Combined genetic and pharmacological inhibition of TRPV1 and P2X3 attenuates colorectal hypersensitivity and afferent sensitization

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Kiyatkin ME, Feng B, Schwartz ES, Gebhart GF. Combined genetic and pharmacological inhibition of TRPV1 and P2X3 attenuates colorectal hypersensitivity and afferent sensitization. Am J Physiol Gastrointest Liver Physiol 305: G638–G648, 2013. First published August 29, 2013; doi:10.1152/ajpgi.00180.2013.—The ligand-gated channels transient receptor potential vanilloid 1 (TRPV1) and P2X3 have been reported to facilitate colorectal afferent neuron sensitization, thus contributing to organ hypersensitivity and pain. In the present study, we hypothesized that TRPV1 and P2X3 cooperate to modulate colorectal nociception and afferent sensitivity. To test this hypothesis, we employed TRPV1-P2X3 double knockout (TPDKO) mice and channel-selective pharmacological antagonists and evaluated combined channel contributions to behavioral responses to colorectal distension (CRD) and afferent fiber responses to colorectal stretch. Baseline responses to CRD were unexpectedly greater in TPDKO compared with control mice, but zymosan-produced CRD hypersensitivity was absent in TPDKO mice. Relative to control mice, proportions of mechanosensitive and -insensitive pelvic nerve afferent classes were not different in TPDKO mice. Responses of mucosal and sensoal class afferents to mechanical probing were unaffected, whereas responses of muscular (but not muscular/mucosal) afferents to stretch were significantly attenuated in TPDKO mice; sensitization of both muscular and muscular/mucosal afferents by inflammatory soup was also significantly attenuated. In pharmacological studies, the TRPV1 antagonist A889425 and P2X3 antagonist TNP-ATP, alone and in combination, applied onto stretch-sensitive afferent endings attenuated responses to stretch; combined antagonism produced greater attenuation. In the aggregate, these observations suggest that 1) genetic manipulation of TRPV1 and P2X3 leads to reduction in colorectal mechanosensation peripherally and compensatory changes and/or dis- inhibition of other channels centrally, 2) combined pharmacological antagonism produces more robust attenuation of mechanosensation peripherally than does antagonism of either channel alone, and 3) the relative importance of these channels appears to be enhanced in colorectal hypersensitivity.

pelvic nerve; purinergic receptor; single fiber; transient receptor potential; visceral pain

CHRONIC ABDOMINAL PAIN is a key feature of irritable bowel syndrome (IBS), which is prevalent, costly, and difficult to manage. An important contributor to pain in IBS is heightened perception of mechanical events in the bowel (i.e., hypersensitivity). Indeed, patients with IBS typically report greater pain and/or reduced response thresholds to rectal balloon distension than control subjects (3, 24, 28, 34, 46). Although central processes contribute to colorectal hypersensitivity, the driving force is increased afferent mechanosensitivity (i.e., sensitization). For example, intrarectal lidocaine reduces pain evoked by rectal distension in healthy subjects as well as ongoing pain and both visceral and somatic (referred) hypersensitivity in patients with IBS (24, 32, 49, 50). Accordingly, reversing or moderating afferent sensitization and hyperexcitability is a clinically important goal, and developing drugs that selectively target colorectal mechanosensation and/or sensitization would improve management of IBS pain. Targets include ion channels expressed in colorectal afferents, two of which are transient receptor potential vanilloid 1 (TRPV1) and P2X3.

TRPV1 is a capsaicin-, heat- and proton-gated ion channel expressed in the majority of colorectal afferents (8, 35). Expression of TRPV1 in colorectal afferents is increased in patients with IBS, and the magnitude of channel expression often positively correlates with the severity of sensory symptoms (1). TRPV1 knockout (KO) mice exhibit reduced responses of stretch-sensitive afferents (20) and decreased behavioral responses to colorectal distension (CRD) (19) in both naive and hypersensitive states. TRPV1 antagonists similarly decrease afferent and behavioral responses to CRD (9, 29, 31, 52).

P2X3 exists in homomeric P2X3 and heteromeric P2X2/3 configurations and is expressed in ~20% of colorectal afferents (6, 40). Both configurations are activated via pressure-dependent mucosal release of ATP (39, 54), which facilitates distension-evoked mechanosensation (54). In agreement, we found that genetic deletion of P2X3 attenuates colorectal mechanosensation and sensitization (39). Furthermore, the expression and function of P2X3 is enhanced in rodent models of IBS (40, 56).

