Acupuncture modulates mechanical responses of smooth muscle produced by transmural nerve stimulation in gastric antrum of genetically hyperglycemic rats

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Abstract

Effects of acupuncture treatment on mechanical responses produced by transmural nerve stimulation (TNS) and acetylcholine (ACh) were investigated in circular smooth muscle preparations isolated from the antrum of the stomach of genetically hyperglycemic rats. While control rats had blood glucose levels of about 140 mg/dl, this was approximately tripled in the genetically hyperglycemic rats, but only doubled in the acupuncture treated genetically hyperglycemic rats. Antrum smooth muscle produced phasic contractions spontaneously, with a similar frequency and amplitude in the three groups of rats. Effects of atropine and Nω-nitro-L-arginine (L-NA) on TNS-induced responses revealed that in the antrum smooth muscle of the control rats, cholinergic excitatory, non-adrenergic non-cholinergic excitatory (NANCE), nitrergic inhibitory and off-responses produced projections: the last projection was considered to be non-adrenergic non-cholinergic non-nitrergic (NANCNN) in nature. In genetically hyperglycemic rats, nitrergic and NANCNN projections were enhanced and NANCE projections were absent. Acupuncture treated genetically hyperglycemic rats showed a reduction of NANCNN projection and enhancement of cholinergic projection, with no alteration to nitrergic projection, but a recovery of NANCE projection. ACh elicited inhibitory responses at low concentrations (1–30 nM) and excitatory responses at high concentrations (100–300 nM), in the three groups of rats. L-NA converted the ACh-induced inhibitory responses to excitatory responses. Immunohistochemical examination indicated no significant difference in the distribution of c-Kit expressing cells in the antrum smooth muscle from the three groups of rats. The results indicated that in antral smooth muscle, hyperglycemia was associated with enhanced activity in nitrergic and NANCNN projections and attenuation of NANCE projections, and that acupuncture treatment caused both a reduced blood glucose level and attenuated NANCNN projections. In genetically hyperglycemic rats, cholinergic responses were enhanced by acupuncture, possibly due to the enhanced cholinergic projections, with no change in the sensitivity of postjunctional muscarinic receptors to ACh.

Key words: acupuncture, gastric muscle, mechanical response, neural projection, hyperglycemia
Introduction

Development of diabetes mellitus is often associated with disorders of gastro-intestinal function, including having altered autonomic nerve projections (Stacher, 2001; Owyang and Hasler, 2002; Ordög, 2008). The cellular mechanism related to the functional alteration of gastrointestinal smooth muscle during development of diabetes mellitus has frequently been investigated using animal models (Srinivasan and Ramarao, 2007). Otsuka Long-Evans Tokushima Fatty (OLETF) rats which elevate blood glucose level spontaneously, have been reported to provide a useful animal model for type-II diabetes mellitus (Kawano et al., 1992; Mori et al., 1992; Kawano et al., 1994). In vitro experiments using OLETF rats indicate that altered spontaneous activity and neuro-muscular transmission are found in smooth muscle preparations isolated from both stomach (Xue and Suzuki, 1996; Takano et al., 1998) and colon (Imaeda et al., 1998).

Acupuncture has been used in Chinese medicine to modulate the physiological condition of intestinal organs, and its effectiveness may be the result of correcting an imbalance of the autonomic nervous system (Iwa and Sakita, 1994; Imai et al., 2008). It is shown in humans that an application of acupuncture on the lower abdomen induces gastric relaxation via the somato-sympathetic pathway (Tada et al., 2003). Reduction of blood glucose levels by acupuncture treatment has also been noted in humans (Nakamura et al., 1983; Nakamura et al., 1994; Nakamura et al., 1999; Kinuta et al., 1999; Yamda et al., 2002). This has also been found to be the case in animal models, as acupuncture treatment results in a reduced blood glucose level in the streptozotocin-induced diabetic model rats (Nakamura et al., 1996), and also in OLETF rats (Nakamura et al., 2008).

In the present study, we have investigated the alteration of nerve stimulation-induced mechanical responses of gastric smooth muscle preparations by acupuncture treatment in OLETF rats. Gastro-intestinal smooth muscle receives cholinergic excitatory, nitrergic inhibitory and non-adrenergic non-cholinergic non-nitrergic (NANCNN) inhibitory nerve projections (Hoyle and Burnstock, 1989; Kuriyama et al., 1998). In streptozotocin-induced diabetic rat, reduction of cholinergic and NANCNN projections has been shown in stomach smooth muscle, with changes in both the amplitude and form of junction potentials evoked by transmural nerve stimulation (TNS) (Xue and Suzuki, 1997). Therefore, it was considered that these attenuated muscle responses produced by nerve excitation may also be found in OLETF rats. Furthermore, if treatment with acupuncture is effective in reducing blood glucose levels in type-II diabetic animal models (Nakamura et al., 2008), an associated recovery of smooth muscle activity in response to nerve excitation might also be expected. The results have indicated that the cholinergic and nitrergic components were attenuated in OLETF rats, and that acupuncture treatment prevented the attenuation of cholinergic excitatory and nitrergic inhibitory projections, with an associated reduction of blood glucose levels. Preliminary results related to the effects of acupuncture on the blood glucose levels in OLETF rats had been reported briefly to the Annual Meeting of the Japan Acupuncture Society (Nakamura et al., 2008).
Materials and Methods

