Effects of Dai-kenchu-to on spontaneous activity in the mouse small intestine

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Abstract

The effects of Dai-kenchu-to (DKT), a Chinese medicine, on spontaneous activity of mouse small intestine were investigated. Experiments were carried out with tension recording and intracellular recording. DKT contracted mouse longitudinal smooth muscles in a dose dependent manner (0.1–10 mg/ml). Low concentration of DKT (0.1 mg/ml) did not contract the longitudinal muscles of mouse small intestine. DKT (0.1 mg/ml) inhibited contraction elicited by transmural nerve stimulation (TNS). DKT (1 mg/ml) evoked relaxation before contraction. The initial relaxation was abolished by Nω-nitro-L-arginine (L-NNA). DKT (10 mg/ml)-induced contraction had two components: a transient rapid contraction and a following slow contraction. Atropine inhibited DKT (1 mg/ml)-induced contraction to about 50% of control. In the presence of atropine, tetrodotoxin (TTX) inhibited the contraction elicited by DKT (1 mg/ml) to about 80%. DKT depolarized the membrane and decreased the amplitude of pacemaker potentials recorded from in situ myenteric interstitial cells of Cajal (ICC-MY) with no alteration to the frequency, duration and maximum rates of rise in the presence of nifedipine and TTX. The same results were obtained in slow waves recorded from circular smooth muscle cells. These results indicate that DKT evoked both contraction and relaxation by releasing acetylcholine, nitric oxide and other excitatory neurotransmitters in mouse small intestine. DKT had no effects on pacemaker mechanisms and electrical coupling between ICC-MY and smooth muscle cells in mouse small intestine. The results also suggest that DKT may contract smooth muscles by depolarizing the membrane directly.

Key words: Dai-kenchu-to, smooth muscle, pacemaker potential, small intestine

Introduction

Smooth muscle tissues isolated from small intestine generate phasic contractions periodically. These mechanical activities are evoked by rhythmical membrane potential changes, termed slow wave recorded from smooth muscle cells (Tomita, 1981). It is generally considered that smooth muscle cells generate slow waves, since slow waves were recorded
equally in any parts of intestinal tissues. Recently it has become apparent that interstitial cells of Cajal distribute in myenteric region (ICC-MY) are the origin of spontaneous electrical activity in small intestine (Ward et al., 1994; Huizinga et al., 1995). ICC-MY are rich in mitochondria and form a network via having close contact with surrounding ICC-MY (Thuneberg, 1982; Komuro et al., 1996). ICC-MY generate pacemaker potentials periodically (Kito and Suzuki, 2003; Kito et al., 2005). Pacemaker potentials are conducted in an electrotonic way to smooth muscle layers (circular and longitudinal layers) to generate slow waves (Cousins et al., 2003; Hirst and Ward, 2003).

Dai-kenchu-to (DKT) is a traditional Chinese herbal medicine containing Zanthoxylum fruit, ginseng, dried ginger root and malt sugar. DKT is used conventionally for abdominal distention in Japan. It has been shown recently that DKT is useful for treatment of postoperative ileus, postoperative intestinal paralysis, irritable bowel syndrome and pediatric constipation (Ito et al., 2002; Ohya et al., 2003; Endo et al., 2006). DKT seems to improve postoperative quality of life (QOL) of patients after surgery for gastrointestinal disorders, since it makes possible for patients to shorten their duration of hospitalization (Endo et al., 2006). DKT ameliorates gastrointestinal dysfunction through various effects, such as contraction and relaxation of smooth muscles (Furukawa et al., 1995; Kurosawa et al., 1998; Hayakawa et al., 1999b; Satoh K et al., 2001a, 2001b; Tulimat et al., 2001), increase of blood flow in mesenteric artery (Murata et al., 2002) and anti-inflammatory effects (Hayakawa et al., 1999a). Thus, DKT has functional improvement in intestine. However there is no report concerning the effects of DKT on pacemaker mechanisms in gastrointestinal tract.

