TRPV2 ion channels expressed in inhibitory motor neurons of gastric myenteric plexus contribute to gastric adaptive relaxation and gastric emptying in mice

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Mihara H, Suzuki N, Yamawaki H, Tominaga M, Sugiyama T. TRPV2 ion channels expressed in inhibitory motor neurons of gastric myenteric plexus contribute to gastric adaptive relaxation and gastric emptying in mice. Am J Physiol Gastrointest Liver Physiol 304: G235–G240, 2013. First published November 29, 2012; doi:10.1152/ajpgi.00256.2012.—Gastric adaptive relaxation (GAR) is impaired in ~40% of functional dyspepsia (FD) patients, and nitric oxide (NO) released from inhibitory motor neurons plays an important role in this relaxation. Although the underlying molecular mechanism of GAR is poorly understood, transient receptor potential channel vanilloid 2 (TRPV2) mechano- and chemoreceptors are expressed in mouse intestinal inhibitory motor neurons and are involved in intestinal relaxation. The aim of this study was to evaluate the distribution of TRPV2 in inhibitory motor neurons throughout the mouse gastrointestinal tract and the contribution of TRPV2 to GAR. RT-PCR and immunohistochemical analyses were used to detect TRPV2 mRNA and protein, respectively. Intragastric pressure was determined with an isolated mouse stomach. Gastric emptying (GE) in vivo was determined using a test meal. TRPV2 mRNA was detected throughout the mouse gastrointestinal tract, and TRPV2 immunoreactivity was detected in 84.3% of neuronal nitric oxide synthase-expressing myenteric neurons in the stomach. GAR, which was expressed as the rate of decline of intragastric pressure in response to volume stimuli, was significantly enhanced by the TRPV2 activator probenecid, and the enhancement was inhibited by the TRPV2 inhibitor tranilast. GE was significantly accelerated by TRPV2 agonist applications, and the probenecid-induced enhancement was significantly inhibited by tranilast coapplication. Mechanosensitive TRPV2 was expressed in inhibitory motor neurons in the mouse stomach and contributed to GAR and GE. TRPV2 may be a promising target for FD patients with impaired GAR.

gastric adaptive relaxation; gastric emptying; nitric oxide; inhibitory motor neuron; transient receptor potential channel vanilloid 2

Gastric adaptive relaxation (GAR) or accommodation (29, 31, 33). Mechanosensitive neurons, neuronal nitric oxide (NO) synthase (nNOS)-positive neurons, and NO release may play important roles in GAR in rats, guinea pigs, and humans (17, 24, 26, 27). Furthermore, any dysfunction in this process may cause functional dyspepsia (FD) (30). While it has been suggested that delayed gastric emptying (GE) was related to symptoms, recent studies have indicated that abnormality in GAR increases intragastric pressure (IGP) and conversely enhances early GE (15, 25). Additional studies have suggested that increased GAR enhances GE (32). The relationship between GAR and emptying and their underlying molecular mechanisms have yet to be elucidated.

The transient receptor potential vanilloid 2 (TRPV2) was originally isolated as a molecule sensitive to temperatures above 52°C (3) but has also been shown to be sensitive to certain chemicals (probenecid, lysophospholipids) (1, 20) and mechanical stimuli (18, 20, 28). It is important to note that TRPV2 knockout mice displayed normal thermal and mechanical nociception (23) and that the positive cardiac inotropic effect of the TRPV2 agonist probenecid was absent in these mice in vivo (10).

We have previously reported that TRPV2 was expressed in inhibitory motor neurons and intrinsic primary afferent neurons in mouse intestine and contributed to intestinal relaxation and transit (18). However, TRPV2 expression in other gastrointestinal regions, including the stomach, and its functions remain unknown. In the present study, we investigated whether TRPV2-positive inhibitory motor neurons contributed to GAR and emptying.

