Properties of *Rikkunshi-to* (TJ-43)-induced relaxation of rat gastric fundus smooth muscles

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*Rikkunshi-to* (TJ-43) is one of the traditional Japanese medicines consisting of eight herbs (*Glycyrrhiza radix*, *Zingiberis rhizoma*, *Atractylodis lanceae* *rhizoma*, *Zizyphi pericarpium*, *Ginseng radix*, *Aurantii nobilis* pericarpium, *Ginseng radix*, *Pinelliae tuber*, and *Hoelen*). In Japan, *Rikkunshi-to* has been used to treat FD (31) and dyspepsia associated with organic disease (32, 35). Recent study has indicated that TJ-43 is also effective in other disease such as gastroesophageal reflux disease (11). TJ-43 is thought to improve FD by reversing an existing impaired adaptive relaxation (2, 9), leading to an improvement of delayed gastric emptying (12). Additionally TJ-43 exerts protective effects on the mucosa (7) and raises levels of somatostatin and gastrin in human plasma (20). Interestingly, TJ-43 improves both cisplatin-induced gastrointestinal dysfunction (30) and selective serotonin reuptake inhibitor-induced anorexia (6) via an increase in the plasma active ghrelin levels. However, the cellular mechanism of TJ-43 on smooth muscle cells of gastric fundus has not yet been fully elucidated in impaired gastric accommodation because most in vitro studies attempting to characterize the main mechanism of TJ-43 action on FD have performed with whole stomach preparations for measurement of intragastric volume with pressure (9) or gastric emptying rate (12).

The present experiments examined the effects of TJ-43 on the contractility and electrical activity of the circular smooth muscles (CSMs) isolated from the rat gastric fundus. Furthermore, the responses to TJ-43 were compared in the rat fundic muscles (CSMs) isolated from the rat gastric fundus. Because TJ-43-evoked relaxation of fundic CSM strips was more potent in diabetic GK rat than in control rat, further analysis of this herb could lead to better treatments of diabetic gastroparesis.

**GASTRIC RESERVOIR FUNCTION** is an important, unique property of the proximal stomach to accommodate the intake of food and liquid (1, 18, 21). It has been reported that this reservoir function of the stomach is mediated by two mechanisms, adaptive and receptive relaxations. Adaptive relaxation is a reflex elicited by dilation of the fundic region in response to small increases in intraluminal pressure when food enters the stomach. Receptive relaxation is a reflex stimulated by dilation of the gastric fundus during swallowing of the food passing down the pharynx and the esophagus. In both cases, nitric oxide (NO) seems to be a final mediator to generate gastric relaxation because inhibitors of NO synthase (NOS) abolish these two types of reflex responses (1, 4).

Disturbance of the adaptive relaxation in the absence of underlying organ disease results in symptoms called functional dyspepsia (FD) (28, 29). The symptom complex includes epigastic pain, bloating, postprandial fullness, early satiety, nausea, vomiting, belching, and anorexia. Therefore, adaptive relaxation is likely to be related to the pathogenesis of FD. Other forms of dyspepsia can be caused by organic diseases such as stomach cancer and stomach ulcer (25) or caused by diabetic mellitus (DM) (27). Although drugs promoting gastric emptying could improve dyspepsia symptoms, trials to evaluate the effects of such drugs are scarce and their results mostly negative. Thus no pharmacological treatment of established efficacy is presently available for patients with gastric dyspepsia (13).

Diabetic male Goto-Kakizaki (GK) rats (GK/Crj) and nondiabetic male Wistar rats were obtained at the age of 8 wk from a commercial breeder (Charles River, Shizuoka, Japan). The rats (age 10–15 wk) were weighed, anesthetized with fluoromethyl 2,2,2-trifluoro-1-(tri-fluoromethyl) ethyl ether (sevoflurane; Maruishi Pharmaceuticals, Osaka, Japan), and then exsanguinated by bleeding from the femoral arteries. The blood glucose level was measured three times using glucose test kit (ACCU-CHEK Aviva; Roche Diagnostics K. K., Osaka, Japan), and then exsanguinated by bleeding from the femoral arteries. The blood glucose level was measured three times using glucose test kit (ACCU-CHEK Aviva; Roche Diagnostics K. K., Tokyo, Japan), and the mean value of the measurement was used. All animals were treated according to the Guidelines for the Care and Use of Laboratory Animals of Nagoya City University Medical School.
accrued by The Physiological Society of Japan, and were approved by Nagoya City University animal center (approval no. H19–36). The stomach was excised and opened by cutting along the small curvature in Krebs solution (see below). The mucosal layers were removed by cutting with fine scissors, and smooth muscle tissues were isolated from the fundic region. For tension measurement, a tissue segment (about 2–3 mm wide and 10–15 mm long) of circular muscle was dissected. For intracellular electrical recordings, the serosal layers and longitudinal layers were carefully peeled away under a dissecting microscope, and small pieces of gastric fundus, ~0.5 mm wide and 0.5 mm long, were dissected.