In the present study, the effects of DKT on spontaneous electrical activity of mouse small intestine were investigated with tension measurement and intracellular recording. DKT modulated mouse intestinal motility by releasing both excitatory and inhibitory transmitters. DKT did not change the frequency, duration and dV/dtmax of pacemaker potentials recorded from in situ ICC-MY, indicating that the fundamental pacemaker mechanism is not affected by DKT. These results are also discussed in relation to the clinical benefits induced by taking DKT in gastrointestinal disease. A part of these results was reported briefly in the 48th Annual Meeting of The Japan Society of Smooth Muscle Research (Kito and Suzuki, 2006).

Methods

Test systems used

BALB/c mice of either sex, aged 4–6 weeks, were anesthetized with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether (sevoflurane, Maruishi Pharm., Osaka, Japan), and sacrificed by cervical dislocation and exsanguination. All animals were treated according to the Guidelines for the Care and Use of Laboratory Animals of Nagoya City University Medical School, accredited by The Physiological Society of Japan. Segments of terminal ileum were removed from animals and opened along the mesenteric border, in Krebs solution (see below).

Abbreviations: DKT, Dai-kenchu-to; TNS, transmural nerve stimulation; L-NNA, N omega-nitro-L-arginine; TTX, tetrodotoxin; ICC-MY, myenteric interstitial cells of Cajal; ACh, acetylcholine; NO, nitric oxide.
The mucosal layer was removed under a dissecting microscope. For tension measurement, a tissue segment (about 1 mm wide and 10 mm long) of longitudinal muscle was dissected. For intracellular electrical recordings, the serosal layer was carefully peeled away under a dissecting microscope and small pieces of small intestine, approximately 0.5 mm wide and 0.5 mm long, was dissected.

**Measurement made**

(1) **Isometric tension measurement**

Longitudinal muscle preparations were transferred to 2 ml organ baths and were superfused with warmed (35°C) physiological saline at a constant flow rate (2 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 the mechanotransducer (FD-pick up TB-612T, Nihon Kohden, Tokyo, Japan). The forces developed between two silk threads were measured isometrically as the tension produced by the longitudinal muscles. A pair of silver plates was fixed at each side of the wall of the recording chamber, and rectangular electrical pulses (50 µs duration, supra maximal intensity) were applied transmurally to exit intramural nerves. The mechanical responses displayed on a pen-writing recorder (VP-6524A, National, Tokyo, Japan) were tested for sensitivity to 5 × 10⁻⁷ M tetrodotoxin (TTX), and those that disappeared were accepted as the nerve-mediated responses.

(2) **Intracellular microelectrode recordings**

For recording of the membrane potential, the 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 (35°C), at a constant flow rate of about 2 ml/min. After 2h equilibration, ICC-MY or circular smooth muscle cells were impaled with glass capillary microelectrodes (outer diameter, 1.2 mm, inner diameter 0.6 mm; Hilgenberg, Germany) filled with 3 M KCl had tip resistances ranging between 50 and 80 MΩ. Electrical responses recorded via a high input impedance amplifier (Axoclamp-2B, Axon Instruments, Inc., Foster City, California, U.S.A.) were displayed on a cathode-ray oscilloscope (SS-7602, Iwatsu, Osaka, Japan) and also stored on a personal computer for later analysis. Experiments were carried out in the presence of 3 µM nifedipine throughout, and this minimized the movement of muscles.

**Data analysis and statistical procedures**

Experimental values were expressed by the mean value ± standard error (S.E.) for mechanical responses and the mean value ± standard deviation (S.D.) for intracellular recordings. Statistical significance was tested using Student’s t-test, and probabilities of less than 5% (P<0.05) were considered significant.

