Inhibition of airway smooth muscle tone by Chinese herbal medicines

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ABSTRACT: The aim of the present study was to elucidate whether Chinese traditional herbal drugs, Gorei-San (TJ-17) and Toki-Shakuyaku-San (TJ-23), affect airway smooth muscle tone and, if so, to determine what the mechanism of action is. Rabbit tracheal segments were isolated and the contractile responses to electrical field stimulation and acetylcholine were measured before and after the application of TJ-17 or TJ-23 under isometric conditions in vitro. Ouabain-sensitive rubidium-86 ($^{86}$Rb) uptake by tissues in response to each drug was also measured.

Each herbal medicine attenuated the contractile responses to electrical field stimulation and acetylcholine in a concentration-dependent manner, the maximal inhibition of acetylcholine-induced contraction being 37.5±4.9% for TJ-17 and 42.4±5.3% for TJ-23 (p<0.05 for each). These effects were not altered by mechanical removal of the epithelium, indomethacin, the nitric oxide synthase inhibitor N$^\bullet$-nitro-L-arginine methyl ester, the cyclic adenosine monophosphate (cAMP)-dependent protein kinase inhibitor adenosine 3′-5′-cyclic monophosphorothioate (Rp-cAMPS), the cyclic guanosine monophosphate (cGMP)-dependent protein kinase inhibitor KT5823, or the calcium (Ca$^{2+}$)-activated potassium (K$^+$) channel inhibitor charybdotoxin, but were greatly inhibited in the presence of the sodium (Na$^+$)-K$^+$ adenosine triphosphatase (ATPase) inhibitor ouabain. Incubation of tissues with TJ-17 and TJ-23 dose dependently increased ouabain-sensitive $^{86}$Rb uptake.

The results of the study suggest that both Gorei-San and Toki-Shakuyaku-San reduce airway smooth muscle tone via a postjunctional mechanism probably through stimulation of the sodium pump and the subsequent hyperpolarization/repolarization of the cell membrane. These effects may contribute to the antiasthmatic properties of these herbal medicines.


Chinese traditional herbal drugs have long been used in the treatment of asthma in Asian countries. Among them, Gorei-San (TJ-17) and Toki-Shakuyaku-San (TJ-23) produce improvements in asthma symptom scores and pulmonary function tests in adult and childhood asthma [1, 2]. Furthermore, long-term administration of these drugs reduce the use of supplemental β$^2$-agonist in steroid-dependent asthma [3]. The mechanisms for the efficacy include inhibition of immunoglobulin-E (IgE)-mediated release of histamine from basophils [4] and platelet-activating factor from neutrophils [5] and prevention of down-regulation of glucocorticoid and β-adrenergic receptor [5]. However, the direct action on airway smooth muscle tone remains unknown.

The sarcolemmal sodium-potassium adenosine triphosphatase (Na$^+$-K$^+$ ATPase) has been implicated in the mechanism of relaxant responses of airway and vascular smooth muscle induced by a β-adrenergic agonist and sodium nitroprusside [6, 7]. Stimulation of enzymatic activity of Na$^+$-K$^+$ ATPase may lead to generation of the sodium gradient necessary to exclude calcium (Ca$^{2+}$) via the Na$^+$/Ca$^{2+}$ exchange or hyperpolarization of the membrane, which in turn reduces Ca$^{2+}$ influx through membrane potential-dependent Ca$^{2+}$ channels [8]. It has recently been shown that TJ-17 and TJ-23 protect brain cells against apoptosis by elevating Na$^+$-K$^+$ ATPase activity of microvascular beds [9]. Therefore, it was hypothesized that if these drugs could stimulate sarcolemmal Na$^+$-K$^+$ ATPase in airway smooth muscle cells, then bronchodilation might occur through the inhibition of Ca$^{2+}$-dependent contraction of airway smooth muscle cells. To test this hypothesis, the effects of these agents on rabbit tracheal segments under isometric conditions were examined in vitro.