**Isometric tension measurement.** Circular muscle preparations were transferred to 2-ml organ baths and were superfused with warmed (36°C) Krebs solution at a constant flow rate (4 ml/min) using peristaltic pump (PO-1; Tokyo Rikakikai, Tokyo, Japan). Silk threads were tied around both ends of a strip; one of them was fixed at the bottom of the organ bath, and the other was connected to an isometric force transducer (FD-pick up TB-612T; Nihon Kohden, Tokyo, Japan). Tissue strips were superfused with the organ bath. The neural selectivity was confirmed by sensitivity to tetrodotoxin (TTX) (1 μM).

**Intracellular microelectrode recordings.** When recording the membrane potential, preparations were pinned out on a silicone rubber plate with the serosal side uppermost, and the plate was fixed at the bottom of an organ bath (8 mm wide, 8 mm deep, 20 mm long). The tissue was superfused with oxygenated Krebs solution (36°C), at a constant flow rate of about 3 ml/min. After 2 h equilibration, CSM cells were impaled with glass capillary microelectrodes (outer diameter 1.2 mm, inner diameter 0.6 mm; Hilgenberg, Germany) filled with 3 M KCl with tip resistances ranging between 50 and 80 MΩ. Electrical responses recorded via a high input impedance amplifier (Axoclamp-2B; Axon Instruments, Foster City, CA) were displayed on a cathode-ray oscilloscope (SS-7602; Iwatsu, Osaka, Japan) and also stored on a personal computer for later analysis. Intramural nerves were selectively stimulated by passing brief pulses of constant current (duration 0.3 ms) between two parallel silver-plated electrodes placed in the organ bath. The neural selectivity was confirmed by sensitivity to tetrodotoxin (TTX) (1 μM).

**General observations.** When enprostil (0.5 μM), a prostaglandin E2 analog (3), was applied to CSM strips of rat gastric fundus, it produced a phasic, followed by a sustained contraction (Fig. 1A). In 10 of 27 preparations, enprostil generated oscillatory contractions during the sustained contraction (see Fig. 7A). An application of TJ-43 (0.1–5 mg/ml) produced a dose-dependent relaxation of the enprostil-induced contraction throughout to minimize the movement of muscles and inhibit neuronal excitation, respectively.

Experimental values were expressed by the means ± SD. Statistical significance was tested using Student’s t-test, and probabilities of less than 5% (P < 0.05) were considered significant.

The ionic composition of the Krebs solution was as follows (in mM): 137.4 Na+, 5.9 K+, 2.5 Ca2+, 1.2 Mg2+, 15.5 HCO3-, 1.2 H2PO4-, 134 Cl−, and 11.5 glucose. The solutions were aerated with O2 containing 5% CO2, and the pH of the solutions was maintained at 7.2–7.3. The concentration of K+ was modified by the isotonic replacement of NaCl with KCl.

Drugs used were nifedipine, Nω-nitro-l-arginine (l-NNa), sodium nitroprusside (SNP), verapamil (from Sigma, St. Louis, MO), apamin (Peptide Institute, Osaka, Japan), 6-[2-(4-[4-fluorophenyl]phenylmethylene]-1-piperidinyl)ethyl]-7-methyl-5H-thiazolo [3,2-a] pyrimidine-5-one (R59022), 1H[1,2,4] oxadiazolo [4,3-a]quinolinaxin-1-one (ODQ) (from Tocris Cookson, Bristol, UK), and hesperidin and TTX (from Wako, Osaka, Japan). Nifedipine, R59022 and verapamil were dissolved in DMSO to make stock solutions and were added to Krebs solution to make the desired concentrations, just before the use. Enprostil was kindly provided from Tanabe Pharmaceutical and dissolved in propylene carbonate. Rikkunshi-to (TJ-43) supplied from Tsumura and Company (Tokyo) was dissolved with Krebs solution directly. Hesperidin was also dissolved in Krebs solution directly. All other drugs were dissolved first in distilled water. The final concentration of the solvent in Krebs solution did not exceed 1/1,000. Addition of these chemicals to Krebs solution did not alter the pH of the solution.

