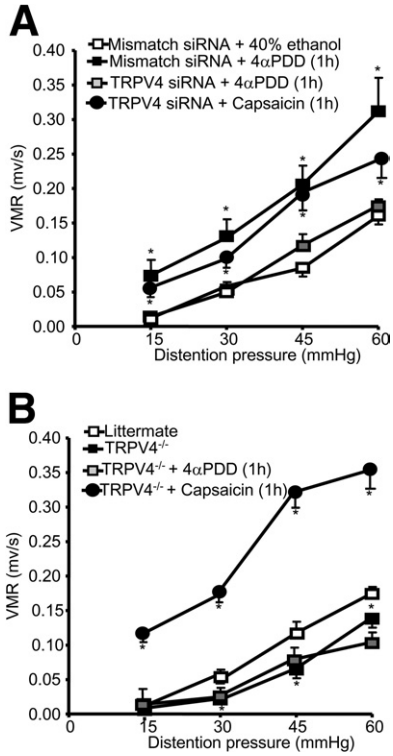

Figure 6. Effect of intracolonic administration of 4αPDD and TRPV4 blockade on visceral sensitivity in response to CRD. (*A*) Mice received an intervertebral administration of siRNA control (*white* and *black squares*) or against TRPV4 (*gray square* and *black circle*) 3 days before the intracolonic administration of 4αPDD (100 μmol/L; *black* and *gray squares*), its vehicle (40% ethanol; *white square*), or capsaicin (100 μmol/L; *black circle*). One hour after the intracolonic administration, CRD was performed and VMR was recorded. Values are mean ± SEM, n = 8 mice. $^{*}P < .05$ significantly different from control values. (*B*) Visceral sensitivity in TRPV4-deficient mice (*black square*) that received or not intracolonic administration of 4αPDD (100 μmol/L; *gray square*) or capsaicin (100 μmol/L; *black circle*) and in littermates (*white square*) in response to CRD. $^{*}P < .05$ significantly different from littermate values.

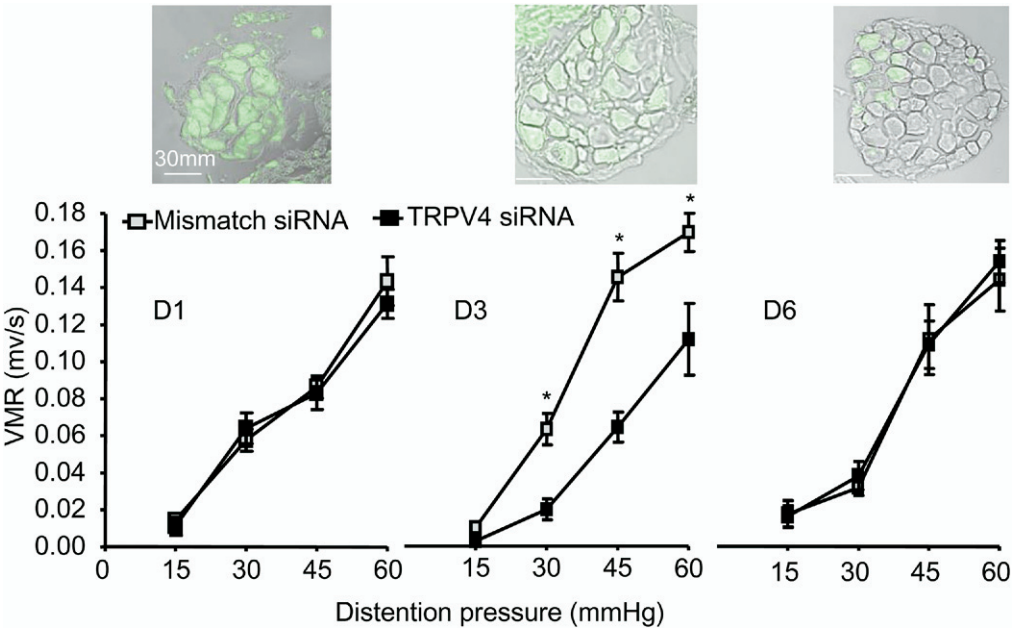

Figure 7. Effect of TRPV4 silencing on VMR to CRD. Mice received an intervertebral administration of siRNA against TRPV4 (*black square*) or mismatch (*gray square*), and CRD was performed from day 1 to 6 after the intervertebral injection. Mice were killed to verify uptake of fluorescent 6-FAM siRNA in the DRGs at each time point (*inserted pictures*). Values are mean ± SEM, n = 8 mice. *$P < .05$ significantly different from mismatch siRNA distention values.