Material and methods

Preparation of tissues

Japanese-white rabbits of either sex, weighing 1.7–2.4 kg, were anaesthetized with pentobarbital sodium (35 mg kg$^{-1}$, i.v.), and the trachea was removed and immersed in oxygenated Krebs-Henseleit (KH) solution consisting of the following (mM): sodium chloride (NaCl), 118; potassium chloride (KCl), 5.9; calcium chloride (CaCl$_2$),
Effects of epithelial removal and pharmacologic blocking agents

To test whether the release of epithelium-derived relaxing factor is involved in the effects of TJ-17 and TJ-23 [10], the effects of the drug (100 µg·mL⁻¹) on acetylcholine (1×10⁻⁵ M)-induced contractions between epithelium-intact tissues and the tissues with their epithelial layer removed were compared. To do so, the epithelial cells were gently removed by passage of a moistened cotton-wrapped pipe cleaner through the tracheal lumen, and the absence of the epithelial layer was confirmed after the experiment by staining tissues with Masson’s trichrome.

The involvement of the activation of Na⁺-K⁺ ATPase [11] and the opening of Ca²⁺-activated K channel [12] was assessed by determining the inhibition of the contractile responses to acetylcholine (1×10⁻⁵ M) by TJ-17 and TJ-23 (100 µg·mL⁻¹) in the absence and presence of ouabain (1×10⁻⁷ M), a Na⁺-K⁺ ATPase inhibitor, or charybdotoxin (1×10⁻⁶ M), a Ca²⁺-activated K⁺ channel blocker [13]. In addition, possible involvement of the generation of inhibitory prostaglandins [14], the release of nitric oxide [15], and the synthesis of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) were likewise assessed by the use of the following pharmacological blocking agents: indomethacin (3×10⁻⁶ M), a cyclooxygenase inhibitor; N⁶,-nitro-L-arginine methyl ester (l-NAME, 1×10⁻⁵ M), an inhibitor of nitric oxide synthase [16]; adenosine 3’,5’-cyclic monophosphorothioate (Rp-cAMPS, 1×10⁻⁴ M), a specific inhibitor of cAMP-dependent protein kinase; and KT5823 (N-methyl-(8R*, 9S*, 11S*)-(-)-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H, 11H-2,7b,11a-triazadibenzo(a,g)cycloocta(cde)trinden-1-one, 2×10⁻⁶ M), a specific inhibitor of cGMP-dependent protein kinase [17].

Measurement of Na⁺-K⁺-ATPase activity

To examine the effects of TJ-17 and TJ23 on sarcoplasmic sodium pump activity, ouabain-sensitive ⁸⁶Rb uptake by epithelium-denuded tracheal rings was measured. After incubation of the tissues for 15 min with various concentrations of each drug (1–1,000 µg·mL⁻¹), rubidium-86 chloride (⁸⁶RbCl) (2 µCi·mL⁻¹) was added, and 10 min later the tissues were washed with ice-cold unlabelled KH solution to remove the radioisotope from the extracellular compartment. The tissues were then blotted on a filter paper, placed in a plastic vial, dried overnight in an oven at 100°C, and the ⁸⁶Rb contents were later, and the contractile responses to acetylcholine before (control) and after the drug addition were compared.

To determine the concentration-dependent effects of TJ-17 and TJ-23, contractile responses to acetylcholine at a concentration that produced approximately 50% of the control maximum contraction (1×10⁻⁴ M) were measured 15 min after the addition of various concentrations of each drug (1–1,000 µg·mL⁻¹). In this experiment, TJ-17 or TJ-23 was cumulatively added after establishing the effect of each concentration.

