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The ion channel TRPA1 is required for normal mechanosensation and is modulated by algescic stimuli

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Abstract

Background & Aims—The transient receptor potential (TRP) channel family includes transducers of mechanical and chemical stimuli for visceral sensory neurons. TRPA1 is implicated in inflammatory pain; it interacts with G-protein-coupled receptors, but little is known about its role in the gastrointestinal (GI) tract. Sensory information from the GI tract is conducted via 5 afferent subtypes along 3 pathways.

Methods—Nodose and dorsal root ganglia (DRG) whose neurons innervate 3 different regions of the GI tract were analyzed from wild-type and TRPA1^{−/−} mice using quantitative RT-PCR, retrograde labeling, and in situ hybridization. Distal colon sections were analyzed by immunohistochemistry. In vitro electrophysiology and pharmacology studies were performed and colorectal distension and visceromotor responses were measured. Colitis was induced by administration of trinitrobenzene sulphonic acid.

Results—TRPA1 is required for normal mechano- and chemosensory function in specific subsets of vagal, splanchnic and pelvic afferents. The behavioral responses to noxious colonic distension were substantially reduced in TRPA1^{−/−} mice. TRPA1 agonists caused mechanical hypersensitivity, which increased in mice with colitis. Colonic afferents were activated by bradykinin and capsaicin, which mimic effects of tissue damage; wild-type and TRPA1^{−/−} mice had similar direct responses to these 2 stimuli. After activation by bradykinin, wild-type afferents had increased mechanosensitivity whereas after capsaicin exposure, mechanosensitivity was reduced—these

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SMB, PAH, AJP, CMM, TOD, NJC, AJH, BA TL and GH acquired and analysed data and designed studies. KYK and DPC created and provided the TRPA1 knockout mouse. GYR provided intellectual input and LAB wrote the manuscript

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changes were absent in TRPA1^{-/-} mice. No interaction between protease receptor (2) activation and TRPA1 was evident.

Conclusion—These findings demonstrate a previously unrecognized role for TRPA1 in normal and inflamed mechanosensory function and nociception within the viscera.

Introduction

Chronic pain and discomfort in functional gastrointestinal disorders represent a major unmet need for treatment and consequent economic impact. A hallmark of these disorders is allodynia and hyperalgesia to mechanical events¹⁻³, and low-grade inflammatory status⁴⁻⁶. Therefore, therapies are needed that reduce signaling of nociceptive mechanosensory and inflammatory events. Normally, there is a constant stream of subliminal information from the gut, which is involved in autonomic reflexes controlling motor and secretory function. It is therefore important to distinguish this information from nociceptive signals in targeting visceral pain.

Gastrointestinal sensory nerves follow three main pathways to the central nervous system - the vagal, splanchnic and pelvic nerves. Vagal afferent fibers have neuronal cell bodies in the nodose and jugular ganglia, whilst the splanchnic and pelvic innervations have cell bodies in spinal thoracolumbar and lumbosacral dorsal root ganglia (DRG) respectively. Sensory afferent fibers within these pathways can be classified into 5 subtypes in the mouse gastrointestinal tract according to the location of their mechanoreceptive fields^{7, 8}. These are: mucosal, muscular (or tension receptor), muscular-mucosal, serosal and mesenteric afferents. Mucosal afferents respond exclusively to fine tactile stimulation of the luminal surface. All others respond to distension, either at physiologic levels (muscular afferents) or noxious levels (serosal and mesenteric afferents). Muscular-mucosal afferents respond to both types of stimulus at low thresholds⁷⁻¹¹. In general, vagal pathways are typically associated with sensations such as satiety and nausea, whereas spinal pathways also innervate pelvic viscera and are associated with sensations of pain, discomfort, bloating, and urgency to void¹². The pelvic pathway contains both non-nociceptive and nociceptive afferents, whilst splanchnic afferents have generally higher mechanical thresholds, with fewer mucosal and muscular afferents, constituting primarily a nociceptive pathway⁷. The reason why these different types of afferents signal different mechanical events is firstly because they end in different layers of the gut wall, and secondly because they utilise different mechanosensory ion channels.

It was recently shown that the transient receptor potential (TRP) channel TRPV4 is required exclusively for mechanotransduction by high-threshold colonic afferents⁹, for their responses to inflammatory proteases¹³, and for visceral pain behavior^{9, 14}. TRPV4 does not account for all aspects of visceral pain, since there are residual mechanonociceptive responses in TRPV4 knockout mice, and other inflammatory stimuli unlikely to act via TRPV4. Interest has therefore broadened to other TRPs such as TRPA1, which is expressed in sensory neurons, primarily in subsets expressing the nociceptive marker TRPV1¹⁵⁻¹⁸. TRPA1 is activated by noxious cold, pungent natural chemicals including mustard oil¹⁹, and by several environmental irritants¹⁷. Numerous endogenous pro-algesic factors interact directly or indirectly with TRPA1 to augment inflammatory pain, such as bradykinin via B₂ receptor activation¹⁹. Conversely, TRPA1 can undergo functional desensitization through modulation by the classical capsaicin receptor TRPV1²⁰.

