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Geissoschizine methyl ether, an indole alkaloid extracted from *Uncariae Ramulus* et Uncus, is a potent vasorelaxant of isolated rat aorta

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Abstract

Effects of geissoschizine methyl ether, an indole alkaloid isolated from the hook of *Uncariae Ramulus* et Uncus, on vascular responses were examined using isolated strips of rat aorta. Geissoschizine methyl ether (10^{-7}–10^{-4} M) relaxed norepinephrine (5×10^{-8} M)-induced contraction in a dose-dependent manner. The potency (50% efficacy concentration, $EC_{50} = 0.744$ μM) was approximately 14 times greater than that ($EC_{50} = 10.6$ μM) of hirsutine, one of the indole alkaloids isolated from *Uncariae Ramulus* et Uncus that demonstrates a vasorelaxant effect by Ca^{2+}-channel blocking. The vasorelaxant effect of geissoschizine methyl ether found at the lower concentrations (10^{-7}–3×10^{-6} M) on the norepinephrine-induced contraction was abolished by pretreatment with N^G-nitro-L-arginine (10^{-4} M), an inhibitor of nitric oxide synthesis, or by denuding aortas of endothelium, while the effects at the higher concentrations (10^{-5}–10^{-4} M) were not completely prevented by either N^G-nitro-L-arginine and deendothelialization. Furthermore, geissoschizine methyl ether did not relax high K^+-, Ca^{2+}- and a Ca^{2+}-channel agonist Bay K8644-induced contractions at the lower concentrations that markedly relaxed the norepinephrine-induced contractions, while the higher concentrations of geissoschizine methyl ether relaxed the high K^+-, Ca^{2+}- and Bay K8644-induced contractions. These results suggest that the vasorelaxant effect of geissoschizine methyl ether is composed of two different mechanisms: endothelial dependency with nitric oxide and endothelial independency with voltage-dependent Ca^{2+}-channel blocking.

Keywords: Ca^{2+} channel; Endothelium; Geissoschizine methyl ether; Nitric oxide (NO); Aorta, rat; Vasorelaxation

1. Introduction

Uncariae Ramulus et Uncus is one of the Chinese plants that has been used empirically for a long time to relieve headache and dizziness resulting from hypertension (Yano, 1987). Recently, evidence of the antihypertensive and vasodilative effects of *Uncariae Ramulus* et Uncus has been accumulated by several in vivo and in vitro studies (Ishii et al., 1987; Kuramochi et al., 1994; Sugimoto et al., 2000; Goto et al., 1999, 2000). These findings suggest that *Uncariae Ramulus* et Uncus contains active substances with vasodilative or vasorelaxant effects. To date, various indole alkaloids have been isolated from *Uncariae Ramulus* et Uncus and identified (Haginiwa et al., 1971, 1973; Endo et al., 1983). The major indole alkaloids, rhynchophylline, isorhynchophylline, corynoxeine, isocorynoxeine, hirsuteine and hirsutine have been demonstrated to possess a vasodilative effect. These alkaloids relax norepinephrine-, high K^+- and Ca^{2+}-induced contractions by inhibition of $^{45}Ca^{2+}$ uptake in the isolated rat thoracic aorta, suggesting that the vasorelaxant mechanism is due to Ca^{2+}-channel blocking activity in the vascular smooth muscle (Yamahara et al., 1987; Horie et al., 1992; Yano et al., 1991). However, the pharmacological effects of geissoschizine methyl ether, the indole alkaloid isolated from *Uncariae Ramulus* et Uncus (Aimi et al., 1977) shown in Fig. 1, have been little investigated (Sakakibara et al., 1997).

In the present study, therefore, the effects of geissoschizine methyl ether on vascular relaxation responses were examined in the isolated rat aorta, and the results were compared with those of hirsutine as a representative *Uncar-*

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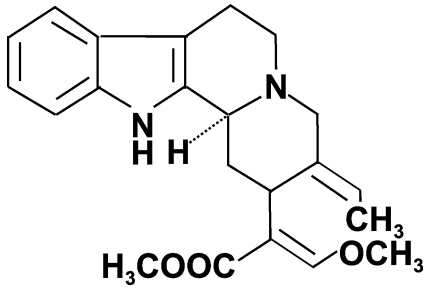

Fig. 1. Chemical structure of geissoschizine methyl ether.

iae Ramulus et Uncus-isolated indole alkaloids with Ca^{2+}-channel blocking effects.