Glossary

GI Gastrointestinal
FD Functional dyspepsia
NO Nitric oxide
TRPV2 Transient receptor potential channel vanilloid 2
RT-PCR Reverse transcription polymerase chain reaction
GE Gastric emptying
GAR Gastric adaptive relaxation
nNOS Neuronal nitric oxide synthase
IGP Intragastric pressure
L-NAME Nω-nitro-L-arginine methyl ester
LPC Lysophosphatidylcholine
ChAT Choline acetyltransferase
IPAN Intrinsic primary afferent neuron
NADPH Nicotinamide adenine dinucleotide phosphate

MATERIALS AND METHODS

Animals. Male C57BL/6 (8-wk-old; SLC) mice were housed in a controlled environment (12:12-h light-dark cycle; room temperature, 22–24°C; 50–60% relative humidity) with free access to food and water. All procedures involving the care and use of animals were

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approved by the Institutional Animal Care and Use Committee of the University of Toyama and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (21).

**RT-PCR analysis.** To examine TRPV2 expression in the murine gut, total RNAs (1 μg) isolated from esophagus (striated muscle), stomach, duodenum, colon, and rectum were used for reverse transcription with the Superscript III first-strand synthesis system for RT-PCR (Invitrogen). PCR was performed using Taq DNA polymerase (TaKaRa) in an iCycler (Bio-Rad) with specific primer sets (Table 1) for the TRPV2 channel and GAPDH. The following PCR conditions were used: 1 cycle at 94°C for 30 s; and 1 cycle at 72°C for 2 min.

**Immunohistochemistry.** The immunohistochemical methods used in the present study have been previously described (18). All experiments were repeated on specimens from at least three mice. Antibody information is summarized in Table 2. Mice were anesthetized with diethyl ether and perfused through the heart with a 4% paraformaldehyde in phosphate buffer solution (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2). Each organ was removed and stored at 4°C overnight. Next, they were embedded in optimum cutting temperature compound (Tissue Tek, Elkhart, IN), and 14-μm thick sections were collected onto slides and dried at room temperature before exposure to primary antibodies. Sections were then incubated with primary antibodies at 4°C overnight and then washed (three times, 10 min each) in PBS and mounted on glass slides. Preparations were analyzed using a confocal laser-scanning microscope (Keyence BZ-8000) and a fluorescent microscope (LSM 700; Carl Zeiss).

**Recordings of IGP.** IGP of isolated mouse stomach were recorded using a modified method (2, 6). Briefly, 8-wk-old male wild-type (WT) mice were killed by cervical dislocation. The whole stomach with esophagus and duodenum was removed and kept in standard bath solution (140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, and 10 mM glucose at pH 7.4, adjusted with NaOH) at 37°C bubbled with 95% O2-5% CO2. An 18-gauge Cathelin needle was inserted from the esophagus to the stomach, and the edges of the esophagus and duodenum were tied with thread. A three-way tap linked the Cathelin needle, a 5-ml syringe, and the pressure transducer, thereby recording IGP. IGP was recorded and viewed in real time using customized PowerLab Chart5 v5.1 software (AD Instruments). After tissue recovery for 3 min, IGP were set at 0 mmHg and recorded in response to stepwise isovolumetric distensions. GAR compliance expressed as the rate of decline of IGP to each volume stimuli (0–20 s) and plateau pressure expressed as plateau values minus basal values were evaluated using the same software. Responses with or without pretreatment with a TRPV2 agonist (probenecid from Sigma) (1, 10, 18, 28), and inhibitory effects of tranilast (a TRPV2 inhibitor, from Kissei) (8), N0- nitro-l-arginine methyl ester [L-NAME, a nitric oxide synthase (NOS) inhibitor], tetrodotoxin (TTX, a voltage-gated sodium channel blocker, from Sangkyo), hexamethonium (a nicotinic ACh receptor antagonist), and nifedipine (an L-type Ca2+ channel blocker) were evaluated. Pretreatment with these inhibitors occurred 3 min before the application of probenecid. Inhibitors were purchased from Sigma, with the exception of tranilast and TTX.

**GE (phenol red method).** The GE assay was modified from that previously described (18) using WT 8-wk-old male mice. Briefly, mice fasted for 14 h with water available ad libitum. A test meal, 5 mg/kg (200 μl), containing phenol red and TRPV2 agonists [1 mM probenecid or 10 μM lysophosphatidylcholine (LPC)] or vehicle (water) with and without the TRPV2 inhibitor tranilast was administered into the stomach using a feeding needle. Mice were killed 15 min later by cervical dislocation, the isolated stomach was minced and transferred to 10 ml 0.1 N NaOH, and the remaining test meal was calculated and expressed as means ± SE in each group.