Experimental animals

Genetically hyperglycemic rats developed in the Research Laboratory of Otsuka Pharmaceutical Co., known as Otsuka Long-Evans Tokushima Fatty (OLETF) rats, and their age-matched control animals, known as Long-Evans Tokushima Otsuka (LETO) rats (Kawano et al., 1992; Mori et al., 1992; Kawano et al., 1994), were supplied from the Research Laboratory of Otsuka Pharmaceutical Co. at Tokushima (Japan) at 5 weeks of age. These were maintained in the Experimental Animal Sciences Center of Nagoya City University Medical School, with free access to water and food for between 42–67 weeks. A group of the OLETF rats were treated with acupuncture twice a week for 41 weeks (AcOLETF rats). The acupuncture was applied to 6 points (CV12, ST25, CV6, BL20, BL22 and BL23), using acupuncture needles with a diameter of 0.12 mm which were inserted into the skin to stimulate underlying muscles with a small degree of pressure (Nakamura et al., 1996). Rats were starved for 20 hours every 4-weeks, after which they were weighed and the blood glucose level in blood collected from the tail artery was measured with a glucose test meter (Accu-Check Aviva, Roche-Diagnostics K.K., Tokyo, Japan). All animals were treated ethically according to the guiding principles for the care and use of experimental animals as approved by The Experimental Animal Committee of the Nagoya City University Medical School.

Isolation of tissues

At between 42–67 weeks, rats were anesthetized with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether (sevoflurane, Maruishi Pharm., Osaka, Japan), and exsanguinated by decapitation and the blood glucose level measured. These procedures were commenced at 9:00 a.m. to adjust the daily rhythms of the rats. The stomach was excised from the animal, and the contents removed by cutting along the lesser curvature. The mucosal layer was removed by cutting with fine scissors, and the smooth muscle preparations isolated from the antrum region.

Measurements of mechanical activity

Segments of antral circular smooth muscle tissue, together with attached longitudinal smooth muscle and myenteric layers, were prepared to be about 1 mm wide and 10 mm long and then tied at both ends with fine thread. The tissue was mounted in a cylindrical organ bath (1.0 cm diameter and 2.5 cm high, capacity about 2.0 ml), with one end of the thread anchored to the bottom of the chamber and the other end connected to the lever of the mechanical-transducer (TB-612T, Nihon Kohden, Tokyo, Japan) for isometric tension recording. The tension responses of smooth muscle were expressed in mN. The segment of tissues was superfused with warm (36°C) Krebs solution, at a constant flow rate of about 2 ml/min using a peristaltic pump (Tubing Pump Rotor, Type 1500N, Taiyo Kagaku Kougyo Co., Tokyo, Japan). A pair of silver plates (width 2 mm, length 15 mm) was attached to the wall of the recording chamber, so as to sandwich the tissue segment and allow transmural electrical stimulation to be applied. Rectangular pulses of 0.1 ms duration and 10–15 V intensity were applied to the tissue segment to stimulate intramural nerves, using an electric stimulator (SEN-6101, Nihon Kohden,
Tokyo, Japan). The mechanical responses of the smooth muscle segments were recorded isometrically and stored in a personal computer for later analysis.

**Histochemistry**

A segment (5 mm wide, 10 mm long) of tissue was isolated from the antrum region, was kept in Krebs solution in a dissecting chamber at room temperature and lightly stretched before being immobilized on the Sylgard plate covering the bottom of the chamber using tiny pins. The tissue was fixed with Zamboni’s fixative (Iino et al., 2007) for 1 hour, and then kept in phosphate-buffered saline at 4°C. The tissue was embedded in Tissue-Tec (miles, Elkhart, IN, USA), and sections (12 µm) cut in a cryostat. Sections were collected on glass slides and preincubated with 5% normal donkey serum before being incubated with rabbit anti-c-Kit antibody (SR-1, 1:5000) (Iino et al., 2007). The specificity of antibodies was checked by immunoblot analysis as reported previously (Iino et al., 2007). Tissues were examined with a TCS-SP2 confocal microscope (Leica, Wetzlar, Germany) and images collected and measured using Leica Confocal Software.

**Solutions and chemicals**

The ionic composition of the Krebs solution was as follows (in mM): Na⁺ 137, K⁺ 5.9, Ca²⁺ 2.5, Mg²⁺ 1.2, H₃PO₄⁻ 1.2, HCO₃⁻ 15.5, Cl⁻ 134 and glucose 11.5. The solution was aerated with O₂ containing 5% CO₂, and the pH of the solution maintained at 7.3–7.4.

