Vasorelaxing Effect of Mesaconitine, an Alkaloid From *Aconitum japonicum*, on Rat Small Gastric Artery: Possible Involvement of Endothelium-Derived Hyperpolarizing Factor

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ABSTRACT—Aconiti tuber, roots of aconite (*Aconitum japonicum*), has been used for centuries in Japan and China to increase peripheral body temperature. We previously reported that mesaconitine, an alkaloid from *Aconitum japonicum*, elicits endothelium-dependent and nitric oxide-mediated relaxation in isolated rat aorta. In the present study, we investigated the effect of mesaconitine on isolated rat small gastric arteries. Mesaconitine elicited a concentration-dependent (10, 30, 100 μM) vasorelaxation in isolated rat gastric artery precontracted with norepinephrine, which was resistant to Nω-nitro-L-arginine (L-NNA) (an inhibitor of nitric oxide synthase) and indomethacin (an inhibitor of cyclooxygenase). The L-NNA- and indomethacin-resistant relaxation by mesaconitine was mainly endothelium-dependent, inhibited by high K⁺ (30 mM), and inhibited by a combination of Ca²⁺-dependent K⁺ channel blockers, charybdotoxin and apamin. The relaxation by mesaconitine was proportional to the external Ca²⁺ concentration. These results suggest that mesaconitine elicits vasorelaxation of isolated rat small gastric artery mainly via release of endothelium-derived hyperpolarizing factor.

Keywords: Mesaconitine, Vasorelaxation, Gastric artery, Endothelium-derived hyperpolarizing factor, *Aconitum japonicum*

Aconiti tuber, roots of aconite (*Aconitum japonicum* or *Aconitum carmichaeli*), is an oriental herbal medicine traditionally used for centuries in Japan and China for analgesic, antirheumatic and neurological indications. The pharmacological effects of aconite alkaloids, including aconitine, have been described as positive inotropic effects (1). The main active constituents are the C₁₉ diterpene alkaloids, mesaconitine being pharmacologically the most active. Mesaconitine is described as the most potent analgesic constituent in Aconiti tuber (2)

Preparations of Aconiti tuber have been therapeutically used to increase peripheral body temperature. It can be anticipated that the effect of Aconiti tuber preparations on peripheral body temperature might be the consequence of improvement of the low blood flow by aconite alkaloids. Indeed, in a previous study we found that mesaconitine elicits a strong relaxation in isolated rat aorta. This relaxation is mainly endothelium-dependent and mediated by nitric oxide (NO) (3). Considering that smaller resistance rather than big conductance vessels are responsible for improvements in peripheral blood circulation, the potential vasodilatory influence of mesaconitine on smaller resistance vessels was investigated in the present study. In addition, the role of the endothelium in this relaxation was studied.

The endothelium modulates vascular tone by releasing NO, but also by releasing other mediators such as prostacyclin and endothelium-derived hyperpolarizing factor(s) (EDHF). While prostacyclin is only involved in a limited number of preparations, the relative contributions of NO and EDHF is likely to be dependent on the size of the vessels; the contribution of NO is the largest in large-diameter vessels, whereas the contribution of EDHF increases with decreasing diameter of vessels, EDHF becoming the dominating relaxing factor in microvessels (4, 5).

The endothelium-dependent, NO synthase- and cyclooxygenase-independent relaxation is considered to be mediated by EDHF (6, 7). The rat gastric artery has been established to show EDHF-mediated hyperpolarization and
vasorelaxation in response to acetylcholine (8). In the present study, we investigated the relaxing influence of mesaconitine on precontracted rat gastric arteries. We also investigated the potential involvement of the endothelium and looked for the different components involved in the endothelium-mediated effect.