2. Materials and methods

2.1. Reagents and drugs

N^G-nitro-L-arginine and norepinephrine were purchased from Wako (Osaka, Japan) and Sigma (St. Louis, USA). Bay K8644 was purchased from Bioscience (Daemstadt, Germany). The other reagents used for analysis were purchased from commercial sources.

A cut and dried hook of *Uncariae Ramulus* et Uncus was made available by Tsumura (Tokyo, Japan). A voucher specimen (no. 16125) has been deposited at the Kampo and Pharmacognosy Laboratories, Tsumura. Rhynchophylline, isorhynchophylline, corynoxeine, isocorynoxeine, hirsutine, hirsuteine and geissoschizine methyl ether were isolated from *Uncariae Ramulus* et Uncus in our laboratory. In brief, 319.4 g of *Uncariae Ramulus* et Uncus dissolved in 2.5 l of distilled water was refluxed at 120 °C for 2 h. The extracted solution was passed through a 100-mesh size stainless steel filter and then lyophilized to give a dried powder (38.2 g). The extract was chromatographed on a Diaion HP-20 (Mitsubishi, Tokyo, Japan), eluted with 2 l of water, 2 l of aqueous methanol (50% v/v) and 1 l of methanol, successively. The methanol eluate was evaporated to remove the solvent and then lyophilized to afford the dried methanol–eluate powder (0.529 g). The indole alkaloids were further isolated from the methanol extract by eluting with 0.05 M ammonium acetate buffer (pH 3.6)–acetonitrile (1:1) on a separation column (ODS, 5 cm i.d. × 30 cm, Inertsil, GL Science, Tokyo, Japan). As a result, 26 mg of rhynchophylline, 12 mg of isorhynchophylline, 18 mg of corynoxeine, 9 mg of isocorynoxeine, 17 mg of hirsuteine, 11 mg of hirsutine and 10 mg of geissoschizine methyl ether were obtained. These isolated compounds were identified by direct comparison with authentic samples.

2.2. Animals

Ten- and eleven-week-old male Wistar rats weighing 280–350 g obtained from Charles River (Yokohama, Japan) were used. The animals were allowed free access to water and standard laboratory food (MF, Oriental Yeast, Tokyo, Japan) and kept in a facility at a temperature of 24 ± 1 °C, relative humidity of 55 ± 5%, and with lights on from 07:00 to 19:00 daily. The animal experimental protocol met the "Guidelines for Animal Experimentation" of the Japanese Association of Laboratory Animal Science.

2.3. Preparation of isolated rat aorta strips

Rats were killed by a blow to the head and exsanguination. Thoracic aortas were isolated, cleaned of nonarterial tissue and immediately immersed in Krebs solution of the following composition (mM): NaCl 135, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 20, glucose 10 and EDTA·2Na 0.026, at pH 7.4. The aortas were cut into helical strips about 2.0 mm in width and 8.0 mm in length. Each strip was mounted in an organ bath containing 20 ml of Krebs solution gassed with 5% CO_2 in O_2 and maintained at 37 °C. One end of the aorta was attached to a force-displacement transducer (San-ei Instrument, Tokyo, Japan) so that its isometric contraction could be recorded (Rika Denki Kogyo, Tokyo, Japan) via an amplifier (San-ei Instrument). The strip was equilibrated for 60 min at an initial resting tension of 2 g prior to the experiments for measurement of relaxation or contraction.

2.4. Measurement of relaxation activity of alkaloids on norepinephrine-induced vasocontraction

Each equilibrated aorta strip was contracted by adding 5×10^{-8} M (final concentration) of norepinephrine to the organ bath. Ten minutes later, various concentrations of indole alkaloids were added into the bath in order to evaluate the vasorelaxation. The relaxant effect was expressed as percentages of abolition of the maximal contractile tension induced by the norepinephrine. The endothelium of some strips was denuded by gently rubbing the intimal surface of the aorta with a cotton swab to examine the effects of endothelium on test substance-induced relaxation. The effect of nitric oxide was examined by adding 10^{-4} M of N^G-nitro-L-arginine 10 min before norepinephrine-induced contraction in intact aorta strip.