Zamboni’s fixative contained 2% paraformaldehyde and 1.5% saturated picric acid solution in phosphate-buffered saline (pH = 7.3). The phosphate-buffered saline contained 24.04 mM NaHPO₄ and 125.65 mM Na₂HPO₄ (pH = 7.4).

Drugs used were acetylcholine chloride (ACh), atropine sulfate, guanethidine and Nω-nitro-L-arginine (L-NA). Drugs were dissolved in distilled water at 1–10 mM, and added to Krebs solution to obtain the desired concentrations. No alteration of the pH of the solution was detected by these procedures.

**Statistics**

The data obtained were expressed as the mean ± standard error (S.E.). Statistical difference was tested using paired and non-paired Student’s *t*-tests, and probabilities of less than 5% (*P*<0.05) were considered to be significant.

**Results**

**Blood glucose level and body weight of experimental animals**

After starvation for 20 hours, the blood glucose level and body weight were measured in LETO, OLETF and acupuncture-treated OLETF (AcOLETF) rats aged at between 29–41 weeks. The blood glucose level was stable at about 100–150 mg/dl throughout in LETO rats, while in OLETF and AcOLETF rats, the value elevated with age, reaching between 500–700 mg/dl at 30–35 weeks. The level of blood glucose was higher in OLETF rats (400–700 mg/dl) than in AcOLETF rats (300–500 mg/dl).

At sacrifice at between 42–67 weeks of age, the body weight and blood glucose level were
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again measured. The body weights between groups were not significantly different \( (P>0.05) \), with the mean and standard deviation being 661 ± 57 g \( (n=10) \) for LETO rats, 557 ± 142 g \( (n=13) \) for OLETF rats and 635 ± 111 g \( (n=10) \) for AcOLETF rats. The blood glucose levels were 139.7 ± 15.6 mg/dl \( (n=10) \) in LETO rats, 404.8 ± 128.5 mg/dl \( (n=13) \) in OLETF rats and 298.5 ± 139.2 \( (n=10) \) in AcOLETF rats. The glucose level was the highest in OLETF rats, and was significantly lower in AcOLETF rats \( (P<0.05) \). The glucose levels of both OLETF and AcOLETF rats were significantly higher than those of LETO rats \( (P<0.05) \).

**Spontaneous mechanical activity of gastric muscles**

Antrum circular smooth muscle segments isolated from the stomach were spontaneously active in LETO, OLETF and AcOLETF rats, with periodic generation of phasic contractions (Fig. 1). The amplitude of phasic contractions varied between preparations, and also between the type of rat, and ranged between 50 mN and 150 mN. The mean value of the amplitude of phasic contraction was 74.7 ± 25.9 mN \( (n=18) \) in LETO rats, and it was similar for OLETF and AcOLETF rats \( (P>0.05) \) (Fig. 1D). The frequency of phasic contractions ranged between 2.5–4 /min, with a mean value of about 3 /min for LETO and OLETF preparations (Fig. 1E). In AcOLETF rats, the amplitude of phasic contractions tended to be larger than that for LETO and OLETF rats, but they were not significantly different \( (P>0.05, \text{Fig. 1D}) \). The frequency of phasic contractions was not significantly altered by acupuncture treatment (Fig. 1E). These results indicate that the amplitude and frequency of spontaneous contractions of antrum smooth muscle
were not significantly altered either by hyperglycemia or by the treatment with acupuncture.

The spontaneous phasic contractions were not altered by either guanethidine (10 µM, up to 60 min) or TTX (0.3 µM) in muscle segments isolated from any type of rat (n=2–3 for each type of rat; data not shown), indicating that the phasic contractions were not related to periodic activity of either adrenergic or any other nerve type.

**Mechanical responses evoked by transmural nerve stimulation (TNS)**

Effects of transmurally applied electrical field stimulation on mechanical responses of circular smooth muscle bundles were examined in segments isolated from the gastric antrum of LETO, OLETF and AcOLETF rats. Segments were stimulated with a train of rectangular pulses (duration, 0.05 ms) for 1 min at a constant frequency of 1 Hz, with supra-maximal intensity (10–15 V). These stimulations elicited different patterns of mechanical response in segments isolated from LETO, OLETF and AcOLETF rats, and all were abolished by 0.3 µM TTX in a reversible manner (data not shown). Thus, we considered that the stimulation selectively excited intramural nerves (i.e., transmural nerve stimulation, TNS).