**RESULTS**

**General observations.** When enprostil (0.5 μM), a prostaglandin E2 analog (3), was applied to CSM strips of rat gastric fundus, it produced a phasic, followed by a sustained contraction (Fig. 1A). In 10 of 27 preparations, enprostil generated oscillatory contractions during the sustained contraction (see Fig. 7A). An application of TJ-43 (0.1–5 mg/ml) produced a dose-dependent relaxation of the enprostil-induced contraction.

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Fig. 1. Effects of 1H[1,2,4] oxadiazolo [4,3-a]quinolinaxin-1-one (ODQ) on relaxation produced by Rikkunshi-to (TJ-43) or sodium nitroprusside (SNP) in rat fundic circular muscles contracted with enprostil. A: TJ-43 (5 mg/ml) and SNP (3 μM) were applied before (a) and after application of ODQ (10 μM) (b). The concentration of enprostil was 0.5 μM. a and b were recorded from the same tissue. B: summary of the relaxation produced by TJ-43 (5 mg/ml) and SNP (3 μM) recorded in the presence (hatched bar) and absence of ODQ (open bar) during the contraction induced by enprostil in rat fundic circular smooth muscle (CSM) strips (n = 5 for TJ-43; n = 4 for SNP). The peak amplitude of the relaxation is expressed as a percentage of maintained tonic contraction induced by enprostil before application of drugs. **P < 0.01, significant difference from control.
The amplitude of relaxation induced by TJ-43 was not affected by 1 μM TTX (control, 74.6 ± 9.0%; with TTX, 72.9 ± 10.1%; n = 3; P > 0.05), indicating that neurogenic responses are not involved in TJ-43-induced relaxation. Therefore, recording of mechanical responses were carried out in the presence of 1 μM TTX to assess TJ-43-induced direct effects on smooth muscle cells (except for Fig. 6).

**Effects of ODQ on TJ-43-induced relaxation.** NO synthesized from L-arginine by neuronal NOS (nNOS) in nonadrenergic, noncholinergic neurons diffuses to smooth muscle cells to generate cGMP by stimulating soluble guanylate cyclase (sGC) (19, 24). It has been reported that a part of improving mechanisms of TJ-43 on gastric dyspepsia may be mediated by NO because TJ-43 contains L-arginine (0.9%) (9, 12). Thus the possible involvement of NO in TJ-43-induced relaxation of CSM strips was examined. In preparations precontracted by enprostil (0.5 μM), TJ-43 (5 mg/ml) suppressed about 70% of the contraction (Fig. 1, Aa and Ab), whereas SNP (3 μM), a NO donor, suppressed the contraction by about 98% (Fig. 1, Aa and Ab). TJ-43-induced relaxation was not influenced by ODQ (10 μM), an inhibitor of sGC, whereas SNP (3 μM)-induced relaxation was inhibited by ODQ (Fig. 1, Ab and B). Another difference of the relaxant responses between TJ-43 and SNP was the time course of the relaxations induced by two drugs.

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Effects of TJ-43 on depolarization produced by enprostil and high K⁺ in rat fundic circular muscles. **A:** TJ-43 (5 mg/ml) was applied before and after application of apamin (1 μM) during the depolarization produced by enprostil (0.5 μM) (a). TJ-43 (5 mg/ml) was applied during the depolarization produced by high K⁺ (29.4 mM [K⁺]o) (b). The resting membrane potentials were as follows: a, −45 mV; b, −46 mV. a and b were recorded from different tissues. **B:** high-speed traces of the effects of 5 mg/ml TJ-43 on depolarization produced by enprostil in the absence (bottom) and presence of 1 μM apamin (top). **C:** summary of repolarization induced by TJ-43 in the presence of enprostil (n = 9), apamin + enprostil (n = 6), or high K⁺ (n = 6). **P < 0.01, significant difference from control; n.s., no significant difference.
TJ-43 caused a stable relaxation during application. On the other hand, SNP-induced relaxations subsided with time (Fig. 1Aa). These results suggest that NO is not involved in TJ-43-induced relaxation of rat fundic strips.

