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Mesaconitine-induced relaxation in rat aorta: role of Na^+/Ca^{2+} exchangers in endothelial cells

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Abstract

Previously, we reported that mesaconitine, an aconite alkaloid, increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) level in endothelium and caused relaxation in rat aorta via nitric oxide production. In the present study, we investigated the mechanisms of increase in the $[Ca^{2+}]_i$ level induced by mesaconitine in rat aorta and in human umbilical vein endothelial cells (HUVECs). Treatment with the low Na^+ buffer delayed the 30 μM mesaconitine-, but not 10 μM acetylcholine-, induced relaxation in rat aorta. Treatments with an inhibitor of Na^+/Ca^{2+} exchangers (20 μM 3′,4′-dichlorobenzamil) and a reversed mode (Ca^{2+} influx) inhibitor of the exchangers (30 μM 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate, KBR7943) showed similar effects. In HUVECs, 30 μM mesaconitine increased the $[Ca^{2+}]_i$ level in the presence of extracellular $CaCl_2$ and NaCl, and the response was inhibited by KBR7943. Mesaconitine increased intracellular Na^+ concentration level in HUVECs. The $[Ca^{2+}]_i$ response by mesaconitine was inhibited by 100 μM D-tubocurarine (an inhibitor of nicotinic acetylcholine receptors), but was not inhibited in the glucose-free buffer and by inhibitors of Na^+/H^+ exchangers. These findings suggest that mesaconitine stimulated Ca^{2+} influx via the Na^+/Ca^{2+} exchangers in endothelial cells and caused relaxation in the aorta. The possibility of D-tubocurarine-sensitive Na^+ channels as target(s) of mesaconitine is discussed.

Keywords: Vasorelaxation; Mesaconitine; Endothelial cell; Na^+/Ca^{2+} exchanger; Na^+ channel

1. Introduction

Aconiti tuber, the roots of aconite (*Aconitum japonium* or *Aconitum carmichaeli*), is an important oriental herbal medicine used for centuries in Japan and China. Aconiti tuber is believed to increase the peripheral temperature, relieve rheumatic pain, and improve the health of persons with a weak constitution and poor metabolism. The main constituents are aconite alkaloids; mesaconitine is pharmacologically the most active component. Mesaconitine was reported to have various pharmacological effects such as positive inotropic effects, analgesic and anti-inflammatory effects, contraction in vas deferens and ileum (see Introduction in Mitamura et al., 2002b). Previously, we reported that mesaconitine induced relaxation in the aorta (Mitamura et al., 2002b) and in small gastric arteries (Mitamura et al., 2002a) in an endothelium- and an extracellular $CaCl_2$-dependent manner in rats.

The regulation of vascular tone by the endothelium is mainly mediated by production of prostacyclin, nitric oxide and endothelium-derived hyperpolarizing factor (EDHF) (Groschner et al., 1994; Prabhakar et al., 1998; Ferro et al., 1999; Fukao et al., 2001). The relative contribution of nitric oxide and EDHF appeared to be dependent on the size of the vessels; the contribution of nitric oxide to the vascular tone is marked in large-diameter vessels such as the aorta, and that of EDHF increases with decreasing diameter of vessels, and that of EDHF is becoming dominant in microvessels such as gastric artery (Shimokawa et al., 1996; Tomioka et al., 1999). Mesaconitine increased the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) level by an influx of Ca^{2+} from extracellular spaces and nitric oxide synthase activity in endothelium, and thus induced relaxation in rat aorta (Mitamura et al., 2002b). In rat small gastric arteries, mesaconitine-induced relaxation

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was inhibited by high K^+ (30 mM) and a combination of Ca^{2+}-dependent K^+ channel inhibitors, but not by nitric oxide synthase inhibitor and/or cyclooxygenase inhibitors (Mitamura et al., 2002a). Thus, mesaconitine appeared to increase Ca^{2+} influx and thus stimulate nitric oxide and EDHF production in the endothelial cells in the aorta and the small gastric arteries, respectively. However, the mechanism(s) of Ca^{2+} influx into the endothelial cells has not been identified.

In the present study, we investigated (1) the effects of extracellular $CaCl_2$ and NaCl concentrations and various inhibitors of ion transport systems on mesaconitine-induced relaxation in rat aortic rings; and (2) the effect of mesaconitine on $[Ca^{2+}]_i$ level and its mechanism in human umbilical vein endothelial cells (HUVECs). The present findings suggest that mesaconitine activates Na^+ influx and a resulting Ca^{2+} influx via Na^+/Ca^{2+}exchangers in endothelial cells, and a resulting relaxation of rat aorta. Mesaconitine-induced increase in $[Ca^{2+}]_i$ level in HUVECs was inhibited by D-tubocurarine. The possible involvement of Na^+ channels such as nicotinic acetylcholine receptors on mesaconitine-induced response in endothelial cells is discussed.