In LETO rats, application of TNS for 1 min at 1 Hz frequency elicited a sustained inhibition of spontaneous contractions, and the tension remained at around the resting level. The cessation of TNS evoked an enhanced amplitude of phasic contraction (the off-response) which returned slowly to the resting condition within 1–5 min (Fig. 2A). The amplitude of the off-response, expressed as relative to that of phasic contractions generated before TNS, was 1.64 ± 0.23 (n=6). In OLETF rats, TNS reduced the level of resting tension, during which no phasic contraction was generated, and the cessation of TNS elicited a large amplitude of off-response. The amplitude of the off-response was about 3.5 times larger than that of spontaneous phasic contractions generated before TNS, and it required 5–8 min for full recovery (Fig. 2B). Thus, the TNS-induced inhibition of phasic contraction and reduction of resting tension level were stronger in OLETF than in LETO rats. The off-response was either a single phasic contraction or a repetitive generation of a group of phasic contractions, with peak amplitudes which were significantly larger in OLETF rats than in LETO rats (Fig. 2E). In AcOLETF rats, TNS again reduced the level of the resting tension, during which no spontaneous phasic contraction was generated (Fig. 2C). The amplitude of the off-response evoked in AcOLETF rats was about 2 times that of the phasic contractions generated before TNS, but it was smaller than that observed in OLETF rats (P<0.05) and identical to that of LETO rats (P>0.05) (Fig. 2E). The recovery from the off-response was quick for AcOLETF rats, compared to OLETF rats (Fig. 2, C vs. B). Thus, these results indicate that TNS-induced inhibition was stronger in OLETF rats than in LETO rats, while the off-response was much more marked in OLETF rats compared to LETO rats. The treatment with acupuncture inhibited the off-response alone, and the sustained inhibition produced by TNS remained unchanged.

The effects of Nω-nitro-L-arginine (L-NA, 30 µM) and atropine (3 µM) on TNS-induced responses were investigated in antrum smooth muscle segments from the three types of rats. In LETO rats, L-NA tended to increase the amplitude and frequency of spontaneous phasic contractions, but this difference was not significant (P>0.05) (Table 1). The amplitude of phasic contraction tended to be decreased by L-NA in OLETF rats, but this difference was again not
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Fig. 2. Mechanical responses produced by transmural nerve stimulation (TNS) in smooth muscle segments isolated from LETO (A), OLETF (B) and AcOLETF (C) rats. TNS: 0.5 ms duration pulses were applied at 1 Hz frequency for 1 min. Mean values of the change of resting tension during TNS (D) and amplitude of off-response (shown as relative to the amplitude of phasic contractions generated before TNS) (E) are shown by the mean ± S.E.M. (n=6 for LETO, 8 for OLETF and 9 for AcOLETF). *, significant difference (P<0.05).

Table 1. Effects of L-NA and atropine on the amplitude and frequency of phasic contractions of antrum smooth muscle segments isolated from LETO, OLETF and AcOLETF rats. Amplitude and frequency of phasic contractions were shown as relative to those generated before application of L-NA (30 µM). Atropine (3 µM) was applied cumulatively after application of L-NA. Mean ± S.E.M. (n-number of observations). *, significantly different to control (P<0.05)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-NA</th>
<th>L-NA + Atropine</th>
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<tr>
<td>(1) Amplitude</td>
<td></td>
<td></td>
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<tr>
<td>LETO</td>
<td>74.7 ± 25.9 (6)</td>
<td>113.6 ± 50.8 (6)</td>
<td>94.2 ± 31.7 (6)</td>
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<tr>
<td>OLETF</td>
<td>76.7 ± 12.9 (8)</td>
<td>57.1 ± 7.6 (8)</td>
<td>39.6 ± 5.8 (8)*</td>
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<tr>
<td>AcOLETF</td>
<td>114.1 ± 16.8 (6)</td>
<td>50.8 ± 7.6 (6)*</td>
<td>54.3 ± 8.4 (6)*</td>
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<tr>
<td>(2) Frequency</td>
<td></td>
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<tr>
<td>LETO</td>
<td>3.04 ± 0.19 (6)</td>
<td>3.16 ± 0.20 (6)</td>
<td>3.12 ± 0.28 (6)</td>
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<tr>
<td>OLETF</td>
<td>3.49 ± 0.15 (8)</td>
<td>3.86 ± 0.15 (8)*</td>
<td>3.92 ± 0.19 (8)*</td>
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<tr>
<td>AcOLETF</td>
<td>3.30 ± 0.19 (6)</td>
<td>3.76 ± 0.19 (6)*</td>
<td>3.74 ± 0.24 (6)*</td>
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significant. However, significant reduction of the amplitude of phasic contraction by L-NA was observed in smooth muscle segments isolated from AcOLETF. The frequency of phasic contractions was increased by L-NA in OLETF rats. In AcOLETF rats, L-NA decreased the amplitude and increased the frequency of phasic contractions. An increase in the frequency of phasic contractions by L-NA was observed both in OLETF and AcOLETF rats, indicating that the acupuncture treatment had not altered the L-NA-induced increase in the frequency of phasic contractions.