Effects of TRPV4 Expression Blockade on Visceral Sensitivity

The VMR to CRD was similar in TRPV4 siRNA-treated or vehicle-treated mice 1 day after the intervertebral injection (Figure 7, *left panel*). At that time point, siRNA-tagged 6-FAM was detected in the DRG neurons (Figure 7, *insert D1*). Three days after intervertebral injection of siRNA, tagged 6-FAM siRNA was still detected in the DRG neurons (Figure 7, *insert D3*). No difference in VMR to CRD at the innocuous pressure of distension (15 mm Hg) was observed in TRPV4-targeted siRNA-treated mice compared with mismatch siRNA–treated mice. In contrast, the nociceptive response to CRD for the noxious pressures of distension (30, 45, and 60 mm Hg) 3 days after the last injection of TRPV4-targeted siRNA was significantly reduced compared with the mismatch-treated group (Figure 7, *insert D3*). Six days after intervertebral injections of siRNA, 6-FAM labeling in the DRG neurons was weak, and no difference in VMR was observed between mismatched and TRPV4-targeted siRNA-treated mice (Figure 7, *insert D6*).

The VMR to 15 mm Hg CRD was not different in TRPV4-deficient mice compared with wild-type mice (Figure 6*B*). However, in response to noxious stimulation (30, 45, and 60 mm Hg), TRPV4$^{-/-}$ mice showed a significant decreased VMR compared with TRPV4$^{+/+}$ mice (Figure 6*B*). Taken together, these data show that decreased expression of TRPV4 is associated with a decreased visceral sensitivity response to noxious but not innocuous stimulus.

Effect of TRPV4 Blockade Against SLIGRL Intracolonic Administration

Intracolonic administration of a subinflammatory dose of PAR_2-activating peptide (SLIGRL-NH_2 150 μmol/L in 100 μL) in C57BL/6 mice enhanced visceral sensitivity, causing an increase in VMR to CRD characteristic of allodynia (at 15 mm Hg) and hyperalgesia (at 30, 45, and 60 mm Hg). The maximal effect of the PAR_2 agonist SLIGRL-NH_2 on VMR was observed 6 hours after its intracolonic administration (Supplementary Figure 2; see supplementary material online at www.gastrojournal.org). Pretreatment of mice by an intervertebral administration of TRPV4-targeted siRNA completely inhibited PAR_2-induced allodynia and hyperalgesia compared with mice that had received an intervertebral injection of mismatched siRNA (Figure 8*A*).

We investigated the possibility that PAR_2 agonist could sensitize TRPV4-induced visceral hypersensitivity, by coadministering doses of SLIGRL-NH_2 and 4αPDD that individually did not cause hyperalgesia. We determined that a concentration of 75 μmol/L of PAR_2-AP did not cause visceral hyperalgesia and allodynia (Supplementary Data; see supplementary material online at www.gastrojournal.org). Intracolonic administration of 10 μmol/L of 4αPDD also did not cause visceral allodynia and hyperalgesia either (Figure 5*B*). However, intracolonic injection of PAR_2-AP (75 μmol/L) 6 hours before CRD and 4αPDD (10 μmol/L) 1 hour before CRD provoked a significant increase in VMR to a CRD of 15 mm Hg, characteristic of allodynia. Furthermore, we observed a significant increase in VMR to a CRD of 30, 45, and 60 mm Hg, characteristic of hyperalgesia (Figure 8*B*). This suggests that PAR_2 activation is able to sensitize the response to low doses of the TRPV4 agonist 4αPDD (Figure 8*D*).