2. Materials and methods

2.1. Drugs

The following drugs and chemicals were used: L-phenylephrine hydrochloride, thapsigargin, D-tubocurarine chloride, mefenamic acid, histamine, monensin, mecamylamine and cimetidine (SIGMA, St. Louis, MO, USA); acetylcholine chloride (Daiichi Seiyaku, Tokyo, Japan); fura-2 acetoxymethyl ester (Fura-2 AM) and clonidine hydrochloride (Wako, Tokyo, Japan); 3′,4′-dichlorobenzamil and sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI-AM, a probe for measurement of intracellular Na^+ concentration ($[Na^+]_i$)) (Molecular Probes, Eugene, OR, USA); 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate (KBR7943, Tocris, Ballwin, MO, USA); 1-[2-(methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1*H*-imidazole (SKF96365, Funakoshi, Tokyo, Japan); nicotine tartrate and hexamethonium chloride (Tokyo Kasei, Tokyo, Japan). The antagonists or inhibitors were used at the reported concentrations in previous studies using endothelial cells.

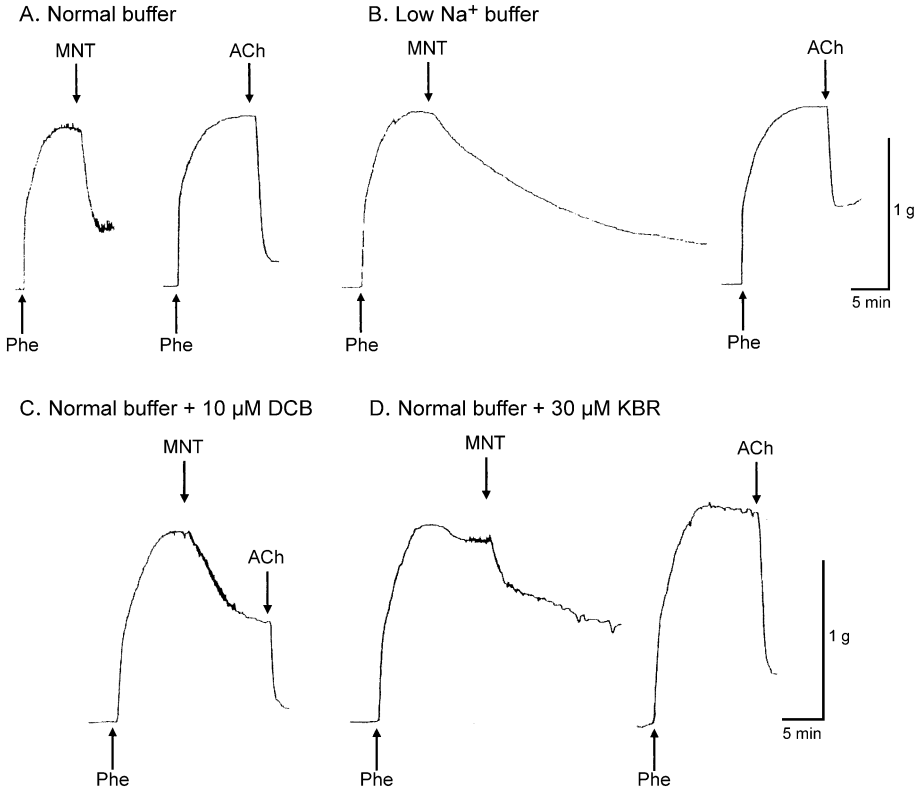

Fig. 1. Typical recordings of the effects of external Na^+ concentrations, 3′,4′-dichlorobenzamil and KBR7943 on mesaconitine- and acetylcholine-induced relaxation in rat aorta. In Panels A and B, rat aortic rings with endothelium were incubated in the normal buffer containing 118 mM NaCl (Panel A) or the low Na^+ buffer containing 118 mM LiCl (Panel B) for 15 min. In Panels C and D, rat aortic rings with endothelium were incubated in the normal buffer containing 118 mM NaCl in the presence of 10 μM 3′,4′-dichlorobenzamil (Panel C, DCB) or 30 μM KBR7943 (Panel D, KBR) for 10 min. Then the contraction was elicited with 3 μM phenylephrine (Phe). Mesaconitine (MNT, 30 μM) or acetylcholine (ACh, 10 μM) was added after contraction by phenylephrine reached a sustained plateau. The data presented are from a typical experiment and are representative of three to four independent experiments. Quantitative analysis of the data is shown in Table 1.

2.2. Animals and measurement of contraction in rat isolated aortic rings

Male Wistar rats (Takasugi Exp. Animals, Kasukabe, Japan) weighing 240–400 g were used. Animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, approved by the Japanese Pharmacological Society. Contraction in isolated rat aortic rings was determined as described previously (Mitamura et al., 2002b). 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). The mechanical activity was recorded isometrically, and 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 low Na^+ buffer was prepared by substituting NaCl with 118 mM LiCl.