We hypothesized that combined inhibition of these two ligand-gated channels should produce greater attenuation of mechanosensation than inhibition of either channel alone. To test this hypothesis, we utilized TRPV1-P2X3 double knockout (TPDKO) mice together with selective pharmacological antagonists to evaluate 1) channel contributions to colorectal nociception and hypersensitivity in vivo and 2) afferent mechanosensation and sensitization in vitro. Portions of these data have been previously reported in abstract form (22).

MATERIALS AND METHODS

Animals. Adult male mice (20–30 g) of the following strains were used: C57BL/6 wild-type control (Taconic, Germantown, NY) and global, nonconditional TPDKO. TPDKO mice were generated by crossing TRPV1 and P2X3 single knockout mice. All knockout mice were backcrossed onto a Taconic C57BL/6 genetic background for >10 generations. TRPV1 single knockout mice were provided by Dr. H. R. Koerber, the University of Pittsburgh. P2X3 single knockout mice were obtained from The Jackson Laboratory (Bar Harbor, ME) with the permission of Dr. D. A. Cockayne, Roche Bioscience, Palo Alto, CA. Genotypes were confirmed by PCR and Southern blot analysis. We used only homozygous TPDKO mice. Although we did

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not test for functional deletion of ion channels in double knockout mice, previous single-fiber and patch-clamp recordings in colorectal afferents from the two parent genotypes TRPV1−/− and P2X3−/− revealed complete insensitivity to the respective channel agonists capsaicin (20) and α,β-meATP (39). All protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Labeling, single-cell reverse transcriptase PCR, and calcium imaging of colorectal DRG neurons. Dorsal root ganglion (DRG) sensory neurons innervating the colorectum (c-DRGs) were labeled, collected, and processed as previously detailed (37, 40). Briefly, a laparotomy was performed on mice anesthetized with isofluorane (Hospira, Lake Forest, IL) to expose the distal colon. Three to six boluses (2–3 μl) of 2% by weight 1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine methanesulfonate (DiI; Molecular Probes, Eugene, OR) dissolved in DMSO were injected into the colon wall ~1.5 cm rostral to the anal verge. Mice were allowed 2 wk for postsurgical recovery and transport of DiI to DRG somata.

Subsequently, mice were overdosed with isofluorane followed by bilateral removal of L6-S2 (LS) DRGs, which principally contribute to the pelvic nerve (PN). DRGs were incubated at 37°C for 10 min in Hanks’ Balanced Salt solution (Sigma-Aldrich, St. Louis, MO) containing l-cysteine (5.5 mM), papain (60 Units; Worthington Biochemical, Lakewood, NJ), and saturated NaHCO3 (2 mM). Collagenase II (4,320 U; Worthington) and dispase II (14 U; Roche Diagnostics, Indianapolis, IN) were added and cells incubated for an additional 20 min before quenching enzyme activity with 10% fetal bovine serum (Sigma). Dissolved in advanced Dulbecco’s modified eagle medium/H11001 (1.5-cm length, 0.9-cm diameter) were inserted transanally 1 cm beyond the anal verge under isofluorane sedation. Mice were placed inside darkened, sound-attenuated plastic cylinders to minimize movement and stress. A 45-min postsisofluorane recovery period preceded CRD. Distension balloons were inflated for 10-s with pressurized nitrogen to 15, 30, 45, or 60 mmHg. Each pressure was tested three times with 4 min between distensions, starting at 15 mmHg (nonnoxious) and ending at 60 mmHg (noxious). Baseline VMRs were recorded 4 days after surgery (day 0), after which intracolonic treatment with either 0.1 ml of normal saline vehicle or zymosan (30 mg/ml; Sigma) was given and repeated daily for two additional consecutive days; VMRs to CRD were recorded again 1 day after the third intracolonic treatment (i.e., day 3) (39). Some TPDKO mice were killed after day 3 CRD for single fiber electrophysiology (described below).

Single-fiber electrophysiology. Mice were killed by CO2 inhalation, and the distal 2–3 cm of the colorectum was dissected out with the PN innervation intact (12). Dissection was performed in ice-cold oxygenated Krebs solution containing 4 μM nifedipine (L-type Ca2+ channel blocker to inhibit spontaneous muscle contraction; Sigma) and 3 μM indomethacin (to inhibit cyclooxygenase; Sigma). The dissected colon-nerve preparation was isolated and continually perfused with Krebs (30 mM KCl in normal bath with 100 mM NaCl to maintain osmolarity), the P2X3 agonist α,β-meATP (100 μM in normal bath; Sigma), and the TRPV1 agonist capsaicin (500 nM in normal bath and <0.01% ethanol; Sigma). All drugs were delivered via a fast-step superfusion system (Model DAD-12; ALA Scientific Instruments, Westbury, NY).