MATERIALS AND METHODS

Tension measurements

Experiments were performed on small gastric arteries from young female Wistar rats (195–270 g). After cervical dislocation, the arteries were dissected free from the animals and mounted in a small vessel myograph using two 40-μm diameter stainless steel wires. One wire was fixed to a force-displacement transducer (model 500 A; J.P. Trading, Aarhus, Denmark) and the other was connected to a micrometer (type SM-13; Newport, Ysselstein, Netherlands). After mounting, the preparations were allowed to equilibrate for more than 30 min in the Krebs-Ringer bicarbonate solution of the following composition: 135 mM NaCl, KCl, 5 mM; NaHCO₃, 20 mM; glucose, 10 mM; 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.2 mM KH₂PO₄, 0.026 mM EDTA. High-K⁺ solution of the following composition: 120 mM KCl, 20 mM NaCl, 5 mM glucose, 200 mM NaHCO₃, 1.2 mM MgSO₄, 2.5 mM CaCl₂ and 5% CO₂ at 37°C. The arteries were then normalized to obtain optimal conditions for active force development (9). On the basis of the relationship between passive wall tension and internal circumference for each vessel, the circumference was set to 90% of the internal circumference that the vessels would have under a passive transmural pressure of 100 mmHg. In the present study, vessels with normalized lumen diameter ranging from 218 to 557 μm were used. After a second equilibration for at least 15 min, the vessels were contracted 3 times with Krebs-Ringer bicarbonate solution containing 10 μM norepinephrine and 120 mM K⁺. The presence of functional endothelium was assessed by determining the ability of 10 μM acetylcholine to induce more than 80% relaxation. In some experiments, endothelium was removed by bubbling the lumen of the preparations for 2 min with 95% O₂ and 5% CO₂ at 37°C. The arteries were then normalized to obtain optimal conditions for active force development (9). On the basis of the relationship between passive wall tension and internal circumference for each vessel, the circumference was set to 90% of the internal circumference that the vessels would have under a passive transmural pressure of 100 mmHg. In the present study, vessels with normalized lumen diameter ranging from 218 to 557 μm were used. After a second equilibration for at least 15 min, the vessels were contracted 3 times with Krebs-Ringer bicarbonate solution containing 10 μM norepinephrine and 120 mM K⁺. The presence of functional endothelium was assessed by determining the ability of 10 μM acetylcholine to induce more than 80% relaxation. In some experiments, endothelium was removed by bubbling the lumen of the preparations for 2 min with 95% O₂ and 5% CO₂. The absence of functional endothelium was assessed by determining the ability of 10 μM acetylcholine to induce no or almost no relaxation. Concentration-response curves were made by cumulative addition of higher concentrations of an agonist after reaching the maximum effect of the previous concentration.

Drugs

The experiments were performed using Krebs-Ringer bicarbonate solution of the following composition: 135 mM NaCl, KCl, 5 mM; NaHCO₃, 20 mM; glucose, 10 mM; 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.2 mM KH₂PO₄, 0.026 mM EDTA. High-K⁺ solution (120 mM and 30 mM) Krebs-Ringer bicarbonate solutions were prepared by equimolar substitution of NaCl with KCl.

Norepinephrine bitartrate, acetylcholine chloride, indomethacin, Nω-nitro-L-arginine (L-NNA), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), charybdotoxin and apamin were all obtained from Sigma (St. Louis, MO, USA). Sodium nitroprusside was obtained from Merck (Darmstadt, Germany). Mesoacitone was a kind gift from Drs. Hiromitsu Takayama and Norio Aimi (Laboratory of Molecular Structure and Biological Function, Graduate School of Pharmaceutical Sciences, Chiba University). All concentrations are expressed as final molar concentrations in the organ bath. Mesoacitone and ODQ were dissolved in dimethylsulfoxide. Acetylcholine was dissolved in phthalate buffer, pH 4.0. Indomethacin was dissolved in ethanol, and other drugs were dissolved in distilled water.

Statistical analyses

Relaxations are expressed as a percentage of the maximum effect on norepinephrine-induced contraction. All data are shown as the mean ± S.E.M. Statistical analyses were performed with the 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 the Bonferroni multiple comparison test for observations for more than three groups. A P value <0.05 was considered statistically significant.

RESULTS

Vasorelaxing effects of mesaconitine, acetylcholine and sodium nitroprusside on isolated rat gastric artery

In isolated rat gastric artery, mesaconitine elicited concentration-dependent (10, 30 and 100 μM) vasorelaxation (that is at least partially transient). The relaxation by mesaconitine was not blocked by nitric oxide synthase inhibitor, L-NNA (100 μM), cyclooxygenase inhibitor, indomethacin (50 μM), and a soluble guanylyl cyclase inhibitor, ODQ (1 μM). The L-NNA- and indomethacin-resistant relaxation by mesaconitine was significantly inhibited by high K⁺ (30 mM) (Figs. 1 and 2A). The same series of experiments were performed using acetylcholine and sodium nitroprusside as vasorelaxing substances. The maximal vasorelaxation by acetylcholine was not influenced by L-NNA, indomethacin and ODQ, and the L-NNA- and indomethacin-resistant relaxation was abolished by high K⁺ (Fig. 2B). Vasorelaxation by sodium nitroprusside was enhanced by administration of L-NNA and indomethacin, and it was slightly inhibited by high K⁺. In the presence of L-NNA and indomethacin, the enhanced sodium nitroprusside-induced relaxation was markedly inhibited by ODQ (Fig. 2C).