**RESULTS**

**Expression of TRPV2 mRNA throughout the mouse gastrointestinal tract.** Because TRPV2 mRNA has been detected in myenteric plexus in mouse intestine, we examined TRPV2 expression in other gastrointestinal regions. TRPV2 and control (GAPDH) mRNAs were detected throughout the gastrointestinal tract (Fig. 1).

**Expression of TRPV2 protein in nNOS-positive neurons and throughout the gastrointestinal tract.** Specificity of the anti-TRPV2 antibody used in the present study was confirmed by the absorption assay using mouse stomach (Fig. 2A) and by absorption assay and Western blotting conducted in our previous study (18). Each region was double-stained with anti-TRPV2 and anti-nNOS antibodies. Most nNOS-immunoreactive (IR) myenteric neurons in the esophagus (striated muscle) (98 of 105 neurons, 93.3 ± 2.5%, n = 3 mice), stomach (436 of 522 neurons, 84.3 ± 3.4%, n = 5 mice), and colon (235 of 466 neurons, 49.9 ± 3.4%, n = 3 mice) expressed TRPV2. The percentage of double-labeled neurons was 84.3 ± 2.5%, 85.3 ± 2.2%, and 49.9 ± 3.4%, respectively, in esophagus, stomach, and colon, respectively.
trypsinogen (both positive/nNOS-negative neurons and fibers may be excitatory involved in other processes such as esophageal movement, although investigation is required to clarify whether TRPV2 is expressed in inhibitory motor neurons of the gastric periphery, as previous studies demonstrated that axons of IPANs are rare or absent in the stomach (4, 5), and because TRPV2 expressed in inhibitory motor neurons (26). Thus, it is likely that the majority of inhibitory motor neurons expressed TRPV2 (37 of 78 neurons, 47.4%). We divided each mouse stomach without the associated forestomach (stratified squamous epithelium) into three equal parts (upper, middle, and lower) and evaluated the TRPV2-positive rate in nNOS-positive neurons. No regional differences were detected (upper 79.3%, middle 76.0%, lower 77.5%).

Effects of TRPV2 modulators on IGP. We hypothesized that TRPV2 expressed in inhibitory motor neurons of the gastric myenteric plexus may detect changes in IGP and enhance gastric compliance. To test this hypothesis, we measured changes in IGP of isolated mouse stomach responding to volume stimuli. Gastric compliances were significantly larger upon pretreatment with the TRPV2 agonist probenecid (1 mM) than with control (Fig. 3, A and B). This effect in the response to 1.0 ml of volume stimuli was diminished by pretreatment with the TRPV2 inhibitor tranilast (75 μM), the NOS inhibitor L-NAME (100 μM), TTX (1 μM), or nifedipine (100 μM) but not by pretreatment with the nicotinic ACh receptor antagonist hexamethonium (100 μM). Plateau IGP's were not affected by pretreatment with probenecid, probenecid + tranilast, or probenecid + L-NAME (Fig. 3, A and C).

Effects of TRPV2 modulators on GE. Although some reports have suggested that abnormalities in GAR increase IGP and enhance GE, others have shown that enhancement of GAR enhances GE. Retention rates of test meals were significantly smaller with not only coadministration of the TRPV2 agonists probenecid (1 mM) or LPC (10 μM) but also the TRPV2 inhibitor tranilast (75 μM) than with control (P < 0.05, Fig. 4A). Addition of the TRPV2 inhibitor tranilast (75 μM) significantly blocked the probenecid-induced acceleration of emptying, but not significantly in the case of LPC (Fig. 4, B and C).