**Drugs, chemicals reagents and other materials**

The ionic composition of the Krebs solution was as follows (mM): Na⁺, 137.4; K⁺, 5.9; Ca²⁺, 2.5; Mg²⁺, 1.2; HCO₃⁻, 15.5; H₂PO₄⁻, 1.2; Cl⁻, 134; and glucose, 11.5. The solutions were aerated with O₂ containing 5% CO₂, and the pH of the solutions was maintained at 7.2–7.3.
Drugs used were atropine, nifedipine (all from Sigma, St. Louis, Missouri, U.S.A.), Nω-nitro-L-arginine (LNNA), substance P and spantide (Peptide Institute, Osaka, Japan), and tetrodotoxin (TTX) (from Wako, Japan). Nifedipine was dissolved in dimethyl sulphoxide (DMSO) to make stock solution, and was added to Krebs solution to make the desired concentrations, just prior to the use. Dai-kenchu-to was kindly provided from Tsumura & Co. (Tokyo) and was dissolved in Krebs solution directly. All other drugs were dissolved in distilled water. The final concentration of the solvent in Krebs solution did not exceed 1/1000. Addition of these chemicals to Krebs solution did not alter the pH of the solution.

**Results**

*Mechanical responses evoked by Dai-kenchu-to*

Longitudinal muscle tissues of the mouse small intestine generated phasic contractions spontaneously with the frequency of 25–35 min⁻¹ (mean, 29.4 ± 1.5 min⁻¹, n=16; where n refers to the number of animals examined), identical to those of pacemaker potentials recorded from *in situ* ICC-MY (Table 1). Dai-kenchu-to (DKT) produced contraction dose dependently (Fig. 1). DKT (0.1 mg/ml) did not change the resting tone of longitudinal preparations (Fig. 1A). Mechanical responses evoked by DKT (1 mg/ml) were biphasic, an initial small relaxation and following large contraction (Fig. 1B, 2A). Application of 10 mg/ml DKT produced two types of contractions; a transient rapid contraction and following slow contraction (Fig. 1C). The frequency of spontaneous contraction was not changed in the presence of DKT (1 mg/ml) (mean, 28.4 ± 1.4 min⁻¹, n=16).

Properties of DKT (1 mg/ml) induced contraction were studied by cumulative application of atropine (3 µM), an inhibitor of muscarinic receptors, L-NNA (100 µM), an inhibitor of NO synthase, spantide (3 µM), an inhibitor of substance P receptor and TTX (3 µM), a blocker of voltage-dependent Na⁺ channels. Atropine (3 µM) reduced the amplitude of contraction evoked by 1 mg/ml DKT by about 50% (Fig. 2B). Additional application of L-NNA (100 µM) abolished
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the initial relaxation and slightly increased the amplitude of contraction (Fig. 2C). Spantide (3 µM) had no effect on DKT (1 mg/ml) induced contraction in the presence of atropine and L-NNA (Fig. 3). Desensitization of substance P receptor by exposing tissues with a high concentration of substance P (3 µM) did not cause any effect on the contraction evoked by DKT. Further application of TTX (3 µM) decreased the contraction evoked by 1 mg/ml DKT to about 80% of control (Fig. 2D). These results indicate that in mouse small intestine DKT (1 mg/ml) produced relaxation and contraction by releasing various transmitters, such as acetylcholine (ACh) and nitric oxide (NO). Substance P does not seem to be involved in the DKT induced contraction. Interestingly DKT produced small contraction even in the presence of TTX (Fig. 2D), suggesting that DKT may have some direct effects on smooth muscles. The effects of atropine, L-NNA, spantide and TTX on the amplitude of DKT induced responses are summarized in Fig. 3.

Effects of Dai-kenchu-to on neurally evoked contractions

In longitudinal muscle preparation of mouse small intestine, TNS (1–30 stimuli at 10 Hz frequency) elicited phasic contraction (Fig. 4A). These contractions were abolished by 1 µM atropine (data not shown). TNS (30 stimuli) elicited L-NNA sensitive relaxation in the presence