TRPA1^{-/-} mice are deficient in behavioral responses to noxious cold, and cutaneous mechanical and chemical stimuli^{17, 18}. TRPA1 contributes to the development of hyperalgesia in numerous inflammatory models^{17, 18, 21}. Many of these conclusions are based on indirect evidence from behavioral models, recombinant systems and isolated neurons. Knowledge is currently lacking on the function of TRPA1 specifically within sensory afferent nerve fiber

endings in their native tissue, and on the roles of TRPA1 in mechanosensation and chemosensation in viscera.

We hypothesized that TRPA1 contributes to mechanosensory function in visceral afferent endings, and underlies alteration of mechanosensory function by algescic stimuli. We show enrichment of TRPA1 in visceral afferents, and correspondingly, alteration of mechanosensory function in specific classes of visceral afferents after disrupting TRPA1, which translate to sensory deficits in whole animals. We also demonstrate that activation of TRPA1 by specific agonists induces mechanical hypersensitivity in these specific afferent subtypes, and that this is exacerbated in inflammatory conditions. We determined the role of TRPA1 in visceral afferent responses to activation of bradykinin, capsaicin, and protease receptors, and its role in altered mechanosensory function after activation of these receptors. Our data indicate that TRPA1 is critical in the viscera for normal mechanosensory function, the signaling of noxious mechanical stimuli and the alteration of mechanical responsiveness of visceral afferent endings by algescic chemical stimuli.

Materials and Methods

All experiments were performed with approval of the Animal Ethics Committees of the Institute for Medical and Veterinary Science and the University of Adelaide, Adelaide, Australia.

Targeted deletion of TRPA1

Mice with disruption to the TRPA1 gene were generated by homologous recombination on a C57/BL6 background as we have described previously in detail¹⁸. Subsequently separate lines of knockout and wild-type mice were bred and maintained, and animals of both sexes used for experiments at 12–16 weeks old.

Quantitative RT-PCR

Nodose ganglia and DRG (T10-L1 and L6-S1), corresponding to the 3 different innervations of the gut were removed from TRPA1^{+/+} and ^{-/-} mice. RNA was isolated from these 3 groups of whole ganglia and QRT-PCR performed using methods described elsewhere²² with specific primers for TRPA1 and a range of other channels and receptors (Supplementary Table 1). Each assay was run in at least triplicate in separate experiments. Control PCRs were performed by substituting RNA template with distilled RNase-free water or by omitting the RT step. The comparative cycle threshold method was used to quantify the abundance of target transcripts in whole DRG from TRPA1^{+/+} or ^{-/-} mice as previously described²². Quantitative data are expressed as mean $\pm$ SD, and significant differences in transcript expression determined by a Mann-Whitney test.

Retrograde labeling and fluorescent in situ hybridization

As described previously^{9, 22}, the fluorescent retrograde neuronal tracer cholera toxin subunit B conjugated to fluorescein isothiocyanate (CTB-FITC) was injected at several sites subserosally and within the muscle layers of the descending colon or proximal stomach. After three days animals were perfuse-fixed and nodose ganglia or DRG (T10-L1 and L6-S1) removed, and sections (12 μ m) cut and post fixed. Digoxigenin-labeled oligonucleotide probe anti-sense to 1571–1618 of murine TRPA1 mRNA was used to target TRPA1. Complementary sense probe was used as a negative control revealing no labeling above background. Digoxigenin was detected using CARD amplification (Perkin-Elmer, USA) combined with streptavidin-conjugated AlexaFluor 546 (SAF546) (Invitrogen, USA). Only cells with intact nuclei were included; data are expressed as % of neurons in the DRG or nodose section in 4–

8 DRG or nodose sections per mouse averaged across 5–6 mice. Unpaired t-tests were used to determine differences.

Immunohistochemistry

Distal colon was removed from mice after transcardial perfusion with 4% paraformaldehyde. Either transverse sections (20 μ m) of whole colon or wholemounts of mucosa-stripped preparations were examined. A goat anti calcitonin gene-related peptide (CGRP 1/200; Abcam, #ab36001) was used overnight at 4°C for sections or 37°C for wholemounts⁹. A rabbit anti-TRPA1 (1/1000; Abcam, #AB58844) was used under the same conditions. Secondary antibodies coupled to AlexaFluor® 488 and AlexaFluor® 546 were used for visualization. Negative controls were prepared as above with the primary antibody omitted or in tissue from TRPA1^{-/-} mice.

In vitro electrophysiology and pharmacology

Recordings of vagal, splanchnic and pelvic afferents were made from TRPA1^{+/+} and ^{-/-} mutant mice using standard protocols (⁷ and supplementary information).