2.3. Culture of HUVECs and measurements of $[Ca^{2+}]_i$ and $[Na^+]_i$ in HUVECs

HUVECs (Dainippon Seiyaku, Tokyo, Japan) were cultured in DMEM/Ham's F12 supplemented with 10% fetal bovine serum (Thermo-Trace, Melbourne, Australia) and 10 ng/ml of recombinant human basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA). For measurement of $[Ca^{2+}]_i$, HUVECs on dishes were loaded with 3 μM Fura-2 AM for 30 min at 37 °C in the modified Tyrode buffer of the following composition (mM): NaCl, 135; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 1.0; glucose, 10.0; and HEPES, 5.0 at pH 7.4 (normal solution). HUVECs were washed and detached from the dish under a gentle stream of buffer. An aliquot of $3–5 \times 10^6$ cells was immediately used for autofluorescence measurements at 37 °C. In some cases, HUVECs were washed with the Na^+-free buffer, and then the $[Ca^{2+}]_i$ responses were measured. The Na^+-free buffer was prepared by substituting LiCl. Fluorescence readings were taken with a Hitachi F-2500 spectrophotometer, as described previously (Naganuma et al., 1999; Mitamura et al., 2002b).

For measurement of $[Na^+]_i$, HUVECs on dishes were loaded with 4 μM SBFI-AM for 90 min at room temperature in the modified Tyrode buffer, as described previously (Touyz et al., 2001; Yang et al., 2001). The loaded cells were washed and incubated for a further 20 min at room temperature. The $[Na^+]_i$ response was measured using an emission wavelength of 520 nm and alternating excitatory wavelengths of 340 and 380 nm. The agents were dissolved in the dimethylsulfoxide for the fluorescence measurement (Dojin, Kumamoto, Japan). Since the change in the ratio was small in the present study conditions, we could not estimate the absolute $[Na^+]_i$ level using the Na^+ calibration curve.

2.4. Statistical analysis

All values are shown as the mean ± S.E.M. Statistical analyses for data between two groups and among three or four groups were performed by Mann–Whitney *U*-test and by one-way analysis of variance (Kruskal–Wallis test) followed by nonparametric Dunnett's multiple comparison test. *P* value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of external NaCl and inhibitors of Na^+/Ca^{2+} exchange system on mesaconitine-induced relaxation in rat aorta

Previously, we reported that mesaconitine induced relaxation in rat aorta in the endothelium- and extracellular $CaCl_2$-dependent manner (Mitamura et al., 2002b). First, we

Table 1
Effects of external Na^+ concentrations, 3′,4′-dichlorobenzamil and KBR7943 on mesaconitine- and acetylcholine-induced relaxation in rat aorta

	Mesaconitine (30 μM)		Acetylcholine (10 μM)	
	$T_{1/2}$ (min)	Relaxation (% of control)	$T_{1/2}$ (min)	Relaxation (% of control)
Experiment I (n = 5)				
Normal Na^+ buffer	1.1 ± 0.2	100	0.3 ± 0.1	100
Low Na^+ buffer	10.7 ± 1.6[a]	127.3 ± 18.3	0.3 ± 0.1	52.9 ± 4.5[b]
Experiment II (n = 4)				
None	0.7 ± 0.1	100	0.2 ± 0.1	100
DCB (10 μM)	1.4 ± 0.3	69.3 ± 6.0[a]	0.2 ± 0.1	105.8 ± 1.6
DCB (20 μM)	2.5 ± 0.5[c]	28.0 ± 5.6[c]	0.5 ± 0.1	41.2 ± 7.2[b]
Experiment III (n = 5)				
None	1.2 ± 0.3	100	0.4 ± 0.1	100
KBR7943 (10 μM)	1.6 ± 0.5	93.8 ± 4.5	0.4 ± 0.1	96.5 ± 2.8
KBR7943 (30 μM)	4.8 ± 1.0[a]	41.4 ± 7.6[b]	0.3 ± 0.1	65.9 ± 7.6[a]

The contraction of rat aorta was measured as described in the legend to Fig. 1. Quantitative analyses of the relaxation induced by mesaconitine and acetylcholine in the normal Na^+ buffer or the low Na^+ buffer (Experiment I), and in the presence of the indicated concentrations of 3′,4′-dichlorobenzamil (DCB, Experiment II) and KBR7943 (Experiment III) are shown. The relaxation at 50 min (Experiment I) or 40 min (Experiments II and III) after the addition of mesaconitine was estimated as the maximum response, and the half-time ($T_{1/2}$) of the maximum relaxation induced by 30 μM mesaconitine or 10 μM acetylcholine was calculated. Each value of relaxation (% of control) is normalized as a percentage of the maximum response induced by mesaconitine and acetylcholine, respectively, in the normal conditions. The ability of 30 μM mesaconitine and 10 μM acetylcholine to induce the relaxation of the rings precontracted with 3 μM phenylephrine were 60.2 ± 10.2% and 88.2 ± 5.2%, respectively. Each value represents the mean ± S.E.M. for four to five animals.