Influence of endothelium removal

In these experiments, the role of the endothelium in the effects of mesaconitine, acetylcholine and sodium nitro-
prusside in isolated rat gastric arteries was investigated. The relaxation was measured before and after the procedure of endothelium removal. The endothelium was removed by bubbling the lumen of the preparations for 2 min with 95% O₂ and 5% CO₂. The effectiveness of the procedure was evaluated by determining the ability of acetylcholine to induce no or almost no relaxation (<10%). All experiments were performed in the presence of L-NNA (100 μM) and indomethacin (50 μM).

The L-NNA- and indomethacin-resistant relaxation by mesaconitine was partly impaired by endothelium removal, especially at a concentration of 30 μM (endothelium (+), 72.8 ± 4.1%; endothelium (−), 30.0 ± 6.4%) (Fig. 3A). The L-NNA- and indomethacin-resistant relaxation by acetylcholine was almost completely suppressed by endothelium removal (maximum relaxation: endothelium (+), 93.6 ± 2.3%; endothelium (−), 8.0 ± 1.8%) (Fig. 3B). The relaxation by sodium nitroprusside was not influenced by endothelium removal (maximum relaxation: endothelium (+), 76.9 ± 1.9%; endothelium (−), 83.3 ± 1.5%) (Fig. 3C).
Mesaconitine-Induced Vasorelaxation

Effects of Ca\(^{2+}\)-dependent K\(^{+}\) channel blockers, charybdotoxin and apamin

The L-NNA- and indomethacin-resistant relaxation by 30 \(\mu\)M mesaconitine was not inhibited by charybdotoxin, an intermediate- and large-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels inhibitor (0.1 \(\mu\)M), but was significantly inhibited by apamin, a small-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels inhibitor (0.1 \(\mu\)M), and by a combination of charybdotoxin and apamin (control, 73.7 \(\pm\) 9.6%; charybdotoxin, 70.4 \(\pm\) 5.9%; apamin, 23.7 \(\pm\) 4.3%; charybdotoxin and apamin, 13.1 \(\pm\) 2.6%) (Fig. 4A). The L-NNA- and indomethacin-resistant relaxation by acetylcholine (10 \(\mu\)M) was inhibited neither by charybdotoxin nor apamin when each was administrated alone; however, it was strongly inhibited when charybdotoxin and apamin were administered together (control, 92.2 \(\pm\) 1.5%; charybdotoxin, 84.5 \(\pm\) 2.9%; apamin, 79.1 \(\pm\) 5.8%; charybdotoxin and apamin, 10.3 \(\pm\) 4.7%) (Fig. 4B). The relaxation by sodium nitroprusside (10 \(\mu\)M) was not inhibited by charybdotoxin, apamin, or by a combination of these blockers (control, 77.1 \(\pm\) 3.3%; charybdotoxin, 76.5 \(\pm\) 6.0%; apamin, 82.9 \(\pm\) 3.1%; charybdotoxin and apamin, 72.3 \(\pm\) 6.7%) (Fig. 4C).
When l-NNA and indomethacin were not administered, the combination of charybdotoxin and apamin did not completely inhibit the relaxation of acetylcholine and mesaconitine. These residual relaxing effects were sensitive to l-NNA and indomethacin (acetylcholine, 10 μM: control, 95.0 ± 2.1%; charybdotoxin and apamin, 31.7 ± 6.2%; charybdotoxin and apamin and l-NNA and indomethacin, 10.3 ± 4.7%; n = 4) (mesaconitine, 30 μM: control, 60.5 ± 12.9%; charybdotoxin and apamin, 25.9 ± 7.5%; charybdotoxin and apamin and l-NNA and indomethacin, 13.1 ± 2.6%; n = 4).

Effect of external Ca\(^{2+}\) concentration
To investigate the role of external Ca\(^{2+}\) in the effects of mesaconitine, acetylcholine and sodium nitroprusside, Krebs-Ringer bicarbonate solutions with various CaCl\(_2\) concentrations (0.3, 1 and 3 mM) were prepared. Norepinephrine-induced precontraction was proportional to the CaCl\(_2\) concentration (CaCl\(_2\): 2.5 mM, 100%; 3 mM, 98.1 ± 9.8%; 1 mM, 87.8 ± 2.3%; 0.3 mM, 57.3 ± 4.6%; n = 12).