Inflammatory model

Trinitrobenzene-sulphonic acid (TNBS, 0.1mL 130 μ L/mL 30% ethanol) was administered intracolonicallly to TRPA1^{+/+} mice¹⁰. The mice were allowed to recover for 7 days before the colon and attached splanchnic nerves were removed and used for *in vitro* electrophysiological experiments. Histological assessment indicated ulceration, crypt destruction, infiltration and edema in colon, as reported previously¹⁰.

Colorectal distension and visceromotor response

To record the visceromotor response to colorectal distension, electromyographic electrodes were surgically implanted in the abdominal musculature (⁹ and supplementary information). Each distension lasted 10sec, and was tested ten times, with 30sec separating each distension.

Results

TRPA1 is expressed in visceral afferent pathways

We used QRT-PCR to determine expression of TRPA1 in extrinsic gastrointestinal sensory neurons - in nodose ganglia, and from two different levels of DRG, specifically the thoracolumbar (T10-L1) and lumbosacral DRG (L6-S1), corresponding to the vagal innervation of the gastroesophageal region and to the splanchnic and pelvic innervations of the colon. TRPA1 transcript expression was detected in all three groups of ganglia from TRPA1^{+/+} mice, whilst TRPA1 transcripts were absent from TRPA1^{-/-} (Figure 1A). Quantitative analysis indicated similar levels of TRPA1 expression between the different sensory ganglia (Figure 1C). However, the innervation of the gut represents <5% of neurons in these ganglia. To specifically identify gut-projecting neurons we injected tracer (CTB-FITC)²², which is retrogradely transported from afferent endings to the cell body. Vagal neurons in nodose ganglia (Figure 1Bi) were identified by tracer injected into the wall of the stomach. Neurons in the thoracolumbar and lumbosacral DRG (Figure 1Bii & iii) were identified by colonic injections. Combined retrograde tracing/fluorescence *in-situ* hybridization allowed us to compare TRPA1 expression in gut-projecting and unlabeled neurons. TRPA1 was expressed in 36 $\pm$ 2 % of unlabeled neurons in nodose ganglia, 42 $\pm$ 2 % in the thoracolumbar DRG and 40 $\pm$ 2% in the lumbosacral DRG. Immunohistochemistry for TRPA1 protein showed similar proportions (not shown). In comparison we found that a significantly higher proportion of gut innervating neurons expressed TRPA1, specifically 55.1 $\pm$ 2% of gastric neurons, 54 $\pm$ 2% of

splanchnic neurons and $58 \pm 2\%$ of pelvic neurons (Figure 1D), indicating abundant expression of TRPA1 transcript within gut innervating neurons in all 3 pathways ($P < 0.001$; t-test).

To determine whether the *TRPA1* mutation interferes with the expression of other receptors and channels implicated in visceral sensory function, we compared their expression in TRPA1 $+/+$ and $-/-$ ganglia. As shown in Figure 1E, the expression levels of these genes were not altered suggesting compensatory changes in alternative transcript expression do not confound our physiologic findings reported below.

Immunoreactivity for TRPA1 protein was colocalized with the sensory neuropeptide CGRP in endings in different layers of the gut from TRPA1 $+/+$ mice (Figure 2). Collaterals of afferent endings within the colonic wall were also immunoreactive for CGRP and TRPA1, but occasionally each label was expressed alone in separate fibers, notably CGRP in the absence of TRPA1 in intramural endings. There was no TRPA1 immunoreactivity in TRPA1 $-/-$ mice, whereas patterns of CGRP labeling were unchanged (Figure 2D). Overall, these data indicate that TRPA1 is well placed to participate in visceral afferent function, and appears to be located preferentially in both mucosal and serosal/mesenteric afferent fibres.

TRPA1 is required for normal mechanosensory function in specific subsets of visceral afferents

In order to test the hypothesis that TRPA1 is required for visceral sensory mechanotransduction in specific afferent subtypes, we investigated all 5 major subtypes of mechanoreceptors in different regions of gut using standardized *in vitro* single fiber recording techniques^{7, 8}. Splanchnic colonic mechanoreceptors with receptive fields on the mesentery and serosa responded to high intensities of mechanical stimulation with static von Frey hairs. Mechanosensory responses of both populations were dramatically reduced in mice lacking TRPA1 (Figure 3A) whilst their mechanosensory thresholds were significantly increased (Supp Figure 1). However, the conduction velocities and electrical activation thresholds of both afferent classes were identical in TRPA1 $+/+$ and $-/-$ mice (Supp Figure 1), indicating no significant change in the electrical properties of TRPA1 $-/-$ afferents.

Deletion of TRPA1 also reduced mechanosensitivity of pelvic serosal afferents (Figure 3Bi), and increased their mechanosensory thresholds (Supp Figure 1). Two other populations of pelvic afferents were affected by loss of TRPA1: significant deficits were seen in the responses to mucosal stroking of pelvic mucosal afferents and muscular/mucosal afferents (Figure 3B). By contrast TRPA1 deletion did not affect the response to muscle stretch in pelvic muscular/mucosal afferents or in pelvic muscular afferents, suggesting TRPA1 is involved in signaling specific modalities.