[a] $P < 0.05$ significantly different from the value in the corresponding control group.

[b] $P < 0.01$ significantly different from the value in the corresponding control group.

[c] $P < 0.001$ significantly different from the value in the corresponding control group.

investigated the effect of mesaconitine on the phenylephrine-induced contraction in the low Na^+ buffer (25 mM $NaHCO_3$ plus 118 mM LiCl). The contraction induced by 3 μM phenylephrine in the low Na^+ buffer was similar to that in the normal Na^+ buffer containing 25 mM $NaHCO_3$ and 118 mM NaCl (Fig. 1, Panels A and B). In the low Na^+ buffer, the rate of relaxation induced by 30 μM mesaconitine was quite slow, and the relaxation reached a plateau at 40–50 min after the addition. The half-time ($T_{1/2}$) of the maximum relaxation by mesaconitine was significantly greater compared with that in the normal Na^+ buffer (Table 1). In the low Na^+ buffer, the degree of the maximum relaxation induced by mesaconitine was 65.5 ± 12.1 (%relaxation against the phenylephrine-induced contraction, $n = 5$), which was almost the same as that in the normal Na^+ buffer (60.2 ± 10.2%, $n = 6$). Although the maximum relaxation induced by 10 μM acetylcholine in the low Na^+ buffer was significantly less than that in the normal Na^+ buffer, the values of $T_{1/2}$ for acetylcholine response in both buffers were quite small (within 30 s) and similar.

3′,4′-Dichlorobenzamil was shown to be a potent and selective inhibitor of Na^+/Ca^{2+} exchangers, and other functions such as membrane integrity, Na^+ and Ca^{2+} conductances and Na^+,K^+-ATPase, etc. were not changed by 25 μM 3′,4′-dichlorobenzamil treatment in endothelial cells (Li and Van Breemen, 1995). In the present study,