Effects of hesperidin and R59022 on enprostil-induced contraction. Several studies have tried to elucidate the mechanisms underlying TJ-43-induced improvement of delayed gastric emptying. To date, hesperidin, which is contained in TJ-43 at a concentration of 0.86% (4.29 mg/500 mg of TJ-43), has been identified as one of the active components in TJ-43. Hesperidin (1 mg/kg po) mimics the effects of TJ-43 (500 mg/kg po), ameliorating the decrease of the gastric emptying rate in rat stomach induced by NOS inhibitor (12). It has also been reported that TJ-43 inhibits the increase in diacylglycerol kinase (DGK) activity observed in streptozotocin-induced diabetic rats (23).

In the following experiments, the effects of hesperidin and R59022, an inhibitor of DGK, were examined in CSM strips precontracted with 0.5 μM enprostil. The relaxant effect of hesperidin (0.1 mg/ml) on enprostil-induced contraction of rat fundic CSM strips was much weaker than that of TJ-43 (5 mg/ml) (Fig. 2). The relaxant effects of R59022 (10 μM) were also relatively weaker than TJ-43 (Fig. 2). These results indicate that TJ-43-induced relaxations of rat fundic strips are unlikely to be generated by a single mechanism.

Effects of TJ-43 on membrane potential. The effects of TJ-43 on the membrane potential of CSM cells of rat gastric fundus were examined with intracellular microelectrode recordings (15). The resting membrane potential (RMP) of CSM cells was −44.8 ± 3.6 mV (n = 23) in the presence of nifedipine (3 μM) and TTX (1 μM). TJ-43 (5 mg/ml) hyperpolarized the membrane by −4.4 ± 1.8 mV (n = 10). Hesperidin had no effect on RMP (control, −44.9 ± 5.0 mV; with hesperidin, −45.0 ± 5.3 mV; n = 4; P > 0.05). To further investigate the mechanisms underlying TJ-43-induced hyperpolarization, TJ-43 was applied in the presence of enprostil. Enprostil (0.5 μM) depolarized the membrane (15.6 ± 2.9 mV; n = 9) (Fig. 3Aa). Application of TJ-43 (5 mg/ml) during the steady-state depolarization induced by enprostil repolarized the membrane to reach a stable potential after 1 min (Fig. 3Aa).

Apamin (1 μM), an inhibitor of small conductance Ca2+ -activated K+ (SK) channel, reduced the repolarization induced by TJ-43 (Fig. 3Aa, B, and C). Apamin (1 μM) had no effect on RMP (control, −47.7 ± 1.7 mV; with apamin, −47.4 ± 1.8 mV; n = 6; P > 0.05) or on enprostil-induced membrane depolarization (control, 15.6 ± 2.9 mV; n = 9; with apamin...
15.6 ± 2.9 mV; n = 6; P > 0.05). Application of high K+ (29.4 mM [K+]o) depolarized the membrane (12.9 ± 1.6 mV, n = 6). TJ-43-induced repolarization was also reduced during depolarization induced by high K+ (Fig. 3, Ab and C).

Role of apamin-sensitive K+ channel in TJ-43-induced relaxation. Because intracellular recording revealed that TJ-43 repolarized the depolarized membrane induced by enprostil through the activation of SK channels (Fig. 3), the role of SK channel in TJ-43-induced relaxation was examined in CSM strips contracted with 0.5 μM enprostil. Figure 4A shows an example of TJ-43-induced relaxation in the presence or absence of apamin (1 μM). The amplitude of the relaxation induced by 5 mg/ml TJ-43 was reduced in the presence of 1 μM apamin (control, 72.2 ± 8.0%; with apamin, 62.3 ± 8.0%; n = 7; P < 0.05).

The relaxation induced by TJ-43 with apamin reached the maximum relaxed level much more slowly than that induced by TJ-43 alone (Fig. 4A). To assess the effects of apamin on the time course of the relaxation, the half-relaxing time (T50, measured as the mean time taken for 50% relaxation in the amplitude of the peak relaxation) was measured (Fig. 4B). Apamin (1 μM) prolonged the T50 induced by TJ-43 (Fig. 4, Ca and D). The T50 induced by TJ-43 was also increased in strips contracted with high K+ (Fig. 4, Cb and D). However, the T50 induced by TJ-43 with apamin in enprostil-precontracted strips was not different from that induced by TJ-43 in high K+-precontracted strips (Fig. 4D). Finally, the slowing of the TJ-43-induced relaxations in the presence of apamin was not arising from some form of desensitization during repetitive applications of TJ-43 because TJ-43 produced reproducible relaxant responses when applied during 10-min intervals (data not shown). These observations indicate that TJ-43-induced fast relaxation seems to be associated with an opening of SK channel.