2.5. Measurement of relaxation effects of geissoschizine methyl ether and hirsutine on high K^+-induced vasocontraction

Each equilibrated aorta strip was contracted by adding a high K^+ solution (final concentration: 60 mM) that substituted KCl for NaCl on an equimolar basis in the Krebs solution. After 10 min, various concentrations of geissoschizine methyl ether or hirsutine were added into the bath in order to evaluate the vasorelaxation. The relaxant effect was expressed as percentages of abolition of the maximal contractile tension induced by the K^+.

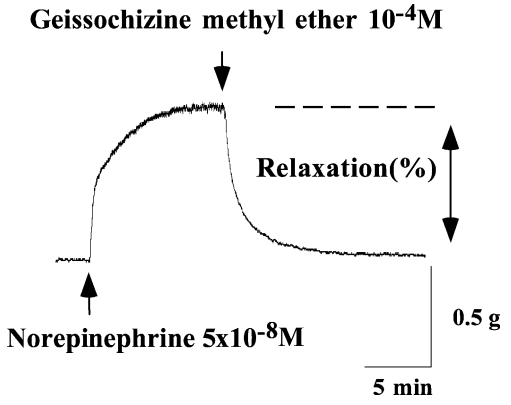

Fig. 2. A typical recording of the vasorelaxant effect of geissoschizine methyl ether on norepinephrine-induced contraction of an isolated rat aorta strip. Geissoschizine methyl ether (10^{-4} M) was applied after norepinephrine (5×10^{-8} M)-induced contraction reached a plateau.

2.6. Measurement of inhibitory effects of geissoschizine methyl ether and hirsutine on Ca^{2+}-induced vasocontraction

Each equilibrated aorta strip was stabilized for 10 min in Ca^{2+}-depleted Krebs solution containing 60 mM KCl. The Ca^{2+}-free solution was prepared by removing $CaCl_2$ and adding 1.0 mM EGTA. Ca^{2+}-induced contractions were elicited by the gradual addition of $CaCl_2$ (final concentration: 10^{-4}–10^{-2} M) to the Ca^{2+}-free Krebs solution, as a control. After the aorta strip was washed three times with the Ca^{2+}-free Krebs solution, the same procedure for the cumulative Ca^{2+}-induced contractions was repeated 10 min after addition of 10^{-6}–10^{-4} M of geissoschizine methyl ether or 3×10^{-6}–10^{-4} M of hirsutine to the bath. The contraction rate was expressed as a percentage of the maximal tension obtained in the control.

2.7. Measurement of inhibitory effects of geissoschizine methyl ether and hirsutine on Ca^{2+}-channel agonist Bay K8644-induced vasocontraction in denuding aortas of endothelium

Each denuding aorta of endothelium was stabilized for 10 min in 15 mM K^+ solution that substituted KCl for NaCl on an equimolar basis in the Krebs solution. The aorta strip was contracted by adding Bay K8644 (final concentration: 0.3 μM). After 10 min, various concentrations of geissoschizine methyl ether or hirsutine were added into the bath in order to evaluate the vasorelaxation. The relaxant effect was expressed as percentages of abolition of the maximal contractile tension induced by Bay K8644.

2.8. Statistics

Each value was expressed as the mean ± S.E.M. The concentration producing a half-maximal response (EC_{50}) was determined graphically by the linear regression of the 20–80% region of the log concentration response curve. Results were statistically evaluated using a one-way analysis of variance coupled with Dunnett's test. Significance was accepted at $p < 0.01$.