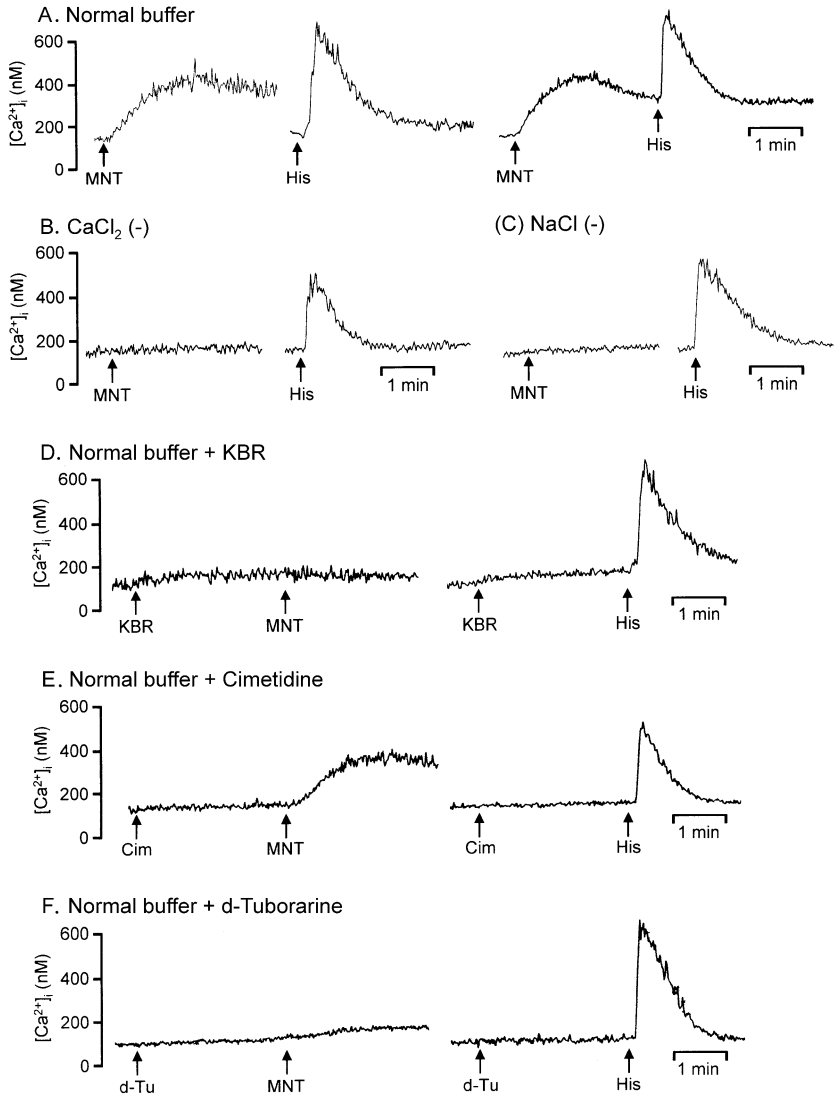

Fig. 2. Typical recordings of the mesaconitine- and histamine-induced increases on $[Ca^{2+}]_i$ level in HUVECs. HUVECs were incubated with 3 μM Fura-2 AM for 30 min. In Panels A, D, E and F, the HUVECs were suspended with the normal buffer containing 135 mM NaCl and 1.8 mM $CaCl_2$. In Panel B, the HUVECs were suspended with the buffer containing 135 mM NaCl without $CaCl_2$. In Panel C, the HUVECs were suspended with the Na^+-free buffer containing 135 mM LiCl and 1.8 mM $CaCl_2$. In some cases, the HUVECs were treated with 50 μM KBR7943 (Panel D, KBR), 3 mM cimetidine (Panel E, Cim) or 100 μM D-tubocurarine (Panel F, D-Tu) for 3 min in the normal buffer. Then, the HUVECs were stimulated with 30 μM mesaconitine (MNT) or 100 μM histamine (His) at the indicated time. The data presented are from a typical experiment and are representative of four independent experiments. Quantitative analysis of the data is shown in Table 2.

treatment with 10 μM 3′,4′-dichlorobenzamil inhibited the maximal relaxation induced by 30 μM mesaconitine, but not by acetylcholine (Panel C; Table 1). In the 20 μM 3′,4′-dichlorobenzamil-treated aorta, the maximum relaxation by 30 μM mesaconitine was inhibited and the response of relaxation was significantly slower than that in the control aorta. KBR7943 is shown to inhibit the reverse mode (intracellular Na^+-dependent Ca^{2+} influx, $[Ca^{2+}]_i$ increase) selectively, not the forward mode (extracellular Na^+-dependent Ca^{2+} efflux, $[Ca^{2+}]_i$ decrease), of Na^+/Ca^{2+} exchange activity in Na^+/Ca^{2+} exchanger 1-trasfected fibroblasts (Iwamoto et al., 1996). Similar results (increase of $T_{1/2}$ and decrease of the maximum relaxation in the mesaconitine response) were obtained in the 30 μM KBR7943-treated aorta (Panel D; Table 1).

Thapsigargin, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase, was reported to cause an increase of $[Ca^{2+}]_i$ and nitric oxide accumulation in endothelial cells and, thus, relaxation of rat aorta (Amerini et al., 1996; Huang et al., 2000). The relaxation induced by the 1 μM thapsigargin was not changed by KBR7943; the relaxation in the vehicle and the 30 μM KBR7943-treated aorta were 85.0 ± 4.6% ($n = 7$) and 81.5 ± 6.3% ($n = 4$) of the phenylephrine response, respectively. Although the maximum relaxation induced by 10 μM acetylcholine was inhibited in the 20 μM 3′,4′-dichlorobenzamil- and 30 μM KBR7943-treated aortas, the $T_{1/2}$ values for acetylcholine response were not modified by these treatments.

It is reported that SKF96365 and mefenamic acid, which are inhibitors of nonselective and/or receptor-operated Ca^{2+} channels (Groschner et al., 1994; Fukao et al., 2001; Ihara et al., 2001) and cyclooxygenase (Dorigo et al., 1997), inhibited the relaxation response in several arteries. These inhibitors showed no effect on mesaconitine response; the relaxations by 30 μM mesaconitine in the 10 μM SKF96365- and 100 μM mefenamic acid-treated aorta were 50.1 ± 11.1% ($n = 6$) and 45.5 ± 10.1% ($n = 4$), respectively, which were similar to that in the control which showed 60.2 ± 10.2% ($n = 6$) of the phenylephrine response.

3.2. Mesaconitine-induced increase in $[Ca^{2+}]_i$ level via Na^+/Ca^{2+} exchange system in HUVECs

Mesaconitine at 30 μM increased the $[Ca^{2+}]_i$ level in endothelium by influx of Ca^{2+} from extracellular spaces in rat aorta (Mitamura et al., 2002b). In the present study, we examined the effect of mesaconitine on the $[Ca^{2+}]_i$ level in HUVECs, which is commonly used as a model cell line of endothelial cells. Addition of 30 μM mesaconitine stimulated the $[Ca^{2+}]_i$ level gradually and the increased level was sustained for at least 5 min (Fig. 2A). The effect of mesaconitine was almost completely dependent on the existence of extracellular $CaCl_2$ (Panel B) and NaCl (Panel C). Quantitative analyses of increases in $[Ca^{2+}]_i$ level induced by mesaconitine are shown in Table 2. It was reported that activation of H_1 histamine receptor stimulated

Table 2
Effects of extracellular $CaCl_2$ and NaCl, KBR7943, cimetidine and D-tubocurarine on mesaconitine-induced increases in $[Ca^{2+}]_i$ level in HUVECs

