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.

0016-5085/08/$34.00
doi:10.1053/j.gastro.2008.05.024

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