CRD. The visceromotor response (VMR) to CRD was measured to assess colorectal nociception and hypersensitivity (7). Briefly, mice were anesthetized (isofluorane), and a pair of electrodes was implanted into the abdominal musculature and exteriorized at the back of the neck for subsequent electromyographic recording of muscle activity in unanesthetized mice. Contraction in response to CRD was quantified with Spike2 software (Cambridge Electronic Design, Cambridge, UK) as electromyographic activity during distension minus predistension resting activity. Polyethylene distension balloons (1.5-cm length, 0.9-cm diameter) were inserted transanally 1 cm beyond the anal verge under isofluorane sedation. Mice were placed inside darkened, sound-attenuated plastic cylinders to minimize movement and stress. A 45-min postsisofluorane recovery period preceded CRD. Distension balloons were inflated for 10-s with pressurized nitrogen to 15, 30, 45, or 60 mmHg. Each pressure was tested three times with 4 min between distensions, starting at 15 mmHg (nonnoxious) and ending at 60 mmHg (noxious). Baseline VMRs were recorded 4 days after surgery (day 0), after which intracolonic treatment with either 0.1 ml of normal saline vehicle or zymosan (30 mg/ml; Sigma) was given and repeated daily for two additional consecutive days; VMRs to CRD were recorded again 1 day after the third intracolonic treatment (i.e., day 3) (39). Some TPDKO mice were killed after day 3 CRD for single fiber electrophysiology (described below).

Table 1. Primer pairs for PCR amplification of TRPV1 and P2X3 mRNA

<table>
<thead>
<tr>
<th>Gene (expected size)</th>
<th>External Primers</th>
<th>Internal Primers</th>
<th>Genebank Number</th>
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<tr>
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<td>NM_001001445.1</td>
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<tr>
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<tr>
<td>(251, 141 bp)</td>
<td>CTGCTGCTGCTGCTGCTGCTGCTG</td>
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Single-cell PCR was performed in dissociated colorectal afferent somata from L6-S2 dorsal root ganglia (LS DRGs). A nested PCR strategy with external and internal cDNA primer pairs was used to amplify reverse-transcribed transient receptor potential vanilloid 1 (TRPV1) and P2X3 mRNA. Sequences for cDNA primers are indicated with the 5′ end on the left. The Genebank reference used for designing primers is indicated at the right. bp, base pairs.
was threaded into a separate oil-filled recording chamber and progressively teased apart into 6–10 bundles (10-μm thick) to isolate single fibers (>3:1 signal-to-noise ratio). Recordings were made by laying bundles atop a platinum-iridium wire extracellular electrode ~100 μm in diameter. Neural activity was amplified (10,000-fold; DAM80; World Precision Instruments, New Haven, CT), filtered (0.3–10 kHz), and sampled (20 kHz) using a 1401 interface (CED) and Spike2.

An electrical search strategy was used for unbiased detection of all excitable afferent receptive endings (REs) and measurement of their electrical activation thresholds (12). All REs were tested for mechanosensitivity as follows: mucosal stroking with a fine brush producing ~0.1 mN of perpendicular force; blunt perpendicular probing (1–80 mN; 5-s duration) and uniform circumferential stretch [0–170 mN, equivalent to 45 mmHg CRD (11, 12), applied as a ramp (5 mN/s, 34 s) or fast step (to 80 or 170 mN in 0.2 s)]. Probing and stretching were performed using a servo-controlled force actuator (Aurora Scientific, Toronto, ON, Canada). Colorectal PN afferents were classified as previously described (12). Briefly, all REs responded to blunt probing except mechanically insensitive afferents (MIs). Muscular afferents also responded to stretch, mucosal afferents also to stroking, and muscular/mucosal afferents also to stretch and stroking. Serosal afferents responded only to probing. On average, 1–6 fibers were studied per mouse.

