

TRPV1 Function in Mouse Colon Sensory Neurons Is Enhanced by Metabotropic 5-Hydroxytryptamine Receptor Activation

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Using whole-cell patch-clamp methods, we examined the hypothesis that serotonin [5-hydroxytryptamine (5-HT)] receptor activation enhances TRPV1 function in mouse colon sensory neurons in lumbosacral dorsal root ganglia, which were identified by retrograde labeling with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine methanesulfonate) injected into multiple sites in the wall of the descending colon. 5-HT increased membrane excitability at a temperature below body temperature in response to thermal ramp stimuli in colon sensory neurons from wild-type mice, but not from TRPV1 knock-out mice. 5-HT significantly enhanced capsaicin-, heat-, and proton-evoked currents with an EC₅₀ value of 2.2 μ M. 5-HT (1 μ M) significantly increased capsaicin-evoked (100 nM) and proton-evoked (pH 5.5) currents 1.6- and 4.7-fold, respectively, and significantly decreased the threshold temperature for heat current activation from 42 to 38°C. The enhancement of TRPV1 by 5-HT was significantly attenuated by selective 5-HT₂ and 5-HT₄ receptor antagonists, but not by a 5-HT₃ receptor antagonist. In support, 5-HT₂ and 5-HT₄ receptor agonists mimicked the facilitating effects of 5-HT on TRPV1 function. Downstream signaling required G-protein activation and phosphorylation as intracellularly administered GDP- β -S [guanosine 5'-O-(2-thiodiphosphate)], protein kinase A inhibitors, and an A-kinase anchoring protein inhibitor significantly blocked serotonergic facilitation of TRPV1 function; 5-HT₂ receptor-mediated facilitation was also inhibited by a PKC inhibitor. We conclude that the facilitation of TRPV1 by metabotropic 5-HT receptor activation may contribute to hypersensitivity of primary afferent neurons in irritable bowel syndrome patients.

Key words: visceral pain; thermal hyperalgesia; DRG; serotonin receptor; capsaicin receptor; DiI

Introduction

Irritable bowel syndrome (IBS) is a chronic disorder characterized by abdominal pain and discomfort and changes in bowel habits in the absence of structural or biochemical abnormalities. IBS is prevalent, with symptoms at least transiently affecting up to one in five Americans each year, and is the second leading cause of workplace absenteeism in the United States after the common cold, resulting in estimated direct and indirect costs exceeding \$30 billion annually (Sandler et al., 2002). The mechanisms leading to the development of functional gastrointestinal disorders such as IBS are poorly understood. However, mounting evidence suggests that changes in visceral sensation (i.e., visceral hypersensitivity) play an important role in the pathogenesis of these disorders. Sensitization of peripheral sensory pathways and altered central processing (central sensitization) both contribute to visceral hypersensitivity. Although changes in ion channel expression during inflammation increase the excitability of primary

afferent neurons, it remains unclear how peripheral mechanisms contribute to altered sensations that arise in the absence of pathology.

The gastrointestinal tract is the richest source of serotonin [5-hydroxytryptamine (5-HT)] in the body, and many studies have focused on 5-HT as a mediator of visceral sensory neuron sensitization (Gershon, 1999). Most intestinal 5-HT is stored in enteroendocrine cells, where it is released locally by mechanical or chemical stimuli and can activate intrinsic and extrinsic sensory neurons, which express a variety of 5-HT receptors. Interestingly, several recent studies have reported an increased number of enteroendocrine cells in IBS patients (Bearcroft et al., 1998; Spiller et al., 2000; Houghton et al., 2003). Furthermore, the peripherally acting 5-HT₃ receptor antagonist alosetron blunts visceral sensation in animal models and humans and has been used clinically in the treatment of IBS (Kozłowski et al., 2000; Camilleri et al., 2001). Although circumstantial in nature, these data raise the question whether and how 5-HT affects visceral sensation and contributes to the development of visceral hypersensitivity.

Serotonin excites mechanosensitive afferent fibers innervating the colon through effects on the ionotropic 5-HT₃ as well as metabotropic 5-HT receptors (Hicks et al., 2002). Although sev-

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eral studies have demonstrated effects of metabotropic 5-HT receptors on voltage-dependent ion channels in neurons (Braha et al., 1993; Torres et al., 1995; Cardenas et al., 2001), the mechanisms by which G-protein-coupled 5-HT receptors trigger action potentials are not known (Christian et al., 1989; Cardenas et al., 1997a,b). An attractive target is the TRPV1 channel, a proton and heat-gated channel that is also activated by endogenous lipid mediators and the pungent vanilloid capsaicin. TRPV1 is present in visceral afferent neurons and is increased in inflammatory diseases of the gastrointestinal tract (Yiangou et al., 2001) and in patients with rectal hypersensitivity (Chan et al., 2003). To test the hypothesis that 5-HT receptor activation enhances TRPV1 function, we performed whole-cell patch-clamp experiments in mouse colon sensory neurons.

Materials and Methods

Male CB57BL/6 mice (20–30 gm; Harlan, Indianapolis, IN) and TRPV1 knock-out mice (TRPV1^{−/−}; 20–30 gm; The Jackson Laboratory, Bar Harbor, ME) were used for all experiments. Mice were fed a standard laboratory diet and maintained on a 12 hr light/dark cycle (lights on 6:00 A.M. to 6:00 P.M.). All experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Iowa.

Labeling of colon sensory neurons. Mice were anesthetized with a mixture of ketamine (17.5 mg/ml) and xylazine (2.5 mg/ml; 5 μ l/gm, i.p.), and the descending colon was exposed through a paramedian incision. One microliter of the dicarbocyanine dye 1,1'-di-octadecyl-3,3',3'-tetramethylindocarbocyanine methanesulfonate [DiI; 100 mg in 2 ml of dimethylsulfoxide (DMSO)] was injected into five sites in the wall of the descending colon using a Hamilton microliter syringe with a 30 gauge needle. Thirty seconds after the injection, the needle was carefully removed, avoiding leakage of dye into the peritoneal cavity. The abdomen was then closed, and mice were allowed to recover. DiI injection sites were confirmed after harvesting dorsal root ganglia (DRGs), and DiI was located in sites ~10–25 mm from the anus.

Cell preparation. Mice were anesthetized with ketamine/xylazine (as above), killed by cervical dislocation, and the L6, S1, and S2 DRGs were quickly removed bilaterally under a dissection microscope. DRGs were transferred into ice-cold culture media and minced with a surgical blade. The tissue was incubated in Neurobasal-A media (Invitrogen, Carlsbad, CA) containing collagenase (type 1A; 2 mg/ml), trypsin (type III; 1 mg/ml), and 3 mM CaCl₂ at 37°C for 50 min. The tissue fragments were then gently triturated with a sterile Pasteur pipette coated with silicon and centrifuged at 800 rpm for 5 min. DRG neurons were resuspended in Neurobasal-A supplemented with 5% B27 supplement, 0.25% L-glutamine (200 mM), and 1% penicillin–streptomycin (all from Invitrogen), plated on poly-D-lysine-coated glass coverslips, and incubated for 2–10 hr at 37°C in a 95%/5% air/CO₂ atmosphere saturated with water vapor.

Whole-cell recordings. Patch pipettes were prepared using borosilicate glass (PG52151-4; World Precision Instruments, Sarasota, FL) with a tip resistance of 2–5 M Ω after fire-polishing. They were filled with an internal solution containing (in mM) 100 KCl, 10 EGTA, 40 HEPES, 5 MgCl₂, and 1 Na₂ATP, adjusted to pH 7.4 with KOH. Extracellular solutions contained (in mM) 128 NaCl, 5.4 KCl, 1.8 CaCl₂, 5 MgCl₂, 5.55 glucose, and 20 HEPES. For solutions below pH 4.5, HEPES was exchanged with 20 mM MES [2-(N-morpholino)-ethanesulfonic acid], pH adjusted with NaOH or HCl. In calcium-free experiments, CaCl₂ was omitted, and 5 mM EGTA was added to the standard solution.

