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Mesaconitine-induced relaxation in rat aorta: involvement of Ca^{2+} influx and nitric-oxide synthase in the endothelium

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Abstract

Aconiti tuber, roots of aconite (*Aconitum japonicum*), is an oriental herbal medicine used for centuries in Japan and China to improve the health of persons with a weak constitution and poor metabolism. We investigated the effects of mesaconitine, one of the aconite alkaloids in Aconiti tuber, on the contraction and free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) level in isolated rat thoracic aorta. Mesaconitine at 30 μM inhibited 3 μM phenylephrine-induced contraction in the endothelium-intact, but not endothelium-denuded, aortic rings. The effect of mesaconitine was dependent on external Ca^{2+} concentrations. The relaxation induced by mesaconitine was abolished by N^{ω}-nitro-L-arginine methyl ester (0.1 mM, an inhibitor of nitric-oxide synthase), as well as the relaxation induced by acetylcholine. Acetylcholine induced relaxation in two phases in our conditions; the initial phase was transient and external Ca^{2+}-independent, and the second phase was sustained and external Ca^{2+}-dependent. Treatment with 100 nM thapsigargin, which depleted intracellular Ca^{2+} stores, inhibited acetylcholine-induced, but not mesaconitine-induced, relaxation. Mesaconitine increased the $[Ca^{2+}]_i$ level in endothelial cells by influx of Ca^{2+} from extracellular spaces. These findings suggest that mesaconitine-induced Ca^{2+} influx and activation of nitric-oxide synthase in endothelial cells and, thus, induced vasorelaxation in rat aorta.

Keywords: Mesaconitine; Aorta; Vasorelaxation; Nitric oxide (No); Aconiti tuber; (*Aconitum japonicum*); (Rat)

1. Introduction

Aconiti tuber, the roots of aconite (*Aconitum japonicum* or *A. carmichaeli*), is an important oriental herbal medicine used for centuries in Japan and China. Aconiti tuber is believed to improve the health of persons with a weak constitution and poor metabolism. It has been therapeutically used to increase the peripheral temperature, and to relieve rheumatic pain. The main constituents are aconite alkaloids; mesaconitine is pharmacologically the most active component. The pharmacological effects of Aconiti tuber and aconite alkaloids, including aconitine, have been described as positive inotropic effects (Tanz et al., 1973; Honerjager and Meissner, 1983). Mesaconitine is also the most potent analgesic constituent in Aconiti tuber (Murayama et al., 1984; Murayama and Hikino, 1985; Hikino and Murayama, 1985), and has been reported to have an anti-inflammatory activity (Hikino et al., 1980; Saito et al., 1982). Mesaconitine induced contractions of the isolated vas deferens and of the isolated ileum of the guinea pig (Sato et al., 1979, 1980). In the present study, we attempted to clarify the effects of aconite alkaloids on the vascular system.

It has been shown that the endothelium plays an important role in controlling vascular tone by releasing nitric oxide (NO) (for review, see Moncada et al., 1991; Cohn, 1999; Muller et al., 2000). It has been reported that an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the endothelium is an essential step in the activation of NO synthase, which results in NO release (for review, see Sase and Michel, 1997; Prabhakar et al., 1998). Several endothelium-dependent vasodilators, such as bradykinin, acetylcholine and histamine, have been reported to elevate $[Ca^{2+}]_i$ levels in endothelial cells (Rotrosen and Gallin, 1986; Danthuluri et al., 1988; Buchan and Martin, 1991; Falcone et al., 1993; Lantoine et al., 1998; Muller et al., 1999). NO acts on vascular smooth muscle and increases the intracellular cyclic GMP concentration, leading to vasorelaxation (Cohn, 1999; Muller et al., 2000).

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It is suggested that the effect of Aconiti tuber on body temperature is explained by the improvement of poor blood circulation by aconite alkaloids. In the present study, we examined the effects of aconite alkaloids on isolated rat aorta, and found that mesaconitine induced endothelium-dependent relaxation in isolated rat aorta. Thus, we further investigated the involvement of NO synthase and $[Ca^{2+}]_i$ in vasorelaxation induced by mesaconitine.

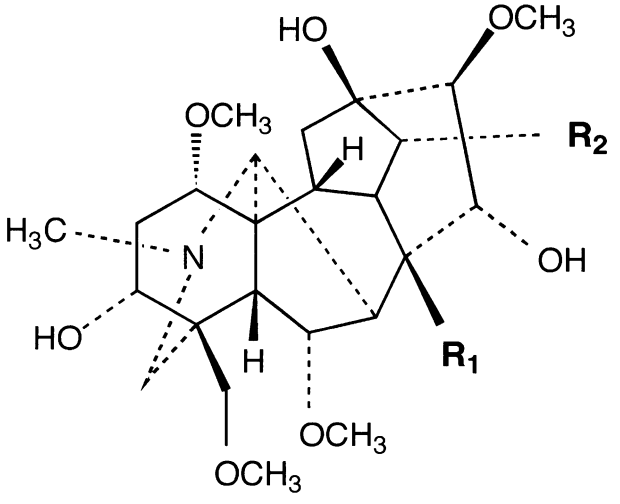
2. Materials and methods

2.1. Drugs

The following drugs and chemicals were used: L-phenylephrine hydrochloride, N^{ω}-nitro-L-arginine methyl ester (L-NAME), L-arginine hydrochloride, carbachol, phorbol 12,13-dibutyrate, thapsigargin (SIGMA, St. Louis, MO, USA); acetylcholine chloride (Daiichi Seiyaku, Tokyo, Japan); atropine sulfate (Nakarai, Kyoto, Japan); tetrodotoxin (Sankyo, Tokyo, Japan); hexamethonium chloride (Tokyo Kasei, Tokyo, Japan) and acetoxymethyl ester of fura-2 (Wako, Tokyo, Japan). Mesaconitine, benzoylmesaconine and mesaconine were from Tsumura (Tokyo, Japan). The structures of these alkaloids are shown in Fig. 1.