Because TRPA1 $-/-$ colonic afferents displayed deficits in mechanosensory function we used a model of colonic pain to determine if changes at the cellular level of the afferent ending translated to alterations in sensory function in the intact animal. The abdominal EMG response to noxious colorectal distension (Figure 3C), was significantly reduced in mice lacking TRPA1, indicating these mice have a reduced ability to detect noxious visceral mechanosensory stimuli.

In the stomach and esophagus TRPA1 deletion caused modest but significant deficits in mucosal receptor function but no change in tension receptor function (Figure 3D), mirroring the effects observed in pelvic colonic mucosal and muscular afferents.

TRPA1 channel agonists evoke mechanical hypersensitivity in specific visceral afferents

To test the hypothesis that activation of TRPA1 increases mechanosensory function, we used the TRPA1 agonists allyl isothiocyanate (AITC; $0.4 - 400 \mu\text{M}$)¹⁹ and *trans*-cinnamaldehyde (TCA; $1 - 1000 \mu\text{M}$)¹⁹. Both agonists caused sensitization of the mechanosensory response of

splanchnic serosal afferents of TRPA1^{+/+} mice (Figure 4A, example in Supp Figure 2), which was dose dependent (data not shown). TRPA1 agonists had no effect on TRPA1^{-/-} serosal afferents.

A key function of TRPA1 is in inflammatory pain^{17, 18, 21}. To determine if TRPA1 function was enhanced in a model of colonic inflammatory hypersensitivity¹⁰, we used TRPA1 agonists in tissue from mice treated with TNBS. TRPA1 agonists caused greater mechanical hypersensitivity in afferents from TNBS-treated compared with untreated mice (Figure 4A, Supp Figure 2) indicating a larger role for TRPA1 in inflammation. Mechanical hypersensitivity was also evoked by these agonists in pelvic serosal and mucosal afferents (Figure 4B). Overall, TRPA1 agonists induced mechanical hypersensitivity only in the corresponding afferent subtype that displayed mechanosensory deficits in TRPA1^{-/-} mice.

Bradykinin-induced mechanical hypersensitivity of colonic splanchnic afferents is mediated via TRPA1

Bradykinin induces mechanical hypersensitivity in visceral afferents via a bradykinin receptor 2-mediated mechanism^{23, 24}. We hypothesized that TRPA1 contributes to this process. Bradykinin elicited a rapid and robust excitation in 50–60% of splanchnic serosal afferent fibers in both TRPA1^{+/+} and ^{-/-} mice (Figure 5A,B,D,E). After bradykinin responses, TRPA1^{+/+} fibers became mechanically hypersensitive, but TRPA1 ^{-/-} fibers did not (Figure 5Ci). Bradykinin-induced hypersensitivity occurred only in fibers that were responsive to bradykinin, not in unresponsive fibers (Figure 5Cii). This mechanism therefore requires bradykinin receptor 2 activation and action potential generation. The direct bradykinin response was almost identical in TRPA1^{+/+} and ^{-/-} fibers (Figure 5D) as was the proportion of bradykinin-responsive afferents (Figure 5E). Overall, these results indicate that TRPA1 is required for bradykinin-induced mechanical hypersensitivity, but does not contribute to the actual chemosensory response elicited by bradykinin.

TRPA1 mediates the capsaicin-induced mechanical desensitization of colonic splanchnic afferents

Splanchnic serosal afferents display mechanical desensitization after TRPV1 activation²³. Previous work indicates cross-interactions between TRPA1 and TRPV1 that may underlie desensitization²⁰. We found capsaicin elicited rapid and robust excitation in 35–40% of the splanchnic fibers tested from both TRPA1^{+/+} and ^{-/-} mice (Figure 6A&B). TRPA1^{+/+} fibers were subsequently desensitized to mechanical stimuli, whereas TRPA1 ^{-/-} fibers were unaffected (Figure 6Ci), as were TRPA1^{+/+} fibers that were unresponsive to capsaicin (Figure 6Cii). Although there was a slightly larger response to capsaicin in TRPA1 ^{-/-} afferents, this was not significant compared with TRPA1^{+/+} (Figure 6D), and the proportion of afferents responsive to capsaicin was similar between genotypes (Figure 6E). These results indicate a requirement for TRPA1 in TRPV1-mediated mechanical desensitization. However, it also suggests that TRPA1 does not contribute to the direct response to capsaicin.