Solutions were applied through a three-barreled glass tubing manifold (inner diameter, 700 μ m) located ~150 μ m from the cell, except for thermal tests. Rapid solution exchange was achieved within 20 msec by movement of the manifold (SF-77B; Warner Instruments, Hamden, CT). To avoid desensitization to capsaicin or protons, we allowed 1 min between solution exchanges, unless noted otherwise. Heat-sensitive currents were evoked once in each cell by superfusing preheated solutions, increasing the bath temperature at a rate of ~1.0–1.5°C/sec. The temperature of the bath solution was monitored with a thermistor placed

~100 μ m from the cell (accuracy, \pm 0.1°C). Colon sensory neurons were identified by their red-orange color under Hoffman Contrast Optics (400 \times) in fluorescent light with a rhodamine filter (excitation wavelength, 546 nm; barrier filter, 580 nm). Only DiI-labeled cells were studied. Whole-cell voltage-clamp and current-clamp recordings were performed with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Recordings were filtered at 5 kHz and digitized at 1 kHz using a Digidata 1320A interface (Axon Instruments). For current-clamp experiments, only neurons with a stable resting membrane potential of at least −40 mV were included in the study. Unless stated otherwise, all experiments were performed at room temperature.

Immunohistochemistry. Acutely dissociated DRG cells (see above) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min. After washing with 0.01 M PBS, the specimens were incubated in 3% normal goat serum (NGS; Sigma, St. Louis, MO) for 30 min at room temperature to reduce nonspecific binding and then with an N-terminal specific polyclonal TRPV1 antibody (rabbit anti-capsaicin receptor at a dilution of 1 μ g/ml; Calbiochem, La Jolla, CA) in 3% NGS at 4°C overnight. Specimens were then washed with 0.01 M PBS and incubated with goat anti-rabbit IgG (1:200; Alexa Fluoro 488; Molecular Probes, Eugene, OR) in 3% NGS for 2 hr at room temperature. After washing, specimens were mounted with Fluoromount G (Electron Microscopy Sciences, Washington, PA) and coverslipped. Control experiments were performed by incubating without primary antibody, or by preincubating primary antibody with the antigenic peptide (capsaicin receptor control peptide; Calbiochem) for 4 hr at 4°C. Slides were examined with a fluorescence microscope (Optiphot; Nikon, Tokyo, Japan) equipped with separate filters. Images were captured with a model 2.3.1 SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Chemicals. 5-HT, (\pm)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), 5-methoxytryptamine (5-MeOT), (1-butyl-4-piperidinyl)methyl-8-amino-7-chloro-1,4-benzodioxane-5-carboxylate hydrochloride (SB204070), guanosine 5'-O-(2-thiodiphosphate) (GDP- β -S), N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide dihydrochloride (H-89), N-amidino-3, 5-diamino-6-chloropyrazinecarboxamide hydrochloride (amiloride), N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide (capsazepine), and 8-methyl-N-vanillyl-trans-6-nonenamide (capsaicin) were purchased from Sigma. 3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,4-[1H,³H]-quinazolinone tartrate (ketanserin), α -methyl-5-hydroxytryptamine maleate (α Me-5-HT), tropanyl 3,5-dichlorobenzoate (MDL-72222), (\pm)-4-amino-5-chloro-N-[1-[(3R*,4S*)-3-(4-fluorophenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide (cisapride), 1-methyl-1H-indole-3-carboxylic acid, and [1-[2-[(methylsulfonyl)amino]ethyl]-4-piperidinyl]methyl ester (GR133808) were purchased from Tocris (Ellisville, MO). The protein kinase A (PKA) inhibitor 6-22 amide, AKAP inhibitor peptide (St-Ht31), and calphostin C were purchased from Calbiochem. All chemicals were dissolved in distilled water or DMSO and applied in a final concentration of <0.1%.

Data analysis. Data are expressed as means \pm SEM. The software package pCLAMP9.0 (Axon Instruments) and SigmaPlot 8.02 and SigmaStat 2.03 (SPSS, Chicago, IL) were used for data acquisition and analysis. Concentration–response curves were fitted by the Hill equation, $I = I_{\text{mini}} + (I_{\text{max}} - I_{\text{mini}}) \times [X]^n / ([EC_{50}]^n + [X]^n)$, where I is the current amplitude ratio triggered by pH 5.5 before and after a given serotonin concentration, $[X]$; 5-HT, or the current density at different proton concentrations, $[X; H^+]$; I_{max} or I_{mini} is the maximum or minimum response of the cell; n is the Hill slope; and EC_{50} is the concentration eliciting a half-maximal response.

Data were compared using Student's two-tailed unpaired or paired t tests or repeated-measures, one-way or two-way ANOVA as appropriate. Statistical significance was determined at $p < 0.05$.

Results

Temperature-dependent activation of colon sensory neurons

In wild-type mice, temperature ramps depolarized all colon sensory neurons tested, triggering bursts of action potentials near 42°C (Fig. 1a,b). When we tested the capsaicin sensitivity of wild-type neurons after temperature ramps, all of the 13 cells tested

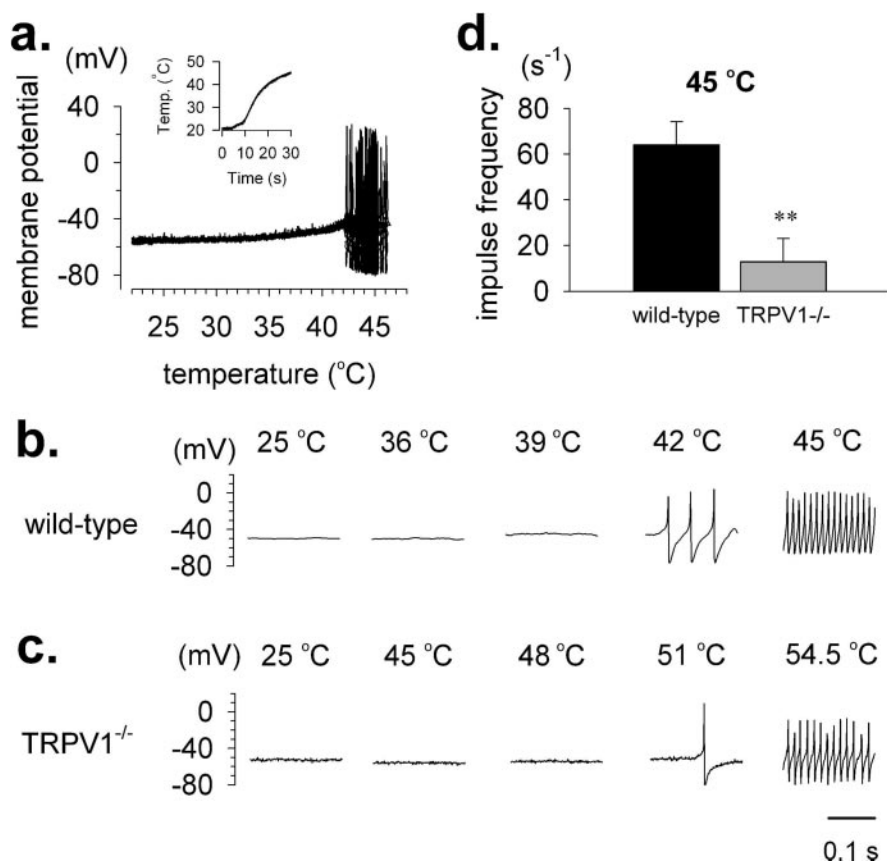


Figure 1. Temperature-dependent excitability of colon sensory neuron from wild-type and TRPV1^{-/-} mice. *a*, Representative responses in current-clamp recording mode to a temperature ramp. Thermal stimuli evoked depolarization and action potentials. *b*, *c*, Representative records from wild-type (*b*) and TRPV1^{-/-} (*c*) neurons at different temperatures show action potential generation at temperatures above 42°C in wild-type neurons and above 50°C in TRPV1^{-/-} neurons. *d*, Mean \pm SEM action potential frequency of colon afferent neurons in response at 45°C in wild-type (black bar; $n = 8$) and TRPV1^{-/-} (gray bar; $n = 6$; $t = 3.49$; $**p < 0.01$) mice.

depolarized in response to 10 μ M capsaicin. Although capsaicin did not trigger action potential in all cells when given after the initial heat stimulus, it elicited spikes when given to untreated cells. In TRPV1^{-/-} mice, temperatures below 50°C typically generated few action potentials, presumably through activation of TRPV2 or other temperature-sensitive currents (Fig. 1*c*), and impulse frequency at 45°C was significantly lower in TRPV1^{-/-} mice than in C57BL/6 mice ($t = 3.49$; $p < 0.01$) (Fig. 1*d*).

