Mechanism of Atropine-Resistant Contraction Induced by Dai-kenchu-to in Guinea Pig Ileum

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ABSTRACT—To clarify the contractile mechanism of Dai-kenchu-to, the effects of hydroxy β-sanshool (an ingredient of Zanthoxylum fruit), Zanthoxylum fruit (a constituent herb of Dai-kenchu-to) and Dai-kenchu-to were studied in mucosa-free longitudinal muscle of guinea pig ileum. Hydroxy β-sanshool at 10^{-7} – 10^{-5} g/ml induced dose-related contractions accompanied by autonomous contraction and produced an initial contraction at a concentration of 10^{-4} g/ml or more. The contraction induced by hydroxy β-sanshool (10^{-5} g/ml) was significantly inhibited by tetrodotoxin or the capsaicin-receptor antagonist capsazepine. Although atropine or the substance P antagonist spantide tended to inhibit the contraction, a combination of atropine and spantide almost abolished the contraction by hydroxy β-sanshool. The P2-purinoceptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid did not affect hydroxy β-sanshool-induced contraction in the presence or absence of spantide. The tonic contractions by Zanthoxylum fruit (2 x 10^{-4} g/ml) and Dai-kenchu-to (10^{-3} g/ml) were significantly inhibited or tended to be inhibited by atropine, spantide, tetrodotoxin or capsazepine and were remarkably suppressed by the combination of atropine and spantide. These results suggested that acetylcholine release from intrinsic cholinergic nerves and tachykinins from sensory neurons are involved in the contractions induced by hydroxy β-sanshool and that tachykinins may be involved in the atropine-resistant contraction by Dai-kenchu-to.

Keywords: Dai-kenchu-to, Zanthoxylum fruit, Hydroxy β-sanshool, Intestinal motility, Tachykinin

Dai-kenchu-to (Da-Jian-Zhong-Tang in Chinese) is a traditional Chinese herbal medicine, called Kampo medicine in Japan, and is a mixture of Zanthoxylum fruit, ginseng, dried ginger root and malt sugar. This formula is known for clinical effects on intestinal obstruction subsequent to laparotomy (1, 2). In vivo studies have demonstrated that Dai-kenchu-to enhanced gastrointestinal motility in dogs and rabbits (2, 3), and prevented intestinal adhesion in rats (4). In experiments using isolated intestines, Dai-kenchu-to induced contractions in rabbit jejunum, guinea pig ileum and colon, and relaxed guinea pig gastric body (5 – 8). These reports indicated that Zanthoxylum fruit induces contraction; however, the other constituent herbs had no significant contractile effect on the isolated intestine. In guinea pig ileum and colon, contractions due to Dai-kenchu-to and Zanthoxylum fruit are mediated by acetylcholine release from the ends of cholinergic nerves, and 5-HT_{1} receptors are involved (7, 8). In addition, since we also noted that a mucosa-free preparation resists the contractile response to atropine (8), it was suggested that other neurotransmitters are involved in the contractile effect of Dai-kenchu-to.

Hydroxy β-sanshool is considered to be one of the active compounds involved in inducing contraction by Zanthoxylum fruit (9, 10). It was reported that the contractile effect is due to the release of acetylcholine and other neurotransmitters (10). Natural pungent substances, capsaicin (red pepper), piperine (black pepper) and shogaol (ginger) have contractile effects on guinea pig ileum, and these effects were induced via release of substance P from sensory nerve endings (11 – 15). Zanthoxylum fruit is called Japanese pepper and has oral pungency. Therefore, it is possible to consider that tachykinins are involved in the action mechanism of contraction by hydroxy β-sanshool.

This study was carried out to investigate the involve-
ment of tachykinins from sensory nerves in the atropine-resistant contractile response to hydroxy β-sanshool to clarify the contractile mechanism of Dai-kenchu-to. For this purpose, we examined the influence of spantide (16, 17), a substance P antagonist; capsaizepine (18), a capsaicin receptor antagonist; and other antagonists on the contractions induced by hydroxy β-sanshool, Zanthoxylum fruit, and Dai-kenchu-to on mucosa-free longitudinal muscle of isolated guinea pig ileum.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs were purchased from Nippon SLC, Inc. (Shizuoka). The animals were housed in an air-conditioned animal room kept at a temperature of 23 – 24°C, a humidity of 50 – 65% and a 12 h light-dark cycle, and had free access to food and water. Well-nourished animals with a body weight of 320 – 500 g were used throughout the experiments.