Effects of TJ-43 on high K+-induced contraction. Preliminary attempts were made to study the likely mechanisms involved in the TJ-43-induced time-dependent slow relaxation in CSM strips. As shown in Fig. 5A, TJ-43 (5 mg/ml) suppressed the high K+ (29.4 mM [K+]o)-induced contraction. The amplitude of the relaxation induced by TJ-43 was comparable to that induced by verapamil (0.5 μM), a blocker of L-type Ca2+ channels (Fig. 5A). However, the half-relaxing time induced by 0.5 μM verapamil was longer than that of TJ-43 in strips contracted with high K+ (Fig. 4, Cb and D). Furthermore, unlike TJ-43, there was a tendency that the amplitude of relaxation to verapamil subsided in enprostil-precontracted strips compared with the sustained relaxations of high K+-precontracted strips (Fig. 5B). These results indicate that TJ-43-induced time-dependent slow relaxation is likely to be mediated by several mechanisms.

Properties of transmural nerve stimulation-induced relaxation in diabetic rat fundus. Dysfunctional nitrergic nerves in gastric fundus result in disordered gastric emptying in patients with DM (27), indicating that NO plays a most important role in adaptive relaxation (4). Therefore, experiments were carried out to examine the influence of DM on the function of nitrergic nerves in gastric fundus of GK rats, a type 2 diabetic rat model (8).

Table 1 summarizes the body weight and glucose level of control Wistar rats and GK rats. In GK rats, blood glucose level...
was significantly higher than in Wistar rats. Body weight of Wistar rats was significantly heavier than that of GK rats.

The contractile response of fundic strips to 1 mM carbachol (CCh) was not significantly different between Wistar and GK rat (Wistar rat, 2,648 ± 564 mg, n = 7; GK rat, 2,610 ± 258 mg, n = 8; P > 0.05). Transmural nerve stimulation (TNS) (repetitive at 10 Hz and 0.3-ms pulse duration) applied during contractions induced by CCh (1 mM) evoked a phasic relaxation in Wistar rat fundic CSM strips. When the number of stimuli was increased in a stepwise manner at a constant stimulus intensity and frequency (10 Hz), the amplitude of the relaxation increased proportionally (Fig. 6A). L-NNA (30 μM), an inhibitor of NOS, reduced the peak amplitude and half-width (the duration of relaxations at the half-amplitude of the peak) of the TNS-induced relaxation in Wistar rat strips (Fig. 6, B and C). In GK rats, the peak amplitudes of the TNS-induced relaxation were not affected by 30 μM L-NNA (Fig. 6B). The half-widths of TNS (10 and 30 stimuli)-induced relaxation were significantly decreased by 30 μM l-NNA in GK rat strips. However, the l-NNA-sensitive component of the half-width of the relaxation induced by TNS (30 stimuli) was much shorter in GK rat strips than in Wistar rat strips (Fig. 6, A and C). These results indicate that the function of nitricergic nerve was impaired in GK rat gastric fundus.

Effects of TJ-43 on CSM strips of GK rat fundus. In the next series of experiments, the effects of TJ-43 on GK rat gastric fundus were examined. Contractile responses to enprostil were not significantly different in gastric fundus of GK rats compared with Wistar rats (Wistar rat, 2,650 ± 632 mg, n = 7; GK rat, 2,780 ± 541 mg, n = 8, P > 0.05). In GK rat fundic strips, TJ-43 (0.1–5.0 mg/ml) produced a dose-dependent relaxation during the contraction induced by 0.5 mM enprostil (Fig. 7, A and B). The maximum relaxation induced by each dose of TJ-43 was significantly larger in GK rat strips than in Wistar rat strips (Fig. 7B). The effects of TJ-43 on the membrane potential were examined in GK rat fundic CSM cells. The RMP of CSM cells of GK rat fundus was 43.7 ± 2.7 mV (n = 11), comparable to that of Wistar rat (P > 0.05). Enprostil (0.5 μM)-induced depolarizations were not significantly dif-

### Table 1. Body weight and blood glucose of the experimental animals

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<th>Body Weight, g</th>
<th>Blood Glucose, mg/dl</th>
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<tr>
<td>Wistar R</td>
<td>352 ± 38</td>
<td>165 ± 10</td>
<td>18</td>
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<tr>
<td>GK</td>
<td>301 ± 17*</td>
<td>280 ± 36†</td>
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Values are means ± SD. n = number of animals. *P < 0.05, †P < 0.01, compared to Wistar rat.