2.2. Animals

Male Wistar rats (Takasugi Exp. Animals, Kasukabe, Japan) weighing 240–400 g were used. The rats were bled to death by severing the carotid artery. Animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, approved by the Japanese Pharmacological Society.



	R_1	R_2
Mesaconitine	$OCOCH_3$	$OCOC_6H_5$
Benzoylmesaconine	OH	$OCOC_6H_5$
Mesaconine	OH	OH

Fig. 1. Chemical structures of mesaconitine, benzoylmesaconine and mesaconine.

2.3. Measurement of contraction in rat isolated aortic rings

Contraction in isolated rat aortic rings was determined as described previously (Yano et al., 1991; Pang et al., 2001). Briefly, the thoracic aorta was removed and placed in the normal Krebs-Henseleit buffer of the following composition (mM): NaCl, 118.1; KCl, 4.7; $CaCl_2$, 1.8; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; and glucose, 11.1, at pH 7.4 (normal solution). Surrounding connective tissue of the aorta was carefully cleaned, and the aorta was cut into rings about 3 mm in length. In some experiments, the endothelium was removed by gently rubbing the inner surface of the vessel with a cotton swab. Krebs-Henseleit buffer was maintained at 37 °C and bubbled with a 95% O_2 and 5% CO_2 mixture. The mechanical activity was recorded isometrically on a recorder (model 056, Hitachi, Tokyo, Japan) via an amplifier (model 1237, NEC San-ei, Tokyo, Japan) by means of a force–displacement transducer (model T7-8-240, Toyo Boldwin, Tokyo, Japan). An initial resting tension of 1 g was applied to the aortic ring. Relaxation is expressed as a percent of the maximum effect induced by 3 μM phenylephrine. The presence of functional endothelium was assessed by determining the ability of 10 μM acetylcholine to induce more than 80% relaxation of rings precontracted with 3 μM phenylephrine. The absence of functional endothelium was assessed by determining the ability of acetylcholine to induce less than 5% relaxation of rings precontracted with 3 μM phenylephrine. The Krebs-Henseleit buffer containing 100 nM and 10 μM Ca^{2+} were prepared by substituting 45 and 450 μM $CaCl_2$, respectively, in the presence of 0.5 mM EGTA.

2.4. Measurement of $[Ca^{2+}]_i$ in rat isolated aorta

$[Ca^{2+}]_i$ in isolated rat aorta was measured with a fluorescent Ca^{2+} indicator, fura-2, as reported previously (Sato et al., 1988, 1990; Suenaga and Kamata, 1999). The aortic strip preparation, not ring preparation, was used for $[Ca^{2+}]_i$ measurement. The endothelium-intact or -denuded aortic strips were treated with 5 μM acetoxymethyl ester of fura-2 for 8–10 h at room temperature in the Krebs-Henseleit buffer containing 0.05% Cremophor EL (nontoxic detergent, Nakarai). The aortic strips were then fixed horizontally in a bath (7 ml, 37 °C) attached to a fluorimeter (model CAF-100, Japan Spectroscopic, Tokyo, Japan). The aortic strips were illuminated from the surface of endothelium alternately (48 Hz) with two excitation wavelengths (340 ± 10 and 380 ± 10 nm). The two intensities of 500 ± 20 nm fluorescence induced by 340 nm excitation (F340) and by 380 nm excitation (F380) were measured, and their ratio (R340/380) was calculated. In the aortic strips loaded with fura-2, an increase in $[Ca^{2+}]_i$ is found to result in an increase in F340, a

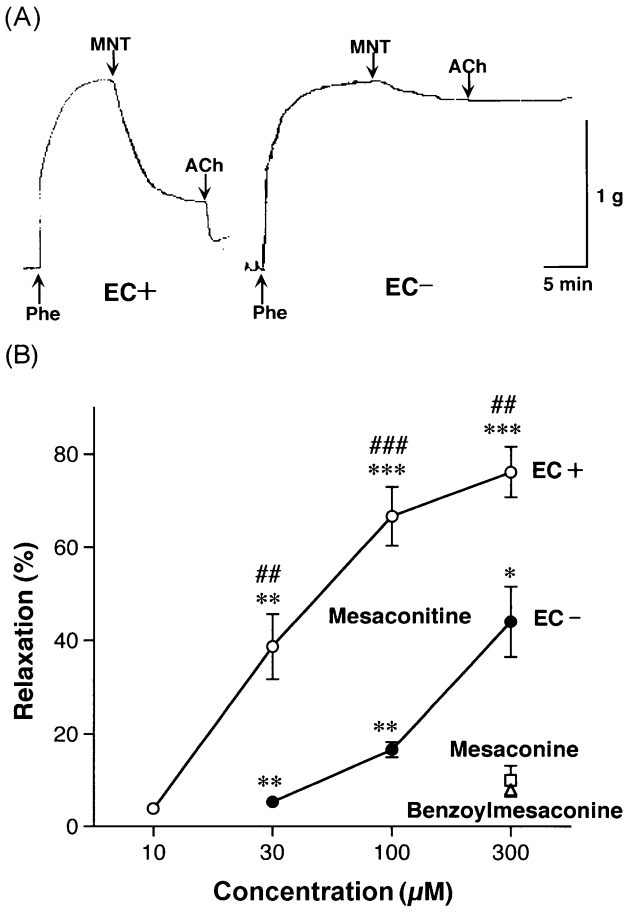


Fig. 2. Effect of mesaconitine on phenylephrine-induced contraction in rat aortic rings with or without endothelium. (A) Typical recordings of the effect of mesaconitine (MNT, 30 μM) and acetylcholine (ACh, 10 μM) on phenylephrine (Phe, 3 μM)-induced contraction in the endothelium-intact (EC+) or -denuded (EC −) rings. (B) Quantitative analysis of the effect of mesaconitine. Indicated concentrations of mesaconitine were added after contraction by phenylephrine (3 μM) reached a sustained plateau in the endothelium-intact (○) or -denuded (●) rings. Benzolymesaconine (△) and mesaconine (□) at 300 μM were added in the endothelium-intact rings. Relaxation is expressed as the percent of the maximum effect induced by phenylephrine. Each value represents the mean ± S.E.M. of three to seven animals. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, significantly different from the value without mesaconitine. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, significantly different from the value in the endothelium-denuded rings.