Drugs

Hydroxy β-sanshool was isolated from the extract of Zanthoxylum fruit (Rutaceae, Zanthoxylum piperitum De Candolle, pericarpium) by the method described previously (10). Dai-kenchu-to extract powder (extracts in water from a 5:3:2 mixture of dried ginger root (Zingiberaceae, Zingiber officinalis Roscoe, rhizoma), ginseng (Araliaceae, Panax ginseng C.A. Meyer, radix) and Zanthoxylum fruit), extract powder of Zanthoxylum fruit and malt sugar (the candy produced from rice, wheat and malt) were manufactured by Tsumura & Co. (Tokyo). Dai-kenchu-to was prepared by mixing Dai-kenchu-to extract powder and malt sugar at a ratio of 1:8. Dai-kenchu-to was used at a concentration identical to that of the powdered extract of Dai-kenchu-to. The other drugs used were: acetylcholine chloride (ACh) (Ovisot Injection; Daiichi Pharmaceutical Co., Tokyo); tetrodotoxin (TTX) (Wako Pure Chemicals, Osaka); spantide (Novabiochem, Läufelfingen, Switzerland); and pyridoxal-phosphate-6-azophenyl-2,4-disulphonic acid tetrasodium (PPADS) (Research Biochemicals International, Natick, MA, USA). Atropine sulfate, capsaicin and capsaizepine were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Powdered extracts were dissolved in Krebs solution. Hydroxy β-sanshool, capsaizepine and capsaicin were dissolved in dimethyl sulphoxide (final concentration was not more than 0.5%). Other drugs were dissolved in distilled water.

Isolated guinea pig ileal preparations

Guinea pigs were killed by decapitation, and their ileum was immediately excised. After removing the mucosa, strips were suspended along the longitudinal muscle by 0.5 g loading in an organ bath with oxygenated (95% O2 and 5% CO2) Krebs solution at 37°C. The composition of Krebs solution was as follows: 118 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l MgSO4, 1.2 mmol/l NaH2PO4, 2.5 mmol/l CaCl2, 25 mmol/l NaHCO3 and 11 mmol/l glucose. Experiments started after 60 min of equilibration. The preparations were exposed to ACh (5.5 × 10−2 mol/l) at the beginning of the experiment. Contractile responses were recorded isotonically.

The dose-related effects of hydroxy β-sanshool (10−5 – 3 × 10−4 g/ml), Zanthoxylum fruit (10−6 – 10−3 g/ml) and Dai-kenchu-to (10−6 – 10−3 g/ml) were examined by single application. These concentrations of hydroxy β-sanshool or Zanthoxylum fruit are approximately in the range of their concentrations in Dai-kenchu-to. ACh (5.5 × 10−7 mol/l) was applied 15 min after the application of hydroxy β-sanshool to evaluate the effect of hydroxy β-sanshool on ACh-induced contraction. Since the mode of contractions was changed by concentrations, the maximal value of the contraction was measured regardless of the mode of contractions.

The effects of pretreatment with capsaizepine (10−4 mol/l), atropine (10−5 mol/l), spantide (10−5 mol/l), PPADS (3 × 10−5 mol/l) or TTX (3 × 10−5 mol/l) on contractions induced by hydroxy β-sanshool (10−5 g/ml) were examined. Each antagonist was applied 10 min before application of hydroxy β-sanshool. To evaluate the combined effect, atropine (10−4 mol/l) or PPADS (3 × 10−3 mol/l) were applied 1 min before spantide (10−5 mol/l).

The effects of pretreatment with capsaizepine (10−5 mol/l), atropine (10−4 mol/l), spantide (10−5 mol/l), or TTX (3 × 10−5 mol/l) on contractions induced by Zanthoxylum fruit (2 × 10−4 g/ml: the concentration corresponding to 10−3 g/ml of Dai-kenchu-to or Dai-kenchu-to (10−4 g/ml) were examined. The combined effects of atropine (10−4 mol/l) and spantide (10−2 mol/l) on these contractions were also examined.

The contractions induced by Dai-kenchu-to (10−3 g/ml) and Zanthoxylum fruit (2 × 10−4 g/ml) have both phasic and tonic components. However, as a phasic component was not observed in the contraction by hydroxy β-sanshool was measured; and since the maximal value in the tonic contraction by Zanthoxylum fruit and Dai-kenchu-to was achieved about 1.5 min after the phasic contraction, the value at 1.5 min after the phasic contraction was measured. Contractile responses were expressed as a percentage of maximal responses of ACh (5.5 × 10−7 mol/l).

All values are expressed as the mean ± S.E.M. Statistical significance was assessed by the unpaired Student’s t-test.
or Fisher’s PLSD test.