3. Results

A typical recording of the vasorelaxant effect of geissoschizine methyl ether on an norepinephrine-induced contraction is shown in Fig. 2. Norepinephrine (5×10^{-8} M) contracted an aorta strip and the contraction reached a plateau. Geissoschizine methyl ether (10^{-4} M) relaxed the norepinephrine-induced contraction. By using the same procedure, the vasorelaxant effects of seven indole alkaloids including geissoschizine methyl ether at various concentrations were examined. All indole alkaloids relaxed the norepinephrine-induced vasocontraction in a dose-dependent manner. Dose response correlative formulas and the 50% efficacy concentration (EC_{50}) for each indole alkaloid are shown in Table 1. The highest potency (EC_{50}) was found in geissoschizine methyl ether (0.744 μM) and followed by hirsuteine (7.566 μM), hirsutine (10.597 μM) and isorhynchophylline (10.477 μM). Smaller potencies were found in rhynchophylline (31.104 μM), isocorynoxeine (34.269 μM) and corynoxeine (37.154 μM), respectively. The potency of geissoschizine methyl ether was approximately 14 times greater than that of hirsutine.

The effects of a nitric oxide synthesis inhibitor N^G-nitro-L-arginine and endothelium on geissoschizine methyl ether or hirsutine-induced vasorelaxant effects are shown in Fig. 3A and B. Geissoschizine methyl ether relaxed the norepinephrine-induced contraction in a dose-dependent manner, at ranges from 10^{-7} to 10^{-4} M. The relaxant effects found at lower concentrations (10^{-7}–3×10^{-6} M) of geissoschizine methyl ether were strongly inhibited by pretreatment with N^G-nitro-L-arginine (10^{-4} M) or endothelium-denuding, while those at higher concentrations (10^{-5}–10^{-4} M) were not prevented by either treatment with N^G-nitro-L-arginine or endothelium removal. On the other hand, the dose-dependent relaxant effects of hirsutine

Table 1
Dose–response correlation and 50% efficacy concentration (EC_{50}) of various indole alkaloids on relaxation of NE-induced contraction in isolated rat aorta

	Correlative formulas	r	EC_{50} (μM)
Geissochizine methylether	$y = 67.5\log(x) + 463.68$	0.999	0.744
Hirsuteine	$y = 52.0\log(x) + 316.30$	0.963	7.566
Hirsutine	$y = 59.0\log(x) + 343.58$	0.963	10.597
Isorhynchophylline	$y = 52.9\log(x) + 313.43$	0.966	10.477
Rhynchophylline	$y = 48.7\log(x) + 269.50$	0.912	31.104
Isocorynoxeine	$y = 47.6\log(x) + 262.54$	0.857	34.269
Corynoxeine	$y = 55.0\log(x) + 293.65$	0.988	37.154

(3×10^{-6}–10^{-4} M) were not completely prevented by either treatment with N^G-nitro-L-arginine or the endothelium removal.

The effects of geissoschizine methyl ether and hirsutine on high K^+-induced vasocontractions are shown in Fig. 4.