decrease in F380 and an increase in R340/380. Since the dissociation constant in endothelium may be different from that in smooth muscle and the fura-2 contents of these two types of cells may be different, it is difficult to calculate the absolute amounts of $[Ca^{2+}]_i$ in endothelium-intact aortic strips. Therefore, we used the R340/380 as an indicator of $[Ca^{2+}]_i$ as reported previously (Ozaki et al., 1987; Sato et al., 1988, 1990; Suenaga and Kamata, 1999).

2.5. Statistical analysis

All values are shown as the mean ± S.E.M. Statistical analyses were performed with two-tailed paired *t*-test for paired observations of two groups, two-tailed Student's *t*-test for unpaired observations of two groups, and one-way analysis of variance followed by Bonferroni multiple comparison test for unpaired observations for more than three groups. P value < 0.05 was considered statistically significant.

3. Results

3.1. Endothelium-dependent relaxation induced by mesaconitine on phenylephrine-induced contraction

Fig. 2A shows the effect of mesaconitine on phenylephrine-induced contraction of isolated rat aortic rings. Addition of 3 μM phenylephrine induced the sustained contraction of the aortic rings. Addition of mesaconitine markedly inhibited phenylephrine-induced contraction in the endothelium-intact rings in a concentration-dependent manner (10–300 μM) (Fig. 2B). Benzoylmesaconine and mesaconine, hydrolyzed compounds of mesaconitine, had no effect on the contraction and relaxation of the endothelium-intact rings even at 300 μM. In the endothelium-denuded rings, mesaconitine at higher concentrations over 30 μM significantly inhibited phenylephrine-induced contraction. However, the effects of mesaconitine at the examined concentrations in the endothelium-intact rings were significantly more than those in the endothelium-denuded rings. In the absence of phenylephrine, 30 μM mesaconitine by itself had no effect on the basal tension both in endo-

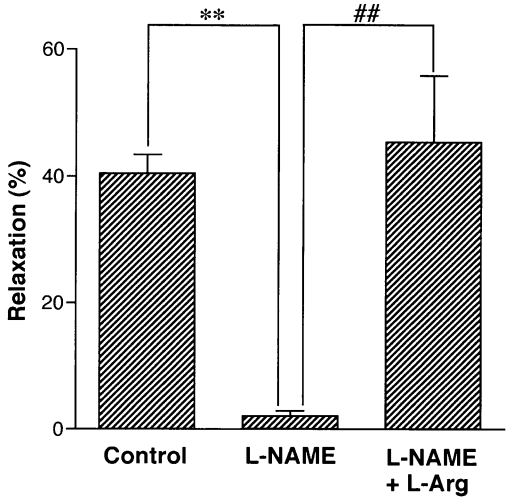


Fig. 3. Effect of L-NAME on vasorelaxation induced by mesaconitine. Rat aortic rings with endothelium were incubated with vehicle, 100 μM L-NAME or combinations of 1 mM L-Arg and 100 μM L-NAME for 15 min, and then a contraction was elicited with phenylephrine (3 μM). Mesaconitine (30 μM) was added after contraction by phenylephrine reached a sustained plateau. Each column represents the mean ± S.E.M. of five animals. ** $P<0.01$, significantly different from the control group. $^{\#\#}P<0.01$, significantly different from the L-NAME-treated group.

thelium-intact and -denuded rings (data not shown). In our preparations, 10 μM acetylcholine also caused the relaxation in the endothelium-intact, but not endothelium-denuded, rings, as described in Materials and Methods. In the endothelium-intact rings, 10 μM acetylcholine applied after 30 μM mesaconitine treatment caused some additional relaxation (Fig. 2A). We used 30 μM mesaconitine in the following experiments in order to focus on the endothelium-dependent relaxant effect of mesaconitine in rat aorta, because the effect of 30 μM mesaconitine in the endothelium-denuded strips was limited.

3.2. Effects of atropine, tetrodotoxin and hexamethonium on endothelium-dependent relaxation induced by mesaconitine

Next, we investigated the involvement of muscarinic acetylcholine receptors and/or neuronal transmission pathway(s) on the relaxation induced by mesaconitine. Pretreatment with 1 μM atropine, a general antagonist of muscarinic acetylcholine receptors, almost completely abolished the relaxation induced by 10 μM acetylcholine: the relaxation (%) was 92.5 ± 4.4 and 4.5 ± 2.5% ($P < 0.001$, $n = 6$) in the absence and presence of 1 μM atropine, respectively. On the