Characterization of TRPV1 function in colon sensory neurons

Inward current in colon DRG neurons developed at $\sim 42^\circ\text{C}$ with an extremely steep temperature dependency (Fig. 2*a*), consistent with our current-clamp data and previous reports in DRG neurons (Sugiura et al., 2002). The mean threshold temperature for activation (defined as the intersection where two lines approximating the stable baseline current and clearly increasing temperature-dependent current) was $41.8 \pm 0.7^\circ\text{C}$. Similar to results obtained with current clamp, colon DRG neurons also responded to subsequent challenge with capsaicin. The amplitude of the capsaicin-evoked current was smaller than that of the heat-evoked current because of desensitization of TRPV1 activation. When given to naive cells, the peak current density evoked by capsaicin (409.8 ± 33.0 pA/pF; $n = 3$) was greater than that of heat (249.1 ± 60.8 pA/pF; $n = 6$). The capsaicin-evoked current showed a lasting desensitization; when applied a second time 1

min after the first application of capsaicin (1 μ M), the response was $77 \pm 4\%$ ($n = 6$) of the first response to capsaicin (Fig. 2*b*) ($F = 50.11$; $p < 0.001$). Only 6 of 42 colon sensory neurons tested did not respond to capsaicin. In two of these capsaicin-insensitive neurons, we tested the response to temperature and only saw inward currents when the bath temperature exceeded 50°C (data not shown). None of the neurons from TRPV1^{-/-} mice responded to capsaicin (10 μ M; $n = 4$) (Fig. 2*f*).

Consistent with previous reports, all capsaicin-sensitive colon sensory neurons tested also responded to protons with slowly activating and sustained inward currents (Fig. 2*b,c,f*) (Caterina et al., 1997). This acid-evoked current did not desensitize significantly when neurons were repeatedly stimulated in the absence of extracellular calcium ($F = 0.57$; $p = 0.7$). However, in the presence of extracellular calcium, repetitive proton applications triggered current responses that desensitized significantly ($F = 2.96$; $p < 0.05$) (Fig. 2*b*). The TRPV1 blocker capsaizepine (10 μ M) significantly attenuated the sustained acid-evoked current (Fig. 2*c*, middle; *d*). In contrast, amiloride (200 μ M) did not significantly affect the acid-evoked inward current (Fig. 2*c*, right; *e*), suggesting that proton-sensitive currents in colon sensory neurons are primarily carried through TRPV1 channels. We therefore examined acid-evoked currents in colon sensory neurons from TRPV1^{-/-} animals. Consistent with the pharmaco-

logical data, cells from knock-out mice did not show significant sustained-type current responses to protons (Fig. 2*f*). To further characterize the properties of acid-evoked currents, we examined the pH dependence of these currents using solutions with pH between 7.0 and 3.5. We allowed 1 min between each 5 sec application to prevent desensitization. As shown in the left trace of Figure 2*f*, the current is activated at pH 5.5 with half-activation at pH 4.57 ± 0.07 (Fig. 2*f*, right) ($n = 8$).

To obtain additional evidence that colon sensory neurons express TRPV1, we examined immunoreactivity (IR) for TRPV1 in acutely dissociated DRG neurons. Figure 3*a* shows double staining of retrograde tracer that was injected into the colon wall and TRPV1-like IR in a DRG neuron. In control experiments performed by omitting the primary antibody (Fig. 3*c*) or preincubating the primary antibody with blocking peptide (Fig. 3*d*), immunohistochemical staining of DRG cells for TRPV1-like IR was abolished.

5-HT enhances excitability of colon sensory neurons through TRPV1 activation

To test the working hypothesis, we next examined the effect of 5-HT (1 μ M; 2 min; bath application) on heat-evoked depolarization and action potential firing in colon sensory neurons. Although neither vehicle nor 5-HT altered resting membrane potential (-49.4 ± 1.8 mV and -46.6 ± 1.5 mV in vehicle- and

5-HT-treated neurons, respectively; $n = 8/\text{group}$), responses to heat were significantly enhanced in the presence of 5-HT in DRG neurons from C57BL/6 mice (Fig. 4a,c). Notably, action potentials were generated by heat at temperatures below core body temperature (Fig. 4c, broken vertical line) in the presence of 5-HT. In contrast, there was no significant temperature dependency of responses in TRPV1^{-/-} mice or effect of 5-HT within the temperature range tested (Fig. 4b,d).

In voltage-clamp experiments, neither vehicle nor 1 μM 5-HT triggered current responses. In contrast to the desensitization seen with repeated capsaicin application (Fig. 2b), pretreatment with 5-HT (1 μM ; 50 sec) between the first and second application of capsaicin not only prevented the desensitization but significantly enhanced the response to the second application of capsaicin more than twofold relative to the typically desensitized response to a second application of capsaicin (Fig. 5a). Similarly, 5-HT enhanced responses to heat. Defining the threshold temperature (T_t) as the intersection where two lines approximating the stable baseline current and the clearly increasing temperature-dependent current cross (Sugiura et al., 2002), 5-HT (1 μM ; 2 min; bath application) pretreatment significantly reduced the mean T_t to $38.1 \pm 1.2^\circ\text{C}$ ($n = 7$; $t = -2.50$; $p < 0.05$) from a control $41.8 \pm 0.7^\circ\text{C}$ ($n = 6$) (Fig. 4b). Likewise, the acid-activated current, pH 5.5, was also significantly enhanced by pretreatment with 5-HT (1 μM ; 50 sec) (Fig. 5c). Consistent with results obtained in neurons from TRPV1^{-/-} mice, the TRPV1 receptor antagonist capsazepine (10 μM) significantly attenuated enhancement of the acid-activated current by 5-HT (to $19.6 \pm 3.8\%$; $n = 3$) relative to pH 5.5 application in the absence of the antagonist ($100.1 \pm 6.8\%$; $n = 8$; $t = 6.86$; $p < 0.001$) (Fig. 5c). To determine the concentration dependency of the enhancing effect of 5-HT, we examined the acid-activated current, pH 5.5, after exposure of cells to various 5-HT concentrations between 1 nM and 10 μM , testing each cell only once to prevent desensitization to 5-HT. Greater concentrations of 5-HT were given in the presence of MDL72222 (3 μM), a selective 5-HT₃ receptor antagonist, to block activation of 5-HT₃ receptors, which caused a decrease in serotonergic enhancement of acid-evoked currents (30 and 100 μM ; data not shown). Within the range between 1 nM and 10 μM , the effect of 5-HT was concentration dependent, with an EC_{50} value of 2.2 μM and Hill coefficient of 1.1 (Fig. 5d). Figure 5e shows the magnitude and duration of 5-HT (1 μM) enhancement of the acid-activated current, pH 5.5; 5-HT was applied once for 50 sec after the first application of acidic solution. The