Discussion

Neurologic mechanisms of visceral nociception and pain differ from those involved in somatic pain.[13] In contrast to somatic pain, visceral pain is diffuse and

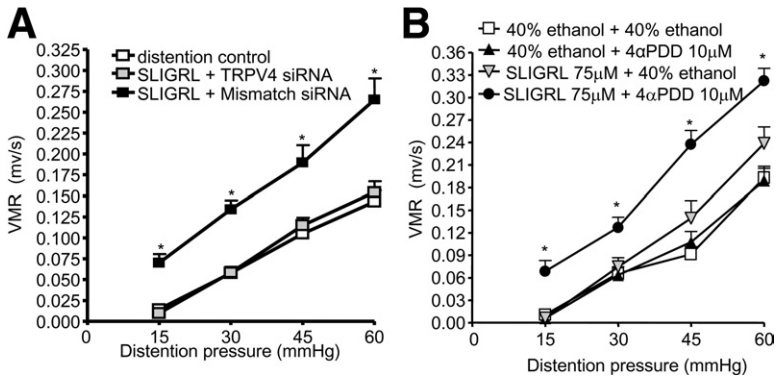

Figure 8. Role of TRPV4 expression in PAR_2-induced visceral hypersensitivity. (*A*) Mice received an intervertebral injection of siRNA control (*white square*) or TRPV4-directed siRNA (*gray square*) 3 days before the intracolonic administration of SLIGRL (150 μmol/L). CRD was performed on naive mice (*white square*) or 6 hour after SLIGRL intracolonic administration. Values are mean ± SEM, n = 8 mice. **P* < .05 significantly different from control distention values. (*B*) Mice received intracolonic administration of SLIGRL (75 μmol/L; *gray triangle* and *black circle*) or its vehicle (40% ethanol; *white signs*). Five hours later, mice received intracolonically 4αPDD (10 μmol/L; *black triangle* and *circle*) or its vehicle (40% ethanol; *gray triangle* and *white square*). CRDs were performed 1 hour after 4αPDD administration and VMRs were recorded. Values are mean ± SEM, n = 8 mice. **P* < .05 significantly different from values in the 40% ethanol group.

poorly localized, and it is not always associated with tissue injury. Therefore, one must be cautious in extrapolating findings of somatic pain research into the field of visceral pain. Although some studies have shown that TRPV4 was expressed on sensory neurons where it caused the release of nociceptive peptides (substance P and calcitonin gene-related peptide)[7] and induced somatic pain,[6,14,15] the same role for TRPV4 might not apply to visceral nociception. However, because TRPV4 can be activated by mechanical stretch,[16] and because one of the most prominent signs of visceral hypersensitivity in patients with IBS is in fact a lower threshold to mechanical distension (stretching) of their colonic lumen, we hypothesized that TRPV4 could be an important actor of visceral nociception and hypersensitivity. Here, we show that TRPV4 expression and function is involved in visceral nociception in response to CRD and that this cation channel plays a prominent role in hypersensitivity symptoms induced by the activation of PAR_2.