PAR₂ activates colonic splanchnic afferent fibers via a TRPA1 independent mechanism

PAR₂-induced activation of visceral¹³ and somatosensory²⁵ pathways is mediated via opening of TRPV4 ion channels. There is also a putative link between PAR₂ and TRPA1²⁶. We asked whether TRPA1 was also involved in PAR₂-activation of visceral afferents. We first confirmed that splanchnic serosal fibers respond to the PAR₂-activating peptide (-AP), SLIGRL (Supp Figure 3); this was similar in TRPA1^{+/+} and ^{-/-} afferents. However, unlike bradykinin or capsaicin, PAR₂-AP did not change mechanosensory function in either TRPA1^{+/+} or ^{-/-}. In addition, TRPA1^{-/-} had similar magnitudes and proportions of afferent response to PAR₂-AP. Therefore, in our system there is no evident interaction between TRPA1 and PAR₂ in splanchnic colonic serosal afferents.

Discussion

Our findings indicate that TRPA1 plays a critical role in the detection of mechanical stimuli by visceral afferent fibers. We found that TRPA1 mRNA expression is enriched within gastrointestinal sensory neurons, whilst in the periphery TRPA1 protein is localized within nerve endings at sites where mechanical stimuli are transduced. Deletion of TRPA1 resulted in highly specific changes in afferent mechanosensory function: firstly TRPA1 contributed to the tactile function of vagal and pelvic mucosal afferents. This was somewhat surprising given the putative role of TRPA1 as a detector of noxious stimuli¹⁸. However, we observed secondly there were significant deficits in the mechanosensory function of nociceptors from the splanchnic and pelvic innervation of the colon and rectum, which translated into a reduced behavioral response of TRPA1^{-/-} mice to noxious visceral mechanosensory stimuli. Our results also show that activation of TRPA1 by selective agonists induces mechanical hypersensitivity of afferent endings. This role of TRPA1 is enhanced in inflammatory conditions associated with visceral hyperalgesia. Our data also indicate that the sensitivity of TRPA1 to mechanical stimuli can be tuned by algescic stimuli in certain subtypes of afferents. Specifically, bradykinin functionally sensitizes TRPA1 to increase mechanosensory function, whilst capsaicin functionally desensitizes TRPA1 to decrease mechanosensory function. By contrast, we found no evidence to support an interaction of TRPA1 and PAR₂ in splanchnic colonic afferents.

TRPA1 involvement in mechanosensation

TRPA1 deficient mice display significant deficits in sensing noxious punctate cutaneous mechanical stimuli. They also had higher thresholds than TRPA1^{+/+} mice and reduced responses to a series of suprathreshold stimuli¹⁸, but the site of the deficit was not determined. In the current study, we determined mechanosensitivity of visceral afferents in their natural environment. Deletion of TRPA1 significantly reduced the mechanosensory responses in four afferent subtypes: mucosal afferents in both upper and lower gut, mesenteric afferents and serosal afferents in colon, and mucosal responses of muscular/mucosal afferents in colon. We also found higher mechanical thresholds on deletion of TRPA1. Correspondingly, TRPA1 agonists induced mechanical hypersensitivity in the same subtypes that were affected by gene deletion, in agreement with recent data showing mustard oil sensitization mainly in higher threshold colonic afferents²⁷. Overall, these alterations implicate TRPA1 directly in the transduction of mechanical stimuli. Our findings represent the most conclusive evidence in mammals to date, and indicate that TRPA1 contributes to the detection of low and high intensity mechanical stimuli depending on the afferent ending in which it is expressed. We investigated the possibility that a broader role of TRPA1 in the regulation of the general excitability of afferent endings could explain our findings. However, in TRPA1^{+/+} and ^{-/-} mice we observed identical conduction velocities and electrical activation thresholds, and we observed almost identical responses to various chemical stimuli, indicating the general excitability of the afferent endings remains extensively unaltered after TRPA1 deletion. Although TRPA1 makes a considerable impact towards the mechanosensory function of specific classes of visceral afferents, residual mechanosensory responses were apparent, and notably some other subtypes were totally unaffected by TRPA1 deletion. Therefore other channels must contribute additionally to visceral mechanosensory function. In this regard ASIC1, 2 and 3 and TRPV4 have select roles as sensors of visceral mechanical stimuli^{9, 28}, however each channel contributes differently to mechanosensory function in individual subtypes of visceral afferents innervating each visceral organ. The roles of these channels differ markedly to that of TRPA1 in aspects of physiological and noxious mechanosensation in different regions, but there is overlap in the contribution of ASIC3, TRPV4 and TRPA1 in colonic high-threshold afferents, and possibly redundancy. Such redundancy is to be expected in a system important to signalling of injury or disease. The picture emerging from these investigations is that different classes of

sensory neuron have unique signatures of channel expression conferring their unique mechanosensory properties, which may vary between different parts of the body and differ between species²⁹. The ability of TRPA1 to act as a mechanosensor and to be tuned by numerous chemical stimuli makes it a key player in visceral allodynia and hyperalgesia.

Augmentation of mechanosensitivity by TRPA1 agonists was greater during colonic inflammation, suggesting a more prominent role of TRPA1 in pathophysiological states. Whether this is due to pharmacological potentiation of TRPA1 by endogenous mediators (see below) or upregulation of TRPA1 expression is the subject of continued investigation. We were struck by the fact that the same afferent subtypes that showed reduced function in TRPA1 $-/-$ here, showed increased function in acute and delayed inflammatory models in our recent investigation¹⁰. Together, these findings provide a strong link between TRPA1 and alteration of afferent function in pathophysiological states.