Atropine, added in the presence of L-NA, did not significantly alter the amplitude and frequency of phasic contractions in LETO rats (Table 1). In OLETF rats, atropine further reduced the amplitude of phasic contractions ($P<0.05$), while it did not appear to do so in AcOLETF rats, suggesting an inhibition of atropine-sensitive projections by the treatment with acupuncture. The frequency of phasic contractions was not further altered by atropine in either the OLETF or AcOLETF rats.

The effects of L-NA on TNS-induced responses are shown in Fig. 3. L-NA changed the TNS-induced inhibitory response to an excitatory one, with an elevation of resting tension during
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TNS in LETO, OLETF and AcOLETF rats (Fig. 3, A, B and C, respectively). The off-response was generated in all types of rats, and quantified data indicated that the amplitude was significantly larger in OLETF rats, but was similar in LETO and AcOLETF rats (Fig. 3E). The recovery from the off-response was facilitated by L-NA in all rat types. In AcOLETF rats, TNS evoked a sustained contraction with reduced or nearly abolished phasic contractions (Fig. 3C). The off-response generated in the presence of L-NA was large in AcOLETF rats, but was reduced to values comparable to those of LETO rats in AcOLETF rats (Fig. 3C). In the presence of L-NA, the TNS-induced responses produced in AcOLETF rats resembled those of LETO rats. The results also indicated that in OLETF rats the inhibition by acupuncture treatment of the neural projections which produced the off-response was not altered by L-NA.

The effects of inhibition of muscarinic receptors were observed by adding atropine in the presence of L-NA. In LETO rats, atropine attenuated the TNS-induced excitatory responses produced in the presence of L-NA, but the resting tension was still elevated by TNS, with an associated generation of phasic responses, followed by off-responses (Fig. 4A). In OLETF rats, the inhibitory responses evoked by TNS were again attenuated by atropine, even in the presence of L-NA, but there was a reduced resting tension and a large off-response (Fig. 4B). In

![Figure 4](image-url)

**Fig. 4.** Effects of atropine on TNS-induced responses in smooth muscle isolated from the stomach antrum of LETO (A), OLETF (B) and AcOLETF (C) rats. Atropine (1 µM) was applied in the presence of 10 µM L-NA. Values of resting tension produced during TNS (D) and amplitude of off-response measured as relative to that of spontaneous phasic contraction generated before TNS (E) were summarized by the mean ± S.E.M. (n=6 for LETO, 8 for OLETF and 6 for AcOLETF rats). *, significant difference (P<0.05).
AcOLETF rats, TNS applied in the presence of L-NA and atropine produced a sustained inhibition of phasic contractions with a reduced resting level (Fig. 4C). The off-responses were still small after adding atropine in AcOLETF rats. The quantified data indicated that the atropine-resistant component of TNS-induced contraction was absent in OLETF and AcOLETF rats (Fig. 4, D and E).

These results indicate that in LETO rats, the antrum smooth muscle receives 4 types of neural projections: cholinergic excitatory projections, nitrergic inhibitory projections, non-adrenergic non-cholinergic non-nitrergic (NANCNN) off-response generating projections and non-adrenergic non-cholinergic non-nitrergic excitatory (NANCE) projections. The distribution of the former three projections was also confirmed in the OLET F rats. Significant difference between LETO and OLETF appeared with the NANCE projections which produced contractile responses as a result of TNS in the presence of L-NA and atropine and NANCNN projections which produced off-response at the cessation of TNS. Acupuncture treatment inhibited the NANCNN projections which generate the off-response, but did not allow the appearance of NANCE projections.

The TNS-induced responses remained unaltered in the presence of guanethidine (10 µM) for over 30 min in LETO, OLETF and AcOLETF rats (n=3, data not shown), suggesting that there were no effective adrenergic projections in the antrum smooth muscle.

Mechanical responses produced by exogenously applied acetylcholine

Possible change in the properties of cholinergic receptors during hyperglycemia was investigated by observing the responses produced by exogenously applied acetylcholine (ACh) in smooth muscle segments isolated from the gastric antrum of LETO and OLETF rats. The amplitude and frequency of phasic contractions produced by stimulation with ACh (1 nM–300 nM) were measured as relative to the spontaneous phasic contractions generated in the absence of ACh. The changes in resting tension produced by ACh were also measured. Stimulation of muscle with low concentrations of ACh (1–10 nM) decreased the amplitude of phasic contractions with no significant change in the frequency, and the resting tension level was unaltered or often reduced (A in Figs. 5 and 6 for LETO and OLETF rats, respectively). In some preparations isolated from either LETO or OLETF rats, an absence of a clear inhibitory response resulted from these concentrations of ACh (an example shown in Fig. 6A). High concentrations of ACh (100–300 nM) elevated the resting tension, and phasic contractions were increased in amplitude, again with no significant change in the frequency, in both LETO and OLETF rats. These properties of ACh-induced responses were not altered by acupuncture in OLETF rats, as summarized in Fig. 7.