Agonists, antagonists, and a sensitizing inflammatory soup (IS) were applied directly atop isolated REs (11). IS was composed of bradykinin, prostaglandin E2, serotonin, and histamine (all at 10 μM) with pH adjusted to 6.0 (20). IS was applied for 3 min followed by mechanical testing 3 min later and then every 5 min until washout (i.e., return to baseline). Channel antagonists were applied for 5 min followed immediately by mechanical testing repeated every 5 min until washout. In our hands, a 5-min intertest interval is sufficient for full recovery of fiber response to mechanical stimulation. To inhibit TRPV1, we utilized A889425 (Abbott Laboratories, Abbott Park, IL), a highly selective competitive antagonist for the capsaicin-binding site with an IC50 of ~300 nM (5, 27). A889425 was dissolved in 1-methyl-2-pyrrolidinone (1M2P) and diluted to a final concentration of 0.3 μM (100, 300 nM). In contrast, only a minority of c-DRGs responded to αβ-meaTP, but the majority of these neurons also responded to capsaicin (500 nM). A total of 14 LS DRG sensory neurons innervating the colorectum (c-DRG) including 1 control from 1 preparation were tested for mRNA expression. A total of 31 LS c-DRGs from 2 preparations was examined for Ca2+ transient responses to αβ-meaTP (100 μM) followed by capsaicin (500 nM). For Ca2+ imaging, only cells that initially responded to 30 mM KCl were treated with agonists.

Response to CRD. C57BL/6 and TPDKO mice responded similarly to CRD (Fig. 1A). However, responses were statistically greater in TPDKO mice at noxious intensities of CRD (i.e., 45 and 60 mmHg). Intracolonic treatment with saline had no effect on responses to CRD in either genotype, whereas treatment with zymosan elicited significant colorectal hypersensitivity in C57BL/6 (as previously reported; e.g., Ref. 14) but not TPDKO mice (Fig. 1, B and C). Statistical details are in the figure legend.

Characterization of colorectal afferents. To explore the contributions of PN afferents to the CRD phenotype and to study in detail the effects of combined ablation of TRPV1 and P2X3 on colorectal afferent mechanosensation, we compared classes of colorectal PN afferents in C57BL/6 and TPDKO mice. Neither the proportions nor the electrical activation thresholds of the five classes of PN afferents differed between the two genotypes (Fig. 2). However, relative to C57BL/6 mice, there was a tendency in TPDKO mice for mucosal and muscular/mucosal afferent REs to be topographically shifted to and concentrated in the distal 1 cm of the colorectum (10/18 mucosal and 15/32 mucosal/mucosal REs in C57BL/6 mice vs. 7/10 mucosal and 13/17 muscular/mucosal REs in TPDKO mice). There were no other differences in topographical distributions of PN afferents between genotypes.

Serosal and mucosal REs in both genotypes gave similar responses to probing (Fig. 3, A and B). Likewise, there was no difference in response to probing between stretch-sensitive muscular and muscular/mucosal afferents (Fig. 3, C and D). As noted previously (e.g., Ref. 12), responses of muscular afferents to stretch in C57BL/6 mice were generally more robust than responses of their muscular/mucosal afferent counterparts. In TPDKO mice, responses of muscular (Fig. 4A), but not muscular/mucosal afferents (Fig. 4B), to stretch were significantly attenuated relative to C57BL/6 mice. Responses to the fast-step stretch protocol did not reveal any genotype differences between muscular and muscular/mucosal afferents (Fig. 4, C and D).

We next examined IS-induced sensitization of stretch-sensitive afferents (stretch-insensitive mucosal and serosal afferents...
ents do not sensitize). IS significantly increased stretch-response functions and decreased response thresholds (i.e., sensitized) of muscular as well as muscular/mucosal afferents in both C57BL/6 and TPDKO mice (Fig. 5). However, both afferent classes sensitized to a significantly lesser degree in TPDKO mice. All fibers tested, regardless of genotype, were also activated by IS, revealing RE chemosensitivity. Recovery (washout) was typically complete in both genotypes within ~20 min after IS.

Single-fiber recordings were also performed in TPDKO mice treated intracolonically with saline (36 fibers from 4 mice) or zymosan (26 fibers from 5 mice). Importantly, none of these mice showed any hypersensitivity in CRD experiments. There were no significant differences between saline- and zymosan-treated mice with respect to either proportions of colorectal afferent classes or the response thresholds or stretch-response functions of stretch-sensitive afferents. This is consistent with the absence of colorectal hypersensitivity in these mice (Fig. 1C) as well as the decreased IS-induced afferent sensitization in naive TPDKO mice (Fig. 5).

Pharmacological antagonism of TRPV1 and P2X3. To pharmacologically validate the TPDKO phenotype and better interpret channel interaction, we applied TRPV1 and/or P2X3 antagonists onto colorectal afferent REs in C57BL/6 mice, focusing on afferent responses to stretch, the stimulus most affected in TPDKO mice and most relevant for colorectal mechanosensation in vivo. Responses to stretch of 34 muscular and 28 muscular/mucosal afferents were examined before (baseline) and after vehicle (Krebs for TNP-ATP and 1M2P in concentrations of 0.01, 0.1, 1.0, and 10.0% for A889425) and incremental antagonist concentrations around their reported IC50s (100 nM and 300 nM and 1, 3, and 10 μM for A889425; 30 nM and 300 nM and 1 and 3 μM for TNP-ATP) to determine effective concentrations. Most REs were exposed to both vehicles and to both antagonists during testing, but not to all concentrations of vehicles or antagonists.