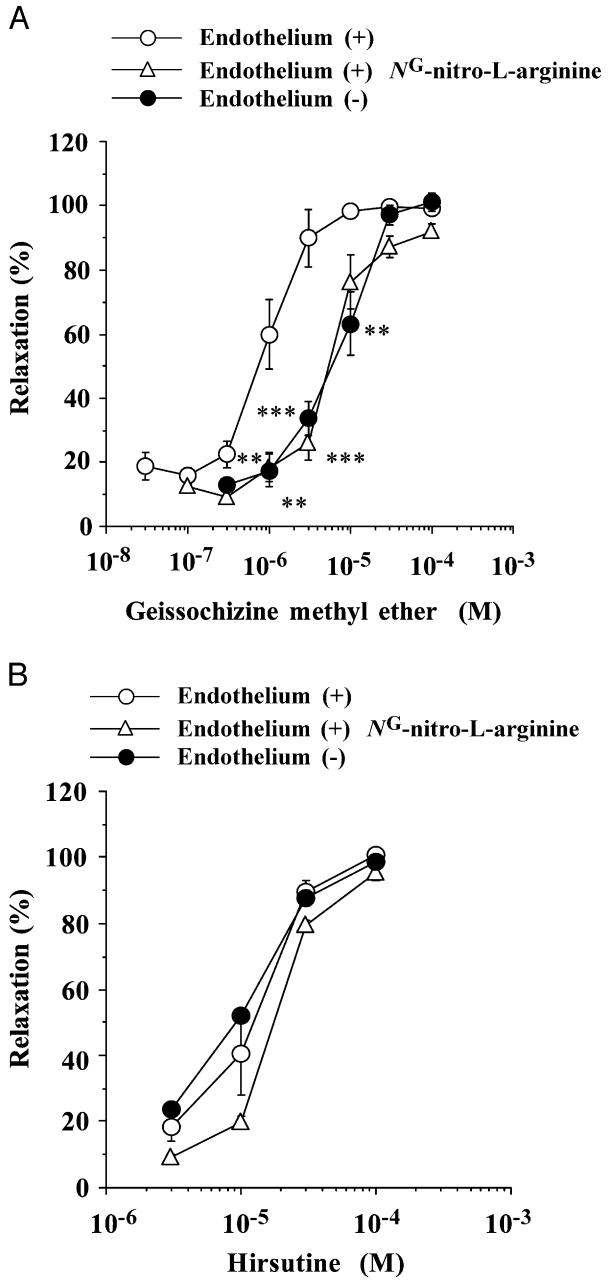

Fig. 3. Effects of endothelium and N^G-nitro-L-arginine on geissoschizine methyl ether (A)-induced or hirsutine (B)-induced vasorelaxant effects. The relaxant effects of geissoschizine methyl ether and hirsutine on norepinephrine-induced vasocontractions were examined in isolated rat aorta strips with (E+) or without (E−) the endothelium. Effect of N^G-nitro-L-arginine was examined in the aorta strips with endothelium. The degree of the relaxation at each concentration of geissoschizine methyl ether or hirsutine in the aorta strips without endothelium or N^G-nitro-L-arginine-treated aorta strips with endothelium was compared with that obtained in the aorta strips with endothelium as control (** $P<0.01$ and *** $P<0.001$).

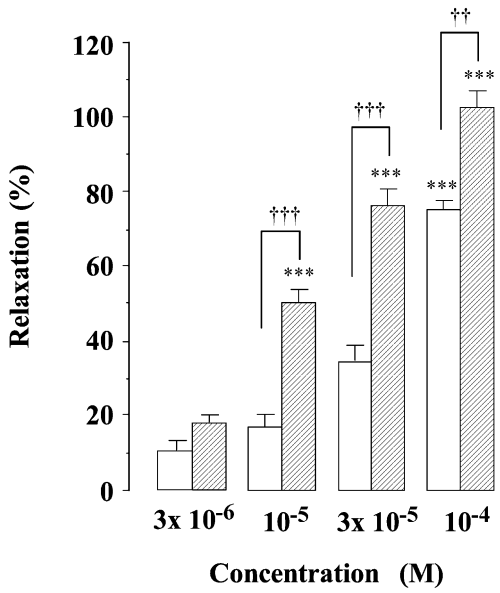

Fig. 4. Relaxant effects of geissoschizine methyl ether (white columns) and hirsutine (hatched columns) on high K^+-induced contractions in isolated rat aorta strips. The relaxant effect was expressed as percentages of abolition of the maximal contractile tension induced by the K^+ (*** $P<0.001$). The significances between the degrees of the relaxant effects obtained at the same concentration of geissoschizine methyl ether and hirsutine were compared ($^{\dagger\dagger}P<0.01$ and $^{\dagger\dagger\dagger}P<0.001$).

High K^+ (60 mM) contracted the aorta strip, and the contraction reached a plateau. Hirsutine relaxed the high K^+-induced contraction in a dose-dependent manner, at ranges from 3×10^{-6} to 10^{-4} M, in a similar way to the norepinephrine-induced contraction in Fig. 3B. On the other hand, geissoschizine methyl ether did not relax the high K^+-induced contraction at concentrations (3×10^{-6}–10^{-5} M) that markedly relaxed the norepinephrine-induced contraction in Fig. 3A, while the higher concentrations (3×10^{-5}–10^{-4} M) of geissoschizine methyl ether relaxed the high K^+-induced contraction. Thus, the degree of relaxation induced by hirsutine was greater than that by geissoschizine methyl ether.