The l-NNA- and indomethacin-resistant relaxation by mesaconitine (30 μM) was significantly inhibited when the external Ca\(^{2+}\) concentration was 1 and 0.3 mM (3 mM CaCl\(_2\), 53.2 ± 13%; 1 mM CaCl\(_2\), 12.7 ± 2.9%; 0.3 mM CaCl\(_2\), 18.9 ± 3.1%) (Fig. 5A). The l-NNA- and indomethacin-resistant relaxation by acetylcholine (10 μM) was proportional to the external Ca\(^{2+}\) concentration and was significantly inhibited at 0.3 mM (3 mM CaCl\(_2\), 85.2 ± 6.4%; 1 mM CaCl\(_2\), 77.0 ± 2.7%; 0.3 mM CaCl\(_2\), 52.7 ± 7.7%) (Fig. 5B). When a lower external Ca\(^{2+}\) concentration (0.1 mM) was used, the norepinephrine-induced contraction was very small (2.5 mM CaCl\(_2\), 100%; 0.1 mM CaCl\(_2\), 37.7 ± 4.4%); however, the relaxation by acetylcholine was further inhibited (29.1 ± 10.4%, n = 4). The vasorelaxation induced by sodium nitroprusside (10 μM) was not influenced by the external Ca\(^{2+}\) concentration (3 mM CaCl\(_2\), 78.3 ± 2.9%; 1 mM CaCl\(_2\), 79.8 ± 3.6%; 0.3 mM CaCl\(_2\), 81.3 ± 2.8%) (Fig. 5C).

DISCUSSION
In isolated rat small gastric artery, the vasorelaxing effect of acetylcholine was not inhibited by an NO synthase inhibitor, l-NNA, and a cyclooxygenase inhibitor, indomethacin. The concentration of l-NNA (100 μM) used in this study can be considered high enough to eliminate the effect of NO because additional application of the guanylyl cyclase inhibitor ODQ did not cause further inhibition. These results confirm previous observations (8) in which most of the endothelium-dependent response to acetylcholine in rat small gastric arteries could be attributed to EDHF.

EDHF is released from vascular endothelium and hyperpolarizes the membrane of smooth muscle cells and elicits vasorelaxation (6, 7). The hyperpolarization and the associated vasorelaxation induced by EDHF have been reported to be impaired by raising extracellular K\(^{+}\) concentration (10, 11). In the present study, the l-NNA- and indomethacin-resistant vasorelaxation by acetylcholine was inhibited by 30 mM K\(^{+}\).

Mesaconitine elicited a concentration-dependent vasorelaxation in isolated rat gastric artery. This relaxation was in part endothelium-dependent and was not inhibited by l-NNA, indomethacin and ODQ. The l-NNA- and indo-
methacin-resistant relaxation by mesaconitine, especially at a concentration of 30 µM, was significantly inhibited by 30 mM K+. These results suggest that EDHF might be partly involved in the vasorelaxing effect of mesaconitine.

The vasorelaxation by sodium nitroprusside was significantly enhanced in the presence of L-NNA and indomethacin, and it was only slightly inhibited by 30 mM K+. The enhancement of sodium nitroprusside-induced relaxation by L-NNA and indomethacin is in line with findings in endothelial NO synthase knockout mouse aorta (12). From their study, Brandes et al. concluded that chronic exposure to endothelium-derived NO desensitizes soluble guanylyl cyclase. Therefore, the lack of NO synthase activity resulted in an enhanced sensitivity to nitrovasodilators. NO is known to open KATP channels and KCl-sensitive channels either directly or indirectly via a second messenger, cyclic GMP (13–16), and causes hyperpolarization of arterial smooth muscle (17, 18). The fact that high K+ slightly inhibited the relaxation by sodium nitroprusside in the present study indicates that K+ channels partly contribute to the vasorelaxing effect of NO together with a cyclic GMP-pathway. These K+ channels are insensitive to apamin and charybdotoxin.