Experiments were carried out to investigate the mechanism producing the ACh-induced inhibition of phasic contractions and the reduction of resting tension in antrum smooth muscle. Figure 8 shows the effects of L-NA (30 µM) on ACh-contractions recorded from antrum smooth muscle segments isolated from the OLETF rats. The inhibitory responses elicited by 10 nM ACh were abolished by L-NA (Fig. 8, A and B), while those elicited by higher concentration of ACh (100 nM) were changed to excitatory responses (with an increase in amplitude of phasic contractions and elevation of resting tension) by L-NA (Fig. 8, C and D). These effects of L-NA
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were also observed in LETO rats, and the mechanical responses produced by ACh (1 μM) were increased by about 1.5 times in the presence of L-NA (data not shown). These results suggested a possible involvement of a nitrergic mechanism in the ACh-induced inhibitory responses in the antrum smooth muscle of the rat stomach. The effects of ACh on the mechanical responses (for both excitatory and inhibitory responses) of antrum smooth muscle were abolished by atropine (1 μM) in any types of rat (data not shown), suggesting that the actions were mediated through activation of muscarinic receptors.

**Distribution of c-Kit expressing cells**

Immunohistochemical examination was carried out to observe the distribution of c-Kit expressing cells in the antral stomach wall of LETO, OLETF and AcOLETF rats. In LETO rats, c-Kit expressing cells were distributed in the myenteric layer and within muscle bundles (Fig. 9A). A similar distributions of c-Kit expressing cells was also found in antral smooth muscle segments isolated from OLETF rats (Fig. 9B), with no detectable alteration in smooth muscle segments isolated from AcOLETF rats (Fig. 9C). These results indicated that the distribution of c-Kit expressing cells was not markedly different between LETO and OLETF rats. The results

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**Fig. 5.** Mechanical responses produced by acetylcholine (ACh) in smooth muscle isolated from a LETO rat. ACh (A, 3 nM; B, 30 nM; C, 300 nM) was applied during the bar under each record, and amplitude of the change in resting tension (D), and amplitude (E) and frequency (F) of phasic contractions generated during stimulation of ACh were shown as a function of concentration of ACh. A–C were recorded from the same tissue. Negative value in D indicates relaxation. Amplitude and frequency of phasic contractions are shown as relative to those generated before stimulation with ACh (mean ± S.E.M., n=6). *, significantly different from that in the absence of ACh (P<0.05).
Discussion

The present experiments showed that in LETO rats, there are 4 neural projections in smooth muscle segments isolated from the gastric antrum. There are cholinergic excitatory projections, non-adrenergic non-cholinergic excitatory (NANCE) projections, nitric inhibitory projections and non-adrenergic non-cholinergic non-nitric (NANCNN) projections which elicited the off-response at the cessation of TNS. Although the nature of the last projection was not identified in the present experiments, similar responses were often evoked in gastrointestinal smooth muscle preparations at the cessation of non-adrenergic non-cholinergic (NANC) inhibitory nerve excitation (Hata et al., 2000; Kato et al., 2007). The excitation of NANC nerves evokes an inhibitory junction potential (i.j.p.) which is blocked by apamin in the guinea-pig stomach (Komori and Suzuki, 1986) or by nicotinamide adenine dinucleotide (NADH) in the rat stomach (McDonnell et al., 2008). The off-response may be produced as a result of rebound excitation elicited at the recovery from the i.j.p.-induced hyperpolarization of

also indicated that the treatment with acupuncture did not alter the distribution of c-Kit expressing cells in OLETF rats.

Fig. 6. Mechanical responses produced by acetylcholine (ACh) in smooth muscle isolated from an OLETF rat. ACh (A, 3 nM; B, 30 nM; C, 300 nM) was applied during the bar under each record, and amplitude of the change in resting tension (D), and amplitude (E) and frequency (F) of phasic contractions generated during stimulation of ACh were shown as a function of concentration of ACh. A–C were recorded from the same tissue. Negative value in D indicated relaxation. Amplitude and frequency of phasic contractions were shown as relative to those generated before stimulation with ACh (mean ± S.E.M., n=8). *, significantly different from that in the absence of ACh (P<0.05).
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In LETO rats, TNS applied in the absence of any inhibitory drugs elicited an inhibition of phasic contractions, which was changed to an excitatory response by adding L-NA, suggesting a predominance of nitrergic projections in the antrum smooth muscle. In the two types of excitatory projections observed in LETO rats, the atropine-sensitive component was not as marked as the atropine-resistant component.