Fig. 2. Effects of atropine, L-NNA and TTX on Dai-kenchu-to evoked contractions in longitudinal smooth muscles of the mouse small intestine. Mechanical responses produced by Dai-kenchu-to (1 mg/ml) were recorded before (A) and after the application of 3 µM atropine (B), after the additional application of 100 µM L-NNA (C), and of 3 µM TTX (D). The effects of TTX were confirmed by transmural nerve stimulation (TNS) (D). A–D were recorded from the same tissue.
of atropine (Fig. 5). These results suggest that TNS elicit contraction and relaxation by releasing ACh from cholinergic nerve and NO from nitrergic nerve, respectively. Amplitude of TNS induced contractions was reduced in the presence of 0.1 mg/ml DKT (Figs. 4 and 5). As shown in Fig. 4, TNS elicited transient relaxation after phasic contraction in the presence of DKT (0.1 mg/ml) in 4 of 10 animals. Unexpectedly, the response to TNS (3 stimuli) was the relaxation and following a transient contraction (off-response) at the cessation of stimuli (Fig. 4B). The inhibitory effects of DKT (0.1 mg/ml) were reversible, requiring 10–20 min for the recovery (Fig. 4C). These results indicate that 0.1 mg/ml DKT inhibits TNS evoked contractions in mouse small intestine.

Effects of Dai-kenchu-to on pacemaker potentials

In intact tissue preparations of mouse small intestine, ICC-MY generate rhythmical large potential changes (Kito and Suzuki, 2003; Kito et al., 2005). Pacemaker potentials have two components: a rapidly rising primary component and following plateau component. The primary component is sensitive to NiCl₂, mibefradil, Ca²⁺ free solution and membrane depolarization by high K solution, while the plateau component is sensitive to 4,4-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS), low Cl⁻ solution and caffeine, indicating that the primary component is generated by voltage-gated Ca²⁺ permeable currents and the plateau component is related to
Ca\textsuperscript{2+} release from internal stores (Kito and Suzuki, 2003; Kito et al., 2005). Attempts were made to study the direct effects of DKT on spontaneous activity of ICC-MY, after inhibiting neural activity with 3 \textmu M TTX.

Application of DKT (1 mg/ml) depolarized the membrane and decreased the amplitude of pacemaker potentials (Table 1, Fig. 6). However DKT had no effects on the frequency, half-width (the duration of potential measured at the half amplitude of the peak) and maximum rates of rise (dV/dt\textsubscript{max}) of pacemaker potentials (Table 1, Fig. 6). These results indicate that DKT has no influence on pacemaker mechanisms in mouse small intestine.

**Effects of Dai-kenchu-to on slow waves**

Slow waves are passive electrotonic potentials conducted from pacemaker potentials through gap junctions (Cousins et al., 2003). In mouse small intestine, both longitudinal muscles and circular muscles generate slow waves with the same frequency (Takano and Suzuki, 2000). However the amplitude of slow waves is larger in circular muscles than that of longitudinal muscles. Furthermore a transient repolarization after the generation of the primary
component, a typical characteristic of intestinal slow waves in mouse, is detected clearly in circular muscles (Kito and Suzuki, 2003; Kito et al., 2005). Therefore the effects of DKT on slow waves were studied in circular muscles.

In the presence of 3 μM TTX, DKT (1 mg/ml) depolarized the membrane by about 4mV and decreased the amplitude of slow waves, with no alteration to the frequency, half-width and \( \frac{dV}{dt_{\text{max}}} \) (Table 1, Fig. 7). These results indicate that the electrical coupling between ICC-MY and circular smooth muscle cells is not significantly affected by this concentration of DKT. DKT induced membrane depolarization may be direct effects of DKT on smooth muscles and contribute to small contraction evoked by DKT in the presence of TTX (Figs. 2D and 3).

