Involvement of TRPV2 Activation in Intestinal Movement through Nitric Oxide Production in Mice

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Transient receptor potential channel vanilloid 2 (TRPV2) can detect various stimuli such as temperature ($>52^{\circ}\text{C}$), stretch, and chemicals, including 2-aminoethoxydiphenyl borate, probenecid, and lysophospholipids. Although expressed in many tissues, including sensory and motor neurons, TRPV2 expression and function in the gastrointestinal tract is poorly understood. Here, we show TRPV2 expression in the murine intestine and its involvement in intestinal function. Almost all mouse intestinal intrinsic sensory and inhibitory motor neurons, both cell bodies and nerve fibers, showed TRPV2 immunoreactivity. Several known TRPV2 activators increased cytosolic Ca^{2+} concentrations and evoked TRPV2-like current responses in dissociated myenteric neurons. Interestingly, mechanical stimuli activated inward currents in a strength-dependent manner, which were inhibited by a TRPV2 inhibitor tranilast. TRPV2 activation in isolated intestine inhibited spontaneous circular muscle contraction, which did not occur in the presence of the TRPV2 antagonist, tetrodotoxin or nitric oxide (NO) synthase pathway inhibitors. Also, increased intestinal NO production was observed in response to a TRPV2 agonist, and gastrointestinal transit *in vivo* was accelerated by TRPV2 agonists or an NO donor. In conclusion, TRPV2 may contribute to intestinal motility through NO production, and TRPV2 is a promising target for controlling intestinal movement.

Introduction

The intestine contains intrinsic primary afferent neurons (IPANs) and a local reflex system (including interneurons, excitatory and inhibitory motor neurons) that play important roles in controlling bowel movement (Furness et al., 2004a; Furness, 2008). Interestingly, many IPANs and some uniaxonal neurons (mainly excitatory and inhibitory motor neurons) appear to be mechanosensitive and activated by muscle contractions in guinea pig intestine (Kunze et al., 1998, 1999). Acetylcholine (ACh) and nitric oxide (NO) are dominant excitatory and inhibitory neurotransmitters, respectively (Harrison and McSwiney, 1936; Rand, 1992), and abnormal NO production or release has been implicated in functional gastrointestinal disorders (Takahashi, 2003).

However, the underlying molecular and cellular mechanisms are poorly understood.

Transient receptor potential vanilloid 2 (TRPV2) was originally isolated as a molecule sensitive to temperatures above 52°C (Caterina et al., 1999) but has since been shown to be sensitive to some chemicals [e.g., 2-aminoethoxydiphenyl borate (2-APB), probenecid (Pro), and lysophospholipids] and mechanical stimuli (Muraki et al., 2003; Hu et al., 2004; Bang et al., 2007; Monet et al., 2009; Shibasaki et al., 2010). TRPV2 expression has been observed in TRPV1-negative medium- to large-diameter neurons of the rat dorsal root and trigeminal ganglia and possibly in thinly myelinated nociceptors (Caterina et al., 1999; Lewinter et al., 2008). However, TRPV2 mRNA or immunoreactivity has also been detected in the brain, autonomic ganglia, spinal cord, skeletal and vascular myocytes, visceral organs (including the intestine, pancreas, spleen, and bladder), and blood cells (Caterina et al., 1999; Ichikawa and Sugimoto, 2001; Muraki et al., 2003; Kashiba et al., 2004; Lewinter et al., 2004; Inada et al., 2006; Lewinter et al., 2008; Hisanaga et al., 2009; Zanou et al., 2009; Link et al., 2010). TRPV2 expression may also be involved in the migration and proliferation of cancer cells (Caprodossi et al., 2008; Monet et al., 2010), and TRPV2-positive neurons observed in the rat myenteric plexus may be IPANs (Kashiba et al., 2004). Mechanosensitivity of TRPV2 was first reported in cardiac myocytes (Muraki et al., 2003), and we have recently reported its expression and mechanosensitivity in developing sensory and motor neurons (Shibasaki et al., 2010).

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Table 1. Characteristics of primary antisera

Tissue antigen	Host	Dilution	Source
TRPV2	Rabbit	1:200	TransGenic
PGP9.5	Mouse	1:200	Serotec, monoclonal antibody (31A3)
GFAP	Mouse	1:100	Sigma, monoclonal antibody (GA5)
KIT	Rat	1:50	BD Biosciences
Neuronal NOS	Sheep	1:2000	P. C. Emson (Williamson et al., 1996)
CGRP	Sheep	1:1000	J. Furness (Qu et al., 2008)
NF145	Mouse	1:300	Millipore, monoclonal antibody (MAB1621)

Table 2. Secondary antibodies used for immunohistochemistry

Antibody label	Dilution	Source
Goat anti-rabbit IgG–Alexa Fluor 488	1:1500	Invitrogen
Goat anti-rabbit IgG–Alexa Fluor 635	1:1500	Invitrogen
Donkey anti-sheep IgG–Alexa Fluor 546	1:1500	Invitrogen
Goat anti-mouse IgG–Alexa Fluor 488	1:1500	Invitrogen
Donkey anti-sheep IgG–Alexa Fluor 488	1:1500	Invitrogen

However, the precise distribution of TRPV2 expression in intestinal neurons and its functional significance remain unknown. In this study, we demonstrate that TRPV2-positive inhibitory motor neurons and IPANs may contribute to the inhibitory modulation of intestinal movement.