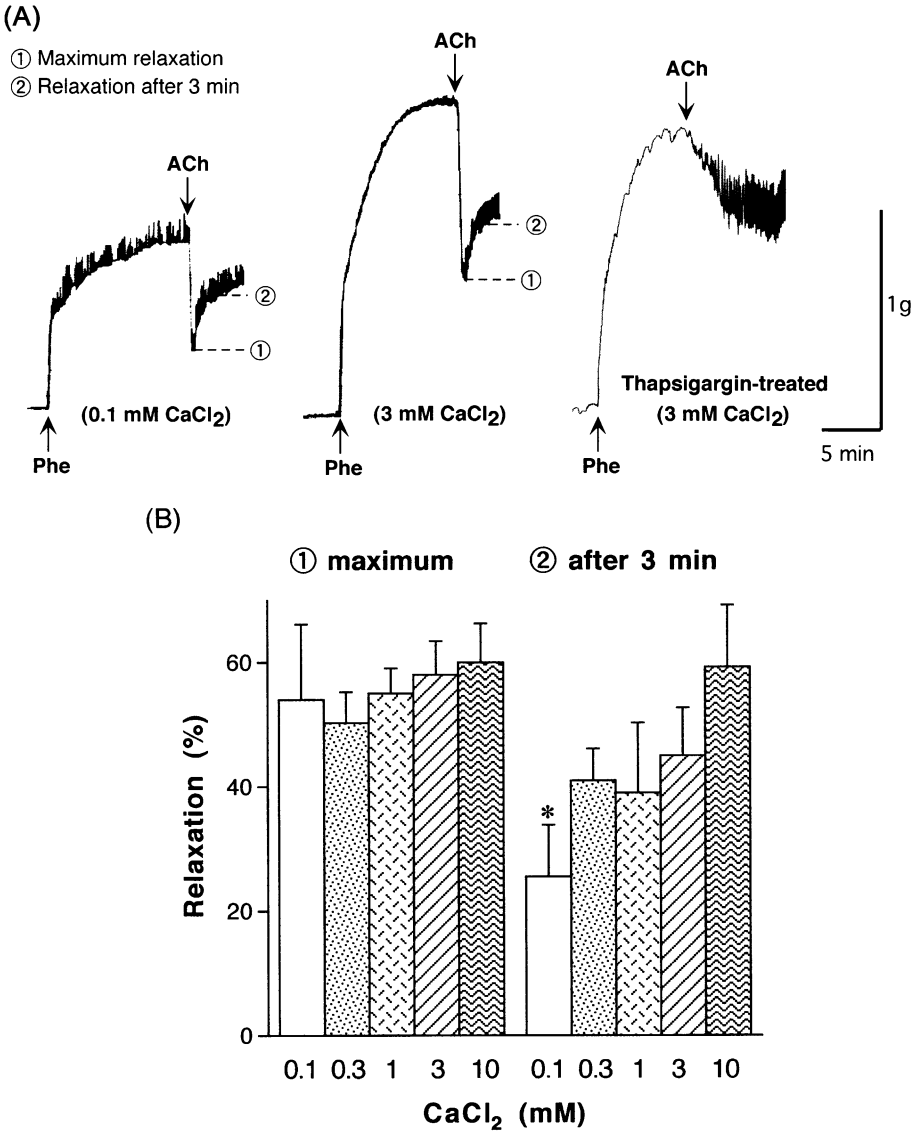


Fig. 4. Effects of external $CaCl_2$ concentrations on vasorelaxation induced by acetylcholine. Rat aortic rings with endothelium were incubated in the nutrient solution containing the indicated concentrations of $CaCl_2$ for 15 min, and then a contraction was elicited with phenylephrine (Phe, 3 μM). Acetylcholine (ACh, 0.1 μM) was added after contraction by phenylephrine reached a sustained plateau. (A) Typical recordings of acetylcholine-induced vasorelaxation at 0.1 (Left) and 3 mM (Middle) external $CaCl_2$ concentrations. Acute and maximum relaxation is indicated by ①. Vasorelaxation at 3 min after the addition of acetylcholine is indicated by ②. In Right panel, a typical recording of acetylcholine-induced vasorelaxation at 3 mM $CaCl_2$ in the thapsigargin-treated ring. The endothelium-intact rings were incubated with 100 nM thapsigargin for 15 min and then a contraction was elicited with phenylephrine. (B) Effects of external $CaCl_2$ concentrations on the maximum vasorelaxation by acetylcholine (shown in ①) and vasorelaxation at 3 min after the addition of acetylcholine (shown in ②). Each value represents the mean ± S.E.M. of four to five animals. * $P < 0.05$, significantly different from the group of $CaCl_2$ concentration at 3 mM.

other hand, 30 μM mesaconitine-induced relaxation was not antagonized by 1 μM atropine; the values were 59.0 ± 7.3 and 70.3 ± 10.6% in the absence and presence of atropine, respectively ($n = 4$). Neither treatment with tetrodotoxin (0.3 μM, an inhibitor of voltage-dependent Na^+ channels) or hexamethonium (100 μM, an antagonist of nicotinic acetylcholine receptors and, thus, a ganglionic blocking agent) showed an inhibitory effect on 30 μM mesaconitine-induced relaxation; the values of relaxation (%) were 61.8 ± 12.5 and 63.3 ± 10.0%, respectively ($n = 4$). These findings suggest that there is no involvement of muscarinic acetylcholine receptors and neuronal transmission pathway(s) in the relaxation induced by mesaconitine.

3.3. Effect of L-NAME on endothelium-dependent relaxation induced by mesaconitine

Relaxation induced by acetylcholine in the rat aorta was established to be diminished by L-NAME, a competitive inhibitor of NO synthase (Wang et al., 1993; Pang et al., 2001). Pretreatment with 100 μM L-NAME almost completely inhibited the relaxation induced by 30 μM mesaconitine in phenylephrine-contracted rat aorta with endothelium (Fig. 3). This inhibition by L-NAME was completely prevented by coadministration of 1 mM L-Arg, a precursor for NO generation in endothelium. The involvement of NO synthase on relaxation induced by mesaconitine was confirmed in experiments as shown in Fig. 5A; the addition of L-NAME reversed the relaxation induced by mesaconitine. These findings suggest that [mesaconitine, like acetylcholine, induced] relaxation in the rat aorta in an endothelium- and NO synthase-dependent manner.