The efficacy of the TRPV1 antagonist A889425 varied considerably and its effects on responses of stretch-sensitive afferents were not apparent at all concentrations tested; 300 nM was most effective in reducing responses of muscular afferents to stretch, whereas 3 μM was most effective in reducing responses of muscular/mucosal afferents (Fig. 6, A and B). Because of intrinsic variability among colorectal afferents and consequent variability in their responses to stretch after exposure to vehicle and because TRPV1 is not expressed in all colorectal afferents (Table 2), we set as an effect criterion a ≥15% posttreatment reduction in afferent response to stretch. Using this criterion (denoted by >15 in Figs. 6 and 7), ~65% of both muscular (9/14) and muscular/mucosal (8/12) afferents exhibited a significant reduction in their response to stretch.

Fig. 1. Visceromotor responses to colorectal distension (CRD). A: relative to C57BL/6 controls, transient receptor potential vanilloid 1 (TRPV1)-P2X3 double knockout (TPDKO) mice exhibited greater baseline responses to CRD (F1/14 = 5.8, *P < 0.05; Holms-Sidak post hoc tests, P < 0.01 at 45 and 60 mmHg CRD). B: intracolonic zymosan, but not saline, produced colorectal hypersensitivity in C57BL/6 mice (F1/24 = 6.4, P < 0.05), but not in TPDKO mice. C: visceromotor responses (not normalized) were used to generate stimulus-response curves in A; normalized responses were used for B and C. n = number of mice/group.

Fig. 2. Codeletion of TRPV1 and P2X3 (i.e., TPDKO) had no effect on proportions (A) or electrical activation thresholds (B) of pelvic nerve colorectal afferents. A total 116 fibers was studied from 23 C57BL/6 mice and 102 fibers from 24 TPDKO mice. musc, muscular; mucos, mucosal; M/M, muscular/mucosal; MIA, mechanically insensitive afferent.
after treatment with 300 nM and 3 μM A889425, respectively. To pharmacologically probe TRPV1 expression in these experiments, we applied capsaicin (3 μM) to eight of the above stretch-sensitive afferents after multiple exposures to A889425. Although washout after A889425 was apparent within ~10 min (i.e., responses to stretch returned to baseline) in the majority of afferents tested, application of capsaicin to REs excited only 2/8 afferents tested (both muscular); however, capsaicin increased response threshold in 6/8 afferents (1/2 muscular/mucosal and 5/6 muscular). In contrast, response thresholds were unaffected by A889425, with or without the effect criterion invoked (Fig. 6, C and D).

Compared with vehicle (Krebs solution), 30–300 nM, 1 and 3 μM concentrations of the P2X antagonist TNP-ATP did not attenuate responses of grouped muscular or muscular/mucosal afferents to stretch (Fig. 7, A and B). Applying the same effect...
criterion as above, ~35% of both muscular and muscular/mucosal afferents demonstrated a ≥15% decrease in their stimulus-response functions after exposure to 3 μM and 300 nM TNP-ATP, respectively. At these concentrations, TNP-ATP significantly attenuated responses to stretch in both muscular and muscular/mucosal afferents. As above with capsaicin, we attempted to pharmacologically establish P2X3 and P2X2/3 expression by applying α,β-meATP to REs after multiple exposures to TNP-ATP; 3/10 REs tested were excited (1/5 muscular/mucosal and 2/5 muscular), whereas 5/10 REs exhibited an increase in response to stretch (1/5 muscular/mucosal and 4/5 muscular). In contrast to the TRPV1 antagonist, TNP-ATP significantly increased response thresholds of muscular/mucosal afferents (Fig. 7D). Antagonist washout was apparent within ~10 min in all muscular and most muscular/mucosal afferents tested.
In another group of 11 stretch-sensitive afferent endings, we tested combined pharmacological antagonism with A889425 plus ATP-TNP at the most effective concentrations determined above (300 nM and 3 μM) and also applied the TRPV1 and P2X3 agonists capsaicin (3 μM) and α,β-meATP (3 mM), respectively. Four out of eleven afferents responded to both agonists (3/6 muscular/mucosal and 1/5 muscular afferents). Of those four afferents, two were activated directly by capsaicin; the other two exhibited increased responses to stretch by 37 ± 109%. Similarly, α,β-meATP activated two afferents directly and sensitized the other two (51 ± 13% increase in response to stretch). Five of the eleven afferents responded to neither agonist; responses to stretch were not changed after application of capsaicin (−3.8 ± 5.1%) or α,β-meATP (−4.0 ± 6.1%). The remaining 2/11 responded only to α,β-meATP (1/6 muscular/mucosal and 1/5 muscular afferents). Importantly, no afferents responded to capsaicin alone, indicating 100% coexpression of TRPV1 with P2X3 and/or P2X2/3. Interestingly, afferents responding to both capsaicin and α,β-meATP had significantly higher response thresholds to stretch than those responding to either agonist (71.5 ± 9.1 vs. 21.1 ± 1.6 mN, t-test, P < 0.001). Figure 8 illustrates that combined antagonist application did not affect responses to stretch in those five afferents that did not respond to either agonist. Co-antagonism with A889425 and TNP-ATP, however, significantly attenuated responses to stretch in those four afferents that responded to both agonists.