Materials and Methods

Animals. Male C57BL/6 (5–12 weeks old; SLC) and ChAT–eGFP (choline acetyltransferase–enhanced green fluorescent protein) [5–8 weeks old; The Jackson Laboratory (Tallini et al., 2006)] mice were used. ChAT–eGFP was used as a marker for IPANs and excitatory motor neurons. Mice were housed in a controlled environment (12 h light/dark cycle; room temperature, 22–24°C; 50–60% relative humidity) with access to food and water *ad libitum*. All procedures involving the care and use of animals were approved by The Institutional Animal Care and Use Committee of National Institutes of Natural Sciences and performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication number 85-23, revised 1985).

Reverse transcription-PCR analysis. To examine TRPV2 expression in the murine gut, total RNAs (1 µg) isolated from whole intestine, whole-mount preparations of longitudinal muscle with myenteric plexus (LMMP) of distal intestine, and primary myenteric culture cells (MCC) were used for reverse transcription with Superscript III first-strand synthesis system for reverse transcription (RT)–PCR (Invitrogen). PCR was performed using rTaq DNA polymerase (TaKaRa) in an iCycler (BioRad) with specific primer sets (supplemental Table 1, available at www.jneurosci.org as supplemental material) for several TRP channels, including TRPV2 (Inada et al., 2006) and the neural marker protein gene product 9.5 (PGP9.5). PCR conditions used were as follows: 1 cycle at 94°C for 2 min; 40 cycles at 94°C for 10 s, 55°C for 10 s, and 72°C for 30 s; and 1 cycle at 72°C for 2 min.

Immunohistochemistry. Immunohistochemical methods were modified from previous reports (Ward et al., 2003; Furness et al., 2004b; Qu et al., 2008). All experiments were repeated on specimens from at least three mice. Antibody information is summarized in Tables 1 and 2.

For LMMP preparation, male mice (8–12 weeks of age) were killed by cervical dislocation after light diethyl ether anesthesia. The gastrointestinal tract from the duodenum to distal colon was removed, the small intestine was opened along the mesenteric border, and the luminal contents was washed with Tyrode's solution. Opened segments were pinned onto the base of an elastomer dish with the mucosal side facing downward for fixation with 4% paraformaldehyde in PBS (0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) at 4°C for 1 h. The mucosa, submucosa, and circular muscle were removed, and LMMPs were prepared. Preparations were cleared of fixative with PBS plus 0.3% Triton X-100 (0.3% PBS-T) (three washes, 5 min each). Nonspecific antibody binding was reduced by incubation with 3% bovine serum albumin (Sigma) in

Figure 1. Expression of TRPV2 genes in isolated whole intestine, LMMP, and MCC. mRNAs of TRPV2, TRPV1, TRPA1, TRPM8, PGP9.5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined with (+) and without (–) RT reaction. Expected sizes of the amplified fragments of TRPV2, TRPV1, TRPA1, TRPM8, PGP9.5, and GAPDH are 552, 548, 577, 412, 413, and 545 bp, respectively.

0.3% PBS-T for 1 h at room temperature before exposure to rabbit anti-TRPV2 antibody and several specific cell type markers [PGP9.5, glial fibrillary acidic protein (GFAP), KIT (CD117)] or different enteric neuron class markers. Anti-neuronal nitric oxide synthase (nNOS) and anti-calcitonin gene-related peptide (CGRP) antibodies were generous gifts from Dr. P. C. Emson (The Babraham Institute, Cambridge, UK) and Dr. J. B. Furness (University of Melbourne, VIC, Australia), respectively. An anti-rat TRPV2 antibody was made by our laboratory and verified by Western blot. LMMP preparations were incubated with primary antibodies at 4°C overnight and then washed (three times, 10 min each) in 0.3% PBS-T before incubation with secondary antibodies at room temperature for 1 h. Preparations were then washed (three times, 10 min each) in PBS and mounted on glass slides using a commercially available mounting medium (Diagnostic BioSystems). To visualize cellular CGRP as an IPAN marker, tissues were incubated in oxygenized DMEM containing colchicine before fixation as described previously (Qu et al., 2008).

For section preparation, three mice were anesthetized with diethyl ether and perfused through the heart with the same fixative as used for immersion fixation. Intestinal tissues were removed and further fixed at 4°C for 6 h. Tissues were washed in PBS (three times, 15 min each), placed in PBS–sucrose (PBS containing 20% sucrose), and stored at 4°C overnight. Next, small intestinal segments were embedded in OCT compound (Tissue Tek), and 14-µm-thick sections were collected onto slides and dried at room temperature for 1 h. Sections were then incubated in primary and secondary antibodies as described above. Preparations were analyzed using a fluorescent microscope and a confocal laser scanning microscope (LSM 510; Carl Zeiss).