Enhanced mechanosensory function of TRPA1 induced by bradykinin

TRPA1 $-/-$ mice show reduced behavioral responses to cutaneous bradykinin injection¹⁸, suggesting that TRPA1 contributes to bradykinin-induced pain^{17, 18}. Somewhat unexpectedly, we observed that the magnitude of direct afferent responses to bradykinin was similar in TRPA1 $+/+$ and $-/-$, as were the proportions of responders and non-responders. Therefore TRPA1 is not required for the direct excitatory response of visceral afferents to bradykinin (mediated via the B₂ receptor²³). This therefore contrasts with other studies of TRPA1 $-/-$ mice *in vivo* or in isolated trigeminal neuron recordings investigating interactions of bradykinin and TRPA1^{17, 18}. However, our data do show that TRPA1 is essential for bradykinin-induced mechanical hypersensitivity, as is the case in the guinea-pig esophagus²⁴. The apparent differences between somatic and visceral pathways may be due to the varying methodologies, but these data raise the possibility that there is differential expression and linkage of channels and receptors between different neuronal populations. Considerable differences are also apparent between different pathways: it is clear from our data that pelvic colonic serosal afferents require TRPA1 as a mechanosensor, yet virtually none of these afferents respond directly to bradykinin, and those that are responsive do not develop mechanical hypersensitivity²³. This obviously contrasts with splanchnic colonic serosal afferents which respond to bradykinin, after which they develop mechanical hypersensitivity. This suggests that whereas B₂ and TRPA1 interact closely in splanchnic afferents, this interaction is lacking in pelvic afferents. Bradykinin receptors may couple via different G α -proteins to phospholipase C (PLC) and protein kinase A (PKA) pathways, which activate divergent intracellular pathways to evoke direct afferent excitation, but they may also sensitize TRPA1 via these pathways with indirect effects³⁰. We suggest that this “optional extra” is perhaps not included in pelvic afferents because they are tuned more towards detection of non-noxious than noxious stimuli⁷. This exemplifies the great biological diversity between afferent populations, even those innervating the same organ.

Reduced mechanosensory function of TRPA1 induced by capsaicin

We found pronounced mechanical desensitization of splanchnic afferents after responses to capsaicin, which was lost in TRPA1 $-/-$. However, the magnitude of direct chemosensory response to capsaicin did not significantly differ between TRPA1 $+/+$ and $-/-$ afferents. We also observed identical proportions of TRPA1 $+/+$ and $-/-$ afferents responding to capsaicin, and there was no change in TRPV1 mRNA expression in TRPA1 $-/-$ mice. Correspondingly, capsaicin-evoked calcium influx into isolated trigeminal neurons appears unaltered in TRPA1 deficient mice¹⁷. However, other data indicate that TRPA1 can undergo pharmacological desensitization that is modulated by TRPV1²⁰. That study proposed a mechanism whereby capsaicin binds to TRPV1, causes channel opening and a rapid, substantial, prolonged rise in intracellular Ca²⁺, which in conjunction with PLC activation, results in depletion of

phosphatidyl 4,5 bisphosphate (PIP2). PIP2 bound to TRPA1 is crucial in maintaining its function^{20, 31}. This leads us to suggest that the mechanical desensitization of splanchnic afferents by capsaicin is the result of a close interaction of TRPV1 desensitizing TRPA1 via PIP2 depletion. Capsaicin desensitization is absent in pelvic colonic serosal afferents, even though their responses to capsaicin are larger than splanchnic afferents³² but they still require TRPA1 for normal mechanosensation, suggesting that TRPA1 and TRPV1 do not interact the same way in pelvic afferents.

TRPA1 does not participate in the PAR₂-AP excitation of visceral afferents

Activation of PAR₂ sensitizes TRPV4 in somatic neurons to cause somatic mechanical hyperalgesia²⁵. We found correspondingly that direct responses of splanchnic colonic afferents to PAR₂ activation are totally lost in TRPV4^{-/-}¹³. A similar mechanism has been proposed by which PAR₂ sensitization of TRPA1 contributes to inflammatory pain²⁶. However, here we observed no change in afferent mechanosensory function after PAR₂-AP. In addition, loss of TRPA1 did not affect the magnitude or proportion of direct responses to PAR₂-AP. Therefore, it would appear there is little if any role for TRPA1 in PAR₂-activation of colonic afferents. However, because PAR₂ activates protein kinases A and C, it engages the same type of transduction mechanisms as the B₂ receptor, and would thus be expected to sensitize TRPA1. So the question arises as to why we did not see involvement of TRPA1 in PAR₂ responses. We suggest that the strong interaction between TRPV4 and PAR₂ in these neurons is due to tight colocalization, whereas TRPA1 and PAR₂ are localized discretely so that TRPA1 is not accessible by downstream products of PAR₂ activation.