Fig. 6. Different properties of transmural nerve stimulation (TNS)-induced relaxation during application of carbachol in fundic circular muscles between Wistar and Goto-Kakizaki (GK) rat. A: repetitive field stimulation of 3, 10, and 30 stimuli at 10 Hz with 0.3-ms pulse duration (arrow) evoked relaxation during the contraction induced by carbachol (1 μM) in Wistar rat (a) or GK rat fundic CSM strips (b). The gray traces show TNS (30 stimuli)-induced relaxation in the presence of N^ω-nitro-l-arginine (l-NNA) (30 μM). B: summary of the relaxation produced by TNS in the presence (hatched bar) and absence of l-NNA (30 μM) (open bar) during the contraction induced by carbachol (1 μM) in Wistar rat (n = 7) or GK rat (n = 8) fundic CSM strips. The peak amplitude of the relaxation is expressed as a percentage of maintained tonic contraction induced by carbachol before application of TNS. C: summary of half-width (s) of TNS-induced relaxation in the presence (hatched bar) and absence of l-NNA (30 μM) (open bar) during the contraction induced by carbachol (1 μM) in Wistar rat (n = 7) or GK rat (n = 8) fundic CSM strips. *P < 0.05, **P < 0.01, significant difference from control. ##P < 0.01, significant difference from Wistar rat.
different between GK rat and Wistar rat (GK rat, 14.8 ± 1.7 mV, n = 9; Wistar rat, 15.6 ± 2.9 mV, n = 9; P > 0.05). TJ-43-induced membrane repolarization in the presence of 0.5 μM enprostil was significantly smaller in GK rats compared with Wistar rats (GK rat, 4.2 ± 1.0 mV, n = 9; Wistar rat, 7.2 ± 0.9 mV, n = 9, P < 0.05) (Fig. 7C). These results indicate that, despite the attenuation of repolarizing effects of TJ-43 on fundic CSM cells in GK rats, TJ-43 produced a greater relaxation in GK rat fundus than in Wistar rat fundus.

DISCUSSION

Rikkunshi-to (TJ-43) is a gastroprotective herbal medicine used for dyspeptic symptoms (31). The main mechanism of TJ-43 on dyspepsia is likely to be due to the recovery of gastric adaptive relaxation (9). However, the effects of TJ-43 on fundal smooth muscle cells, which regulate gastric motility, have not been assessed. In the present study, it was found that TJ-43 suppressed the contraction of rat fundic CSM strips via two mechanisms, causing an initial, fast relaxation upon the opening of apamin-sensitive K⁺ channels, followed by a time-dependent slow relaxation, apparently independent from the membrane potential.

The main finding of the present study is that TJ-43 produced the fast relaxation in precontracted fundic tissues by hyperpolarizing the membrane. This membrane hyperpolarization was likely to be mediated by the opening of apamin-sensitive SK channels. TJ-43 quickly generated a sustained relaxation in the absence of apamin. On the other hand, in the presence of apamin, the TJ-43-induced relaxation developed gradually, reaching its maximum ~7 min later (Fig. 5A). Thus SK channel plays an important role in the fundic CSM strips to cause quick, large relaxations induced by TJ-43. The TJ-43-induced membrane hyperpolarization is presumably closing voltage-dependent L-type Ca²⁺ channels, which are contributing to the tonic contraction induced by enprostil. It has been thought that SK channels are expressed in smooth muscle cells and are directly involved in the generation of fast inhibitory junction potentials induced by ATP in gastrointestinal tract (10, 17). However, recent studies have shown that SK3 is expressed only in fibroblast-like cells in mouse small intestine (5, 14, 16, 33). If this is the case for rat gastric fundus, it is possible that the membrane hyperpolarization generated in fibroblast-like cells by TJ-43 spreads to the surrounding CSM cells electrotonically, causing membrane hyperpolarization in CSM cells.