### Table 1. Effects of Dai-kenchu-to on electrical properties of pacemaker potentials and slow waves

<table>
<thead>
<tr>
<th></th>
<th>Membrane potential (primary), mV</th>
<th>Amplitude (plateau), mV</th>
<th>Frequency, ( \text{min}^{-1} )</th>
<th>half-width, s</th>
<th>( \frac{dV}{dt_{\text{max}}} ), V s(^{-2} )</th>
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<tr>
<td><strong>Pacemaker potential</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>−70.0 ± 3.8</td>
<td>56.2 ± 5.8</td>
<td>26.1 ± 1.9</td>
<td>0.78 ± 0.09</td>
<td>2.18 ± 0.42</td>
<td>7</td>
</tr>
<tr>
<td>Dai-kenchu-to 1 mg/ml</td>
<td>−66.3 ± 4.8*</td>
<td>52.7 ± 6.7*</td>
<td>25.0 ± 1.3</td>
<td>0.70 ± 0.07</td>
<td>1.94 ± 0.38</td>
<td>7</td>
</tr>
<tr>
<td><strong>Slow wave</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>−67.6 ± 3.9</td>
<td>36.0 ± 2.3</td>
<td>31.7 ± 2.2</td>
<td>26.8 ± 1.7</td>
<td>0.75 ± 0.10</td>
<td>6</td>
</tr>
<tr>
<td>Dai-kenchu-to 1 mg/ml</td>
<td>−63.9 ± 4.9*</td>
<td>31.4 ± 2.5*</td>
<td>27.2 ± 2.4*</td>
<td>24.8 ± 2.2</td>
<td>0.70 ± 0.05</td>
<td>6</td>
</tr>
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</table>

Values are means ± S.D. n = number of animals. *P<0.05, compared to control.
Fig. 6. Effect of Dai-kenchu-to on pacemaker potentials recorded from mouse small intestine. A, pacemaker potentials were recorded before (a) and during application of Dai-kenchu-to (1 mg/ml) (b). B, high speed traces of pacemaker potentials recorded in the absence (a) and presence of 1 mg/ml Dai-kenchu-to (b). The resting membrane potential was –72 mV.

Fig. 7. Effect of Dai-kenchu-to on slow waves recorded from mouse small intestine. A, slow waves were recorded before (a) and during application of Dai-kenchu-to (1 mg/ml) (b). B, high speed traces of slow waves recorded in the absence (a) and presence of 1 mg/ml Dai-kenchu-to (b). The resting membrane potential was –70 mV.
Discussion

Dai-kenchu-to (DKT) is reported to be effective in treating uncomplicated postoperative intestinal obstruction (Ito et al., 2002; Ohya et al., 2003; Endo et al., 2006). It has been considered that DKT improves postoperative ileus by exerting gastroprokinetics effects on gastrointestinal tract. However the precise mechanism for the DKT induced ileus-improving effect remains unsolved. In animal experiments, DKT increases dog intestinal motility in vivo (Furukawa et al., 1995). DKT evokes contractions in isolated intestine of rabbit and guinea pig (Kurosawa et al., 1998; Hayakawa et al., 1999b; Satoh et al., 2001a, 2001b). In contrast, DKT inhibits carbachol induced contraction in rat colon (Tulimat et al., 2001). Thus there are species and regional differences in the action of DKT.

The present study indicated that DKT (1 mg/ml) evoked four steps of mechanical responses in mouse small intestine: initial L-NNA-sensitive relaxation, atropine-sensitive contraction, atropine-resistant contraction and TTX-resistant contraction. DKT releases ACh from cholinergic nerve endings via activation of 5HT4 receptors in guinea pig ileum (Satoh et al., 2001b). Since 5HT4 receptors are also expressed in enteric nervous system in mouse small intestine (Liu et al., 2005), DKT may also activate 5HT4 receptors at the ends of cholinergic nerve to enhance the release of ACh in mouse small intestine. TTX inhibited atropine-resistance contraction by about 30%, suggesting that there are additional excitatory transmitters contributing to this contraction. Both spantide and desensitization of receptors with high concentration of substance P were unable to inhibit atropine-resistant contraction, indicating that substance P was not involved in this remaining contraction.