The L-NNA- and indomethacin-resistant vasorelaxation by acetylcholine was abolished after endothelium removal, confirming that this relaxing effect is endothelium-dependent as is well established. The L-NNA- and indomethacin-resistant vasorelaxation by mesaconitine was significantly decreased by removal of the endothelium, suggesting that the relaxation by mesaconitine is in part endothelium-dependent, but in part also endothelium-independent. The endothelium-dependent part is considered more important in the effect of mesaconitine at a low concentration such as 30 µM. The observation that the endothelium-dependent part of relaxation by mesaconitine seems to correspond to the high K+-sensitive part of the relaxation suggests that a main part of the vasorelaxation by mesaconitine is EDHF-mediated.

The mechanisms of EDHF responses have not yet been established. Much evidence suggests that endothelial and/or smooth muscle cell Ca2+-activated K+ channels are somehow involved in EDHF responses (19–21). The EDHF-mediated vasorelaxation and hyperpolarization can be inhibited by a combination of charybdotoxin, an intermediate- and large-conductance Ca2+-activated K+ channels inhibitor, and apamin, a small-conductance Ca2+-activated K+ channels inhibitor (22, 23). We confirmed that either charybdotoxin or apamin alone had little inhibiting effect on vasorelaxation by acetylcholine in rat gastric artery; however, a combination of charybdotoxin and apamin showed a strong inhibiting effect. Mesoacitnine-induced, L-NNA- and indomethacin-resistant relaxation was also inhibited by a combination of charybdotoxin and apamin, which seemed to be mainly dependent on apamin-sensitive Ca2+-activated K+ channels. The difference for apamin sensitivity between acetylcholine and mesaconitine is not clear. It should however be noted that EDHF-mediated vasodilators have been shown to activate different types of Ca2+-activated K+ currents in endothelial cells (24). It has been assumed that the target(s) of charybdotoxin and apamin is on the vascular smooth muscle, since the increase in endothelial intracellular Ca2+ by acetylcholine was not affected by these blockers (25). However, Ca2+-activated K+ channels are also expressed in endothelial cells (26).

Increased cytosolic Ca2+ concentration in endothelial cells is required to produce and/or release EDHF (27). To investigate the influence of external Ca2+ concentration on vasorelaxation by mesaconitine, acetylcholine and sodium nitroprusside, Krebs-Ringer bicarbonate solution containing 0.3, 1 and 3 mM CaCl2 were used. Vasorelaxation by mesaconitine and acetylcholine, but not by sodium nitroprusside, were sensitive to changes of the external Ca2+ concentration. The elevation of cytosolic Ca2+ concentration in endothelial cells induced by agonists such as acetylcholine is the result of Ca2+ release from intracellular stores and of transmembrane Ca2+ influx from the extracellular space (28). The present findings confirm that the Ca2+ influx is an essential step in the EDHF-pathway activated by acetylcholine and mesaconitine. The higher sensitivity of mesaconitine to lowering of external Ca2+ concentration suggests that Ca2+ influx might contribute more to the increase in cytosolic Ca2+ level in response to mesaconitine than in response to acetylcholine. It might also be due to an influence of Ca2+-lowering on the endothelium-independent part of mesaconitine-induced relaxation.

In the present study, relaxation was measured in the presence of L-NNA and indomethacin to investigate the effect of NO- and prostanoids-independent factor(s), EDHF. Although the vasorelaxation by mesaconitine and acetylcholine was not influenced by administration of L-NNA, indomethacin and ODQ, it should not be concluded that NO has no physiological role in rat gastric artery. It has been shown that NO and cyclic GMP can impair endothelium-dependent relaxation mediated by EDHF (29), and that EDHF-induced relaxation can be enhanced when NO synthesis is failing (30, 31). In our study, a combination of charybdotoxin and apamin did not completely inhibit the relaxation of acetylcholine and mesaconitine in the absence of L-NNA and indomethacin. These residual relaxing effects were sensitive to L-NNA and indomethacin. These findings suggest that NO also acts as a relaxing factor in response to both acetylcholine and mesaconitine in rat gastric artery. From the present study, the relaxation mediated by NO (sensitive to L-NNA and indomethacin, but not to charybdotoxin and apamin) accounts for approximately 20% of the maximum relaxation by acetylcholine. This agrees with the idea that EDHF is the most prominent
endothelial relaxing factor in small resistance arteries (4, 5).

In conclusion, the present study demonstrates for the first time that mesaconitine elicits a mainly EDHF-mediated endothelium-dependent relaxation in an isolated small artery. Such a vasorelaxing effect of mesaconitine may contribute to the beneficial effects of Aconiti tuber on microvascular circulation.

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