IBS is the most common gastrointestinal disorder in developed countries.[17–19] It is characterized by abdominal discomfort and pain, associated with altered bowel functions. The most patients with IBS report hypersensitivity symptoms in response to CRD. Recent studies have characterized the role of mediators released from tissues of patients with IBS.[9,20] Those studies have shown that proteases released from colonic tissues of patients with IBS are responsible for the activation of nociceptive visceral sensory nerves[20] and spinal afferents[9] and for the generation of hypersensitivity symptoms in mice.[9] Further, it was shown that the pronociceptive effects and the induction of visceral hypersensitivity by mediators released from the tissues of patients with IBS was completely inhibited in PAR_2-deficient tissues or mice.[9] Taken together, those studies highlighted proteases and the activation of PAR_2 as central mediators of hypersensitivity symptoms associated with IBS. However, the mechanisms by which proteases and PAR_2 activation generated visceral hypersensitivity still needed to be clarified. Here, we show that PAR_2-induced hypersensitivity is in fact mediated by the activation of TRPV4. We also show here that TRPV4 response can be enhanced by PAR_2 activation, because sub-nociceptive doses of 4αPDD started to induce hypersensitivity symptoms after an exposure to PAR_2 agonists. These results are in accord with other results generated with cultures of primary afferents, in which pre-exposure of DRG neurons to PAR_2 agonists was able to induce a larger calcium mobilization in response to 4αPDD.[7] Potentiation of 4αPDD-induced mechanical somatic hyperalgesia was also observed in vivo in that study.[7] Here, we confirm that the same is true in visceral nociception and that TRPV4 activation in the gut can be sensitized by PAR_2 agonists. However, we also provide evidence that in DRG neurons, PAR_2-activating peptide-induced calcium mobilization is not inhibited by lower expression of TRPV4 (Supplementary Figure 3; see supplemental material online at www/gastrojournal.org), which suggests parallel, although additive, mechanisms for PAR_2 and TRPV4 at a cellular level.

Although 4αPDD is considered as the most specific agonist for TRPV4 activation,[6,21] phorbol esters can display nonspecific properties, particularly when used in vivo. One of the important aspects of the present study is the validation of the use of a selective agonist for TRPV4 activation in cultured sensory neurons as well as in vivo in a model of visceral nociception. By performing intervertebral injections of TRPV4-targeted siRNA, we have shown that down-regulation of the expression of TRPV4 in sensory neurons inhibited the response of those neurons to 4αPDD in vitro in primary cultures of DRG neurons, but it also inhibited the visceral hypersensitivity response to 4αPDD in vivo. The specificity of 4αPDD as a TRPV4 agonist was further con-

firmed by its lack of effect in TRPV4-deficient mice. Another important methodologic advance arising from the present study is the use of intervertebral injection of TRPV4-targeted siRNA to down-regulate TRPV4 expression in DRG neurons. As seen in other studies,[22] we observed that this type of injection targets only DRG neurons, provoking a decreased TRPV4 expression after TRPV4 SiRNA treatment.

Our results show that TRPV4 was present and functional in sensory neurons projecting from the colon of mice. Further, we showed that peripheral (colonic) activation of TRPV4 by intracolonic administration of the TRPV4 agonist 4αPDD caused visceral allodynia and hyperalgesia. Blockade of TRPV4 expression in the DRG neurons by intervertebral injections of TRPV4 siRNA and its consequences on the inhibition of 4αPDD-induced hypersensitivity suggests that TRPV4 expressed on the DRG neurons was responsible for the pronociceptive effects of TRPV4 peripheral activation. However, we cannot rule out the possibility that TRPV4 expressed at the colonic level could participate in the transmission of the nociceptive message. Comparative studies investigating the expression and functions of TRPV4 in all the actors of the enteric nervous system (both extrinsic and intrinsic) and on epithelial cells will be necessary to further investigate the effects of TRPV4 activation from colonic tissues.

Taken together, the results generated in the present study show that colonic activation of TRPV4 induces visceral hypersensitivity symptoms and that TRPV4 activation is a major component of visceral nociception. Because of the central role shown for PAR_2 in IBS-related pain symptoms,[9] the fact that TRPV4 mediates PAR_2-induced hypersensitivity highlights TRPV4 as a possible common mediator on a clinical feature reported in all patients with IBS: visceral hypersensitivity. Importantly, our results suggest that TRPV4 could be a new potential therapeutic target in patients with IBS for the treatment of abdominal pain.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.05.024.