Comparison of the TNS-induced responses between LETO and OLETF rats revealed that the nitrergic inhibitory projections and NANCNN inhibitory projections were dominant while the NANCE projections were absent in OLETF rats. A reduction in the density of nerves which express neuronal NO synthase (nNOS) and substance P is produced in human subjects suffered from type 2 diabetes mellitus (Iwasaki et al., 2006), but the present experiments indicated an increased nitrergic projection in OLETF rats. Measurement of the membrane potential from gastric smooth muscle cells indicated that the ACh-induced depolarization was attenuated in OLETF rats (Takano et al., 1998). The present experiments indicated that ACh activated production of NO which had inhibitory effects on the mechanical responses of the smooth muscle. Although no direct measurement was made on the effects of L-NA on the ACh-induced depolarization in the gastric smooth muscle of OLETF rats, a possible inhibitory contribution of

Fig. 7. Mechanical responses produced by acetylcholine (ACh) in smooth muscle isolated from an AcOLETF rat. ACh (A, 3 nM; B, 30 nM; C, 300 nM) was applied during the bar under each record, and amplitude of the change in resting tension (D), and amplitude (E) and frequency (F) of phasic contractions generated during stimulation of ACh are shown as a function of concentration of ACh. A–C were recorded from the same tissue. Negative value in D indicated relaxation. Amplitude and frequency of phasic contractions are shown as relative to those generated before stimulation with ACh (mean ± S.E.M., n=6). *, significantly different from that in the absence of ACh (P<0.05).

the membrane (Hoyle and Burnstock, 1989). In LETO rats, TNS applied in the absence of any inhibitory drugs elicited an inhibition of phasic contractions, which was changed to an excitatory response by adding L-NA, suggesting a predominance of nitrergic projections in the antrum smooth muscle. In the two types of excitatory projections observed in LETO rats, the atropine-sensitive component was not as marked as the atropine-resistant component.
NO on the ACh-induced depolarization was considered. Although the quantification of cholinergic projections was not easy because of weak potency, the present results suggested a difference in the alteration of the neural components in the gastric smooth muscle between streptozotocin-induced diabetic rats and OLETF rats. In the former, the development of diabetes mellitus was associated with a reduction of cholinergic e.j.p. (Xue and Suzuki, 1997), while the present experiments indicated no marked change in the cholinergic projections in the latter. The nature of NANCE projections remained unclear, and the possible involvement of peptidergic nerves was considered, since gastric smooth muscle receives a rich distribution of excitatory neurons which contain substance P or neurotensin.

**Fig. 8.** Effects of L-NA on the ACh-induced mechanical responses in smooth muscle isolated from the antrum of a OLETF rat stomach. ACh (A and B, 10 nM; C and D, 100 nM) was applied at the bar under each record, in the absence (A, C) and presence of 30 µM L-NA (B, D). All responses were recorded from the same tissue.

**Fig. 9.** Distribution of c-Kit expressing cells in the stomach antrum. Cross sections obtained from a LETO (A), OLETF (B) and AcOLETF rat (C). Green color shows c-Kit expressing cells. Red color stained with propidium iodide indicates nuclei. Calibration 100 µm.
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(Lindh et al., 1983; Hoyle and Burnstock, 1989). The NANCNN projections which produced the off-response differed between LETO and OLETF rats, with the off-response only being marked in OLETF rats. Unaltered i.j.p. have been recorded in gastric smooth muscle of streptozotocin-induced diabetic rats (Xue and Suzuki, 1997) and also in OLETF rats (Takano et al., 1998), suggesting an alteration of the relationship between membrane electrical activity and mechanical responses during hyperglycemia.

Acupuncture has been used in the treatment of gastrointestinal diseases which are caused by the autonomic dysfunctions. Although the beneficial effects of acupuncture may be the result of the modulation of an imbalance in the autonomic nervous system (Iwa and Sakita, 1994; Imai et al., 2008), the cellular mechanism still remains unclear. The effects of acupuncture on gastrointestinal function are mostly investigated using electric stimulation (Lin et al., 1997), and in conscious dogs application of electro-acupuncture to points ST-36 and PC-6 has been shown to enhance the gastric migrating motor complex and acceleration of liquid gastric emptying through enhanced vagal activity, thus improving gastric slow wave rhythm and enhancing antral contractile activity (Qian et al., 1999). The effects of acupuncture also vary depending on the points where it is applied, with site specific inhibitory or stimulatory effects of acupuncture on gastric motility being noted (Kametani et al., 1979; Sato et al., 1993). The PC-6 point at the wrist and the ST-36 point on the hind limb are the common loci used for the acupuncture treatment of gastrointestinal symptoms such as nausea and vomiting (Al-Sadi et al., 1997), suggesting that acupuncture at these points may stimulate gastric motility. In contrast, application of acupuncture to the abdomen has been used for the treatment of abdominal pain (Diehl, 1999; Gu, 1992), suggesting that the application of acupuncture to this point may inhibit gastric motility and reduce gastrospasm. It was shown in streptozotocin-treated rats that application of acupuncture treatment to 6 points (CV12, ST25, CV6, BL20, BL22 and BL23) was effective in reducing the glucose level in the blood, with an associated inhibition of the loss of body weight (Nakamura et al., 1998). The present experiments revealed that acupuncture treatment altered the activity of neural projections to gastric muscles in rats, with both the inhibition of NANCNN projections which produced off-response and the enhancement of cholinergic projections, but with no marked change in nitrergic projections. Application of acupuncture significantly reduced the blood glucose level and increased the body weight in OLETF rats (Nakamura et al., 2008), and the possible involvement of a causal relationship between the altered neural projections and the reduction of the blood glucose level was considered.