Primary culture of myenteric neurons. The method was modified from a previous report (Bian and Galligan, 2007). Male wild-type (WT) and ChATBAC–eGFP mice (5 weeks of age) were killed by cervical dislocation after light diethyl ether anesthesia. The entire length of small intestine was placed in cold (4°C) PBS. LMMP was stripped free and cut into 5 mm pieces. Tissues were divided into two aliquots, with each aliquot incubated in 1 ml of trypsin solution (Invitrogen) at 37°C for 15 min. Tissues were then gently triturated 30 times using a Pasteur pipette and centrifuged at 900 × g for 5 min. Pellets were resuspended and incubated (15 min, 37°C) in Tyrode's solution containing 1 mg/ml collagenase (Sigma). Suspensions were triturated again 30 times and centrifuged at 900 × g for 5 min after cell strainer (Falcon; BD Biosciences Discovery Labware) filtering. Pellets were suspended in MEM containing 10% fetal bovine serum, NGF (100 ng/ml), penicillin (100 U/ml), and streptomycin (50 µg/ml). Cells were plated on glass coverslips coated with poly-D-lysine (50 µg/ml for 2 h) and incubated at 37°C with 5% CO₂.

In vitro electroporation. Dominant-negative (DN) TRPV2 plasmid DNA (Nagasawa et al., 2007) or pcDNA3.1 vector (final concentration, 5 µg/100 µl) along with Discosoma Red cDNA was electroporated using a pulse generator Neon (Invitrogen) into myenteric neurons isolated from ChAT–eGFP mice (5 weeks of age, both sexes).

Ca²⁺ imaging. Fura-2 fluorescence was measured in cultured myenteric cells using standard bath solution (in mM: 140 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose at pH 7.4, adjusted with NaOH). Ratio of fluorescence intensities obtained with fura-2 emissions at 340 and 380 nm were calculated. TRPV2 agonists 2-APB, probenecid, and lysophosphatidylcholine (LPC) (all from Sigma) and TRPV2 antagonists [a broad TRP channel blocker, ruthenium red (RR), and a TRPV2-specific antagonist, tranilast (Tra) (Hisanaga et al., 2009) (provided by Kissei)] were used. To identify myenteric neurons, high-potassium (50 mM) solution was applied. All experiments were performed at room temperature (25°C). Neuronal sensitivity to the various compounds (responders) was defined as an increase in 340/380 ratio >0.25, except for *in vitro* electroporation studies in which a ratio increase >0.1 was used.

Electrophysiology. The standard bath solution for the patch-clamp experiments was the same as that used in the Ca²⁺-imaging experiments. Pipette solution for whole-cell recordings contained 140 mM CsCl, 5 mM EGTA, and 10 mM HEPES, pH 7.4. Mechanical stimulus was achieved by applying positive and negative pressures (3–10 cm H₂O) to the pipette. Data from whole-cell voltage-clamp recordings were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pClamp software; Molecular Devices). Voltage ramp pulses from –100 to +100 mV (500 ms) were applied every 4–6 s to generate an current–voltage (*I*–*V*) curve. All the experiments were performed at room temperature (25°C).

Tension recording of isolated intestines. Spontaneous intestinal contractions of circular muscle were recorded as described previously (Penueles et al., 2007; Boudaka et al., 2009). Briefly, 8- to 12-week-old male WT mice were killed by cervical dislocation. Whole-intestinal segments were removed and kept in Tyrode's solution bubbled with 95% O₂/5% CO₂. Lengths of ~1.0 cm of cylindrical intestine were placed under a 0.5 g load in a 10 ml organ bath filled with Tyrode's solution at 30–33°C and continuously bubbled with 95% O₂/5% CO₂. Spontaneous contractions were monitored using an isotonic transducer. After tissue recovery for 30 min, responses to TRPV2 agonists [probenecid, LPC, and lysophosphatidylcholine (LPI) (Sigma)] and an NO donor [sodium nitroprusside (SNP) (Wako)] were examined. Lysophosphatidic acid (LPA) (Sigma) was used as a negative control (Monet et al., 2009). Tetrodotoxin (TTX), *N*^G-nitro-L-arginine methyl ester, hydrochloride (L-NAME), *N*^ω-nitro-L-arginine (L-NNA) (an NO scavenger), hemoglobin (Hb), and the guanylate cyclase inhibitor methylene blue (all from Sigma, except for TTX, which is from Sankyo) were used as 10 min pretreatments before agonist application. Each response was expressed as the ratio of late values (mean of contractile amplitudes at 2–3 or 5–6 min) to basal values (1 min) using PowerLab Chart5 version 5.1 (AD Instruments).

Measurement of NO release (Griess assay). The method was modified from previous reports (Korenaga et al., 2006; Wehner et al., 2009). Whole-intestine tissues were washed and kept in Tyrode's solution bubbled with 95% O₂/5% CO₂. Small segments (0.5 mm each) were trans-

Figure 2. TRPV2 immunoreactivity in murine myenteric plexus neurons. **A**, TRPV2-immunoreactive cells (green) were observed in PGP9.5-immunoreactive myenteric neurons (red). **B**, TRPV2 immunoreactivity in the same section was abolished with antigenic TRPV2 peptide. **C**, **D**, GFAP (red in **C**) or KIT (red in **D**) were not coexpressed with TRPV2 (green). **E**, Staining of TRPV2 (left, green) and nNOS (middle, red) in the same LMMP preparation. **F**, Staining of TRPV2 (left, green) and CGRP (red) in the same LMMP preparation. Merged images (far right) indicate coexpression of nNOS (**E**) or CGRP (**F**) in TRPV2-positive cells. **G**, Costaining of NF145 (red) and TRPV2 (green) in the LMMP preparation. **H**, Merged images of TRPV2 (green)- and nNOS (red)-IR nerve fibers in the LMMP preparation (left, plexus area; right, muscle area). All nNOS-IR fibers show TRPV2 immunoreactivity, and some TRPV2-IR fibers show no nNOS-immunoreactivity. Scale bars, 50 μ m.