	Net increase in $[Ca^{2+}]_i$ (nM)	
	Mesaconitine	Histamine
Experiment I (with 135 mM NaCl)		
10 μM Ca^{2+}	10 ± 2[a]	297 ± 20[a]
0.1 mM $CaCl_2$	10 ± 5[a]	370 ± 34
0.5 mM $CaCl_2$	62 ± 4[a]	Not determined
1.8 mM $CaCl_2$	361 ± 19	428 ± 10
Experiment II (with 1.8 mM $CaCl_2$)		
135 mM NaCl	356 ± 48	430 ± 24
135 mM LiCl	78 ± 25[a]	442 ± 36
Experiment III (with 135 mM NaCl and 1.8 mM $CaCl_2$)		
Vehicle	327 ± 26	448 ± 21
KBR7943	11 ± 8[a]	458 ± 48
Cimetidine	310 ± 24	410 ± 22
D-Tubocurarine	73 ± 8[a]	492 ± 54

The $[Ca^{2+}]_i$ response was measured as described in the legend to Fig. 2. Quantitative analyses of increases in $[Ca^{2+}]_i$ level induced by mesaconitine and histamine are shown. In Experiment I, Fura-2 AM-loaded HUVECs were suspended in the buffer containing 135 mM NaCl and the indicated concentrations of $CaCl_2$. The buffer containing 10 μM Ca^{2+} was prepared by adding 450 μM $CaCl_2$ in the presence of 0.5 mM EGTA. In Experiment II, the HUVECs were suspended with the normal buffer or the NaCl-free buffer containing 135 mM LiCl in the presence of 1.8 mM $CaCl_2$. In Experiment III, the HUVECs were suspended in the normal buffer with 1.8 mM $CaCl_2$, and incubated with vehicle, 50 μM KBR7943, 3 mM cimetidine or 100 μM D-tubocurarine for 3 min. Then, the cells were stimulated with 30 μM mesaconitine or 100 μM histamine. The data presented are the maximal $[Ca^{2+}]_i$ increases induced by the stimulants. The data are means ± S.E.M. for four independent experiments.

[a] $P < 0.05$, significantly different from the value in the corresponding control group (the value in the buffer containing 1.8 mM $CaCl_2$ in Experiment I, the value in the buffer containing 135 mM NaCl in Experiment II or the value with vehicle in Experiment III).

an increase in $[Ca^{2+}]_i$ level from intracellular Ca^{2+} pools in HUVECs (Ehringer et al., 1996; Lantoine et al., 1998; Ferro et al., 1999). In the present study, the increase in $[Ca^{2+}]_i$ level induced by 100 μM histamine was fast and transient, and the response was still observed in the absence of $CaCl_2$ and NaCl. The histamine response was observed in the mesaconitine-treated HUVECs (Panel A, right trace). The mesaconitine response in $[Ca^{2+}]_i$ level was abolished in the Na^+-free buffer containing choline chloride instead of LiCl (data not shown). Treatment with 30 μM KBR7943 for 3 min, which alone showed no or marginal effect, completely inhibited the mesaconitine response without the change of histamine response (Panel D). We could not examine the effect of 3′,4′-dichlorobenzamil, since the agent interfered with the fluorometric detection of $[Ca^{2+}]_i$ in HUVECs, as described previously (Teubl et al., 1999). These findings suggest that mesaconitine increased the $[Ca^{2+}]_i$ level via Ca^{2+} influx mediated by the reverse mode of the Na^+/Ca^{2+} exchange system in HUVECs.

3.3. Effects of agents regulating Na^+ influx on mesaconitine-induced increase in the $[Ca^{2+}]_i$ level in HUVECs

Cells including endothelial cells and HUVECs can stimulate Na^+ influx in several pathways (Yang et al., 2001; Berna et al., 2002). Amiloride, which is a structural analog of 3′,4′-dichlorobenzamil and inhibits Na^+/H^+ exchangers, also interfered with the fluorometric detection. It was reported that cimetidine and clonidine acted as inhibitors of Na^+/H^+ exchangers at high concentrations (Turner et al., 2000; Németh et al., 2002). Treatment with 3 mM cimetidine slightly not significantly inhibited the mesaconitine and histamine responses in HUVECs (Fig. 2, Panel E; Table 2). In addition, treatment with 3 mM clonidine did not inhibit the mesaconitine response (data not shown). Interestingly, treatment with 100 μM D-tubocurarine, an inhibitor of nicotinic acetylcholine receptors, significantly inhibited the 30 μM mesaconitine-, but not the 100 μM histamine-, induced increase in the $[Ca^{2+}]_i$ level in HUVECs (Panel F). Treatment with 10 μM mecamylamine and 100 μM hexamethonium did not inhibit the mesaconitine response; the increases in $[Ca^{2+}]_i$ levels induced by 30 μM mesaconitine were 300–350 nM in the mecamylamine- and hexamethonium-treated cells. The effect of mesaconitine on $[Ca^{2+}]_i$ level was observed in the buffer without glucose (data not shown).