TJ-43 produced a time-dependent slow relaxation in the presence of apamin as mentioned above. This relaxation was associated with a little change of membrane potential. Because both TJ-43 and verapamil evoked monophasic slow relaxations (Fig. 4Cb), it seems reasonable to speculate that TJ-43 suppresses the muscle contraction induced by high K⁺ via blocking of voltage-dependent L-type Ca²⁺ channels. However, the relaxing effect of TJ-43 was faster than verapamil, suggesting that the effects of TJ-43 on high K⁺-induced precontracted strips are not necessarily identical to those of verapamil. TJ-43 may cause relaxation via one or more additional mechanisms in addition to the inhibition of L-type Ca²⁺ channel opening. In fact, the relaxant responses to TJ-43 were much greater than verapamil in strips contracted with enprostil (Fig. 5B). TJ-43 may relax verapamil-insensitive contraction by inhibiting enprostil-induced G protein-coupled signaling pathways such as activation of myosin light-chain kinase by Ca²⁺ binding to calmodulin (26). Alternatively, TJ-43 may inhibit Ca²⁺ sensitization in enprostil-induced contraction although there is no
direct evidence that enprostil evoked Ca$^{2+}$ sensitization in rat gastric fundus. Further studies are required to elucidate TJ-43-induced time-dependent slow relaxation.

Hesperidin caused relaxation without affecting the membrane potential in rat gastric fundus. Although the active ingredients responsible for TJ-43-induced relaxation have not been conclusively identified, this study establishes that hesperidin may be responsible for the membrane potential-independent TJ-43-induced slow relaxation. TJ-43 is composed of eight kinds of herbs. Thus further investigations of the effects of each herb on fundic CSM cells will be required to determine the precise mechanism underlying TJ-43-induced time-dependent slow relaxation.

Gastric motor dysfunction occurs in about half of diabetic patients, which may contribute to poor glycemic control (27). Although the precise mechanism through which diabetes develops gastroparesis is unclear, autonomic nervous dysfunction seems to be directly related to gastric dysfunction in diabetic patients. It has been reported that a selective loss of nNOS protein expression was found in type 1 (insulin-dependent) DM model (34). Dysregulation of nNOS in gastric fundus results in a diminution of NO-dependent relaxation of fundic muscle, leading to gastric dyspepsia, because NO plays an important role in adaptive relaxation (4). In the present study, nitricergic nervous function was also impaired in GK rat, type 2 (noninsulin-dependent) DM. Interestingly, GK rats develop gastric enlargement as has been reported in diabetic mice (34) (Y. Kito, unpublished observation). This enlargement may reflect delayed gastric emptying. Despite the dysfunction of nitricergic nerves in GK rat fundus, TJ-43 produced greater relaxation in GK rat strips compared with control rat strips. It has been shown that PKC activity in smooth muscle is increased in diabetic rat (22). If the same thing happens in CSM of GK rat gastric fundus, increased PKC activity would contribute to G protein-coupled Ca$^{2+}$ sensitization in smooth muscles (26). It is possible that TJ-43 relaxed enprostil-induced contraction via inhibition of enprostil-induced Ca$^{2+}$ sensitization directly (Fig. 5). Thus the simple explanation is that TJ-43 relaxed gastric fundus more effectively by inhibiting Ca$^{2+}$ sensitization increased in GK rat than Wistar rats. However, TJ-43-induced repolarization in the presence of enprostil were attenuated in GK rat strips than that in control rat strips, making it quite difficult to understand the mechanisms underlying TJ-43-induced relaxation in diabetic rat strips. Thus it is important to isolate the active compounds in TJ-43, particularly those active under diabetic conditions. Identification of such compounds will facilitate the usefulness of TJ-43. Nevertheless, the results presented here raise the possibility that TJ-43 may be a useful drug to treat gastric dyspepsia in diabetic gastroparesis.

In conclusion, the present study demonstrated that, in isolated rat fundus smooth muscle, TJ-43 evoked relaxation with two components, an initial fast component followed by a second relaxation with a slower time course. The fast relaxation was likely to be caused by the activation of SK channels. However, it is still unclear as to how TJ-43 produces the following slow relaxation, even though it is evident that these mechanisms do not involve changes in the membrane potential and the active agent may be within the herb hesperidin. The fact that TJ-43-induced relaxations were more effective in diabetic rat strips compared with control rats points to a potential application of TJ-43, or its active ingredients, as a selective treatment of diabetic gastroparesis.

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DISCLOSURES

The authors are not aware of financial conflict(s) with the subject matter or materials discussed in this article with any of the authors, or any of the authors’ academic institutions or employers.

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