The effects of geissoschizine methyl ether and hirsutine on Ca^{2+}-induced contraction are shown in Fig. 5A and B, respectively. In a preliminary study, we examined the effects of Ca^{2+} concentrations on high K^+-induced vasocontraction. The high K^+-induced contraction was not induced entirely in Ca^{2+}-free Krebs buffer. Cumulatively increasing concentrations of Ca^{2+} induced the contraction, and the maximum contraction was found in concentrations of Ca^{2+} higher than 10^{-3} M. Geissoschizine methyl ether inhibited the Ca^{2+}-induced contractions at higher concentrations (10^{-5}–10^{-4} M) but not at the lower concentration at 3×10^{-6} (Fig. 5A). In contrast, hirsutine inhibited the Ca^{2+}-induced contractions at concentrations from 3×10^{-6} to 10^{-4} M (Fig. 5B).

The effects of geissoschizine methyl ether and hirsutine on a specific Ca^{2+}-channel agonist Bay K8644-induced

vasocontractions in denuding aortas of endothelium are shown in Fig. 6. Hirsutine relaxed the Bay K8644-induced contraction in a dose-dependent manner, at ranges from 3×10^{-6} to 10^{-4} M. On the other hand, geissoschizine methyl ether did not relax the Bay K8644-induced contraction at the lower concentrations (3×10^{-6}–10^{-5} M) that markedly relaxed the norepinephrine-induced contraction in Fig. 3A, while the higher concentrations (3×10^{-5}–10^{-4} M) of geissoschizine methyl ether relaxed the Bay K8466-induced contraction.

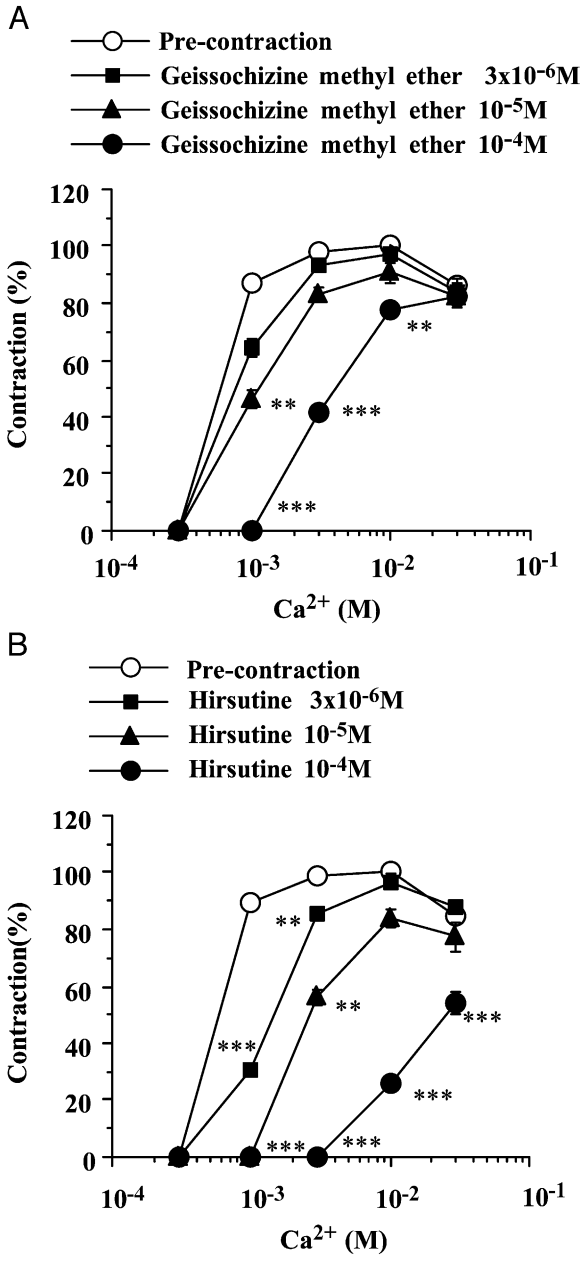

Fig. 5. Relaxant effects of various concentrations of geissoschizine methyl ether (A) and hirsutine (B) on the cumulative Ca^{2+}-induced contractions in isolated rat aorta strips. Effects of each concentration of geissoschizine methyl ether or hirsutine on the Ca^{2+}-induced contraction was compared with the degree of the contraction induced by the same concentration of Ca^{2+} prior to application of these drugs as a control (** $P<0.01$ and *** $P<0.001$).