Effects of EFS- and acetylcholine-induced contraction

TJ-17 and TJ-23 were assessed for their affect on neurally-mediated smooth muscle contraction, by determining contractile responses to EFS before and after the addition of the drugs (100 µg·mL⁻¹). The control responses to EFS were first obtained at increasing frequencies of stimulation (1–50 Hz); each drug was added to the chamber, and after a 15-min incubation the measurements were repeated; the EFS-induced contraction before (control) and after the drug addition were then compared. In a preliminary study carried out by the authors, addition of the solvent (KH solution) alone had no effect on the contractile responses to EFS. Moreover, this incubation period was chosen because in the separate experiment the EFS (10 Hz)-induced contraction was determined at 1, 5, 15, and 45 min after the addition of TJ-17 or TJ-23, and it was found that the inhibition of the contraction reached a plateau at 15 min for each drug.

In order to identify whether the site of drug action is pre- or postjunctional in the cholinergic motor pathway, the effect on the contractile responses to exogenously applied acetylcholine (1×10⁻⁵–1×10⁻³ M) was also examined. After establishing the first concentration-response curves, tissues were washed with KH solution until the tension returned to the resting levels. Then, TJ-17 or TJ-23 (100 µg·mL⁻¹) was added, the second concentration-response curves for acetylcholine were generated 15 min postjunctional in the cholinergic motor pathway, and after a 15-min incubation the measurements were repeated; the EFS-induced contraction before (control) and after the drug addition were then compared. In a preliminary study carried out by the authors, addition of the solvent (KH solution) alone had no effect on the contractile responses to EFS. Moreover, this incubation period was chosen because in the separate experiment the EFS (10 Hz)-induced contraction was determined at 1, 5, 15, and 45 min after the addition of TJ-17 or TJ-23, and it was found that the inhibition of the contraction reached a plateau at 15 min for each drug.

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To determine the concentration-dependent effects of TJ-17 and TJ-23, contractile responses to acetylcholine at a concentration that produced approximately 50% of the control maximum contraction (1×10⁻⁴ M) were measured 15 min after the addition of various concentrations of each drug (1–1,000 µg·mL⁻¹). In this experiment, TJ-17 or TJ-23 was cumulatively added after establishing the effect of each concentration.
determined by gamma counting. To determine ouabain-sensitive 86Rb uptake, the maximally effective concentration of ouabain (2 x 10^{-4} M) was added 10 min before the introduction of 86RbCl into the medium. The ouabain-sensitive portion of the 86Rb uptake, an index of sodium pump activity [18], was calculated by subtracting the ouabain-insensitive 86Rb uptake from the total 86Rb uptake, and the data were expressed as nmol-min^-1 per mg of tissue dry weight.

Drugs

The following drugs were used: acetylcholine chloride, indomethacin, ouabain, l-NAME (Sigma Chemical Co., St. Louis, MO, USA); charybdotoxin (Peptide Institute Ltd., Osaka, Japan); and Rp-cAMPS (BIOLOG Life Science Institute, Bremen, Germany). KT 5823 was a gift from Kamiya Biomedical Company (Thousand Oaks, CA, USA). TJ-17 and TJ-23 were obtained from Tsumura Co. (Tokyo, Japan). 86RbCl was obtained from Amersham (Tokyo, Japan). Indomethacin was dissolved in absolute ethanol at 1 x 10^{-3} M and subsequently diluted in KH solution, and other drugs were dissolved in KH solution. Working solutions of all drugs were kept on ice and added to the organ chamber in 140-μL aliquots.

Statistical analysis

All values were expressed as mean±SEM. Statistical analysis was performed by ANOVA followed by either Turkey’s test for multiple comparison or by a paired t-test, and a p-value of <0.05 was considered statistically significant.

Results

Contractile responses of tracheal segments

Addition of TJ-17 and TJ-23 at concentrations used in the present experiments did not alter the resting tension of rabbit tracheal segments. However, as demonstrated in figure 1, incubation of tissues for 15 min with each drug at a concentration of 100 μg·mL^{-1} significantly reduced the contractile responses to EFS at almost all the stimulus frequencies, so that the frequency-contractile response curves were displaced to the right.