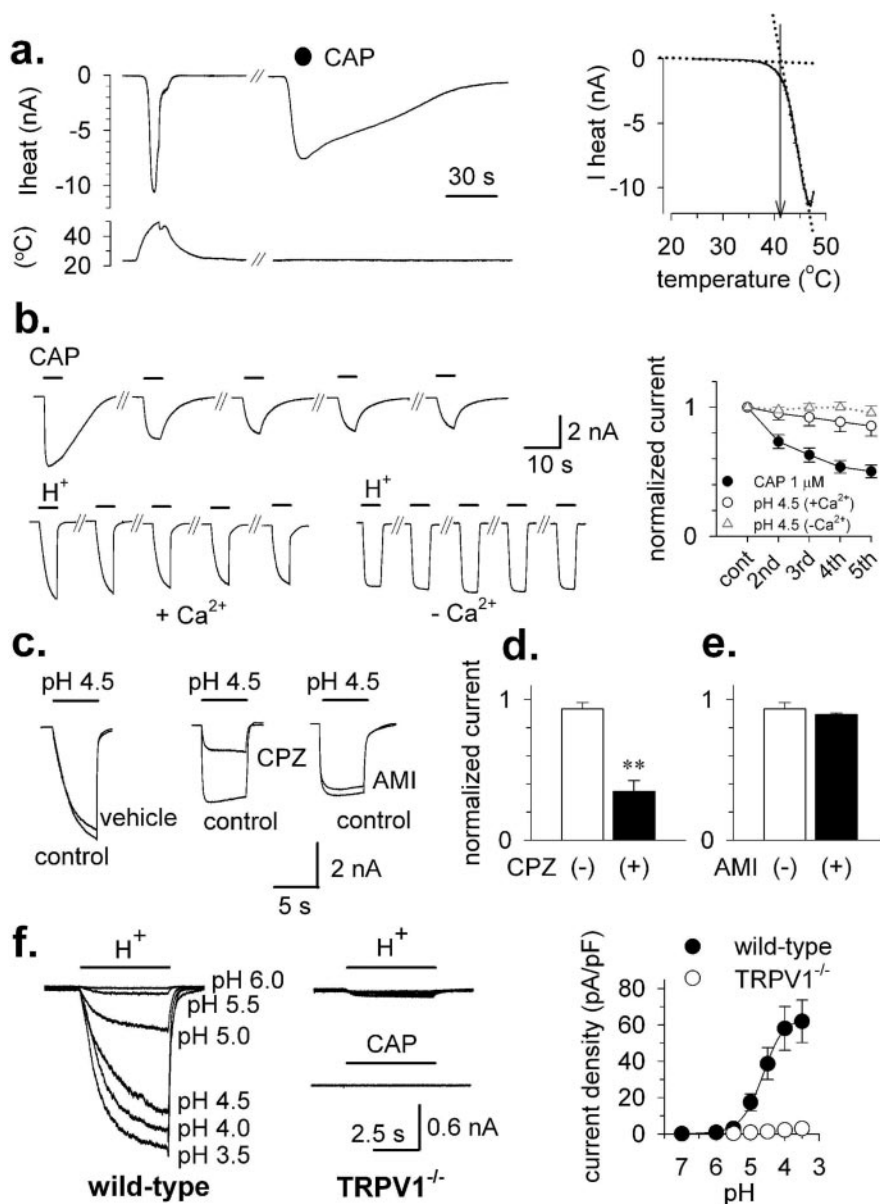


Figure 2. Characteristics of TRPV1 function in colon afferent neurons. *a*, Representative records of heat-evoked (I_{heat}) and capsaicin-evoked (CAP; 10 μM ; filled circle) currents. The right panel shows the threshold temperature for heat activation using heat ramps as illustrated in the left panel (see Materials and Methods for details). *b*, Repeat application of capsaicin (CAP; 1 μM ; 5 sec, 1 min interval; $n = 6$; $F = 50.11$; $p < 0.001$) and acid (H^+ ; pH 4.5; $n = 5$; $F = 2.96$; $p < 0.05$) in the presence of extracellular calcium are associated with significant desensitization of current responses as shown in sample tracings (left); data are summarized in a graph (right). Acid-evoked currents did not desensitize significantly in calcium-free solutions ($-\text{Ca}^{2+}$). *c*, Representative records of acid-evoked currents, pH 4.5, in the presence of vehicle, capsazepine (CZP; 10 μM), or amiloride (AMI; 200 μM). Data are summarized for vehicle ($n = 6$), capsazepine ($n = 9$; $t = 5.81$; $**p < 0.01$), and amiloride ($n = 9$; $t = 0.93$; not significant) in *d* and *e*, respectively. *f*, Representative records of acid-evoked sustained currents in wild-type (left) and TRPV1^{-/-} (middle) mice. None of the four TRPV1^{-/-} neurons tested responded to capsaicin (CAP; bottom record; middle). Data for current responses to different proton concentrations are summarized in the right panel for wild-type mice (filled circles; $n = 8$) and TRPV1^{-/-} mice (unfilled circles; $n = 11$).

effect of the single application of 5-HT lasted at least 7 min ($n = 7$; $p < 0.05$; vs vehicle, $n = 7$). When tested at a greater concentration in the same protocol, 5-HT (3 μM) enhanced the pH-activated current for at least 20 min (10 min: 2.11 ± 0.60 -fold, $n = 6$; 20 min: 1.97 ± 0.48 -fold, $n = 3$). As shown in Figure 5f, 5-HT (1 μM) significantly shifted the pH of half-activation from 4.57 ± 0.07 ($n = 8$) to 5.14 ± 0.09 ($n = 7$; $t = 5.06$; $p < 0.001$) and Hill coefficient of 1.69 ± 0.35 and 3.86 ± 0.45 , respectively.

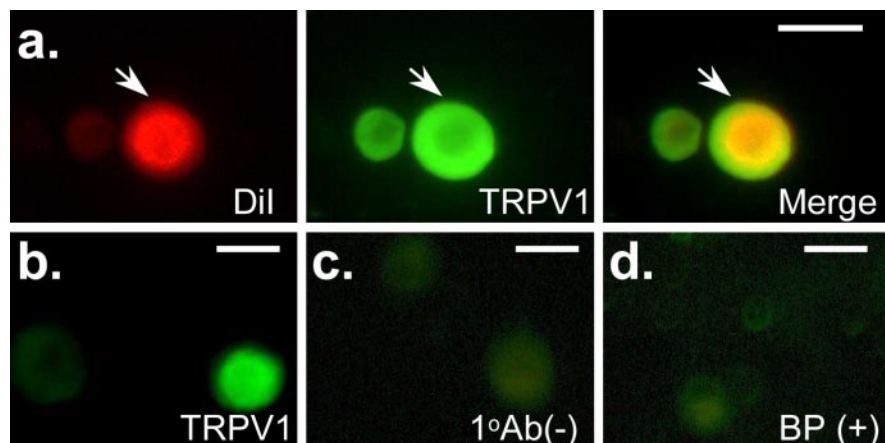


Figure 3. Immunohistochemical evidence of TRPV1 expression in colon DRG neurons. *a*, The left panel shows a Dil-labeled neuron visualized with fluorescent light at an excitation wavelength of 546 nm. When excited with fluorescent light with a wavelength of 488 nm, two cells with TRPV1-like IR can be seen in the same field (middle). The merged image in the right panel demonstrates the colocalization of the retrograde label Dil and TRPV1-like IR in one neuron marked by the arrow. TRPV1-like IR (*b*) was not detected in the absence of the primary antibody [$1^\circ\text{Ab}(-)$]; *c* or after preincubation of the primary antibody with the blocking peptide [BP(+); *d*]. Scale bar, 30 μm .