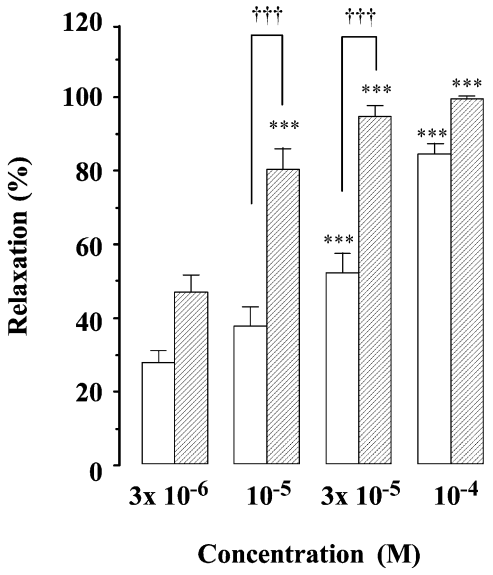

Fig. 6. Effects of geissoschizine methyl ether (white columns) and hirsutine (hatched columns) on a Ca^{2+} channel agonist, Bay K8644-induced contraction in denuding aortas of endothelium. The degrees of relaxation at various concentrations of geissoschizine methyl ether and hirsutine were compared with the degree of Bay K8644 (0.3 M)-induced contraction prior to application of these drugs (*** $P<0.001$). The significances between the degrees of the relaxant effects obtained at the same concentration of geissoschizine methyl ether and hirsutine were compared ($^{\dagger\dagger\dagger}P<0.001$).

4. Discussion

In the present study, we demonstrated in isolated strips of rat aorta that an indole alkaloid, geissoschizine methyl ether, extracted from *Uncariae Ramulus* et Uncus possessed a potent vasorelaxant activity. To date, it has been reported that several indole alkaloids extracted from *Uncariae Ramulus* et Uncus such as hirsutine, hirsuteine, rhynchophylline, isorhynchophylline, corynoxeine and isocorynoxeine show relaxant effects on norepinephrine-induced vasocontractile response in isolated rat aortas (Yamahara et al., 1987). The vasorelaxant activity of hirsutine especially has been demonstrated to be more potent than those of the other products such as rhynchophylline and isorhynchophylline (Yamahara et al., 1987; Yano et al., 1991; Horie et al., 1992). We confirmed the potency of hirsutine, and demonstrated for the first time that the vasorelaxant potency of geissoschizine methyl ether on norepinephrine-induced vasocontractive response was 14 times greater than that of hirsutine.

It is generally accepted that contraction of vascular smooth muscle requires an increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). This occurs by raising the Ca^{2+} influx through two types of Ca^{2+} channels—the voltage-dependent Ca^{2+} channel and the receptor-operated Ca^{2+} channel—and

releasing intracellularly stored Ca^{2+} (Karaki and Weiss, 1980, 1988; Sato et al., 1988). In contrast, vascular endothelium releases an endothelium-derived relaxing factor (the substance appears to be nitric oxide synthesized from L-arginine by nitric oxide synthase), which in turn dilates underlying vascular smooth muscle via a cGMP-mediated process (Rapoport and Murad, 1983; Moncada et al., 1991). In rat aorta, it has been proposed that the two types of Ca^{2+} channels may not be completely separate but function more interdependently, in contrast with the finding that the two types of Ca^{2+} channels are independent in rabbit aorta (Karaki and Weiss, 1984; Weiss et al., 1987; Sadjarwo et al., 1992). Norepinephrine, an α_1-adrenoceptor agonist, used as the contractive stimulant of vascular smooth muscle in the present study has been suggested to open both the receptor-operated Ca^{2+} and the voltage-dependent Ca^{2+} channels in the rat aorta (Karaki, 1990; Karaki and Weiss, 1984; Ko et al., 1992)