**DISCUSSION**

In agreement with previous studies in rat lumbar DRG neurons (16, 41, 48), the present work demonstrates that a substantial proportion of mouse PN colorectal afferent somata coexpress TRPV1 with P2X3. Previously, we reported deficits in colorectal mechanosensation and sensitization with single deletion of either TRPV1 or P2X3 (19, 20, 39). Given the coexpression reported here and elsewhere, we expected similar, if not greater, effects in TRPV1-P2X3 double knockout mice. As expected, colorectal hypersensitivity (i.e., enhanced VMR to balloon distension) did not develop in TPDKO mice. Unexpectedly, basal VMRs to CRD were greater in TPDKO than C57BL/6 mice despite significantly reduced responses of muscular afferents to stretch and unchanged responses of other afferent classes in TPDKO mice.

There are a number of possible explanations for the greater basal VMRs to CRD noted in TPDKO relative to C57BL/6 mice. One possibility is disinhibition or compensatory overexpression of ion channels such as TRPV4, TRPA1, or homomeric P2X2 in the peripheral and/or central terminals of primary afferents or in second-order spinal neurons. Of the two channels studied, P2X3 may be most contributory to enhanced VMRs in TPDKO mice because P2X3 single knockout mice have been reported to exhibit pronociceptive behaviors, including enhanced avoidance of noxious thermal stimuli (38). An alternate explanation for increased basal VMR is the redistribution we noted in TPDKO mice of mucosal and muscular/mucosal afferent REs to the distal 1 cm of the colorectum. In our experience, greater VMRs to CRD are evoked with more distal positioning of the distension balloon. This is due, in part, to greater relative excitability of mouse PN afferent endings in the more muscular, distal ~1 cm of the colorectum (11), a finding also noted in guinea pig rectum (26). Because the proportions of each class of afferent were the same in both genotypes, this caudal transposition in TPDKO mice was unlikely a result of conversion of one class of afferent into another. In support, there were no significant differences between genotypes with respect to probing-response functions of any type of afferent. In other words, the same length, equally positioned CRD balloon in TPDKO mice may stimulate a greater number and proportion of lower threshold and/or more excitable distal PN afferent endings, which may allow central...
sumption. This, of course, in no way discounts greater VMRs and increased inhibitory rectovesicular reflexes (53) with longer-length CRD balloons.

Codetection of TRPV1 and P2X3 reduced responses of muscular afferents to stretch but had no effect on muscular/mucosal afferents. Responses of muscular and muscular/mucosal afferents to stretch in P2X3-null mice did not differ from responses in C57BL/6 mice (39). In contrast, responses of muscular/mucosal afferents to stretch in mice lacking TRPV1 were significantly attenuated relative to C57BL/6 mice; responses of muscular afferents were unexpectedly approximately twice those of control although not statistically significant (20). Collectively, it appears that deletion of either TRPV1 or P2X3 alone does not alter net peripheral input from PN afferents in response to stretch, whereas codetection of both channels results in a net decrease in peripheral input from stretch-sensitive afferents.

The TRPV1 antagonist A889425 significantly attenuated responses of muscular and muscular/mucosal afferents to stretch in C57BL/6 mice. Consistent with previous (8) and current (Table 2) reports of TRPV1 expression in 60–70% of mouse colorectal afferents, A889425 was effective in ~65% of stretch-sensitive afferents, and capsaicin treatment increased response thresholds (i.e., desensitized) in ~75% of this same afferent population. The latter capsaicin-induced desensitization is a well-recognized phenomenon noted in both human patients and rodents (2, 6). It is unclear why capsaicin reduced responses to stretch in some fibers while it mechanically sensitized or activated others. This could be speculated to be dependent on different pretreatment cellular contents and states of individual afferents. It is also unclear why the dose-response curves for A889425 differed between muscular and muscular/mucosal afferents. It is certainly possible that expression of TRPV1 is more robust in one class of afferent than it is in the other. It is difficult to quantify this given the inability to distinguish muscular vs. muscular/mucosal afferents with more quantitative techniques such as patch-clamp electrophysiology in dissociated colorectal afferent somata.