3.4. Mesaconitine-induced increase in $[Na^+]_i$ level in HUVECs

Fig. 3 shows representative fluorescence tracing of $[Na^+]_i$ level in HUVECs loaded with SBFI-AM. Addition of 100 μM mesaconitine induced a small increase in $[Na^+]_i$ level in HUVECs, although the effect was markedly less than that by 20 μM monensin, a Na^+ ionophore. Although we could not detect the effect of 30 μM mesaconitine on the $[Na^+]_i$ level in the present conditions, the effect of 100 μM mesaconitine was abolished in the 100 μM D-tubocurarine-treated HUVECs (data not shown).

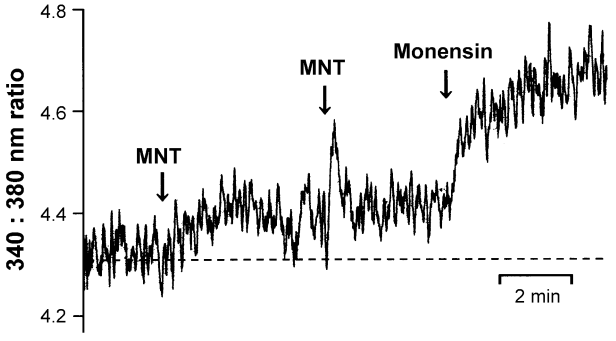

Fig. 3. An increase in $[Na^+]_i$ level caused by mesaconitine in HUVECs. HUVECs were incubated with the Na^+-sensitive fluorescent dye, 4 μM SBFI for 90 min. The HUVECs were suspended with the normal buffer containing NaCl and $CaCl_2$, and stimulated with 100 μM mesaconitine (MNT) twice and 20 μM monensin at the indicated time points. The *y*-axis is the 340:380 nm fluorescence excitation ratio. The data presented are from a typical experiment and are representative of three independent experiments.

4. Discussion

4.1. Effect of extracellular Na^+ on mesaconitine-induced relaxation in rat aorta and increase in the $[Ca^{2+}]_i$ level in HUVECs

Previously, we reported that mesaconitine stimulated Ca^{2+} influx into the endothelium and caused relaxation in rat aorta (Mitamura et al., 2002b). In the present study, we showed that mesaconitine responses (relaxation in rat aorta and increase in $[Ca^{2+}]_i$ level in HUVECs) were dependent on extracellular Na^+. One possible mechanism of Ca^{2+} influx into endothelial cells is an activation of Na^+/Ca^{2+} exchangers. Since the activities of the Na^+/Ca^{2+} exchangers are critically dependent on the electrochemical Na^+ gradient across the cell membrane, removal of external Na^+ would reverse the Na^+ gradient and intracellular Na^+ loss. A decrease in the Na^+ concentration in the buffer slowed the relaxation response induced by mesaconitine in rat aorta; an increase of the $T_{1/2}$ value without a change in the maximum response. In HUVECs, the mesaconitine-induced increase in the $[Ca^{2+}]_i$ level was completely dependent on extracellular Na^+. Li^+ is known to have an inhibitory effect on the hydrolysis of inositol trisphosphate, which causes Ca^{2+} release from intracellular Ca^{2+} stores. However, the Li^+ substitution for Na^+ did not change the $T_{1/2}$ value for acetylcholine-induced relaxation in rat aorta, although the maximum relaxation was inhibited (Table 1). In HUVECs, mesaconitine-induced increase in $[Ca^{2+}]_i$ level was completely dependent on extracellular $CaCl_2$, not from the intracellular Ca^{2+} stores (Fig. 2B). The Li^+ substitution did not change the basal $[Ca^{2+}]_i$ level and the histamine response in the HUVECs (Fig. 2C). In addition, choline chloride substitution for Na^+ abolished the mesaconitine response. Thus, the inhibition of mesaconitine responses in the low Na^+ and Na^+-free buffers is not likely due to the Li^+ substitution.

4.2. Involvement of the reverse mode of Na^+/Ca^{2+} exchange system on the mesaconitine responses

The presence of a Na^+/Ca^{2+} exchanger in endothelial cells including HUVECs has been demonstrated by immunoblotting and by the detection of cDNA coding for the type 1 exchanger (Juhaszova et al., 1994; Quednau et al., 1997). Teubl et al. (1999) reported that treatment with 30 μM 3′,4′-dichlorobenzamil inhibited Ca^{2+}-induced nitric oxide synthase activation in endothelial cells. In the present study, treatment with 20 μM 3′,4′-dichlorobenzamil inhibited the mesaconitine response (both the maximum relaxation and its $T_{1/2}$ value) in rat aorta. Treatment with KBR7943, an inhibitor of the reverse mode of the Na^+/Ca^{2+} exchanger, inhibited mesaconitine-induced relaxation in rat aorta and increase in