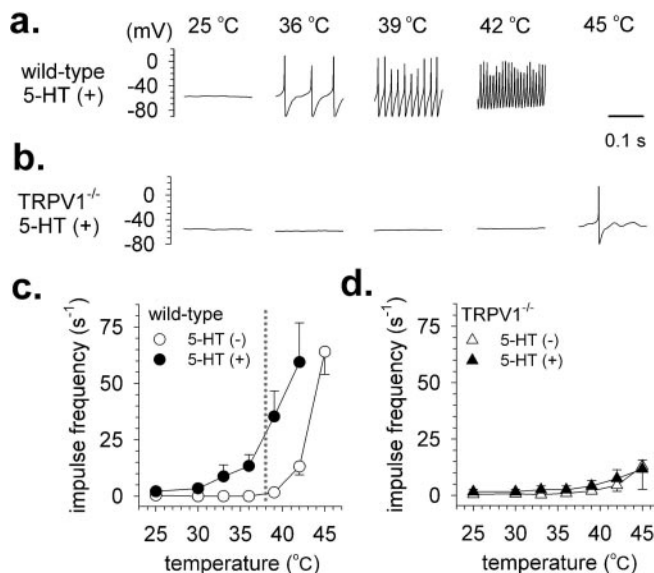


Figure 4. 5-HT enhances excitability of a colon sensory neuron in response to thermal stimulation. Representative records from wild-type (*a*) and TRPV1 $^{-/-}$ (*b*) neurons at different temperatures in the presence of 5-HT ($1\ \mu\text{M}$ pretreatment for 2 min) are shown. Action potentials in neurons from wild-type mice were generated at significantly lower temperatures (36°C) in the presence of 5-HT (*c*; compare with Fig. 1*b*). In neurons from TRPV1 $^{-/-}$ mice, action potentials were generated only at temperatures $>45^\circ\text{C}$ in the presence of 5-HT. Temperature-dependent action potential firing after exposure to vehicle (unfilled symbols) or $1\ \mu\text{M}$ 5-HT (filled symbols) is summarized for wild-type (*c*) and TRPV1 $^{-/-}$ mice (*d*). Compared with vehicle, 5-HT significantly shifted the response to temperature in neurons from wild-type mice (5-HT, $n = 8$; vehicle, $n = 8$; $F = 19.40$; $p < 0.001$). The dotted vertical line in *c* indicates the normal core temperature of wild-type mice.

Pharmacology of serotonergic enhancement of TRPV1 function

Visceral sensory neurons express several 5-HT receptors (Grundt et al., 1998; Hicks et al., 2002; Mazzia et al., 2003), and we therefore examined the role of different 5-HT receptors in the modulation of TRPV1 function using pharmacological tools. As described above, concentrations of 5-HT $\geq 1.0\ \mu\text{M}$ did not affect resting membrane potential or baseline currents. However, 3 μM

5-HT evoked a slowly activating current in seven of eight colon sensory neurons, which increased in amplitude and showed a more rapid activation and desensitization at higher concentrations of 5-HT, consistent with the expression of the ligand-gated 5-HT $_3$ receptor (Fig. 6*a*). To confirm this, we used the selective 5-HT $_3$ receptor antagonist MDL72222 ($0.6\ \mu\text{M}$), which completely and reversibly blocked the inward current produced by $10\ \mu\text{M}$ 5-HT in colon neurons ($8.4 \pm 3.6\%$ of control; $n = 6$; $t = 25.3$; $p < 0.001$) (Fig. 6*b*). MDL72222 ($0.6\ \mu\text{M}$), however, did not affect serotonergic enhancement of the acid-activated inward current in colon sensory neurons (Fig. 6*c*), revealing that metabotropic 5-HT receptors mediate this effect.

Six families of G-protein-coupled 5-HT receptors with several members have been characterized (Barnes and Sharp, 1999). Of those, 5-HT $_1$, 5-HT $_2$, and 5-HT $_4$ have been identified in DRG neurons (Nicholson et al., 2003). Because 5-HT $_1$ receptors preferentially couple to phosphodiesterase and 5-HT $_2$ and 5-HT $_4$ receptors couple to G_s and $G_{q/11}$, we focused on 5-HT $_2$ and 5-HT $_4$ receptors using selective receptor agonists and antagonists. Colon sensory neurons were pretreated with ketanserin (a 5-HT $_2$ receptor-selective antagonist) or 5-HT $_4$ receptor-selective antagonists SB204070 or GR113808 before administration of 5-HT ($1\ \mu\text{M}$) for 50 sec. No detectable effects of these antagonists (all at $10\ \mu\text{M}$) were observed (data not shown). In contrast to the 5-HT $_3$ receptor-selective antagonist MDL72222 (Fig. 6*c*), ketanserin, SB204070, and GR113808 each significantly attenuated 5-HT-induced enhancement of the acid-activated current, pH 5.5, in the presence of 5-HT (Fig. 7*a,c*). Moreover, 5-HT $_2$ receptor agonists DOI and $\alpha\text{Met-5-HT}$ or the 5-HT $_4$ receptor agonist 5-MeOT mimicked 5-HT-mediated facilitation of TRPV1 function in colon sensory neurons (Fig. 7*b,d*). Similar to 5-HT, the effect of 5-MeOT was attenuated by SB204070 ($n = 4$; data not shown).

To determine whether 5-HT-mediated enhancement of TRPV1 function involves activation of G-proteins in colon sensory neurons, we examined the effects of GDP- β -S ($0.5\ \text{mM}$ within the internal pipette solution). In the presence of GDP- β -S, 5-HT-induced enhancement of the acid-activated current was significantly reduced (Fig. 8*a,d*). Because 5-HT $_2$ and 5-HT $_4$ receptors are linked with either $G_{q/11}$, which is known to stimulate phospholipase C (PLC) and result in activation of PKC, or G_s , which is known to stimulate adenylate cyclase and result in activation of PKA, respectively, we examined the involvement of protein kinase pathways by adding selective PKA or PKC inhibitors to the internal pipette solution. The PKA inhibitors H-89 and PKI $_{6-22}$ blocked the 5-HT-induced enhancement of TRPV1 function (Fig. 8*a,d*). Similarly, the PKC inhibitor calphostin C reduced serotonergic enhancement of TRPV1 function (Fig. 8*a,d*).

To examine the contribution of PKA and PKC in 5-HT-mediated facilitation of TRPV1 function in more detail, we tested selective 5-HT $_2$ or 5-HT $_4$ agonist-mediated facilitation. Consistent with the finding linking the 5-HT $_2$ receptor with PLC and PKC, the selective 5-HT $_2$ receptor agonist DOI-mediated enhancement of TRPV1 function was attenuated by calphostin C

(Fig. 8*b,e*). Interestingly, PKI_{6–22} and A-kinase anchoring protein inhibitor St-Ht-31 also significantly attenuated DOI-mediated enhancement of TRPV1 function (Fig. 8*b,e*). Similarly, enhancement of TRPV1 by the selective 5-HT₄ receptor agonist 5-MeOT was blocked by PKI_{6–22} and AKAP inhibitor peptide, but not by calphostin C, consistent with the known coupling between the 5-HT₄ receptor and adenylylate cyclase (Fig. 8*c,f*).

Mouse core temperature

Considering the shift in temperature-dependent action potential firing of colon sensory neurons by 5-HT, we measured colon temperature in C57BL/6 mice. The rectally obtained core temperature of $37.8 \pm 0.3^\circ\text{C}$ ($n = 5$) falls within the range of a significant increase in spontaneous firing because of facilitation of TRPV1 function.

Discussion

The extrinsic, afferent innervation of the gastrointestinal tract conveys information to the CNS that gives rise to the sensations of pain and discomfort. Most afferent input from the viscera, however, is not consciously perceived under normal circumstances. However, patients with functional disorders such as IBS, in which pain and discomfort are common, experience these sensations in the absence of noxious intensities of stimulation or apparent pathology, suggesting the presence of hypersensitivity. In whole-cell patch-clamp recordings from identified colon sensory neurons, we demonstrated a significant leftward shift in the temperature sensitivity of TRPV1 by 5-HT to include activation of TRPV1 at normal body temperature. This suggests that 5-HT release from enteroendocrine cells by normal mechanical or chemical stimuli could play a role in development of the altered sensations that arise in IBS patients.

Although we did not use heterologous expression systems, the present results are interpreted in the context of TRPV1 (proton, heat, and capsaicin activation) and supported by use of TRPV1^{−/−} mice, previously reported to exhibit attenuated thermal responsiveness (Caterina et al., 2000; Davis et al., 2000) and immunocytochemistry. Although there are many receptors that transduce thermal energy (Patapoutian, 2003), enhancement of responses to temperature, protons, and capsaicin by 5-HT was absent in TRPV1^{−/−} mice, pointing at an important role of this channel in modulating colon sensory neuron excitability.

