Transient Receptor Potential Ankyrin 1 Is Expressed by Inhibitory Motoneurons of the Mouse Intestine

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BACKGROUND & AIMS: Transient receptor potential ankyrin (TRPA) 1, an excitatory ion channel expressed by sensory neurons, mediates somatic and visceral pain in response to direct activation or noxious mechanical stimulation. Although the intestine is routinely exposed to irritant alimentary compounds and inflammatory mediators that activate TRPA1, there is no direct evidence for functional TRPA1 receptors on enteric neurons, and the effects of TRPA1 activation on intestinal function have not been determined. We characterized expression of TRPA1 by enteric neurons and determined its involvement in the control of intestinal contractility and transit. **METHODS:** TRPA1 expression was characterized by reverse-transcription polymerase chain reaction and immunofluorescence analyses. TRPA1 function was examined by Ca^{2+} imaging and by assays of contractile activity and transit. **RESULTS:** We detected *TRPA1* messenger RNA in the mouse intestine and TRPA1 immunoreactivity in enteric neurons. The cecum and colon had immunoreactivity for neuronal TRPA1, but the duodenum did not. TRPA1 immunoreactivity was also detected in inhibitory motoneurons and descending interneurons, cholinergic neurons, and intrinsic primary afferent neurons. TRPA1 activators, including cinnamaldehyde, allyl isothiocyanate (AITC), and 4-hydroxynonenal, increased $[Ca^{2+}]_i$ in myenteric neurons. These were reduced by a TRPA1 antagonist (HC-030031) or deletion of *Trpa1*. TRPA1 activation inhibited contractility of the segments of colon but not stomach or small intestine of $Trpa1^{+/+}$ but not $Trpa1^{-/-}$ mice; this effect was reduced by tetrodotoxin or N^G-nitro-L-arginine methyl ester. Administration of AITC by gavage did not alter gastric emptying or small intestinal transit, but luminal AITC inhibited colonic transit via TRPA1. **CONCLUSIONS: Functional TRPA1 is expressed by enteric neurons, and activation of neuronal TRPA1 inhibits spontaneous neurogenic contractions and transit of the colon.**

Keywords: Enteric Nervous System; TRP Channels; Motility.

The transient receptor potential excitatory cation channels are expressed by primary sensory neurons and detect noxious chemical, mechanical, and thermal stimuli. Transient receptor potential ankyrin 1 (TRPA1) is a recently identified channel of primary sensory neurons.[1–3] TRPA1 responds to exogenous alimentary (eg, allyl isothiocyanate [AITC, mustard oil], cinnamaldehyde [CMA], allicin) and environmental (acrolein, formaldehyde) irritants.[4] Endogenous activators include inflammatory mediators produced during oxidative stress (4-hydroxynonenal [4-HNE], 4-oxononenal)[5,6] or prostaglandin (PG) metabolism (eg, 15-delta PGJ_2).[7,8] These compounds activate TRPA1 via covalent modification of free sulfhydryl groups of N-terminal cysteine residues by nucleophilic addition or redox reactions, including nitrosylation and peroxidation. Agonists of G protein–coupled receptors for inflammatory mediators, including the B_2 bradykinin receptor[9] and protease-activated receptor 2,[10–12] also indirectly sensitize TRPA1 by depletion of phosphatidylinositol 4,5-bisphosphate and through activation of second messenger kinases. Thus, TRPA1 is activated and sensitized by inflammatory mediators.

The function of TRPA1 in primary sensory neurons has been extensively studied. TRPA1 is expressed in nociceptive neurons of dorsal root, trigeminal, and nodose ganglia,[13] where activation enhances their excitability.[14] TRPA1 mediates mechanosensory transduction by cutaneous and visceral afferent neurons.[10,15–17] Little is known about the expression and function of TRPA1 in enteric neurons.

TRPA1 agonists are present in commonly ingested foods (eg, cinnamon, garlic, mustard, wasabi, ginger, cloves), and endogenous agonists (eg, 4-HNE, PGs) are produced in the inflamed intestine.[18,19] Therefore, it is important to define the localization of TRPA1 in enteric neurons and to examine the effects of TRPA1 activation on normal digestive functions. Because enteric neurons are intrinsic to the gut wall, they are uniquely placed to respond to TRPA1 agonists from digesta or the inflammatory milieu, and activation may have marked effects on digestion. TRPA1 messenger RNA (mRNA) is expressed in

Abbreviations used in this paper: AITC, allyl isothiocyanate; CCh, carbachol; CMA, cinnamaldehyde; GE, gastric emptying; IR, immunoreactivity; 4-HNE, 4-hydroxynonenal; L-NAME, N^G-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; SIT, small intestinal transit; TRPA, transient receptor potential ankyrin.

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the intestine[20,21] and is up-regulated by mustard oil–induced colitis.[22] TRPA1 immunoreactivity (IR) is present in colonic myenteric neurons,[23] and agonists such as mustard oil affect gastric and intestinal motility by unknown mechanisms.[20,21,24] Although these reports suggest that TRPA1 is expressed by enteric neurons and regulates motility, there is no direct functional evidence for TRPA1 expression by myenteric neurons, and the neuronal subtypes expressing TRPA1 and the mechanisms by which the specific activation of TRPA1 controls motility are unknown.

We examined TRPA1 expression and localization in subtypes of enteric neurons and investigated the function of TRPA1 by ratiometric Ca^{2+} imaging of myenteric neurons and measurement of contractility and transit. We report the novel finding that TRPA1-IR is present in intrinsic neurons, where activation by exogenous and endogenous agonists inhibits contractility of the colon via a nitrergic mechanism, and inhibits colonic transit.