ferred to microtubes containing 0.5 ml of Tyrode's solution with or without probenecid. Response to 10 μ M nicotine was used as a positive control (Patel et al., 2008). After 30 min incubation at 30°C, microtubes were centrifuged at 132,000 rpm at 4°C for 20 min. Tissues were removed and dried overnight to measure dry weight. Supernatants were centrifuged with Amicon Ultra-4 Centrifugal Filter Units with Ultracel-1 membranes (7000 $\times$ g, 4°C, 20 min) to remove proteins. NO₂ and NO₃ concentrations were detected with an NO₂/NO₃ Assay kit (DOJINDO) using a fluoromicroplate reader (Hitachi) with excitation at 365 nm and emission at 450 nm under basic pH. NO concentrations were determined

Figure 3. TRPV2-mediated cytosolic Ca^{2+} increase in myenteric culture neurons. **A**, Changes in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) indicated by the fura-2 ratio, with pseudocolor expression, in response to 30 μM LPC and 50 mM high K^+ solution in primary myenteric culture neurons. Scale bar, 50 μM . **B**, Traces of $[\text{Ca}^{2+}]_i$ changes (340/380 ratio) in response to three TRPV2 agonists (2-APB, Pro, and LPC) in mouse myenteric culture neurons (mean $\pm$ SEM). Bars indicate the period of chemical application. LPC-induced $[\text{Ca}^{2+}]_i$ increase is not observed in the absence of extracellular Ca^{2+} [LPC, Ca^{2+} (–), mean $\pm$ SEM]. Data are from 27–111 cells. **C**, Traces of $[\text{Ca}^{2+}]_i$ changes in response to LPC with two TRPV2 inhibitors (RR at 10 μM and Tra at 75 μM) in mouse myenteric culture neurons (mean $\pm$ SEM). Data are from 10 cells. **D**, Percentage of responders (see Materials and Methods) to TRPV2 agonists (LPC at 30 μM , Pro at 1 mM, and 2-APB at 500 μM) with or without TRPV2 inhibitors (RR at 10 μM and Tra at 75 μM) in the presence [Ca^{2+} (+)] or absence [Ca^{2+} (–)] of extracellular Ca^{2+} . Responder rates in cells treated with LPC in Ca^{2+} (–) ($6.0 \pm 3.2\%$, $n = 85$) and cells treated with RR ($16.7 \pm 6.9\%$, $n = 51$) or Tra ($4.4 \pm 2.7\%$, $n = 133$) in Ca^{2+} (+) are significantly ($*p < 0.05$) smaller than in cells treated with LPC in Ca^{2+} (+) ($63.2 \pm 5.3\%$, $n = 178$). Responder rates in cells treated with Pro in the presence of Tra ($2.0 \pm 0.2\%$, $n = 155$) are significantly ($*p < 0.05$) smaller than in cells treated with Pro ($3.7 \pm 1.4\%$, $n = 98$; $34.4 \pm 10.8\%$, $n = 167$; $57.9 \pm 10.4\%$, $n = 70$ for 300 μM , 1 mM, and 3 mM, respectively). **E**, Responder rates in cells responding to 2-APB (500 μM) with (+) GFP expression (8.4%) are significantly ($**p < 0.01$) smaller than in cells without (–) GFP expression (32.7%). Numbers in parentheses indicate cells examined. Data are from 12 samples. **F**, Traces of $[\text{Ca}^{2+}]_i$ changes in response to 3 mM Pro in mouse myenteric culture neurons (mean $\pm$ SEM) without (–; $n = 17$) or with (+; $n = 25$) expression of DN-TRPV2. **G**, Responder rate in cells responding to Pro (3 mM) with expression of DN-TRPV2 (+; 16.0%) is significantly ($*p < 0.05$) smaller than in cells without DN-TRPV2 expression (–; 53.3%). Numbers in parentheses indicate number of cells examined.

by interpolation of a standard NaNO concentration curve against fluorescence intensity. Total nitrite production was calculated as amount per gram intestinal tissue.

Gastrointestinal tract transit assay. The gastrointestinal tract transit assay was performed as described previously (Firpo et al., 2005) using WT 8- to 12-week-old male mice. Briefly, mice were fasted for 14 h with water available *ad libitum*, and then 5 mg/kg (200 μl) of a test meal containing phenol red along with the TRPV2 agonists 10 μM SNP or vehicle only was administered into the stomach using a feeding needle.

Thirty minutes later, mice were killed by cervical dislocation, the gastrointestinal tract was removed, and then the small intestine was divided into four equal-length pieces. Stomach and intestine tissues were minced, transferred to 10 ml of 0.1 N NaOH, and homogenized at room temperature for 1 h. Phenol red was not detected in any colorectal samples. Supernatant aliquots (1 ml) were transferred into 0.1 ml of 20% trichloroacetic acid solution to precipitate proteins and centrifuged at 3000 rpm for 20 min, and 250 μl of the supernatant was added to 1 ml of 0.5 N NaOH to develop the maximum color intensity. Phenol red concentration was measured by absorbance at 560 nm using a spectrophotometer and expressed as mean $\pm$ SEM in each group.