TNP-ATP was not as broadly effective as A889425 on stretch-sensitive afferents, consistent with the expression of P2X channels in c-DRG somata. Fewer afferent responses met the effect criterion (≥15% reduction) when exposed to TNP-ATP, but those that did (33% of the afferents tested) exhibited attenuated responses to stretch. This correlates well with the proportions of c-DRG somata expressing P2X3 reported here (Table 2) and elsewhere (6, 40). TNP-ATP additionally increased response thresholds in muscular/mucosal afferents, supporting the view that P2X3 and/or P2X2/3 are important primarily for the initiation of nociception (18) and suggesting a greater contribution to mechanosensation by these channels in muscular/mucosal vs. muscular afferents. It should be appreciated that TNP-ATP is not selective for P2X3 and can act as a competitive antagonist at P2X1, P2X3, and P2X2/3 receptors. In previous work (40), we found evidence for P2X3 homomer (33%) and P2X2/3 heteromer (20%) expression in c-DRG neurons; <5% of all c-DRG neurons exhibited P2X2 homomer expression. These data were supported by electrophysiological (whole cell patch clamp) evidence; no P2X1-type inward currents were noted. Thus, although TNP-ATP is not P2X3 selective, the virtual absence of P2X1 receptors in mouse c-DRG neurons limits the activity of TNP-ATP to c-DRG neurons expressing P2X3 receptors, either in homomer or heteromer configurations.

With regard to the effect criterion used to define treatment effect, we chose a ≥15% change from pretreatment response or threshold because in our hands this was greater than the inherent response variability with the same, repeated stimulus in the same fiber. This percent change was also greater than the inherent variability noted after application of vehicle. Supporting use of this criterion as well as antagonist selectivity, in single antagonist/agonist experiments, of the 6/8 stretch-sensitive fibers demonstrating a ≥15% capsaicin-induced increase in response threshold, five of the six fibers exhibited a ≥15% A889425-induced decrease in response to stretch. Likewise, of the 5/10 stretch-sensitive fibers that showed a ≥15% α,β-meATP-induced increase in stimulus response, 2/5 exhibited a ≥15% TNP-ATP-induced decrease in stimulus response or a ≥15% increase in response threshold. The reasons for incomplete association between agonist-antagonist-induced changes in afferent responses to stretch are unclear but may reflect inadequate washout of antagonists before application of agonists.
Combined antagonism of TRPV1 and P2X3 significantly attenuated responses to stretch in colorectal afferents that responded to both capsaicin and α,β-meATP but had no effect in afferents that did not respond to either agonist. The results suggest an additive, if not greater than additive, attenuation of afferent response to stretch by simultaneous antagonism of both channels. This may be secondary to a combined effect of two independent processes, or it may indicate an interaction that could be mediated through shared Ca\(^{2+}\)-dependent signaling cascades (10, 21, 33, 36, 47) or modulatory conformational spread (4) via direct physical association of TRPV1 and P2X3 as demonstrated previously (41). Importantly, this combined antagonist/agonist experiment additionally confers pharmacological validity to the selectivity of the drugs used (e.g., TNP-ATP has effects only on REs that respond to α,β-meATP). Why both antagonists alone or in combination attenuated muscular/mucosal as well as muscular afferents while combined genetic deletion of TRPV1 and P2X3 affected only muscular fibers is unclear. This may be secondary to dis inhibition, recruitment, or compensatory overexpression of other ion channels as discussed above. It is not uncommon for discrepancies to exist between knockout and pharmacological models, and, for this reason, the effects of single vs. combined channel manipulation are compared within rather than across experimental models.