Gastrointestinal dysfunction appearing in diabetic patients is often causally related to autonomic neuropathy (Stacher, 2001; Chandrasekharan and Srinivasan, 2007). However, an involvement of any alteration of the properties in gastrointestinal smooth muscle remains unclear. Gastric smooth muscle cells are spontaneously active with periodic generation of slow waves, and these electrical activities elicit phasic contractions (Tomita, 1981). Slow waves originate as a result of the activity of ICC distributed in smooth muscle tissue (Sanders, 1986), and these potentials trigger phasic contractions (Tomita, 1981). Distinctive morphologic abnormality in the intestinal smooth muscle cells and interstitial cells of Cajal (ICC) has been noted in diabetic patients (Owyang and Hasler, 2002; Ordög et al., 2008). In OLETF rats, the frequency of slow waves is irregular (Takano et al., 1998), and the possible alteration of the
properties of ICC during the development of diabetes mellitus has been considered (Ordög, 2008). However, the present results did not show any causal relationship between blood glucose level and frequency of phasic contractions, and the generation of regular phasic contractions was observed in any of the rat types. The discrepancy between electrical and mechanical activities remains unclear, and a possible involvement of the difference in size of smooth muscle preparation to be employed in the experiments was considered, as the electrical responses were recorded in tiny smooth muscle segments while the mechanical responses were recorded from segments of smooth muscle which were much larger than the former. In small segments of smooth muscle, the ICC which are considered to pace smooth muscle activity (Sanders, 1996) are few in number and thus the alteration of individual cells could directly reflect the electrical activity of smooth muscle cells, while in large segments of tissue, a large population of active ICC may mask irregular activities produced by a limited number of ICC and as a consequence the contraction of the whole tissue segment is synchronized to the activity of the active ICC.

There are many types of ICC in gastrointestinal tissues, and a group of ICC distributed within muscle bundles (i.e., ICC-IM) has a role in the transmission of neural signals to smooth muscle cells in an electrotonic manner through gap junctions (Ward and Sanders, 2001). If this is indeed the case, it is reasonable to presume that TNS modulates not only the amplitude but also the frequency of phasic contractions. The results indicated that TNS reduced the amplitude or sometimes abolished phasic contractions in LETO and OLETF rats, with no marked change in the frequency. Treatment with acupuncture did not alter these effects of TNS on phasic contractions. It is therefore considered that the regulation system of the pacing mechanism is not as simple as that proposed by Ward and Sanders (2001). The present experiments also revealed no marked change in the distribution of ICC that express c-Kit immunoreactivity in either OLETF or AcOLETF rats. These results support the evidence that the amplitude and frequency of phasic contractions were similar in LETO, OLETF and AcOLETF rats.

The effects of ACh on gastric smooth muscle were tested on the resting tension and on both the amplitude and frequency of spontaneous phasic contractions. ACh produced differential responses in gastric smooth muscle, in a concentration-dependent way: low concentrations produced an inhibition of phasic contractions with a reduction in resting tension, while high concentrations increased the amplitude of phasic contractions and elevated the resting tension. The ACh-induced inhibitory responses were converted to excitatory responses by L-NA, suggested an involvement of the production of NO in antrum smooth muscle. No alteration of the responses of gastric smooth muscle to ACh was observed in either OLETF or AcOLETF rats, suggested that the sensitivity of muscarinic receptors and also the production of NO by muscarinic stimulation were not altered either by hyperglycemia or by the treatment with acupuncture. These observations appear to be the opposite of those which indicated an enhancement of cholinergic projections in AcOLETF rats. The discrepancy could be interpreted if the treatment with acupuncture improved the activity of vagal nerves but not the properties of postjunctional muscarinic receptors including the sensitivity to ACh.

We summarize that in LETO rats, there are 4 different types of neural projections as follows: cholinergic excitatory, non-adrenergic non-cholinergic excitatory (NANCE), nitrergic inhibitory
and non-adrenergic non-cholinergic non-nitrergic (NANCNN) inhibitory projections. In OLETF rats which are used as the animal model for type 2 diabetes mellitus, nitrergic and NANCNN projections are enhanced and NANCE projections are diminished. The treatment of OLETF rats with acupuncture results in an enhanced activity of cholinergic excitatory projections and an attenuation of NANCNN inhibitory projections, with no recovery of NANCE projections. Mechanical responses produced by ACh are similar in OLETF and AcOLETF rats, suggesting that the enhanced activity resulting from acupuncture treatment of cholinergic projections appears to be mainly on vagal nerves, but not on postjunctional muscarinic receptors.

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