Data analysis. Ca^{2+} imaging, patch-clamp recordings, tension recordings, Griess assay, and *in vivo* study values were calculated as means $\pm$ SEM from three or more independent experiments. Student's *t* test, nonparametric Bonferroni's-type multiple comparison, or χ^2 test was used. $p < 0.05$ was considered statistically significant.

Results

Expression of TRPV2 mRNA in intestine myenteric neurons

Because TRPV2 was shown to be expressed in the rat myenteric plexus and to have some mechanosensitivity (Muraki et al., 2003; Kashiba et al., 2004; Shibasaki et al., 2010), we examined TRPV2 expression in the small intestine. mRNA expression of TRPV2 and the control PGP9.5 (a neuron marker) was detected in whole small intestine, longitudinal muscle with myenteric plexus (LMMP), and intestinal MCC (Fig. 1). TRPV1 and transient receptor potential melastatin 8 (TRPM8) mRNA were not detected, although TRP ankyrin 1 (TRPA1) mRNA was detected in the whole intestine but not in LMMP or MCC, which is consistent with the observation of functional TRPA1 expression in enterochromaffin cells and the involvement of TRPA1 in murine intestinal contractions (Nozawa et al., 2009).

Expression of TRPV2 protein in intestine myenteric neurons

Specificity of the anti-TRPV2 antibody was confirmed by Western blot analysis and absorption experiments in HEK293 cells expressing mouse TRPV2 (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) and by

absorption experiments in the immunocytochemical analysis with mouse myenteric plexus neurons (Fig. 2B). Similar to the rat intestine (Kashiba et al., 2004), TRPV2-immunoreactive (IR) cells costained with the neuron marker PGP9.5 (Fig. 2A). Approximately half (197 of 396 neurons, $49.7 \pm 3.4\%$, mean $\pm$ SEM; $n = 3$ mice) of the PGP9.5-IR neurons were TRPV2 positive. TRPV2 immunoreactivity was not observed in GFAP-IR

Figure 4. TRPV2 agonists-induced current responses in mouse myenteric culture neurons and TRPV2 mechanosensitivity in both native neurons and HEK293 cells. **A**, 2-APB (1 mM), LPC (30 μ M), and Pro (1 mM) induced inward currents in cells held at -60 mV (mean $\pm$ SEM, 127.8 ± 34.4 pA, $n = 9$ for 2-APB; 114.7 ± 11.3 pA, $n = 3$ for LPC; and 622.6 ± 267.7 pA, $n = 6$ for Pro). The shown I - V curves are from the indicated points (a–c) in each left trace and show outward rectification. RR (10 μ M) inhibited Pro-evoked current responses. **B**, Positive pressure (10 cm H₂O) to the pipette solution also induced an inward current at -60 mV with an outwardly rectifying I - V relation, which was blocked by 75 μ M Tra. Similar current responses were observed for three cells (316.5 ± 89.6 pA). **C**, Similar positive pressure-induced inward currents were blocked by Tra (75 μ M) in mouse TRPV2-transfected HEK293 cells. Similar current responses were observed in 5 of 10 cells (75.2 ± 39.1 pA/pF) but not in 13 nontransfected HEK293 cells (3.7 ± 2.8 pA/pF). **D**, Current densities for 3, 5, and 10 cm H₂O pressure-induced responses in mouse TRPV2-transfected HEK293 cells were 21.9 ± 19.8 ($n = 7$), 67.2 ± 43.8 ($n = 9$), and 75.2 ± 39.1 ($n = 10$) pA/pF, respectively. Pressure-induced TRPV2-mediated responses (10 cm H₂O) were significantly larger than those in 0 cm H₂O pressure-induced responses in TRPV2-expressing cells ($^{\#}p < 0.05$) and than those in 10 cm H₂O pressure-induced responses in cells not expressing TRPV2 ($^{*}p < 0.05$).

enteroglia or KIT-IR interstitial cells of Cajal (Fig. 2C,D). Expression of several neural subtype markers was examined to determine which neurons expressed TRPV2. Some TRPV2-IR neurons costained with an anti-nNOS antibody (Fig. 2E) and most nNOS-IR neurons (606 of 682 neurons, $88.9 \pm 0.9\%$, mean $\pm$ SEM; $n = 3$ mice) were TRPV2 positive.

It has been reported that 79.3% of nNOS-IR neurons are inhibitory circular muscle motor neurons, 10.3% are inhibitory longitudinal muscle motor neurons, and 10.3% are descending interneurons (Qu et al., 2008). Thus, it is likely that the majority of the nNOS-IR neurons observed in our study were inhibitory circular muscle motor neurons, whereas the TRPV2-negative nNOS-IR neurons may be descending interneurons. To test whether TRPV2-IR, nNOS-negative neurons (Fig. 2E) could be regarded as IPANs, we performed double staining for TRPV2 and IPAN markers CGRP or neurofilament 145 (NF145) in the mouse myenteric plexus (Qu et al., 2008) (Fig. 2F,G). Most TRPV2-IR neurons costained with CGRP and NF145, whereas $\sim 80\%$ of CGRP-IR neurons (245 of 296 neurons, $82.4 \pm 5.2\%$, mean $\pm$ SEM; $n = 3$ mice) and NF145-IR neurons (103 of 129

neurons, $81.0 \pm 3.8\%$, mean $\pm$ SEM; $n = 3$ mice) were TRPV2 positive. This suggested that TRPV2-IR neurons were inhibitory motor neurons (nNOS positive) or IPANs (CGRP or NF145 positive). When nNOS- and/or TRPV2-positive neurons in the area of plexus (Fig. 2H, left) and periphery (Fig. 2H, right) were examined carefully, again almost all the nNOS-positive neurons expressed TRPV2, and TRPV2-IR was observed on the nerve fibers that probably covered muscle tissue.