References

1. Owsianik G, Talavera K, Voets T, Nilius B. Permeation and selectivity of TRP channels. Annu Rev Physiol 2006;68:685–717.
2. Nilius B, Prenen J, Wissenbach U, Bodding M, Droogmans G. Differential activation of the volume-sensitive cation channel TRP12 (OTRPC4) and volume-regulated anion currents in HEK-293 cells. Pflugers Arch 2001;443:227–233.
3. Smith PL, Maloney KN, Pothen RG, Clardy J, Clapham DE. Bisandrographolide from Andrographis paniculata activates TRPV4 channels. J Biol Chem 2006;281:29897–904.
4. Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. Nature 2003;424:434–438.
5. Levine JD, Alessandri-Haber N. TRP channels: targets for the relief of pain. Biochim Biophys Acta 2007;1772:989–1003.
6. Suzuki M, Mizuno A, Kodaira K, Imai M. Impaired pressure sensation in mice lacking TRPV4. J Biol Chem 2003;278:22664–22668.
7. Grant AD, Cottrell GS, Amadesi S, et al. Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. J Physiol 2007;578:715–733.
8. Alessandri-Haber N, Dina OA, Yeh JJ, Parada CA, Reichling DB, Levine JD. Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat. J Neurosci 2004;24:4444–4452.
9. Cenac N, Andrews CN, Holzhausen M, et al. Role for protease activity in visceral pain in irritable bowel syndrome. J Clin Invest 2007;117:636–647.
10. Altier C, Dale CS, Kisilevsky AE, et al. Differential role of N-type calcium channel splice isoforms in pain. J Neurosci 2007;27:6363–6373.
11. Altier C, Khosravani H, Evans RM, et al. ORL1 receptor-mediated internalization of N-type calcium channels. Nat Neurosci 2006;9:31–40.
12. Watanabe H, Vriens J, Suh SH, Benham CD, Droogmans G, Nilius B. Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. J Biol Chem 2002;277:47044–47051.
13. Cervero F, Laird JM. Visceral pain. Lancet 1999;353:2145–2148.
14. Alessandri-Haber N, Yeh JJ, Boyd AE, et al. Hypotonicity induces TRPV4-mediated nociception in rat. Neuron 2003;39:497–511.
15. Alessandri-Haber N, Joseph E, Dina OA, Liedtke W, Levine JD. TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator. Pain 2005;118:70–79.
16. Hamanaka K, Jian MY, Weber DS, et al. TRPV4 initiates the acute calcium-dependent permeability increase during ventilator-induced lung injury in isolated mouse lungs. Am J Physiol Lung Cell Mol Physiol 2007;293:L923–932.
17. Camilleri M. Mechanisms in IBS: something old, something new, something borrowed. Neurogastroenterol Motil 2005;17:311–6.
18. Spiller RC. Inflammation as a basis for functional GI disorders. Best Pract Res Clin Gastroenterol 2004;18:641–661.
19. Collins SM. Peripheral mechanisms of symptom generation in irritable bowel syndrome. Can J Gastroenterol 2001;15(suppl B):14B–16B.
20. Barbara G, Wang B, Stanghellini V, et al. Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. Gastroenterology 2007;132:26–37.
21. Vriens J, Owsianik G, Janssens A, Voets T, Nilius B. Determinants of 4 alpha-phorbol sensitivity in transmembrane domains 3 and 4 of the cation channel TRPV4. J Biol Chem 2007;282:12796–12803.
22. Bourinet E, Alloui A, Monteil A, et al. Silencing of the Cav3.2 T-type calcium channel gene in sensory neurons demonstrates its major role in nociception. EMBO J 2005;24:315–324.