3.4. Effects of external $CaCl_2$ concentrations on endothelium-dependent relaxation induced by mesaconitine

NO synthase in endothelium is activated by Ca^{2+} and calmodulin (Bredt and Snyder, 1990; Sase and Michel, 1997; Prabhakar et al., 1998). Next, we investigated the effect of external $CaCl_2$ concentrations on acetylcholine- and mesaconitine-induced relaxation. Phenylephrine-induced contraction in the presence of 0.1 mM $CaCl_2$ was 70.7 ± 2.1% ($n = 6$) of that in the presence of 1.8 mM $CaCl_2$. As shown in a typical recording (Fig. 4, Panel A), the relaxation induced by 0.1 μM acetylcholine was transient and maximum in the first phase and was sustained in the second phase. A transient and maximum relaxation in the first phase induced by acetylcholine was not affected by external $CaCl_2$ concentration (Fig. 4, Panel B). On the other hand, the sustained relaxation in the second phase (3 min after the addition) was dependent on external $CaCl_2$ concentration. The degree of relaxation in the presence of 0.1 mM $CaCl_2$ was much less than that in the presence of 3 mM $CaCl_2$, which was close to the physiological Ca^{2+} concentration.

A vasorelaxation induced by mesaconitine had a different $CaCl_2$ sensitivity from that by acetylcholine (Fig. 5). As

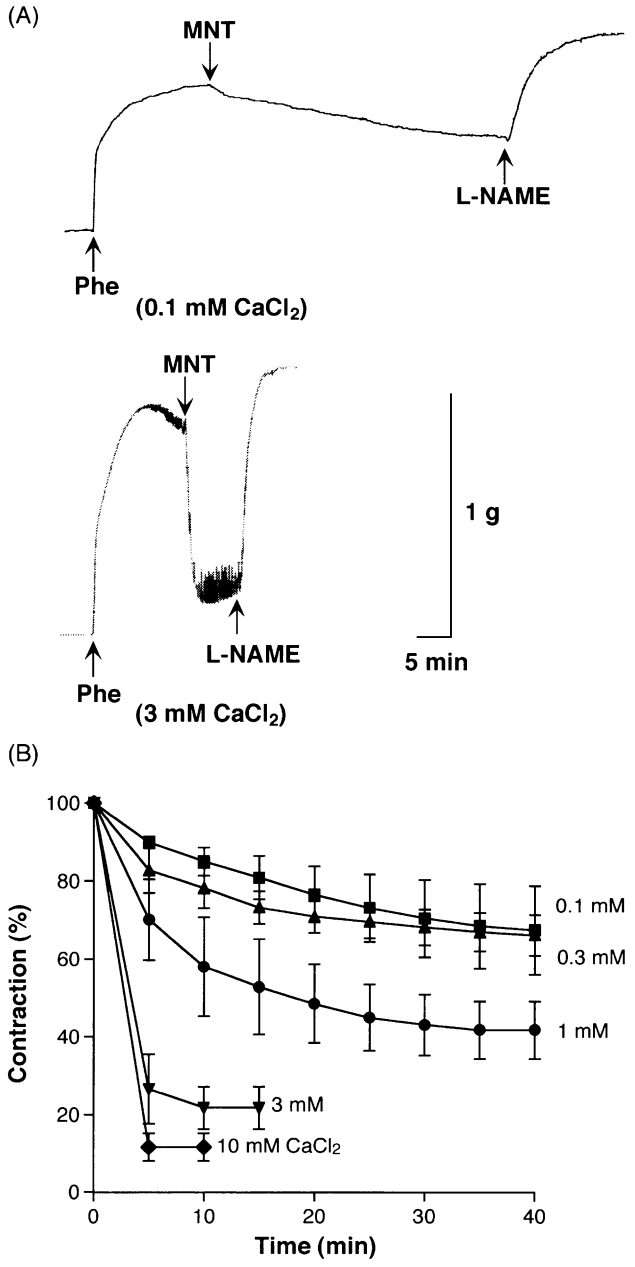


Fig. 5. Effects of external $CaCl_2$ concentrations on vasorelaxation induced by mesaconitine. Rat aortic rings with endothelium were incubated in the nutrient solution containing various $CaCl_2$ concentrations (0.1–10 mM) for 15 min, and then the contraction was elicited with phenylephrine (Phe, 3 μM). Mesaconitine (MNT, 30 μM) was added after the contraction by phenylephrine reached a sustained plateau. (A) Typical recordings of mesaconitine-induced vasorelaxation at 0.1 and 3 mM external $CaCl_2$ concentrations. (B) Time course of mesaconitine-induced vasorelaxation at various $CaCl_2$ concentrations. Each value represents the mean ± S.E.M. of four to five animals.

shown in Panels A (and in Fig. 2A), 30 μM mesaconitine produced a relatively rapid relaxation at higher $CaCl_2$ concentrations (3 and 10 mM), and a gradual relaxation at lower $CaCl_2$ concentrations (0.1 and 0.3 mM). The maximum relaxation induced by mesaconitine was dependent on the external $CaCl_2$ concentration.

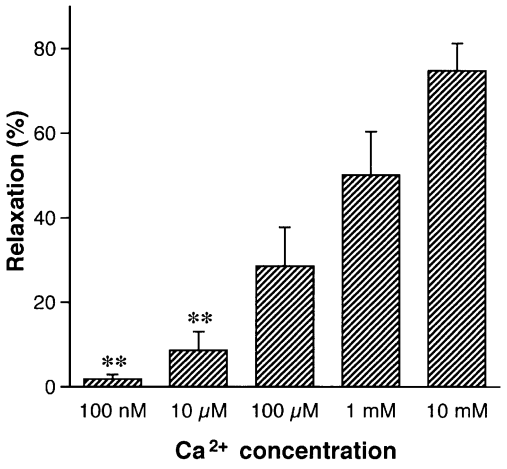


Fig. 6. Effects of external Ca^{2+} concentrations on mesaconitine-induced vasorelaxation on phorbol 12,13-dibutyrate-induced contraction. Rat aortic rings with endothelium were incubated in the nutrient solution containing various Ca^{2+} concentrations (100 nM–10 mM) for 15 min, and then the contraction was elicited with phorbol 12,13-dibutyrate (0.3 μM). Mesaconitine (30 μM) was added after contraction by phorbol 12,13-dibutyrate reached a sustained plateau. The nutrient solution containing 100 nM and 10 μM Ca^{2+} were prepared by adding 45 and 450 μM $CaCl_2$, respectively, in the presence of 0.5 mM EGTA. Relaxation by mesaconitine is expressed as the percent of the maximum effect induced by phorbol 12,13-dibutyrate. Each column represents the mean + S.E.M. of four to five animals. ** $P<0.01$, significantly different from the group with the Ca^{2+} concentration at 1 mM.