In conclusion, TRPA1 contributes substantially to mechanosensory function in gastrointestinal afferents, and tuning of this channel by chemical mediators can alter mechanosensory function. Because altered visceral sensory function is a hallmark of functional gastrointestinal disorders, TRPA1 represents a novel target for therapies that reduce signaling of specific mechanical and inflammatory stimuli from the gut to the central nervous system and a key target for the reduction of visceral pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CTB-FITC	cholera toxin subunit B/fluorescein isothiocyanate conjugate
DRG	dorsal root ganglia
QRT-PCR	quantitative reverse transcription polymerase chain reaction
TL	thoracolumbar
LS	lumbosacral
TRPA1	transient receptor potential channel - ankyrin 1
TRPV	transient receptor potential channel - vanilloid

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Figure 1. Expression of TRPA1 in visceral sensory pathways

A. Agarose gel electrophoresis of amplified RT-PCR products from TRPA1 wild-type (+/+) and null-mutant (-/-) mice using primers specific for TRPA1 and β-actin. Amplified TRPA1 transcripts were detected in nodose ganglia (NG), thoracolumbar (TL; T10-L1) and lumbosacral (LS; L6-S1) dorsal root ganglia (DRG) from TRPA1+/+ mice. TRPA1 transcripts were absent in ganglia from TRPA1-/- mice, whereas β-actin was present. (no = no RNA template added).

B. Fluorescence in situ hybridization of TRPA1 mRNA expression (red) in TRPA1+/+ nodose ganglia (i), thoracolumbar (ii) and lumbosacral DRG (iii), combined with retrograde labeling of visceral sensory neurons from stomach (i), and distal colon (ii&iii) with CTB-FITC (green).

Arrows indicate neurons showing retrograde labeling from the gut: blue arrows indicate those positive for TRPA1, and yellow arrows denote TRPA1-negative neurons. (Scale bar 25 μ m).

C. Quantitative RT-PCR analysis indicated a similar level of TRPA1 transcript expression between whole NG and thoracolumbar and lumbosacral DRG.

D. Neuronal counts of the proportion of neurons expressing TRPA1 in either whole ganglia or in retrogradely labeled neurons from viscera. Graphs indicate that a similar proportion of neurons in the general population (non-labeled neurons, open bars) express TRPA1 in the different ganglia. However, significantly more neurons innervating the stomach and colon (filled bars) express TRPA1. ($P < 0.001$ retrogradely labeled gut neurons vs. general population, t-test).

E. We have previously shown that ASIC1,2,3 and TRPV4 contribute in various ways to colonic mechanosensory function. To determine if compensatory changes in the expression of these transcripts occurs in TRPA1 $^{-/-}$ mice which may explain the changes observed in TRPA1 $^{-/-}$ mechanosensory function we performed quantitative RT-PCR analysis. This analysis revealed that there were no significant changes in ASIC1,2,3 or TRPV4 expression between whole TL DRG in TRPA1 $^{+/+}$ and $^{-/-}$ mice. Moreover, there were no significant changes in TRPV1, Bradykinin 2, TRPV2, TREK-1, or NaV1.8 receptor expression.

Figure 2. Expression of TRPA1 protein in peripheral fibers in mouse colon

A. Immunohistochemical analysis of a colonic section from a TRPA1^{+/+} mouse showing co-localization of the sensory marker TRPA1 exclusively with CGRP in a nerve fiber within the colonic mucosa.

B. Wholemount of mesenteric blood vessels from a TRPA1^{+/+} mouse also showing co-localization of CGRP and TRPA1 in dense nerve fiber networks.

C. Wholemount of colonic wall from a TRPA1^{+/+} mouse showing co-localization of CGRP and TRPA1 in a single colonic afferent with morphology and location consistent with serosal afferents.

D. Wholemount of colonic wall from a TRPA1^{-/-} mouse showing that the pattern of CGRP expression shown in C was also observed whereas TRPA1 expression was absent.

E. Wholemount of colonic wall from a TRPA1^{+/+} mouse showing an example of colocalization of CGRP and TRPA1 in mesenteric blood vessels (BV), but not in endings within muscle.

Colonic Mechanosensory Function

A) Splanchnic Afferents

B) Pelvic Afferents

C) Visceromotor response to colorectal distension

Gastroesophageal Mechanosensory Function

D) Vagal afferents

Figure 3. TRPA1 $^{-/-}$ mice display selective deficits in visceral afferent function

A Splanchnic **i)** Mesenteric and **ii)** Serosal colonic afferents showed dramatically reduced stimulus response functions to focal compression of mechanoreceptive fields with static von Frey hair (vfh) application (70mg–2000mg) in TRPA1 $^{-/-}$ mice ($P < 0.0001$, 2-way ANOVA). This was also significant at most individual stimulus intensities (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Bonferroni post-hoc test).

