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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1Department of Gastroenterology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan; 2Division of Cell Signaling, Okazaki Institute for Integrative Bioscience (National Institute for Physiological Sciences), National Institutes of Natural Sciences, Okazaki, Japan; and 3Department of Physiological Sciences, The Graduate University for Advanced Studies, Okazaki, Japan

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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 probenecid 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

THE GASTRIC DISTENTION STIMULUS that triggers visceral perception in humans is normally a low-pressure mechanostimulus arising from food ingestion. Such a low-pressure stimulus triggers a tone reaction in the upper stomach, primarily the fundus, that enables the upper area of the stomach to receive, retain, and digest food. This is referred to as 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
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 rTaq 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 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. 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 further fixed at 4°C for 6 h. Organs were washed in PBS (three times, 15 min each), placed in PBS-sucrose (PBS containing 20% sucrose), and stored at 4°C overnight. Next, they were embedded in optimum cutting temperature compound (Tissue Tek, Elkhart, IN), cut into 15 min each, placed in PBS-sucrose (PBS containing 20% sucrose), and stored at 4°C overnight. Then, they were sectioned in the horizontal plane at 8 µm thickness with a cryostat. Sections were then incubated with 3% bovine serum albumin (Sigma) in PBS-T 0.3% for 1 h at room temperature before exposure to primary antibodies. Sections were then incubated with the primary antibodies at 4°C overnight and then washed (three times, 10 min each) in PBS-T 0.3% 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. Preparations were analyzed using a fluorescent microscope (Keyence BZ-8000) and a confocal laser-scanning microscope (LSM 700; Carl Zeiss).

Recordings of IGP. IGFs 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, IGFs were set at 0 mmHg and recorded in response to stepwise isovolumetric distensions. GAR compliance expressed as the rate of decline of IGFs 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 Kissel) (8), N⁶-nitro-L-arginine methyl ester [L-NAME, a nitric oxide synthase (NOS) inhibitor], tetrodotoxin (TTX, a voltage-gated sodium channel blocker, from San-kyo), 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 adminis-

Table 2. Characteristics of primary and secondary antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Tissue Antigen</th>
<th>Host</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>TRPV2</td>
<td>Rabbit</td>
<td>1:200</td>
<td>TransGenic</td>
</tr>
<tr>
<td>Neuronal NOS</td>
<td>Sheep</td>
<td>1:2000</td>
<td>P.C. Emson (34)</td>
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<tr>
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<td>IgG-Alexa488</td>
<td>1:1,500</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Donkey anti-sheep</td>
<td>IgG-Alexa546</td>
<td>1:1,500</td>
<td>Molecular Probes</td>
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NOS, nitric oxide synthase.
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 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 (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 (stratified 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 neu-
rons 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 de-
tected. The discrepancy may be attributable to anatomical
differences between human and mouse (foresomach) or ex-
trinsic 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

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**Fig. 3.** Representative traces and accumulated data of volume-induced intragastric pressure (IGP) increases. A: traces of IGP in control (Cont), pretreatment with probenecid (Prob), or both tranilast (Tra) and probenecid, and points at which volume was altered. Black slopes and gray bars indicate the rate of decline of IGP to each volume stimuli or the plateau pressure, respectively. B: pretreatment with 1 mM of probenecid significantly increased the rates of decline of IGP (mmHg/s) to 0.5- or 1.0-ml volume stimuli. The increases in response to 1.0-ml volume stimuli were inhibited by coadministration with the TRPV2 inhibitors tranilast, Nω-nitro-L-arginine methyl ester (L-NAME), tetrodotoxin (TTX), or nifedipine (Nife) but not with the nicotinic ACh receptor antagonist hexamethonium (Hexa). C: plateau IGP was not affected by pretreatment with probenecid and/or tranilast.

**Fig. 4.** Increased gastric emptying by TRPV2 agonists. A: retention rates following treatment with control, probenecid (1 mM), lyso phosphatidylcholine (LPC, 10 μM), or tranilast (75 μM). These were significantly smaller in mice treated with probenecid, LPC, or tranilast than in mice without treatment (*P < 0.05). B: tranilast (75 μM) cotreatment significantly abolished the probenecid-induced exacerbation of gastric emptying. C: cotreatment with tranilast inhibited LPC-induced exacerbation. Values are means ± SE; n = 6–13 experiments.

**Fig. 5.** Schematic diagram of TRPV2-induced gastric adaptive relaxation and emptying enhancement. Mechanical stimuli with food intake could activate TRPV2 expressed in inhibitory motor neurons of the gastric myenteric plexus. Nitric oxide (NO) released from inhibitory motor neurons relaxes smooth muscle, enhancing gastric adaptive relaxation. These mechanisms might contribute to gastric emptying.
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 IGP$s were not positive inotrope acting via cardiac TRPV2 stimulation. J Mol Cell Cardiol 53: 134–144, 2012.