Both TJ-17 and TJ-23 (100 μg·mL^{-1}) also attenuated the acetylcholine-induced contraction (fig. 2). The inhibitory effects of TJ-17 and TJ-23 on the acetylcholine (1 x 10^{-5} M)-induced contraction were concentration-dependent, the maximal inhibition being 37.5±4.9% for TJ-17 (p<0.05, n=6) and 42.4±5.3% for TJ-23 (p<0.05, n=6) observed at a concentration of 1,000 μg·mL^{-1} (fig. 3a).

Effects of epithelial removal and pharmacological blocking agents

Incubation of the tissues whose epithelial cells had been mechanically removed for 15 min with TJ-17 and TJ-23 (100 μg·mL^{-1}) reduced the contractile responses to acetylcholine (1 x 10^{-5} M), where the magnitude of inhibition was not significantly different from that observed with epithelium-intact tissues (fig. 3b).

The effects of ouabain and charybdotoxin on the contractile responses to acetylcholine were then examined. As shown in figure 4, pretreatment of tissues with ouabain attenuated the inhibitory effects of TJ-17 and TJ-23 (100 μg·mL^{-1}), the inhibition of acetylcholine (1 x 10^{-5} M)-induced contraction being decreased from 31.8±4.9 to 12.5±3.2% (p<0.05, n=9) and from 37.3±4.6 to 14.0±4.0% (p<0.05, n=9), respectively. In contrast, pretreatment with charybdotoxin did not alter the effects of TJ-17 and TJ-23. Likewise, other pharmacological blocking agents including indomethacin, l-NAME, Rp-cAMPS, and KT5823 had no effect on the inhibition of acetylcholine (1 x 10^{-5} M)-induced contraction by TJ-17 and TJ-23 (100 μg·mL^{-1}) (n=6–8 for each, data not shown).

Ouabain-sensitive rubidium-86 uptake

Incubation of rabbit tracheal segments for 15 min with TJ-17 and TJ-23 increased the ouabain-sensitive portion of the 86Rb uptake, which comprised of ~40% of the total portion of the 86Rb uptake, in a concentration-dependent manner (fig. 5). The significant responses of ouabain-sensitive 86Rb uptake were observed at concentrations of
Discussion

The in vitro studies demonstrate that the traditional Chinese herbal drugs, TJ-17 and TJ-23, inhibit the contractile responses of rabbit tracheal smooth muscle to EFS and acetylcholine presumably by stimulating the sarcolemmal sodium pump. Thus, these drugs possess a protective action against bronchoconstriction, an effect that could account for the efficacy in the treatment of asthma.

Airway smooth muscle contraction is generally associated with intracellular Ca\(^{2+}\) mobilization and membrane depolarization. The responses induced by EFS and exogenously applied acetylcholine are mediated through prejunctional and postjunctional mechanisms, respectively. In the present study, TJ-17 and TJ-23 attenuated the EFS-induced contraction with the same magnitude as observed with the acetylcholine-induced contraction, implying that the site of action of these drugs is most likely postjunctional. This suggests that TJ-17 and TJ-23 may have exerted their effects by directly acting on airway smooth muscle cells rather than by modulating the release of acetylcholine from cholinergic nerve terminals.