TRPV2-mediated cytosolic Ca²⁺ increase in intestinal inhibitory motor neurons

To confirm functional TRPV2 expression in myenteric neurons, we examined the response to reported TRPV2 agonists (2-APB, probenecid and LPC) (Hu et al., 2004; Bang et al., 2007; Monet et al., 2009) in isolated myenteric neurons using a fluorescent Ca²⁺-imaging system (10 μ M fura-2 AM). Representative pseudocolor images and typical ratio traces are shown in Figure 3, A and B. All three agonists increased intracellular Ca²⁺ concentrations ($[Ca^{2+}]_i$), which suggested that TRPV2 is functionally expressed in myenteric neurons. In addition, the finding that

Figure 5. TRPV2 is involved in the inhibition of spontaneous circular muscle contraction. **A**, A representative trace of dose-dependent Pro-induced inhibition of spontaneous contractions of mouse intestinal circular muscle. **B**, Dose-dependent curve for Pro-induced inhibition with an IC_{50} value of $95.4 \mu M$ ($n = 6-17$ for each concentration). Data are presented as mean $\pm$ SEM ($n = 6-17$). **C**, Relative mean contractions normalized to basal values. SNP ($10 \mu M$) caused relaxation ($46.3 \pm 8.7\%$, $n = 6$). Pro ($100 \mu M$)-induced inhibition ($48.2 \pm 8.7\%$ contraction, $n = 8$) was significantly ($*p < 0.05$) reversed by TTX ($1 \mu M$, $107.2 \pm 6.8\%$, $n = 10$), L-NAME ($100 \mu M$, $94.1 \pm 8.2\%$, $n = 13$), Hb (2 mM , $128.2 \pm 26.1\%$, $n = 10$), methylene blue (MB; 10 mM , $117.3 \pm 18.7\%$, $n = 9$), and Tra ($75 \mu M$, $108.6 \pm 5.9\%$, $n = 8$). LPC ($10 \mu M$, $73.4 \pm 8.1\%$, $n = 12$) and LPI ($10 \mu M$, $71.0 \pm 6.4\%$, $n = 6$), but not LPA ($10 \mu M$, $108.2 \pm 7.2\%$, $n = 9$), inhibited the spontaneous contractions similar to Pro ($*p < 0.05$ vs control and LPA).

LPC-induced $[Ca^{2+}]_i$ increase was abolished in the absence of extracellular Ca^{2+} [$Ca^{2+}(-)$] suggested that the $[Ca^{2+}]_i$ increase was caused by Ca^{2+} influx through the plasma membrane. Figure 3C shows that both RR, a broad TRP channel inhibitor ($10 \mu M$), and Tra ($75 \mu M$), reported to inhibit TRPV2-mediated responses (Hisanaga et al., 2009), inhibited the LPC-induced $[Ca^{2+}]_i$ increase, further supporting TRPV2 involvement. LPC-sensitive cells (as defined in Materials and Methods) were significantly reduced in the absence of $Ca^{2+}(-)$ or by RR or Tra treatment (Fig. 3D). Pro, another TRPV2 agonist, also increased responder rates in a dose-dependent manner, and Pro-induced responses were significantly reduced by Tra pretreatment (Fig. 3D). In addition, 2-APB increased $[Ca^{2+}]_i$ in 33.2% of the examined cells. Together, we concluded that TRPV2 is involved in the LPC-, Pro-, or 2-APB-induced $[Ca^{2+}]_i$ increase.

TRPV2 was expressed in either inhibitory motor neurons or IPANs (Fig. 2). To more effectively detect TRPV2-positive cells, we used ChAT-eGFP mice that express GFP mainly in IPANs or excitatory motor neurons (Tallini et al., 2006; Qu et al., 2008). When we compared 2-APB responses in myenteric neurons with and without GFP expression, the rates of 2-APB-sensitive cells were significantly larger in GFP-negative cells than in GFP-positive cells, which suggested that TRPV2 functions mainly in GFP-negative inhibitory motor neurons (Fig. 3E). To further explore the involvement of endogenous TRPV2 in the $[Ca^{2+}]_i$ in-

creases observed above, we examined the effects of DN-TRPV2 that drastically reduces TRPV2 function in myenteric neurons (Nagasawa et al., 2007; Shibasaki et al., 2010). Using ChAT-eGFP-negative myenteric neurons, Pro-induced $[Ca^{2+}]_i$ increases were reduced in DN-TRPV2-expressing cells, whereas Pro-responder rates were significantly smaller in DN-TRPV2-expressing cells than in cells transfected with pcDNA3.1 alone (Fig. 3F, G). This supported the involvement of endogenous TRPV2 in the $[Ca^{2+}]_i$ increases.