$[Ca^{2+}]_i$ level in HUVECs. The effect of KBR7943 in rat aorta was selective, since the relaxation induced by thapsigargin was not modified by KBR7943. These two inhibitors of Na^+/Ca^{2+} exchangers, similar to treatment with low Na^+ buffer, reduced the maximum relaxation without a change in the $T_{1/2}$ value in the acetylcholine-induced response. The involvement of Na^+/Ca^{2+} exchange systems on acetylcholine response in some parts in aorta was reported (Schneider et al., 2002). From the findings using SKF96365 and mefenamic acid, Ca^{2+} influx via nonselective and/or receptor-operated Ca^{2+} channels did not appear to be involved in the mesaconitine response in rat aorta. These findings suggest that the reverse mode activity of Na^+/Ca^{2+} exchangers have a critical role on mesaconitine-induced relaxation in rat aorta and an increase in $[Ca^{2+}]_i$ level in HUVECs.

The present findings are consistent with the recent findings showing the physiological role of Na^+/Ca^{2+} exchangers on nitric oxide production in the endothelial cells (Teubl et al., 1999; Schneider et al., 2002). It is reported that the Na^+/Ca^{2+} exchange system was involved in regulation of $[Ca^{2+}]_i$ level in HUVECs (Paltauf-Doburzynska et al., 2000; Berna et al., 2002). Na^+/Ca^{2+} exchanger proteins are predominantly localized in caveolae and thus in close proximity of endothelial nitric oxide synthase in endothelial cells (Prabhakar et al., 1998; Teubl et al., 1999). Thereby, Na^+/Ca^{2+} exchanger contributes to local Ca^{2+} homeostasis and cellular control of endothelial nitric oxide synthase activity in the endothelium in rat aorta.

4.3. Possible mechanisms for mesaconitine-induced Na^+ influx in endothelial cells

The effects of mesaconitine in rat aorta and HUVECs were dependent on extracellular Na^+. Mesaconitine increased $[Na^+]_i$ level in HUVECs, although the degree was small. There are several pathways causing Na^+ influx in the endothelial cells and HUVECs (Paltauf-Doburzynska et al., 2000; Touyz et al., 2001; Berna et al., 2002). Na^+/H^+ exchangers are a family of proteins that catalyze the exchange of extracellular Na^+ for intracellular H^+. Although Na^+/H^+ exchangers have been shown to be activated by various stimuli and regulate cell functions in endothelial cells including HUVECs (Németh et al., 2002), inhibitors of Na^+/H^+ exchangers did not modify the mesaconitine-induced increase in $[Ca^{2+}]_i$ level in HUVECs. The mesaconitine response in HUVECs was observed in the glucose-free buffer. Thus, Na^+/H^+ exchangers and Na^+–glucose cotransporters are not likely to contribute to Na^+ influx in HUVECs.

Nicotinic acetylcholine receptors are classified into muscle type and neuronal type. The expressions of neuronal nicotinic acetylcholine receptors are also confirmed in the non-neuronal cells including endothelial cells (McGehee and Role, 1995; Itier and Bertrand, 2001), and HUVECs express nicotinic acetylcholine receptors consisting of α3 or α7 subunits (Wang et al., 2001). Interestingly, the increase in $[Ca^{2+}]_i$ level induced by mesaconitine in HUVECs was inhibited by D-tubocurarine, although no definitive conclusion concerning the effect of D-tubocurarine on the mesaconitine response in rat aorta was revealed. A plant alkaloid methyllycaconitine was suggested to be a selective antagonist of nicotinic acetylcholine receptor, at nM order for the receptor consisting of α7 subunit (Wang et al., 2001; Mogg et al., 2002) and at μM order for that having α3 subunit (Bryant et al., 2002). The present findings suggest that mesaconitine acts as an agonist for nicotinic acetylcholine receptors in endothelial cells.

4.4. Summary

In the present study, we showed that mesaconitine activated the reverse mode activity (Ca^{2+} influx) of the Na^+/Ca^{2+} exchange systems, probably via an influx of extracellular Na^+, in endothelial cells. Then, mesaconitine induced relaxation of aorta via nitric oxide formation activated by Ca^{2+} influx in the endothelium (Mitamura et al., 2002b). In addition, an involvement of the Na^+/Ca^{2+} exchange system on the mesaconitine-induced increase in the $[Ca^{2+}]_i$ level was confirmed in the cultured endothelial cells, HUVECs. One of the possible targets of mesaconitine in HUVECs may be a D-tubocurarine-sensitive Na^+ channel such as nicotinic acetylcholine receptor. Although the nicotinic acetylcholine receptors are configured in clusters on the neuronal cell surface (Horch and Sargent, 1995; Feng et al., 1998), the clustering and/or localization of nicotinic acetylcholine receptors on the regions such as caveolae in endothelial cells has not been studied. The identification and the localization of target molecule(s) of mesaconitine in the endothelial cells should be determined in future.

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