To further confirm the role of external Ca^{2+} concentrations in mesaconitine-induced relaxation, phorbol 12,13-dibutyrate, which is an activator of protein kinase C and induces contraction in rat aorta in Ca^{2+}-free buffer (Ahn et al., 1997; López et al., 2000), was used instead of phenylephrine (Fig. 6). Even in an external Ca^{2+} concentration of 100 nM, the phorbol 12,13-dibutyrate (0.3 μM)-induced contraction remained; 56.6 ± 5.2% ($n=5$) of the contraction that was obtained at 1.8 mM external $CaCl_2$ concentration. In the same conditions, 3 μM phenylephrine-induced contraction was transient and quite limited; thus, it was impossible to examine the role of external Ca^{2+}. Relaxation induced by 30 μM mesaconitine in phorbol 12,13-dibutyrate-contracted preparation was also dependent on external Ca^{2+} concentrations, and mesaconitine-induced relaxation was significantly inhibited at 10 μM Ca^{2+} and almost completely abolished at 100 nM Ca^{2+}. On the other hand, acetylcholine-induced relaxation of phorbol 12,13-dibutyrate-induced contraction at 10 μM Ca^{2+} was 31.7 ± 14.1% ($n=5$), which was similar to that at 1 mM $CaCl_2$ concentration (47.0 ± 4.5%, $n=5$). These findings suggest that mesaconitine-induced relaxation is dependent on the external Ca^{2+} concentration.

3.5. Effect of thapsigargin on mesaconitine-induced relaxation

Thapsigargin, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPases, was known to cause an increase of $[Ca^{2+}]_i$ and NO accumulation and, thus, the relaxation in aorta (Amerini et al., 1996; Huang et al., 2000). Pretreatment with thapsigargin, however, was reported to deplete intracellular Ca^{2+} store(s) and, thus, inhibit the relaxation induced by acetylcholine in the aorta (Amerini et al., 1996; Huang et al., 2000). Also, in our experiments, pretreatment with 100 nM thapsigargin abolished the first phase of acetylcholine-induced relaxation (Fig. 4A, Right Panel). And the acetylcholine-induced relaxation decreased by pretreatment with thapsigargin in concentration-dependent manner (Fig. 7). In contrast, the mesaconitine-induced relaxation was less sensitive to thapsigargin than that induced by acetylcholine. Although treatment with 30 nM thapsigargin significantly inhibited the relaxation induced by acetylcholine, the treatment slightly enhanced the relaxation induced by mesaconitine. Treatment with 100 nM thapsigargin inhibited acetylcholine-induced, but not mesaconitine-induced, relaxation. These findings suggest that Ca^{2+} release from intracellular store(s) plays an important role in acetylcholine-induced vasorelaxation in the first phase, but has little effect in mesaconitine-induced vasorelaxation.

3.6. Effects of mesaconitine on $[Ca^{2+}]_i$ level in rat aortic strips

The effect of mesaconitine on $[Ca^{2+}]_i$ in rat aorta strip preparation was measured using fura-2 (Fig. 8). In Panel A, the $[Ca^{2+}]_i$ level was measured in endothelium-intact aortic strips, representing a net increase in smooth muscle cells

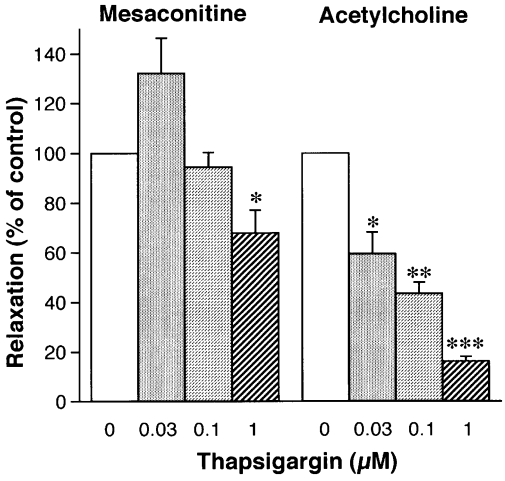


Fig. 7. Effects of thapsigargin treatment on vasorelaxation induced by mesaconitine and acetylcholine. Rat aortic rings with endothelium were incubated with vehicle or thapsigargin (30 nM–1 μM) for 15 min, and then a contraction was elicited with phenylephrine (3 μM). Mesaconitine (30 μM) and acetylcholine (10 μM) were added after contraction by phenylephrine reached a sustained plateau. Each value is normalized as a percentage of the control relaxation by mesaconitine or acetylcholine without thapsigargin treatment, and represents the mean ± S.E.M. of four animals. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, significantly different from the corresponding control group without thapsigargin.