TRPV2-mediated current responses in intestinal inhibitory motor neurons

We next performed patch-clamp experiments with ChAT-eGFP-negative myenteric neurons to further confirm functional TRPV2 expression in intestinal inhibitory motor neurons. Both 2-APB (1 mM) and LPC ($30 \mu M$) evoked inward currents at -60 mV , showing an outwardly rectifying $I-V$ relationship (Fig. 4A). In addition, Pro (1 mM) also caused activation of inward currents with a similar $I-V$ relationship, which were blocked by RR ($10 \mu M$). These patch-clamp experiment results were consistent with the Ca^{2+} -imaging results (Fig. 3). Mechanical stimulus with $10 \text{ cm H}_2\text{O}$ pressure evoked an inward current activation with an outwardly rectifying $I-V$ relationship, which was inhibited by Tra ($75 \mu M$) (Fig. 4B). Similar mechanical stimulus ($10 \text{ cm H}_2\text{O}$ pressure)-evoked current responses with outward rectification were observed in

HEK293 cells expressing mouse TRPV2 [TRPV2(+)] but not in nontransfected HEK293 cells [TRPV2(-)] (Fig. 4C), which indicated that mechanical stimulus-evoked responses required TRPV2 expression. Positive pressure stimulus did not affect cell size (expressed by cell capacitance) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). TRPV2-mediated currents became larger as pressures increased (Fig. 4D). Negative pressure also evoked TRPV2-mediated current responses (data not shown). Even $3 \text{ cm H}_2\text{O}$ pressure evoked TRPV2-mediated current responses, although it was not statistically significant, which suggested that enteric neurons expressing TRPV2 could respond to weak intestinal distortion *in vivo*.

Relaxation of mouse intestine in response to TRPV2 agonists and its blockade by the TRPV2 antagonist TTX or NOS pathway inhibitors

The above results suggested TRPV2 involvement in intestinal function, especially relaxation. To examine this possibility, we measured spontaneous contractions of the circular muscle, using an isometric transducer. System reliability was confirmed using ACh and SNP that caused a contraction and a prolonged relaxation, respectively, as measured by changes in the height of the spontaneous basal contractions (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Pro ($10 \mu M$ to 3 mM) treatment of mouse intestine preparations caused

Figure 6. Increased NO production in whole intestine by probenecid. **A**, NO production was significantly increased in the intestine in response to Pro (100 μM, 93.9 ± 9.0 μmol/g, $n = 10$) compared with no treatment (51.9 ± 6.8 μmol/g, $n = 10$, $*p < 0.05$). **B**, Pro-induced increase in NO production was dose dependent (1.0 ± 0.2, $n = 40$; 2.0 ± 0.5, $n = 31$; and 2.7 ± 0.4, $n = 27$ for 10, 100, and 1000 μM, respectively). Data are expressed as mean ± SEM after normalization to baseline values. $^{\#}p < 0.05$ versus Pro (10 μM).

concentration-dependent relaxation, with a half-inhibition concentration (IC₅₀) value of 95.4 ± 27.2 μM, that recovered after washout (Fig. 5A, B). Pro at 100 μM caused 48.2 ± 8.7% relaxation, a value similar to that obtained using SNP (10 μM, 46.3 ± 8.7%) (Fig. 5C). Pro-induced relaxation was significantly inhibited by TTX (1 μM), which indicated involvement of neural activity in the relaxation. After pretreatment with several NO pathway inhibitors (100 μM L-NAME, 2 mM Hb, or 10 mM methylene blue), Pro did not cause significant relaxation (Fig. 5C), which suggested that the NO pathway was involved in Pro-induced relaxation, which is supported by a similar effect obtained using the NOS inhibitor L-NNA (data not shown). Pro-induced relaxation was also inhibited by Tra (75 μM) (Fig. 5C). Furthermore, TRPV2 activators LPC (10 μM) and LPI (10 μM) (Monet et al., 2009) also inhibited spontaneous contractions. In contrast, LPA (10 μM), a related compound that does not activate TRPV2, did not cause significant relaxation (Fig. 5C), consistent with a role for TRPV2 activation in relaxation.

Effects of probenecid on intestinal NO release

Given the apparent involvement of TRPV2 in NO-mediated intestinal relaxation, we examined NO production from small intestinal specimens stimulated by a TRPV2 agonist by the Griess reaction. As shown in Figure 6, A and B, supernatant NO₂ + NO₃ levels significantly increased from basal values (51.9 ± 6.8 μmol/g) to 93.9 ± 9.0 μmol/g after 30 min Pro (100 μM) stimulation in a dose-dependent manner. This suggested that TRPV2 activation causes NO production, probably through Ca²⁺ influx.