Consistent with documentation of TRPV1-like IR in mouse colon neurons (Robinson et al., 2004), >80% of the colon DRG neurons tested here responded to capsaicin or generated slowly

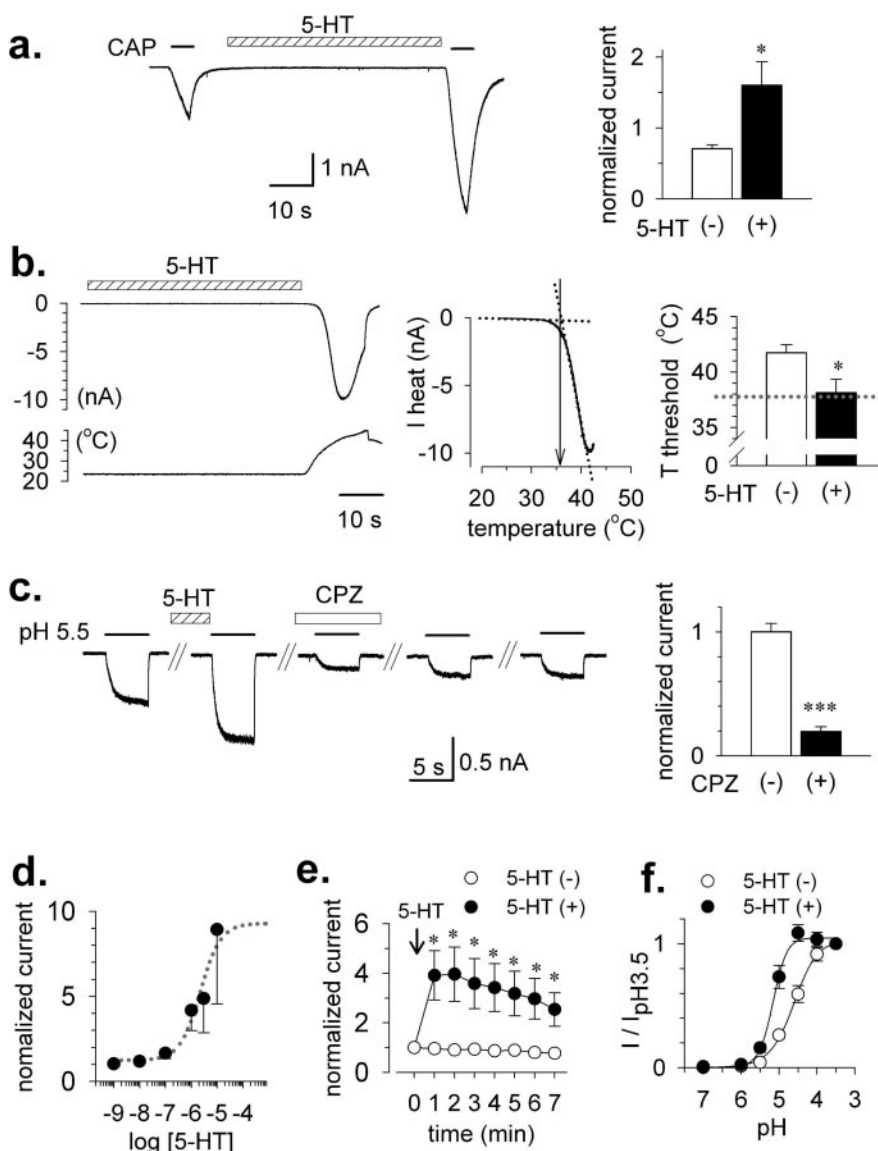


Figure 5. 5-HT enhances capsaicin-, heat-, and proton-evoked currents in colon sensory neurons. *a*, Representative records of the current evoked by capsaicin (100 nM) after exposure to 5-HT (1 μM ; left). Summary data for the second response to capsaicin, normalized to the initial response, are shown to the right (filled bar: 1 μM 5-HT, $n = 7$; unfilled bar: vehicle, $n = 6$; $t = 2.46$; $*p < 0.05$). *b*, Representative heat-evoked current after 2 min pretreatment with 5-HT (1 μM). The middle record shows the threshold temperature determined in this neuron; data are summarized in the right panel for 5-HT (filled bar; $n = 7$) and vehicle (unfilled bar; $n = 6$; $t = 2.50$; $*p < 0.05$, 5-HT vs vehicle). The dotted vertical line indicates the normal core temperature of wild-type mice. *c*, Representative records of acid-evoked currents before and after exposure to 5-HT (1 μM) (right; unfilled bar; $n = 8$), the TRPV1 antagonist capsazepine (CPZ; 10 μM ; filled bar) significantly attenuated serotonergic facilitation of the acid-evoked current ($n = 3$; $t = 6.86$; $***p < 0.001$). *d*, The normalized current in response to protons, pH 5.5, is plotted as a function of 5-HT concentration before the second acid stimulus and fitted to the Hill equation (data from 47 cells). MDL72222 (3 μM) was added in experiments with 10 μM of 5-HT. *e*, The time course of changes in the acid-evoked current after 1 min exposure to 5-HT (1 μM ; filled circles; $n = 7$) or vehicle (unfilled circles; $n = 7$) ($*p < 0.05$). *f*, pH dependence of the acid-evoked current after 1 min exposure to 5-HT (1 μM ; filled circles; $n = 7$) or vehicle (unfilled circles; $n = 8$). The data were fitted to the Hill equation; the difference in pH_{50} between the groups was significant ($t = 5.06$; $p < 0.001$).

sustained currents in response to acid that were blocked by capsazepine. This differs from results obtained in unidentified DRG neurons, in which <50% of cells responded to capsaicin (Dirajlal et al., 2003), consistent with studies showing mRNA or IR for TRPV1 in <50% of DRG neurons (Caterina et al., 1997; Michael and Priestley 1999; Robinson et al., 2004). Similarly, the response to acid appears to be distinct between identified visceral DRG neurons and unidentified DRG neurons. In colon sensory neurons from C57BL/6 mice, protons almost exclusively activated

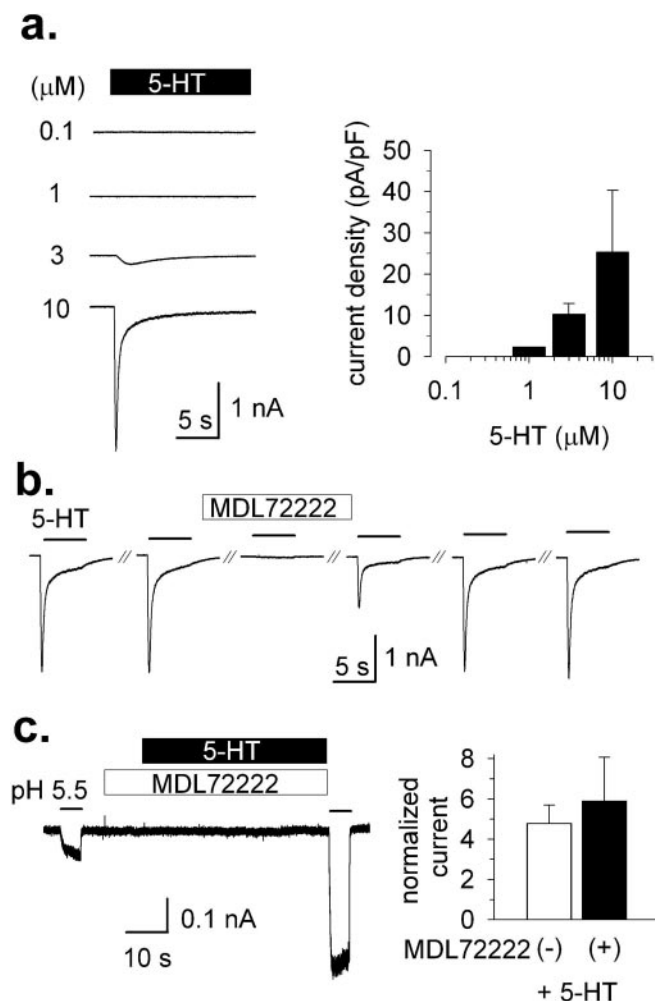


Figure 6. 5-HT₃ channel is activated by 5-HT in colon afferent neurons but does not contribute to serotonergic facilitation of TRPV1 function. *a*, Representative currents activated by increasing 5-HT concentrations (0.1, 1, 3, and 10 μM). The data were normalized to cell capacitance and are summarized in the right panel (0.1 μM , $n = 6$; 1 μM , $n = 9$; 3 μM , $n = 8$; 10 μM , $n = 8$). *b*, The selective 5-HT₃ antagonist MDL72222 (600 nM) reversibly blocked the inward current triggered by 10 μM 5-HT ($n = 6$; $t = 25.3$; $p < 0.001$). *c*, The 5-HT₃ antagonist MDL72222 did not block facilitation by 5-HT (1 μM) of the acid-evoked current. The data summarized to the right for vehicle (unfilled bar; $n = 11$) and MDL72222 (filled bar; $n = 8$; $t = -0.52$; not significant) treatments are shown.