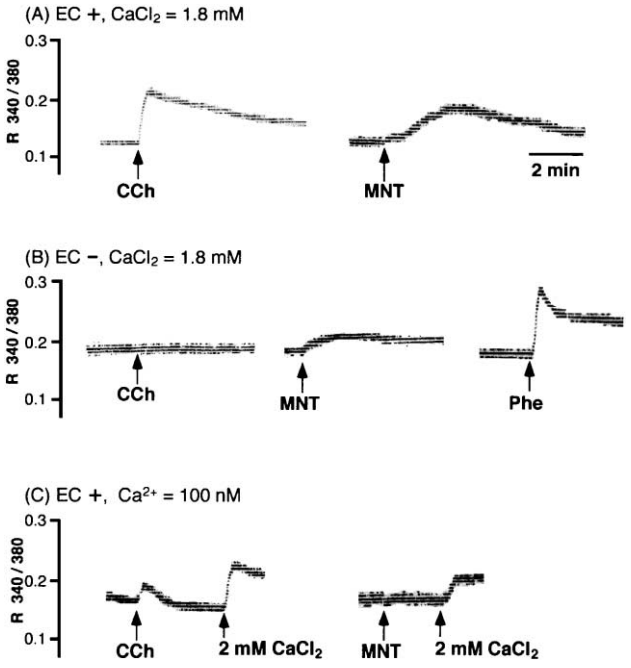


Fig. 8. Typical recordings of the effects of carbachol, mesaconitine and phenylephrine on $[Ca^{2+}]_i$ in rat aortic strips with (EC+, Panels A and C) or without endothelium (EC −, Panel B). (A) and (B) show the experiment achieved in the nutrient solution containing 1.8 mM $CaCl_2$. The experiment of (C) was performed in the nutrient solution containing 100 nM Ca^{2+}, prepared by adding 45 μM $CaCl_2$ in the presence of 0.5 mM EGTA. Carbachol (CCh, 10 μM), mesaconitine (MNT, 30 μM), phenylephrine (Phe, 3 μM) and $CaCl_2$ (2 mM) were added at the indicated points. The traces shown are representatives of 5–12 preparations of different animals.

and endothelial cells. In Panel B, the $[Ca^{2+}]_i$ level was measured in endothelium-denuded aortic strips, representing an increase in smooth muscle cells alone. Addition of 10 μM carbachol induced an elevation of $[Ca^{2+}]_i$ in endothelium-intact strips (Panel A), and had no effect on endothelium-denuded strips (Panel B) in the buffer containing 1.8 mM $CaCl_2$, suggesting that carbachol induced an elevation of $[Ca^{2+}]_i$ in endothelial cells, as described previously (Sato et al., 1990). Mesaconitine (30 μM) induced an elevation of $[Ca^{2+}]_i$ in endothelium-intact strips (Panel A); a net increase of ratio (R340/380) by mesaconitine was 0.025 ± 0.004 ($n = 12$). Although mesaconitine (30 μM) induced a slight elevation of $[Ca^{2+}]_i$ in endothelium-denuded strips, the elevation was smaller than that induced by 3 μM phenylephrine (Panel B); a net increase of R340/380 was 0.007 ± 0.003 ($n = 6$), which was significantly lower than that in endothelium-intact strips ($P < 0.05$). In the buffer containing 100 nM Ca^{2+}, mesaconitine-induced elevation of $[Ca^{2+}]_i$ was quite small (Panel C); a net increase of R340/380 was 0.005 ± 0.001 ($n = 5$), which was significantly lower than the value with 1.8 mM $CaCl_2$ ($P < 0.01$). A subsequent addition of 2 mM $CaCl_2$ to the buffer induced a sustained elevation of $[Ca^{2+}]_i$ in the mesaconitine-treated strips. In the same conditions, carbachol induced a transient elevation of $[Ca^{2+}]_i$, and subsequent addition of 2 mM $CaCl_2$ to the buffer induced sustained elevation of $[Ca^{2+}]_i$ in endothelium-intact strips. These findings suggest that mesaconitine induces an elevation in $[Ca^{2+}]_i$ in endothelial cells by influx of Ca^{2+} from the external buffer.

4. Discussion

4.1. Mesaconitine-induced relaxation in rat aortic rings with endothelium

In the present study, we found that mesaconitine, an aconite alkaloid, induced a relaxation in phenylephrine-contracted rat aorta with endothelium in a concentration-dependent manner (Fig. 2). Benzoylmesaconine and mesaconine, the hydrolyzed compounds of mesaconitine, did not elicit relaxation even at 300 μM. These findings suggest that the acetyl group in the structure of mesaconitine plays an essential role in the vasorelaxant effect of mesaconitine. Relaxation of rat aorta by 30 μM mesaconitine was significantly, almost completely, diminished when endothelium was removed, indicating that the relaxant effect of mesaconitine in that concentration on rat aorta was almost completely endothelium-dependent. In the present study, we investigated the effects of 30 μM mesaconitine in order to focus on the effects of mesaconitine on the endothelium-dependent relaxation in rat aorta.

Acetylcholine produces endothelium-dependent relaxation through NO production (Moncada et al., 1991; Cohn, 1999; Muller et al., 1999, 2000). In the present conditions, acetylcholine-induced relaxation was completely inhibited by pretreatment with an inhibitor of NO synthase (100 μM L-NAME) and the inhibition was significantly prevented by 1 mM L-Arg as described previously (Wang et al., 1993; Pang et al., 2001). Similarly, L-NAME completely inhibited mesaconitine-induced relaxation, and this inhibition was significantly prevented by coadministration of 1 mM L-Arg (Fig. 3). These findings suggest that the relaxation induced by mesaconitine is mediated by NO release from endothelium.

The relaxant effect of mesaconitine was not affected by atropine, indicating that muscarinic receptors were not involved in the effect of mesaconitine. The relaxant effect of mesaconitine was also not affected by tetrodotoxin or by hexamethonium, suggesting that neuron conduction and voltage-dependent Na^+ channels do not mediate the effect of mesaconitine.