Effects of intragastric TRPV2 agonists on gastrointestinal tract transit

Finally, to evaluate possible TRPV2-mediated regulation of intestinal transit *in vivo*, we examined dye movement attributable to peristaltic propulsion (coordinated intestinal contraction and relaxation). Dye concentrations at the distal intestine (Fig. 7A, arrow) were used as a measure of transit speed. Significantly higher dye concentrations were observed after intragastric administration of TRPV2 agonists (1 mM Pro and 10 μM LPC) but not after LPA (10 μM) (Fig. 7B). Furthermore, the Pro-induced increase in dye concentration was blocked by coadministration of Tra (75 μM). Together with the finding that intragastric SNP administration also accelerated intestinal movement (Fig. 7B), our results

Figure 7. Increased gastrointestinal transit by TRPV2 agonists and SNP. **A**, Mouse small intestine was divided into four segments (shown by black bars), and dye concentrations in the fourth segment (shown as an arrow) were measured. **B**, Dye concentrations in mice treated with Pro (1 mM, 174.1 ± 26.5 μg/L, $n = 8$) were significantly higher ($*p < 0.05$) than in mice without treatment (29.7 ± 13.0 μg/L, $n = 10$). Tra (75 μM) treatment abolished the Pro-induced increase in dye concentration (31.2 ± 7.6 μg/L, $n = 7$). LPC (10 μM, 180.1 ± 49.8 μg/L, $n = 5$), but not LPA (10 μM, 22.3 ± 16.7 μg/L, $n = 5$), significantly ($*p < 0.05$) increased dye concentration. SNP (10 μM) showed similar effects (273.5 ± 109.8 μg/L, $n = 5$, $*p < 0.05$). Values are mean ± SEM, $n = 5$ –10.

suggested that TRPV2 accelerates intestinal movement through NO production.

Discussion

We identified anatomical and functional TRPV2 expression in murine intestinal myenteric neurons, especially in inhibitory motor neurons and/or IPANs (Figs. 1–4). TRPV2 activation induced intestinal relaxation through NO production, probably via cytosolic Ca²⁺ increase, leading to gut transit acceleration (Figs. 5–7). Our results strongly suggested that both inhibitory motor neurons and IPANs were involved in the observed TRPV2-mediated peristaltic propulsion, although we cannot rule out the involvement of TRPV2 expressed in the sensory neurons whose cell bodies are located in the nodose ganglion. Because probenecid did not increase contraction in the presence of L-NAME (Fig. 5C) or L-NNA, IPAN involvement is unlikely, suggesting instead the predominant involvement of inhibitory motor neurons. Additional experiments are needed to determine to what extent TRPV2-expressing IPANs are involved in the regulation of intestinal movement. In addition, L-NAME did not completely reverse TRPV2-induced inhibition of contraction (Fig. 5C), which suggests the existence of other inhibitory neurotransmitters. Indeed, other inhibitory neurotransmitters, such as ATP, vasoactive intestinal polypeptide, β-nicotinamide adenine dinucleotide, hydrogen sulfide, and carbon monoxide, have been reported (Burnstock et al., 1970; Fahrenkrug et al., 1979; Miller et al., 2001; Mutafova-Yambolieva et al., 2007; Linden et al., 2008).

In the present study, intestinal relaxation caused accelerated gut transit (Figs. 5, 7). Enterochromaffin cells are believed to sense mechanical stretch, leading to IPAN activation followed by enhanced inhibitory motor neuron activity in the aboral regions. TRPV2-mediated NO release from inhibitory motor neurons could support this mechanism to increase transit. The link between TRPV2 activation and NO release in intestinal movement was supported by the finding that SNP alone accelerated intestinal transit (Fig. 7B).

In our study, we used a mechanical stimulus to activate TRPV2, because TRPV2 has been shown to be mechanosensitive in cardiac myocytes and in sensory and spinal motor neurons

(Muraki et al., 2003; Shibasaki et al., 2010). Indeed, many IPANs and some uniaxonal neurons are also reported to be mechanosensitive and activated by muscle contractions in guinea pig intestine (Kunze et al., 1998, 1999). Mechanosensitive channels in IPANs and some uniaxonal neurons appear to be Gd^{2+} insensitive and not pure Ca^{2+} channels (Furness et al., 2004b), properties similar to those of TRPV2 channels. In addition, the distribution of the reported mechanosensitive channels matches the distribution of TRPV2 observed in this study. The finding that TRPV2 expression with nNOS immunoreactivity was observed not only in the plexus but also in the nerve fibers probably covering muscle layers (Fig. 2) suggests a model in which inhibitory motor neurons, especially nerve fibers, expressing TRPV2 directly detect muscle distortion and then release NO for intestinal relaxation at the site of mechanical stimulus. Basal amounts of $\text{NO}_2 + \text{NO}_3$ (Fig. 6) suggested continuous basal NO release, although the physiological significance of this is unknown. Basal activation of TRPV2 by endogenous mechanical stimulus could cause NO release via Ca^{2+} influx, leading to regulation of intestinal movement by inhibiting overaction. Luminal extension with food in the intestine might then activate TRPV2 mechanically followed by aboral intestinal relaxation. These mechanisms could play pivotal roles in intestinal relaxation in concert with the enterochromaffin cell-mediated pathway.

In addition to mechanical stimulus, TRPV2 has several known chemical activators, including endogenous lysophospholipids LPC and LPI that cause relaxation (Fig. 5C) and enhance transit (Fig. 7B) after TRPV2 activation in myenteric neurons (Figs. 3, 4A). LPC is a major phospholipid component of atherogenic oxidized low density lipoprotein and is present (micromolar range) in some tissues (Okita et al., 1997; Ishii et al., 2004). Therefore, it is tempting to speculate that TRPV2 could be activated by both mechanical and chemical stimuli synergistically to regulate intestinal contractions in physiological and pathological conditions.

Because NO is a major inhibitory neurotransmitter and its abnormal production and release may be involved in functional gastrointestinal disorders (Takahashi, 2003), development of compounds acting on TRPV2 channels could be useful.

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