Materials and Methods

Animals

C57Bl6 mice (6–8 weeks old, male and female) were from Charles River Laboratories (Hollister, CA). Wild-type (*trpa1*$^{+/+}$) and knockout (*trpa1*$^{-/-}$) mice[14] were maintained as heterozygotes, and age- and sex-matched littermates were studied. Mice were anesthetized with sodium pentobarbital (200 mg/kg intraperitoneally) and killed by bilateral thoracotomy. The UCSF Institutional Animal Care and Use Committee approved all procedures.

Reagents

4-HNE was from Enzo Life Sciences (Plymouth Meeting, PA). HC-030031 was from P. Geppetti (University of Florence, Florence, Italy). Substance P was from Bachem (Torrance, CA). Other reagents were from Sigma-Aldrich (St Louis, MO) unless stated otherwise.

Detection of TRPA1 in the Mouse Gastrointestinal Tract by Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated and reverse transcribed, and TRPA1 was amplified using primers for mouse TRPA1 (forward, 5′-GAAGCATGCGTCATTGAAGAGGAT-3′; reverse, 5′-TCCATTTCCAAGCATGTGTCAATG-3′). Control reactions omitted reverse transcriptase. Products were separated by electrophoresis, detected with ethidium bromide, and sequenced.

Immunofluorescence

Whole mounts of enteric ganglia and tissue sections were prepared as described.[25] Tissues were incubated with primary antibodies (Supplementary Table 1; overnight, 4°C), washed, and incubated with fluorescent secondary antibodies (1:200, 1 hour, room temperature; Jackson ImmunoResearch, West Grove, PA). TRPA1 antibody specificity was determined by preadsorption with immunizing peptide (100 μmol/L, 24 hours, 4°C) and by staining HEK-FLPTREX cell lines stably transfected with either TRPA1 with a C-terminal hemagglutinin epitope tag or empty vector (pcDNA5/FRT/TO).[6] Specimens were examined using a Zeiss LSM510 META confocal microscope. Images (1024 × 1024 pixels) were obtained using Zeiss Plan-Neofluar 40 × 1.3 or Plan-Apochromat 63 × 1.4 objectives (Carl Zeiss, Thornwood, NY). TRPA1 localization to neuronal subtypes was investigated by colabeling with neurochemical markers.[26] Twenty or more neurons were analyzed per preparation from ≥3 mice per marker and region.

Culture of Myenteric Neurons

Myenteric neurons of small intestine or colon were enzymatically dispersed and cultured as described.[27] Neurons were studied after 7 to 14 days in culture, either to localize TRPA1-IR by immunofluorescence or to assess expression of functional TRPA1 by ratiometric determination of $[Ca^{2+}]_i$.

Ratiometric Ca^{2+} Imaging

$[Ca^{2+}]_i$ was determined in individual neurons by ratiometric imaging of Fura2-AM (Invitrogen, Carlsbad, CA) as described.[27] Neurons were challenged with TRPA1 activators (AITC, 100 μmol/L; CMA, 10 or 100 μmol/L; 4-HNE, 100 μmol/L) or vehicle (0.01% dimethyl sulfoxide), followed by substance P (100 nmol/L), carbachol (CCh; 1 μmol/L), and KCl (50 mmol/L). Neurons were identified by size, morphology, and KCl responsiveness. The proportion of responsive neurons relative and the change in 340:380 nm emission ratio were determined.

Contractility

Segments of gastric antrum, duodenum, ileum, or proximal colon (whole tissue) were placed in modified Krebs' buffer (37°C, 5% CO_2/95% O_2) under 1 g tension to record isotonic contractions of longitudinal muscle.[28] Tissues were equilibrated (30 minutes), and viability was tested at the beginning and end of experiments by application of CCh (1 μmol/L). Mean contraction amplitude was quantified and expressed relative to pretreatment values (normalized to 1).

Gastric Emptying and Small Intestinal Transit

Phenol red (0.07% in 0.9% NaCl) alone (control) or containing AITC (0.0125 or 0.25 μmol) or vehicle (0.5% dimethyl sulfoxide) was administered by gavage (0.25 mL). After 30 minutes, mice were killed, the stomach and small intestine were removed, and the small intestine was divided into 3 equal segments. Tissues were homogenized in 0.5 mL of 0.1N NaOH and 5.5 mL of H_2O and centrifuged. Supernatant (1.5 mL) was mixed with trichloroacetic acid (0.15 mL, 30%) and centrifuged. Supernatants (1 mL) were mixed with NaOH (1 mL), and absorbance was measured at 570 nm. Gastric emptying (GE) was determined as the proportion of administered phenol red remaining in the stomach after 30 minutes (% GE = [(Administered Phenol Red – Gastric Phenol Red)/Administered Phenol Red] × 100). Small intestinal transit (SIT) was evaluated as the geometric center of the distribution of the marker (Geometric Center = [(Phenol Red In Each Segment) × (Segment Number)]/Administered Phenol Red).

Colonic Transit

Colonic transit was measured using a bead expulsion test as described.[29] AITC (0.5% in olive oil, 100 μL) or vehicle was administered intracolonically by catheter. After 5 minutes, a glass bead (3-mm diameter) was inserted into the colon (2 cm). The time until bead expulsion was measured.

Statistics

Data are expressed as the mean ± SEM and were analyzed using *t* test or one-way analysis of variance with Newman–