sustained currents that were blocked by capazepine, but not by amiloride, and thus likely are attributable to activation of TRPV1. A previous study of unidentified DRG neurons reported a mixture of transient and sustained acid-sensitive currents, both of which were significantly inhibited by amiloride (Dirajlal et al., 2003), suggesting a significant contribution of ASICs. In addition, the majority of the mouse colon DRG neurons in the present study responded to heat, whereas in a previous study only about half of unidentified DRG neurons were heat sensitive (Nagy and Rang, 1999). Because the fraction of capsaicin-sensitive neurons innervating the rat colon is lower (Su et al., 1999), direct comparisons between identified somatic and visceral neurons in the same species are necessary to determine whether colon sensory neurons actually have properties distinct from other, somatic DRG neurons.

The present results point at a potential role of TRPV1 as a modulator of neuron excitability. Changes in TRPV1 function through phosphorylation or release from inhibition through

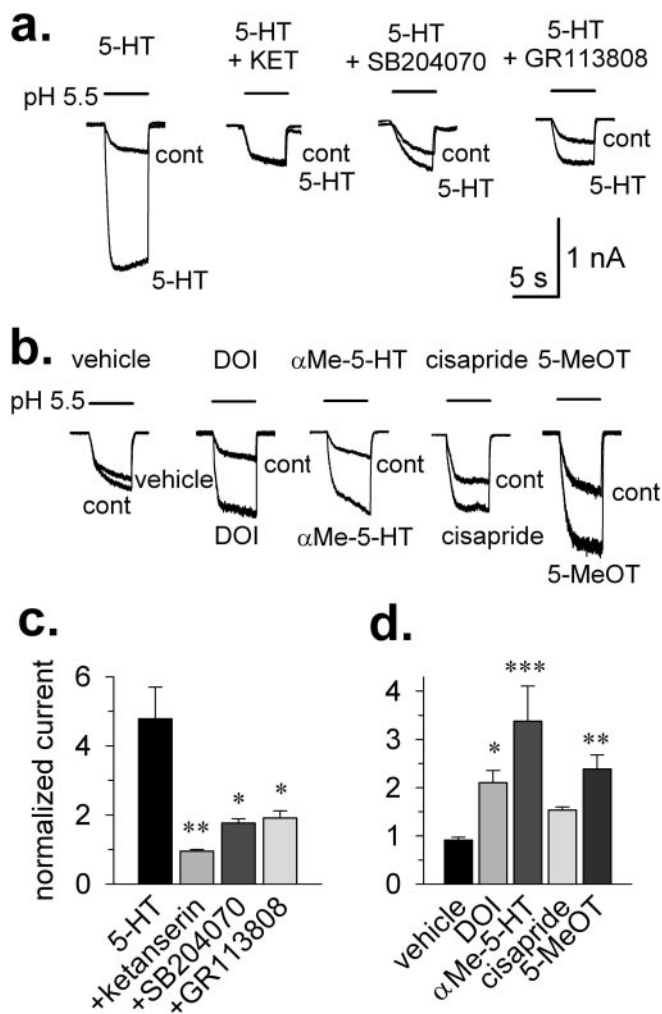


Figure 7. Metabotropic 5-HT receptors are involved in serotonergic facilitation of TRPV1 activation in colon afferent neurons. *a*, Representative records of 5-HT-produced facilitation of acid-evoked currents before (cont) and after treatment with selective 5-HT₂ or 5-HT₄ receptor antagonists. Ketanserin (KET; 10 μM), SB204070 (10 μM), or GR113808 (10 μM) were added 5 sec before application of 5-HT (1 μM). *b*, Representative records of acid-elicited currents before (cont) and after treatment with vehicle or selective 5-HT₂ or 5-HT₄ receptor agonists. DOI (1 μM), $\alpha\text{Me-5-HT}$ (1 μM), cisapride (1 μM), or 5-MeOT (1 μM) were given for 50 sec before the second acid stimulation. The data for antagonists (*c*) and agonists (*d*) are summarized as responses normalized to the amplitude of the initial proton-evoked current [$n = 6-11$; $p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs 5-HT (*c*) or vehicle (*d*); 1-way ANOVA with Bonferroni *t* test].

lipid mediators have previously been reported in response to bradykinin, ATP, nerve growth factor, and prostaglandin (Chuang et al., 2001; Tominaga et al., 2001; Bhavé et al., 2003). Although these mediators play an important role in changes attributable to injury and inflammation, the effects of 5-HT on colon sensory neurons provides a mechanism by which physiological stimuli can trigger enhanced responses in normal tissue. Mucosal stimuli trigger 5-HT release from enteroendocrine cells, which in turn activates intrinsic and extrinsic primary afferent neurons within the gut wall (Pan and Gershon, 2000; Hicks et al., 2002). Given the apparent expression of TRPV1 in nearly all C57BL/6 colon DRG neurons, and the generation of spontaneous action potentials at normal body temperature after exposure to 5-HT, the activation of peripheral afferents may contribute to sensation and even conscious perception of visceral stimuli in the uninflamed gut.