RESULTS

Contractile effect of hydroxy β-sanshool, Zanthoxylum fruit and Dai-kenchu-to

Figure 1 shows the maximal contractions induced by hydroxy β-sanshool (10⁻⁷ – 3 × 10⁻⁵ g/ml), Dai-kenchu-to (10⁻⁸ – 10⁻⁷ g/ml) and Zanthoxylum fruit (10⁻⁸ – 10⁻³ g/ml). Hydroxy β-sanshool at 10⁻⁷ – 10⁻⁵ g/ml induced dose-related contractions accompanied by autonomous contraction (Fig. 2), and it did not inhibit ACh (5.5 × 10⁻⁷ M)-induced contraction; the amplitudes were as follows (% of ACh-induced precontraction): hydroxy β-sanshool 10⁻⁷ g/ml, 115.9 ± 13.7%; 10⁻⁶ g/ml, 118.9 ± 5.7%; 10⁻⁵ g/ml, 116.7 ± 5.2%; vehicle, 117.3 ± 12.5% (N = 3, respectively). At a concentration of 10⁻⁴ g/ml or more, hydroxy β-sanshool produced an initial contraction and subsequently suppressed autonomous contraction. Hydroxy β-sanshool completely abolished ACh-induced contraction at this high concentration (N = 4, respectively). Zanthoxylum fruit and Dai-kenchu-to also induced dose-related contraction. Zanthoxylum fruit (2 × 10⁻⁴ g/ml) and Dai-kenchu-to (10⁻³ g/ml) induced a two-phase contraction: a fast phasic component followed by a slower, more sustained tonic component (Fig. 3). Tonic contractions induced by Dai-kenchu-to (10⁻³ g/ml), Zanthoxylum fruit (2 × 10⁻⁴ g/ml) and hydroxy β-sanshool (10⁻⁷ g/ml) were about the same amplitude.

Effects of antagonists on contraction induced by hydroxy β-sanshool (10⁻⁵ g/ml)

Capsazepine (10⁻⁵ mol/l), which completely inhibited capsaicin (10⁻⁶ mol/l)-induced contraction, significantly inhibited the contraction by hydroxy β-sanshool (10⁻³ g/ml) (Fig. 4). Atropine (10⁻⁶ mol/l) remarkably inhibited the autonomous contraction and showed a trend to inhibit the amplitude of contraction (Fig. 5). Although spantide (10⁻⁵ mol/l) inhibited the contraction slightly, the contractile effect was abolished by a combination of atropine and spantide (Figs. 5 and 6). The hydroxy β-sanshool-induced contraction was significantly inhibited by TTX (3 × 10⁻⁶ mol/l), but not by PPADS (3 × 10⁻⁵ mol/l) or a combination of PPADS and spantide (Fig. 6).
Mechanism of Ileal Contraction by Dai-kenchu-to

Effects of antagonists on contraction induced by Zanthoxylum fruit (2 × 10⁻⁴ g/ml) and Dai-kenchu-to (10⁻³ g/ml)

Capsazepine (10⁻⁵ mol/l) showed an inhibitory trend to the contraction by Zanthoxylum fruit (2 × 10⁻⁴ g/ml) and Dai-kenchu-to (10⁻³ g/ml) (Fig. 4). The Dai-kenchu-to-induced contraction tended to be inhibited by spantide, but it was significantly inhibited by atropine, TTX or combined treatment with atropine and spantide (Fig. 7). The Zanthoxylum fruit-induced contraction also tended to be inhibited by spantide, atropine or TTX, but it was significantly inhibited by the combined treatment with atropine and spantide (Fig. 7).
DISCUSSION

These data provide evidence that tachykinins are involved in the contractile effect of hydroxy  β-sanshool in isolated guinea pig longitudinal muscle. Under the conditions of the present study, the response to hydroxy  β-sanshool was inhibited by capsazepine and almost abolished by the combination of atropine and spantide. This probably indicates that tachykinins released by hydroxy  β-sanshool are from sensory nerve endings.

Capsaicin is a pungent compound and is well-known to stimulate capsaicin (vanilloid) receptor on the sensory nerves and to release substance P and calcitonin gene-related peptide in guinea pig ileum (19). In addition, it was suggested that ATP may be involved in the nontachykininergic activation of cholinergic neurons in the course of the capsaicin-induced contraction (20). Other pungent-tasting compounds such as piperine and zingerone are considered natural analogues of capsaicin (21). However, in psychophysical taste experiments and whole cell patch-clamp studies, similarities and differences among these pungent compounds were reported (21). Olvanil is a non-pungent capsaicin analogue, but stimulates the efferent function of cutaneous sensory nerves in a more potent manner than capsaicin (22). There are additional differences among the peptides released from rat dorsal horn by capsaicin analogues (23). These reports likely indicate the presence of subtypes of vanilloid receptors (21, 24). In the present study, hydroxy  β-sanshool induced ileal contraction, which was inhibited by TTX, capsazepine and combined treatment with atropine and spantide. However, the combination of PPADS and spantide did not significantly inhibit hydroxy  β-sanshool-induced contraction, although the experimental condition was different from that used by Barthó et al. (20). These results suggested that ATP may not be involved in the nontachykininergic activation of cholinergic neurons in the course of the hydroxy  β-sanshool-induced contraction. In addition, it was reported that hydroxy  β-sanshool is nonpungent (25). Therefore, hydroxy  β-sanshool might activate different subtypes of vanilloid receptors compared with capsaicin.