B. i) Pelvic serosal colonic afferents were hyposensitive TRPA1 $^{-/-}$ mice. **ii)** Mucosal afferents also displayed reduced stimulus response functions in TRPA1 $^{-/-}$ mice. **iii)** Muscular/mucosal afferents, which respond to both low intensity mucosal stroking and low intensity stretch, only displayed significant deficits in the mucosal component ($P < 0.001$, 2-way ANOVA; * $P < 0.05$,

*** $P < 0.001$, Bonferroni test). **iv**) Mechanosensory function of stretch sensitive muscular afferents was unaltered in TRPA1^{-/-}. Changes in length of tissue in response to stretch (1–15g) were similar in both genotypes (not shown).

C. Abdominal EMG responses of conscious mice to colorectal balloon distensions (5×80mmHg) were significantly reduced in TRPA1^{-/-} mice (**N=5, $P < 0.01$), implicating TRPA1 in the signaling of colonic pain.

D. Two classes of gastroesophageal vagal afferents were recorded; mucosal and tension receptors. The deletion of TRPA1 caused modest but significant deficits in **i**) mucosal mechanosensory function ($P < 0.01$, 2-way ANOVA).

A) Splanchnic colonic afferents

B) Pelvic colonic afferents

Figure 4. TRPA1 agonists induce mechanical hypersensitivity

A. i) In untreated TRPA1+/+ splanchnic colonic afferents the TRPA1 agonists allyl-isothiocyanate (AITC; 40μM) or *trans*-cinnamaldehyde (TCA; 100μM) induced mechanical hypersensitivity of serosal fibers to a 2000mg vfh, typically by 25%. **ii)** Mechanical hypersensitivity was absent in TRPA1-/- serosal afferents. **iii)** The mechanical hypersensitivity evoked by both agonists is significantly enhanced in acute TNBS inflammation. **iv)** Change in mechanosensory response in inflammation compared with control (calculated from i) & iii). The selective TRPA1 antagonist HC-030031 (10μM for 10mins) had similar effects on mechanosensory responses to gene deletion (not shown)

B. In pelvic colonic afferents 100 μ M TCA also induced significant mechanical hypersensitivity of TRPA1^{+/+} **i)** serosal and **iii)** mucosal colonic afferents which was lost in TRPA1^{-/-} mice (**ii & iv**).

Figure 5. TRPA1 mediates the bradykinin-induced mechanical hypersensitivity of splanchnic colonic afferents

A. TRPA1 $+/+$ splanchnic colonic serosal afferent responding to bradykinin ($1\mu\text{M}$; for 2mins). Note increased mechanosensory response to a 2000mg vfh after the chemosensory response to bradykinin.

B. TRPA1 $-/-$ splanchnic serosal afferent responding to bradykinin ($1\mu\text{M}$; for 2mins). Note mechanosensory response is unchanged after bradykinin.

C. i) TRPA1 $+/+$ serosal fibers that responded to bradykinin subsequently displayed mechanical hypersensitivity ($*P<0.05$, paired t-test). This effect was not observed in the corresponding TRPA1 $-/-$ serosal afferents that responded to bradykinin. **ii)** Mechanical hypersensitivity after bradykinin application only occurred in TRPA1 $+/+$ afferents that responded to bradykinin and was not observed in unresponsive fibers (NS , $P>0.05$, paired t-test).

D. The chemosensory response bradykinin was similar in TRPA1 $+/+$ and $-/-$ fibers (NS , $P>0.05$, t-test).

E. The proportion of serosal afferents responding to bradykinin was similar in TRPA1 $+/+$ and $-/-$ mice (NS , $P>0.05$, Fisher's exact test).

Figure 6. TRPA1 mediates the capsaicin-induced mechanical desensitization of splanchnic colonic afferents

A. TRPA1 $+/+$ splanchnic serosal afferent responding to capsaicin ($3\mu\text{M}$; 2mins). Note decreased mechanosensory response to 2000mg vfh after chemosensory response to capsaicin.

B. TRPA1 $-/-$ splanchnic serosal afferent responding to capsaicin ($3\mu\text{M}$; 2mins). Note mechanosensory response is unchanged after capsaicin.

C i) TRPA1 $+/+$ serosal fibers responding to capsaicin subsequently displayed mechanical desensitization (* $P < 0.05$; paired t-test). This effect was not observed in TRPA1 $-/-$ serosal afferents that responded to capsaicin (NS, $P > 0.05$; paired t-test). ii) Mechanical desensitization after capsaicin application only occurred in TRPA1 $+/+$ afferents that responded to capsaicin and was not observed in unresponsive fibers.

D. Magnitude of chemosensory response to capsaicin was similar in TRPA1 $+/+$ and $-/-$ fibers (NS, $P > 0.05$, paired t-test).

E. The proportion of serosal afferents responding to capsaicin in either TRPA1 $+/+$ or $-/-$ mice was unchanged (NS, $P > 0.05$, Fisher's exact test).