To further explore the contributions of TRPV1 and P2X3 to colorectal afferent mechanosensation, we evaluated in TPDKO mice acute sensitization of stretch-sensitive afferents by IS. In both TRPV1- and P2X3-null mice, IS sensitized muscular/mucosal but not muscular afferent endings (20, 39). In TPDKO mice, however, both afferent classes were sensitized by IS although the magnitude of sensitization was significantly reduced relative to sensitization in C57BL/6 mice. Differences in outcomes between single and double knockout mice may be explained by compensatory changes in protein expression as noted above. In muscular/mucosal afferents, sensitization may cause upregulation or enhanced functioning of TRPV1 and P2X3 (17) as well as other ion channels. Therefore, if only one channel is deleted, functioning of the remaining channels may be sufficiently enhanced when sensitized such that no loss-of-function phenotype is apparent. Only when both channels are eliminated is a reduction in afferent responses to stretch evident, and even then some sensitization is still present. In muscular afferents, on the other hand, deletion of either or both TRPV1 and P2X3 is sufficient to produce a loss-of-function phenotype, suggesting that both channels are critical and/or redundant for mechanical sensitization in this afferent class.

Although we collected only limited single-fiber data in zymosan-treated TPDKO mice, there was no significant sensitization of stretch-sensitive afferents, consistent with the absence of CRD hypersensitivity and attenuated IS-induced sensitization. This is in contrast to C57BL/6 mice, which exhibit significant zymosan-induced CRD hypersensitivity and sensitization of muscular/mucosal afferents (14). Moreover, consistent with previous work in C57BL/6 mice (13, 14, 43), IS treatment in TPDKO mice produced a greater magnitude of sensitization of stretch-sensitive afferents than did zymosan treatment. This may be explained by the concentration of several inflammatory mediators in IS, which was designed to maximally sensitize afferents. Therefore, the persistence of IS-induced afferent sensitization in TPDKO mice is not inconsistent with the absence of behavioral and afferent sensitization in these same mice with milder, more physiological zymosan sensitization.

We made several additional notable observations. First, responses of muscular afferents to ramped stretch were significantly attenuated in TPDKO mice, whereas responses to stepped stretch or probing were not. This suggests a stimulus-specific contribution of TRPV1 and/or P2X3 or P2X2/3 to afferent mechanosensation. It is possible that only slow ramps or repeated steps of increasing stimulus intensity [as performed in our previous studies (20, 39)] are sufficient to recruit these channels because these stimuli allow sufficient time for biochemical generation of ATP and endogenous lipid ligands of TRPV1. Second, TRPV1 and P2X3 appear to be more important for mechanosensitivity/sensitization in muscular/mucosal afferents than in other colorectal afferent classes. Coincidently, zymosan-induced colorectal hypersensitivity to CRD is associated with sensitization of muscular/mucosal afferents (14). Thus TRPV1 and P2X3 may be important in the development and/or maintenance of zymosan-induced colorectal hypersensitivity and, by extension, IBS pain. Third, deletion of both channels abolished zymosan-induced recruitment of MIAs, which also has been observed in C57BL/6 mice (14). Considering that very few colorectal PN MIAs respond to capsaicin (12), our observation in TPDKO mice suggests that P2X3 may be important for mechanical sensitization of MIAs.

In summary, the present study confirms the importance of TRPV1 and P2X3 for colorectal mechanosensation and hypersensitivity at the levels of the primary afferent as well as the whole organism. By evaluating inhibition of both channels simultaneously, this work also contributes to the growing appreciation of the interaction and cooperation between distinct ion channels, which is not uncommon [e.g., TRPV1-TRPA1 (42) and P2X3-GABAA (44)] and may prove important for guiding drug development. With regard to limitations, the single-fiber work focused exclusively on PN colorectal afferents to build on previous studies in this same afferent population (19, 20, 39). Although the PN is necessary and sufficient for mediating colorectal nociception (23), the other source of extrinsic colorectal innervation, the lumbar splanchnic nerve (LuSN), was not evaluated here. Because the LuSN pathway may be relevant for chemosensation (6) and/or central sensitization (45), a TRPV1-P2X3 interaction in LuSN afferents or thoracolumbar spinal cord may reveal unexpected contributions to mechanosensation. For example, it has been reported that activity in PN colorectal afferents actively modulates thoracolumbar dorsal horn neuron processing of the same distending colorectal stimulus through a supraspinal loop (51). Indeed, we previously concluded that P2X3 contributed to colorectal hypersensitivity at both peripheral and central sites (39). Because increased afferent mechanosensitivity is considered key to IBS, we focused here on the TRPV1-P2X3 interaction in PN afferents, but afferent input has obvious central implications that were not experimentally addressed here. The results in TPDKO mice are generally supportive of previous studies in single knockout mice as well as pharmacological antagonism. Differences in outcomes between single and double knockout mice could be due to functional redundancy of TRPV1 and P2X3 in some afferents and compensation in others. The relative importance of these channels appears to be enhanced in hypersensitivity, suggesting their importance in colorectal mechanosensation and potential combined pharmacological...
antagonism as a strategy for improving treatment for IBS pain and hypersensitivity.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