**DISCUSSION**

We observed anatomical TRPV2 expression in nNOS-positive neurons (primarily inhibitory motor neurons) throughout the mouse gastrointestinal tract. Myenteric NADPH-positive neurons in the stomach represented 50–60% of the neurons in all three regions (fundus, body, and antrum) in humans (17) and 29% in guinea pigs (27). In these species, the percentages of NADPH-reactive motor, nonmotor, and multitargeted neurons were reportedly 57, 39, and 4%, respectively. Additionally, the majority of NADPH-reactive motor (86%) and nonmotor (86%) neurons had descending projections, indicating that NADPH-reactive neurons in the stomach are primarily descending neurons that may mediate descending relaxation (26). Thus, it is likely that the majority of inhibitory motor neurons and descending interneurons in mice express TRPV2. TRPV2-IR was observed in nerve fibers that may cover muscle tissue, suggesting that TRPV2 could support excitability in the periphery, as previous studies demonstrated that axons of motor neurons in muscle are not mechanosensitive (12). TRPV2 was also expressed in nNOS-positive neurons in the esophagus (striated muscle), duodenum, and colorectum. Further investigation is required to clarify whether TRPV2 is involved in other processes such as esophageal movement, stool transit, and defecation (9, 14, 35). Observed TRPV2-positive/nNOS-negative neurons and fibers may be excitatory motor neurons or extrinsic fibers, since most evidence suggests that IPANs are rare or absent in the stomach (4, 5), and because TRPV2 is expressed in TRPV1-negative medium-to-large-
diameter neurons of the rat dorsal root and trigeminal ganglia (3, 16). The functions of TRPV2-positive/nNOS-negative neurons and fibers should be the focus of further investigations.

Probenecid-induced enhancement of gastric compliance was inhibited by the TRPV2 inhibitor, the NOS inhibitor, TTX, or nifedipine, but not by hexamethonium, suggesting that these enhancements were mediated by TRPV2 channel and neural activities but not by nicotinic receptors. These results support the hypothesis that TRPV2-positive inhibitory motor neurons are involved in the probenecid-induced enhancement of gastric compliance, since IGP measurement in vitro can exclude the contribution of the reflex mediated by extrinsic neurons (Fig. 5). Although a tone reaction, especially in the fundus, is essential for the adaptive relaxation, no regional differences were detected. The discrepancy may be attributable to anatomical differences between human and mouse (forestomach) or extrinsic reflex modification. The involvement of TRPV2-positive extrinsic neurons in GE in vivo cannot be ruled out, since TRPV2 expression was also observed in TRPV1-negative medium-to-large-diameter neurons of the rat dorsal root and trigeminal ganglia (3, 16). Additionally, the TRPV2 inhibitor tranilast only enhanced GE (Fig. 4A), suggesting that the pyloric sphincter is not involved in probenecid-induced GE enhancement and that suitable TRPV2 activation might be important for physiological GE.

IGPs in the present study ranged between 0 and 12 mmHg (0–16.3 cmH2O). This range is similar to that required to
activate TRPV2 in the heterologous expression system (3–10 cmH2O) (18), suggesting that TRPV2, a mechanosensor in the intestine, may also be a mechanosensor in the stomach. This hypothesis is supported by findings indicating that mechanosensitive channels expressed by myenteric neurons in the guinea pig exhibit a similar property to that of TRPV2 (11, 13, 18), although the properties of mechanosensors in the stomach have not been evaluated thus far. Plateau IGPs were not affected by TRPV2 modulators. In addition to mechanical stimuli, TRPV2 has several known chemical activators, including endogenous lysophospholipids (LPC, etc.) that enhance oxidized low-density lipoprotein and has been reported in some tissues (7, 22). The compound acting on the TRPV2 ion channel could be a novel therapeutic target for gastric relaxation impairment with implications for those with FD.

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DISCLOSURES

The authors have declared that no conflicts of interest exist.

AUTHOR CONTRIBUTIONS

Author contributions: H.M., M.T., and T.S. conception and design of research; H.M., N.S., and H.Y. performed experiments; H.M. and N.S. analyzed data; H.M., N.S., M.T., and T.S. interpreted results of experiments; H.M. prepared figures; H.M., M.T., and T.S. drafted manuscript; H.M., M.T., and T.S. edited and revised manuscript; H.M., N.S., H.Y., M.T., and T.S. approved final version of manuscript.

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