In the present study, the contraction of a rat aorta strip induced by norepinephrine was relaxed by hirsutine in a dose-dependent manner regardless of the presence or absence of the endothelium. Geissoschizine methyl ether also relaxed the contraction of the aorta with intact endothelium induced by norepinephrine in a dose-dependent manner, as well as hirsutine did. However, the dose-dependent vasorelaxation produced by the lower concentrations of geissoschizine methyl ether were completely inhibited by removing the endothelium, while those produced by the higher concentrations of geissoschizine methyl ether were not completely abolished by de-endothelialization. In order to investigate the mechanism of the endothelium-dependent relaxation induced by geissoschizine methyl ether, the effects of N^G-nitro-L-arginine, a known inhibitor of nitric oxide synthesis (Palmar et al., 1988; Lamb and Barna, 1998), were examined in intact aortas. N^G-nitro-L-arginine inhibited the relaxation found at the lower concentrations, but not at the higher concentrations of geissoschizine methyl ether. In contrast, N^G-nitro-L-arginine did not inhibit the dose-dependent relaxant effect of hirsutine. Taken together, these results suggest that the relaxation by geissoschizine methyl ether is composed of two different mechanisms: endothelial dependency with nitric oxide at lower concentrations and endothelium/nitric oxide-independency at higher concentrations, and that the relaxation by hirsutine is considered to be the endothelium/nitric oxide-independent vasorelaxant mechanism.

To clarify the mechanism of the endothelium/nitric oxide-independent vasorelaxation induced by geissoschizine methyl ether, effects of geissoschizine methyl ether on high K^+- and Ca^{2+}-induced contractions were examined in intact aorta strips, and were compared to those of hirsutine with the Ca^{2+}-channel blocking effect. It has been demonstrated that high K^+-induced vasocontraction is the result of an increase in Ca^{2+} influx through voltage-gated Ca^{2+} channels in vascular smooth muscle but not in the endothelium (Kanamori et al., 1981; Sadjarwo et al., 1992; Sato et al., 1988; Ozaki et al., 1990). We also confirmed that the isolated aorta strip in Ca^{2+}-depleted K^+-depolarizing solution was contracted by adding Ca^{2+}, suggesting that high K^+- and Ca^{2+}-induced contractions mainly depend on the influx of Ca^{2+} into the vascular smooth muscle cells from the external medium. Yano et al. (1991) have demonstrated in rat aorta that hirsutine produces a dose-dependent relaxation of the isolated rat aorta contracted by norepinephrine and high K^+ with and without the endothelium. Horie et al. (1992) have demonstrated that hirsutine reduces the increase in cytosolic $[Ca^{2+}]_i$ induced by norepinephrine or high K^+. In the present study, we demonstrated that hirsutine dose-dependently inhibited not only high K^+- and Ca^{2+}-induced contractions in intact aorta strip but also Bay K8644 (Schramm et al., 1983; Hattori et al., 1986), a specific Ca^{2+}-channel agonist-induced contractions in denuding aortas of endothelium, also supporting the idea that hirsutine inhibits Ca^{2+} influx through a voltage-dependent Ca^{2+} channel in smooth muscle as reported previously (Yano et al., 1991; Horie et al., 1992). On the other hand, geissoschizine methyl ether did not relax not only the high K^+- and Ca^{2+}-induced contractions in intact aorta strip but also Bay K8644-induced contractions in denuding aortas of endothelium at the lower concentrations that markedly relaxed the norepinephrine-induced contractions. However, the higher concentrations of geissoschizine methyl ether relaxed the high K^+-, Ca^{2+}- and Bay K8644-induced contractions. These results suggest that the relaxations of norepinephrine-induced contractions observed at lower concentrations of geissoschizine methyl ether are independent on the voltage-dependent Ca^{2+} channel, while the relaxations observed at higher concentrations of geissoschizine methyl ether are dependent on the voltage-dependent Ca^{2+}-channel blocking that is similar to hirsutine.

In conclusion, we first demonstrated in isolated rat aorta strips that an indole alkaloid, geissoschizine methyl ether, isolated from *Uncariae Ramulus* et Uncus possessed a potent vasorelaxant activity. The relaxation by geissoschizine methyl ether is considered to be composed of two different mechanisms: endothelium dependency with nitric oxide and endothelium independency with voltage-dependent Ca^{2+}-channel blocking. Geissoschizine methyl ether might be a candidate for vasodilative or antihypertensive medicines.

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