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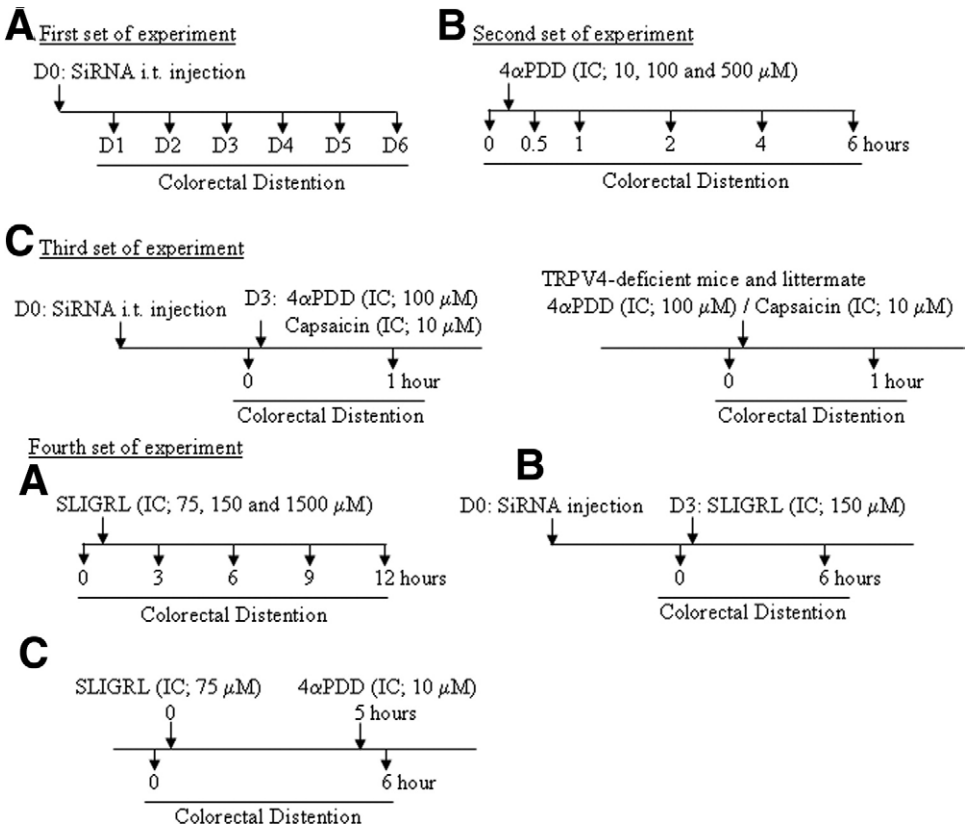
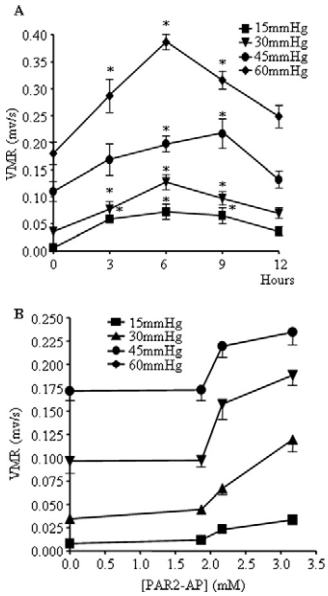
N.C. and C.A. contributed equally to this work.

All authors declare that they have no conflict of interest to disclose.



Supplementary Figure 1

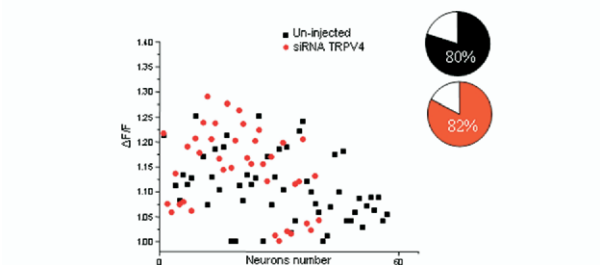


Mice received an intracolonic administration of SLIGRL (150μM) and viscero-motor response (VMR) to colorectal distension (CRD) was recorded at 3, 6, 9 and 12 hours. Time 0 corresponds to VMR to CRD performed before SLIGRL administration, and is used as control distension values. Values are mean + SEM, n=8 mice per group, *P<.05 compared to control distension values.

Dose response curve of viscero-motor response (VMR) to colo-rectal distension (CRD) after the intracolonic administration of the PAR-2-activating peptide SLIGRL or its vehicle (40 % ethanol). Values are mean + SEM, n=8 mice per group, * significantly different from control (vehicle) group for p<.05.

Supplementary Figure 2

Calcium response of DRG neurons harvested from naive mice (black) or from mice that have received inter-vertebral injections of TRPV4-targeted siRNA (red), and those cultured neurons are exposed to 10 μM SLIGRL-NH_2.



Supplementary Figure 3