There might be several mechanisms by which TJ-17 and TJ-23 inhibited airway contraction. First, airway epithelial cells can inhibit bronchoconstriction through a release of the inhibitory prostaglandins and epithelium-derived relaxing factor [10]. However, because mechanical removal of the epithelium did not alter the effects of TJ-17 and TJ-23, involvement of airway epithelial cells in the drug action seems unlikely. Second, TJ-17 and TJ-23 could have stimulated airway smooth muscle cells to release prostaglandin E\(_2\) [14], but this possibility is also unlikely because of the lack of effect of indomethacin pretreatment. Third, airway cells such as nerve fibres and epithelial cells are constitutively generating nitric oxide (NO) [19, 20], which in turn inhibits bronchoconstrictor...
effects of TJ-17 and TJ-23 on the contractile responses to ATPase inhibitor ouabain substantially attenuated the and Na+-K+ ATPase, a enzyme responsible for the synthesis and/or release of NO can be excluded. Finally, pretreatment of tissues with Rp-cAMPS, a cAMP-dependent protein kinase inhibitor, or KT5823, a cGMP-dependent protein kinase inhibitor [13], had no effect on the TJ-17- and TJ-23-induced inhibition, indicating that the involvement of these cyclic nucleotides is not likely.

There is ample evidence that Ca2+-activated K+ channel and Na+-K+ ATPase, an enzyme responsible for the electrochemical gradient of Na+ and K+ across the cell membrane, are present on airway smooth muscle cells and play an important role in the regulation of the contractility [7, 8]. The opening of Ca2+-activated K+ channels and stimulation of Na+-K+ ATPase activity may reduce smooth muscle contraction by increasing the Na+/Ca2+ exchange and inhibiting the Ca2+ influx through the membrane potential-dependent Ca2+ channel [22]. In the present study, incubation of tissues with the Na+-K+ ATPase inhibitor ouabain substantially attenuated the effects of TJ-17 and TJ-23 on the contractile responses to acetylcholine, whereas prior blockade of Ca2+-activated K+ channel by charybdotoxin [13] was without effect. Therefore, the observed effects of the herbal drugs may have derived from the stimulation of Na+-K+ ATPase activity.

The sensitivity of Na+-K+ ATPase to ouabain is variable depending on the tissues and animal species [23], and it is uncertain whether the concentration of ouabain used in the contraction studies (1×10^{-7} M) is sufficient to completely inhibit this enzyme in rabbit tracheal smooth muscle cells. However, this concentration was chosen because it did not alter the baseline tension and the contractile responses to acetylcholine, and because a previous study showed that K+ loss and Na+ accumulation were produced by 50 nM ouabain in other tissues [24]. To further support the notion that Na+-K+ ATPase is involved, ouabain-sensitive 86Rb uptake by tracheal tissues, an index of Na+-K+ ATPase activity [18], was measured, and it was found that incubation of tissues with TJ-17 and TJ-23 increased ouabain-sensitive 86Rb uptake in a concentration-dependent manner. Moreover, it should be noted that the concentrations of the herbal drugs required to significantly increase ouabain-sensitive 86Rb uptake (100 µg·mL^{-1}) were identical to those observed with the experiment on the acetylcholine-induced contraction. Because the tracheal rings also contain cartilage and connective tissues, 86Rb uptake measured in the present experiment does not necessarily reflect smooth muscle Na+-K+ ATPase activity. However, there has been no evidence that alterations in Na+-K+ ATPase activity of cells other than smooth muscle and epithelium affects airway contraction. Thus it is speculated that sarcolemmal Na+-K+ ATPase may be most probably involved in the effects of TJ-17 and TJ-23.

Chinese traditional medicine generally consists of several ingredients extracted from the roots and nutlets of plants. For example, there are six and seven ingredients in TJ-17 and TJ-23, respectively, where Alismatis rhizoma, Hoelen, and Atractylodis lanceae rhizoma are common constituents of these drugs. Further studies are needed to determine what constituent is responsible for the observed effects of these herbal drugs, and it will be necessary to determine if there are interactions between the constituents. To conclude, traditional Chinese herbal drugs Gorei-San and Toki-Shakuyaku-san, inhibit airway smooth muscle contraction, thereby probably protecting against bronchoconstriction in asthma. This inhibitory effect may be associated with stimulation of sarcolemmal sodium-potassium adenosine triphosphatase activity and the consequent membrane hyperpolarization/repolarization of airway smooth muscle cells.

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References