Serotonin enhanced responses to TRPV1 activation at con-

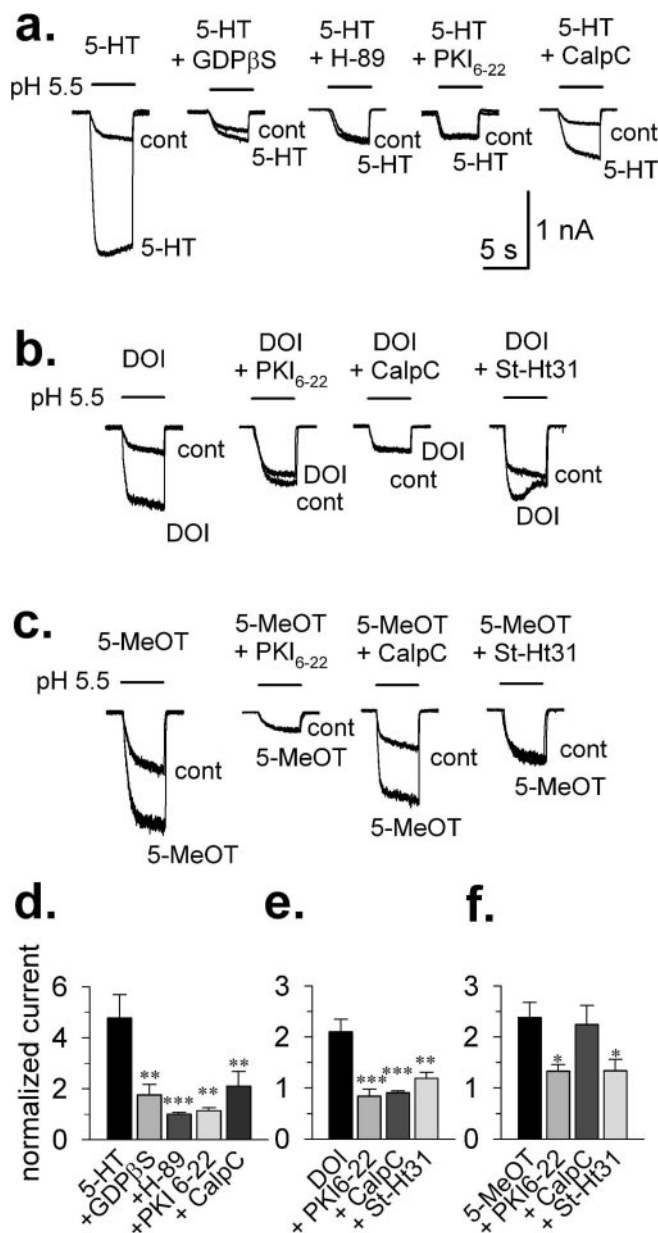


Figure 8. Intracellular signal transduction involved in serotonergic facilitation of TRPV1 activation in colon afferent neurons. *a*, Representative records of acid-elicited current before (cont) and after treatment with 5-HT in the absence or presence of inhibitors of intracellular signaling pathways. GDP- β S (GDP β S; 0.5 mM), H-89 (1 μ M), protein kinase inhibitor 6–22 amide (PKI₆₋₂₂; 5 μ M), or calphostin C (CalpC; 1 μ M) was added to the intracellular solution. *b*, *c*, Representative records of acid-evoked currents before (cont) and after treatment with DOI (*b*) or 5-MeOT (*c*) in the absence or presence of intracellular pathway inhibitors. PKI₆₋₂₂ (5 μ M), CalpC (1 μ M), or AKAP inhibitor St-Ht31 (5 μ M) was added to the intracellular solution. The data are summarized for effects on 5-HT (*d*), DOI (*e*), and 5-MeOT (*f*) ($n = 6–11$; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; 1-way ANOVA with Bonferroni *t* test).

centrations below 1 μ M and without changes in baseline current, arguing against an involvement of the ligand-gated 5-HT₃ receptor. Consistent with these results, selective 5-HT₃ receptor antagonists did not inhibit enhancement of TRPV1 function by 5-HT, revealing that activation of metabotropic 5-HT receptors facilitates TRPV1 responses. To establish this experimentally, we blocked G-protein-coupled signaling pathways by adding GDP- β -S to the pipette solution, which abolished TRPV1 modulation by 5-HT. We found that activation of both 5-HT₂ and 5-HT₄

receptors similarly enhanced TRPV1 function in colon sensory neurons. Whereas 5-HT₂ receptor agonists mimicked the facilitating action of 5-HT on TRPV1 function in response to acid, the 5-HT₄ receptor agonists cisapride and 5-MeOT had potencies lower than that of 5-HT, thus behaving only as partial agonists. The structures of these compounds and/or their receptor coupling efficiencies might explain the different potencies (Leung et al., 1996). In addition, these agonists may affect other 5-HT receptors (Hensley and Cohen, 1992; Sebben et al., 1994; Tsou et al., 1994). However, because the agonist-induced facilitation of TRPV1 function was significantly reversed by SB204070, a selective 5-HT₄ receptor antagonist, we consider their effects to represent actions at the 5-HT₄ receptor.

Phosphorylation of TRPV1 by PKA (Lopshire and Nicol, 1998; De Petrocellis et al., 2001) and/or PKC (Numazaki et al., 2002; Bhavé et al., 2003) and/or regulation by PLC (Chuang et al., 2001) are mechanisms of sensitization of TRPV1 function. Metabotropic 5-HT receptors couple to G_{q/11} or G_s proteins, and their downstream effectors PLC or adenylyl cyclase (Raymund et al., 2001). 5-HT₂ receptor activation can lead to activation of G_{q/11}, resulting in activation of PLC and PKC (Conn and Sanders-Bush, 1984; Roth et al., 1986), and 5-HT₄ receptor activation can lead to activation of G_s and an increase in cAMP levels (Boess and Martin, 1994). Using selective protein kinase inhibitors, we found that 5-HT₂ receptor activation involved not only PKA but also PKC. Similarly, the effects of 5-HT₄ activation were attenuated by inhibitors of PKA and inhibitors of the interaction between PKA binding to the scaffold protein AKAP (Rathee et al., 2002). Surprisingly, 5-HT not only shifted the proton dose-dependent curve to the left but also affected its slope. PKA (Mohanapatra and Nau, 2003) has been reported to shift the capsaicin concentration–response relationship to the left in an approximately parallel manner. The reason for this discrepancy between two different ligands (capsaicin and proton) is unclear, but it might reflect the extracellular binding domain for protons in contrast to the intracellular binding site for capsaicin. Alternatively, the difference reflects use of a heterologous expression system and a Ca²⁺-free solution as opposed to native DRG cells studied in the presence of Ca²⁺. The current observations suggest that enhancement of proton-evoked responses by 5-HT change the proton-binding kinetics to TRPV1 in colon DRG neurons.

Several signaling pathways converge on TRPV1 to modulate its activity and, as shown in this and previous studies, alter neuron excitability (Bhavé et al., 2003; Carr et al., 2003; Moriyama et al., 2003; Dai et al., 2004; Ferreira et al., 2004; Liu et al., 2004; Premkumar et al., 2004; Puntambekar et al., 2004). The potential role of TRPV1 in integrating different physical, chemical, and inflammatory signals and the comparatively high number of capsaicin-responsive colon sensory neurons support the relevance of this channel in sensation and visceral nociception. Consistent with these considerations, luminal application of capsaicin or capsaicin injection into the gut wall lowers sensory threshold or triggers pain in humans (Drewes et al., 2003; Schmulson et al., 2003; Lee et al., 2004). Interestingly, prolonged administration of red pepper, which contains capsaicin, improved symptoms in patients with functional dyspepsia (Bortolotti et al., 2002), likely because of neurotoxic effects of persistent TRPV1 stimulation that leads to transient analgesia in experimental models of neuropathic or inflammatory pain (Karai et al., 2004).

Previous studies demonstrated the presence of metabotropic 5-HT receptors in intestinal afferent neurons and suggest that only a subgroup express 5-HT₃ receptors (Hillsley and Grundy,

1998; Hicks et al., 2002). In addition to differential expression of 5-HT receptors, the onset and duration of 5-HT effects differed with stimulation of metabotropic receptors, causing a slower and longer-lasting stimulation. The modulation of TRPV1 function by 5-HT reported here may increase afferent input from the colon and thus provide a peripheral mechanism for the development of symptoms in the absence of inflammation or injury. Although the present results suggest that activation of 5-HT₂ and 5-HT₄ receptors may affect visceral sensation, the multiplicity of 5-HT receptors and their expression in gastrointestinal smooth muscle cells and intrinsic neurons complicates the interpretation of 5-HT effects (Cooke et al., 1997; Jin et al., 1999; Michel et al., 1997; Borman et al., 2002). Spinal 5-HT receptors also play important roles in descending modulation of pain, including visceral pain (Urban and Gebhart, 1999), and drugs that cross the blood brain barrier may exert both peripheral and central effects. The peripherally acting 5-HT₃ receptor antagonist alosetron blunts responses to colorectal distension in experimental animals and improves discomfort and pain in a subgroup of patients with functional bowel disorders (Camilleri et al., 1999; Kozłowski et al., 2000). Interestingly, other 5-HT₃ receptor antagonists do not consistently affect visceral pain in experimental animals or humans (Langlois et al., 1996; Kim and Camilleri, 2000). Less is known about the effects of agonists and antagonists of metabotropic 5-HT receptors on visceral sensation. The recently introduced 5-HT₄ receptor agonist tegaserod accelerates colonic transit but does not consistently affect sensory function in humans (Coffin et al., 2003; Lacy and Yu, 2002). Although a peripherally acting 5-HT₄ receptor antagonist has been reported to blunt somatic and visceral pain in mice, sensory thresholds to colorectal distension were not affected by inhibition of the 5-HT₄ receptor in humans (Espejo and Gil, 1998; Bharucha et al., 2000).

The present findings provide new information about a potential peripheral mechanism that contributes to visceral hypersensitivity. Additional studies *in vitro* and *in vivo* are needed to determine whether inhibition of TRPV1 channels or their modulation through 5-HT and/or other signaling pathways may be useful therapeutic targets in the treatment of visceral pain syndromes.

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