The initial mechanical responses elicited by 1 mg/ml DKT was the reduction of the basal tone. This inhibitory effect was abolished by application of L-NNA, suggesting an involvement of NO in the DKT-induced initial relaxation. TNS evoked phasic contraction, which turned to relaxation by application of atropine (Fig. 5), indicating that at least cholinergic and nitrergic nerves distribute on mouse small intestine. In general ACh contracts and NO relaxes gastrointestinal smooth muscle cells (Kuriyama et al., 1998). It is interesting that DKT produce two opposing effects (cholinergic excitatory and nitrergic inhibitory) on intestinal motility. DKT may put a brake on smooth muscles not to contract too much. The relaxation evoked by DKT may be effective when intestinal muscles generate excessive contraction in some symptoms. Taken together the gastroprokinetic effects by DKT is not a simple enhancement of intestinal contraction. It seems likely that DKT regulate intestinal motility by maintaining the balance between contraction and relaxation of smooth muscles.

The present study found that low concentration of DKT inhibited TNS evoked phasic contraction. In some preparations, DKT (0.1 mg/ml) not only reduced the amplitude of TNS induced contraction but also increased the amplitude of relaxation (Fig. 4B). However these effects were also observed even in the presence of L-NNA (unpublished observation). Therefore it seems that DKT induced inhibitory actions were not mediated by a simple enhancement of NO release. These results raise the possibility that DKT inhibited TNS evoked contraction mainly through the reduction of ACh release from cholinergic nerve endings. DKT consists of Zanthoxylum fruit, ginseng, dried ginger root and malt sugar. In some animals, the
effect of each herb has been investigated. Zanthoxylum fruit contracted smooth muscle preparations of rabbit jejunum and guinea pig ileum through ACh release (Kurosawa et al., 1998; Hayakawa et al., 1999b; Satoh et al., 2001a). On the other hand dried ginger root relaxed rabbit jejunum (Hayakawa et al., 1999b). Therefore multiple actions of DKT may be produced as a result of mixture of these herbs. Although no direct evidence was obtained from the present study, dried ginger root might have big contribution to DKT induced inhibitory effect in mouse small intestine.

There was no significant change of spontaneous electrical activity by application of 1 mg/ml DKT in mouse small intestine. DKT depolarized the membrane and decreased the amplitude of pacemaker potentials recorded from ICC-MY in the presence of nifedipine and TTX, without changing the frequency, duration and dV/dt_max. These results indicate that DKT has no significant effect on the pacemaker mechanisms in mouse small intestine. As ICC-MY are the origin of spontaneous electrical activity in small intestine (Sanders et al., 1999), activation of ICC-MY would be effective to enhance the intestinal motility. DKT seems to exert a fine regulation of intestinal motility safely by eliciting both contraction (excitatory nerves) and relaxation (inhibitory nerves) of smooth muscles without changing the properties of pacemaker potentials. Furthermore DKT increase blood flow in mesenteric artery via releasing CGRP (Murata et al., 2002) and inhibit COX_2 activity (Hayakawa et al., 1999a). These effects may protect ICC from inflammation and maintain the normal function of ICC in gastrointestinal disease (Lu et al., 1997). Taken together DKT seems to act as a gastroprokinetic and protective agent in gastrointestinal disorders.

Membrane depolarization induced by DKT was also detected in circular muscles. DKT produced contraction even in the presence of TTX. Thus DKT induced membrane depolarization may be involved in TTX-resistant contraction. It is reported that cisapride, an agonist of 5HT_3 receptors, depolarized the membrane of esophageal smooth muscles via blocking HERG-like K^- channels (Akbarali et al., 1999). It may be possible that DKT also depolarized the membrane by inhibiting K^- channels contributing to the resting membrane potential.

In conclusion, DKT exerted its gastroprokinetic actions by releasing ACh, NO and other transmitters in mouse small intestine. The absence of any effects on ICC-MY may be one of the useful merits of DKT to apply to gastrointestinal disorders, since it has been reported that a number of ICC is decreased in many kinds of gastrointestinal disease (Vanderwinden and Rumessen, 1999). DKT seems to improve gastrointestinal symptoms by exerting multiple actions on intestinal motility.

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