Substance P has at least two sites of action in guinea pig ileum, NK₁ receptor on the longitudinal smooth muscles and NK₁ receptor on cholinergic nerves causing release of acetylcholine (16, 26). When substance P is released from intrinsic nerves, substance P stimulates NK₁ receptor on the longitudinal smooth muscles and induces contraction even with an inhibition of muscarinic receptors. On the other hand, with an inhibition of substance P receptors on smooth muscles, substance P enhances ACh release by stimulating the receptors on cholinergic nerves and induces ileal contraction. Thus, regardless of the fact that a significant inhibitory action could not be obtained by a single treatment with atropine or spantide, a remarkable inhibition was observed by combined treatment with both. In the present study, capsazepine failed to completely inhibit the contraction by hydroxy  β-sanshool, although TTX almost abolished the contraction. Accordingly, it was also considered that hydroxy  β-sanshool stimulates cholinergic nerves or tachykinergic nerves directly in addition to sensory nerves.

At high concentrations (10⁻⁴ g/ml or more), hydroxy  β-sanshool inhibited autonomous contraction and ACh-induced contraction after transient contraction. It was reported that at high concentrations, capsaicin and piperine produced a non-specific smooth muscle depressant effect (15, 27). Thus, the inhibitory effect of hydroxy  β-sanshool is thought to be non-specific, similar to that of high concentrations of capsaicin or piperine.

In the experiment using whole ileum, contractions by Dai-kenchu-to or Zanthoxylum fruit was almost abolished by atropine (8), whereas under the conditions of the present study using a mucosa-free ileum preparation, atropine-resistant contraction by Dai-kenchu-to or Zanthoxylum fruit were observed. It might be considered that this effect occurs because the influence of a neurotransmitter or endogenous factor from the mucosa was removed.

Although capsazepine tended to inhibit the contraction by Dai-kenchu-to and Zanthoxylum fruit, these contractions were significantly inhibited by combined treatment with atropine and spantide. Therefore, it was considered that release of both ACh and substance P from the intrinsic nerves is involved in the contractions induced by Dai-kenchu-to or Zanthoxylum fruit, and 5-HT₁ receptors are involved in ACh release (8). The initial contraction including phasic contraction by Dai-kenchu-to or Zanthoxylum fruit was not completely inhibited by TTX or the combination of spantide and atropine in this study. As Dai-kenchu-to and Zanthoxylum fruit are extracts from herbal medicines, numerous inorganic salts and various unknown compounds are thought to be included. When several fractions from Zanthoxylum fruit were evaluated to explore the component involved in the intestinal contraction, unsaturated aliphatic acid amides and aromatic compounds were isolated from the methanol fraction of Zanthoxylum fruit (10).

Although aromatic compounds contracted ileum but relaxed distal colon, unsaturated aliphatic acid amides contracted the isolated ileum and distal colon. Thus, in this study we examined hydroxy  β-sanshool, which is the most abundant unsaturated aliphatic acid amide in the methanol fraction of Zanthoxylum fruit. Accordingly, hydroxy  β-sanshool is considered to be one of the active compounds involved in inducing the contraction by Zanthoxylum fruit, but may not necessarily be the representative of Zanthoxylum fruit or Dai-kenchu-to. From these circumstances, although the contractile response induced by hydroxy  β-
sanshool is mainly mediated by a neurotransmitter, it was considered that two factors, a factor directly affecting muscles and a neural factor, are involved in the contractions induced by Dai-kenchu-to or Zanthoxylum fruit. Many factors that cause direct action on the muscles are considered, for instance, non-specific depolarization, stimulation of the receptors other than the muscarinic and tachykinergic receptors, or activation of contractile protein.

In conclusion, it is suggested that the release of ACh from intrinsic cholinergic nerves and tachykinins from the sensory neurons are involved in the contractions induced by hydroxy β-sanshool, one of the ingredients of Dai-kenchu-to. This effect may influence the contractile mechanism of Dai-kenchu-to, and tachykinins from sensory neurons may be involved in the atropine-resistant contraction by Dai-kenchu-to.

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