4.2. Different effects of internal Ca^{2+} mobilization and external $CaCl_2$ concentrations on acetylcholine- and mesaconitine-induced relaxation

Increases in endothelial $[Ca^{2+}]_i$ trigger the activation of NO synthase and NO release (Griffith et al., 1896; Korenaga et al., 1993; Sase and Michel, 1997; Prabhakar et al., 1998). To clarify the role of $[Ca^{2+}]_i$ in the effects of mesaconitine, we investigated the mesaconitine-induced relaxation (1) in

buffer with different $CaCl_2$ contents and (2) in thapsigargin-treated aorta, in comparison with acetylcholine. In this series of experiments, we compared the effects of 30 μM mesaconitine and 0.1 μM acetylcholine, both of which can introduce an equivalent relaxation in the normal (1.8 mM $CaCl_2$-containing) buffer. Acetylcholine induced rapid relaxation at each $CaCl_2$ concentration, and external $CaCl_2$ did not affect the maximum relaxation by acetylcholine (Fig. 4B). Interestingly, the relaxation induced by acetylcholine was transient and decreased gradually in our conditions. When evaluated 3 min after acetylcholine addition, the relaxation was dependent on external $CaCl_2$ concentrations. On the other hand, mesaconitine showed a different dependency on external Ca^{2+} concentrations. Mesaconitine elicited rapid relaxation at high external $CaCl_2$ concentrations (3–10 mM), but slow relaxation at low external $CaCl_2$ concentrations (0.1–0.3 mM). The maximum effect of mesaconitine was proportional to the external $CaCl_2$ concentrations (Fig. 5). The relaxation induced by 30 μM mesaconitine in phorbol 12,13-dibutyrate-induced contraction was also decreased in the low Ca^{2+} buffer (Fig. 6).

To further exclude the possible involvement of intracellular Ca^{2+} stores on the relaxation, experiments were performed with thapsigargin. Pretreatment with thapsigargin, which depleted intracellular Ca^{2+} store(s) and inhibited the relaxation induced by acetylcholine, but does not interfere with the relaxation induced by endothelium-independent agents (Amerini et al., 1996; Huang et al., 2000). In our studies, the first phase of acetylcholine-induced relaxation was significantly reduced by pretreatment with 100 nM thapsigargin (Figs. 4A, Right Panel, and 7). The mesaconitine-induced relaxation was not affected by 100 nM thapsigargin treatment. It has been reported that an increase in $[Ca^{2+}]_i$ induced by receptor stimulation with acetylcholine in endothelial cells showed biphasic responses (a transient peak followed by a sustained elevation), which corresponded to Ca^{2+} released from intracellular Ca^{2+} stores and Ca^{2+} influx from the extracellular space, respectively (Schilling and Elliott, 1992; Falcone et al., 1993; Lantoine et al., 1998; Muller et al., 1999). Taken together with our present findings, transient relaxation in a first phase by acetylcholine could be ascribed to NO synthase activated by Ca^{2+} release from intracellular Ca^{2+} store(s), so that it is not affected by external Ca^{2+} concentrations. Low external Ca^{2+} concentrations may make mesaconitine-induced Ca^{2+} influx and the resulting relaxation slower and smaller.

4.3. Increase in $[Ca^{2+}]_i$ in endothelial cells by mesaconitine in rat aortic strips

Carbachol increased $[Ca^{2+}]_i$ in endothelium-intact aortic strips, but not in endothelium-denuded strips. Since carbachol elicited a transient increase in $[Ca^{2+}]_i$ in endothelium-intact aortic strips at an external concentration of 100 nM Ca^{2+}, the increase appeared to be derived from intracellular Ca^{2+} stores, as reported previously (Danthuluri et al., 1988; Buchan and Martin, 1991; Muller et al., 1999). On the other hand, mesaconitine increased $[Ca^{2+}]_i$ level in the endothelium-intact strips in the buffer containing 1.8 mM $CaCl_2$, but not in the buffer containing 100 nM Ca^{2+} (Fig. 8). These results also support the mechanism of mesaconitine-induced relaxation, which is dependent on influx of external Ca^{2+}. The removal of endothelium produced marked reduction of mesaconitine-induced increase in $[Ca^{2+}]_i$, but a little increase in $[Ca^{2+}]_i$ was observed in the endothelium-denuded strips, probably in the smooth muscle cells (Fig. 8). However, the increase by mesaconitine in the smooth muscle cells was much smaller than that induced by phenylephrine. This may explain that mesaconitine alone had no contractile effect.

4.4. Problems to be solved and possibility of oriental herbal medicines as therapeutics for the vascular system

As shown in Fig. 2, mesaconitine at higher concentrations over 30 μM induced relaxation in the endothelium-denuded strips. The reason(s) of this effect is not clear at present. Mesaconitine is proposed to be an activator of Na^+ channel and increase synaptosomal Na^+ concentrations (Friese et al., 1997). In our preliminary experiment, treatment with ouabain, an inhibitor of Na^+/K^+-ATPase(s), decreased the relaxation induced by mesaconitine in the endothelium-denuded strips (data not shown). A resulting activation of Na^+/K^+-ATPase(s) after an increase of intracellular Na^+ and, thus, hyperpolarization may be involved in the relaxation induced by mesaconitine in the endothelium-denuded strips.

In conclusion, the present study on rat aorta suggests that mesaconitine elevates $[Ca^{2+}]_i$ in endothelial cells, by influx of external Ca^{2+}, leading to a NO-mediated vasorelaxation. The vasorelaxant effect of mesaconitine may contribute to the therapeutical effect of the Aconiti tuber on persons with a weak constitution and poor metabolism by improving peripheral blood flow/circulation. Wang et al. (1996, 1999) reported that rutaecarpine, an alkaloid isolated from *Evodia rutaecarpa*, increased $[Ca^{2+}]_i$ in endothelial cells and activated the endothelial Ca^{2+}-NO-cyclic GMP pathway. *E. rutaecarpa* also has been therapeutically used in Japan and China to increase the peripheral body temperature. The effects of these alkaloids on Ca^{2+} mobilization in the endothelium may be involved in the therapeutical effects of these oriental herbal medicines.

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